Neural mechanisms underlying the buildup of semantic memory networks

Anumita Samanta

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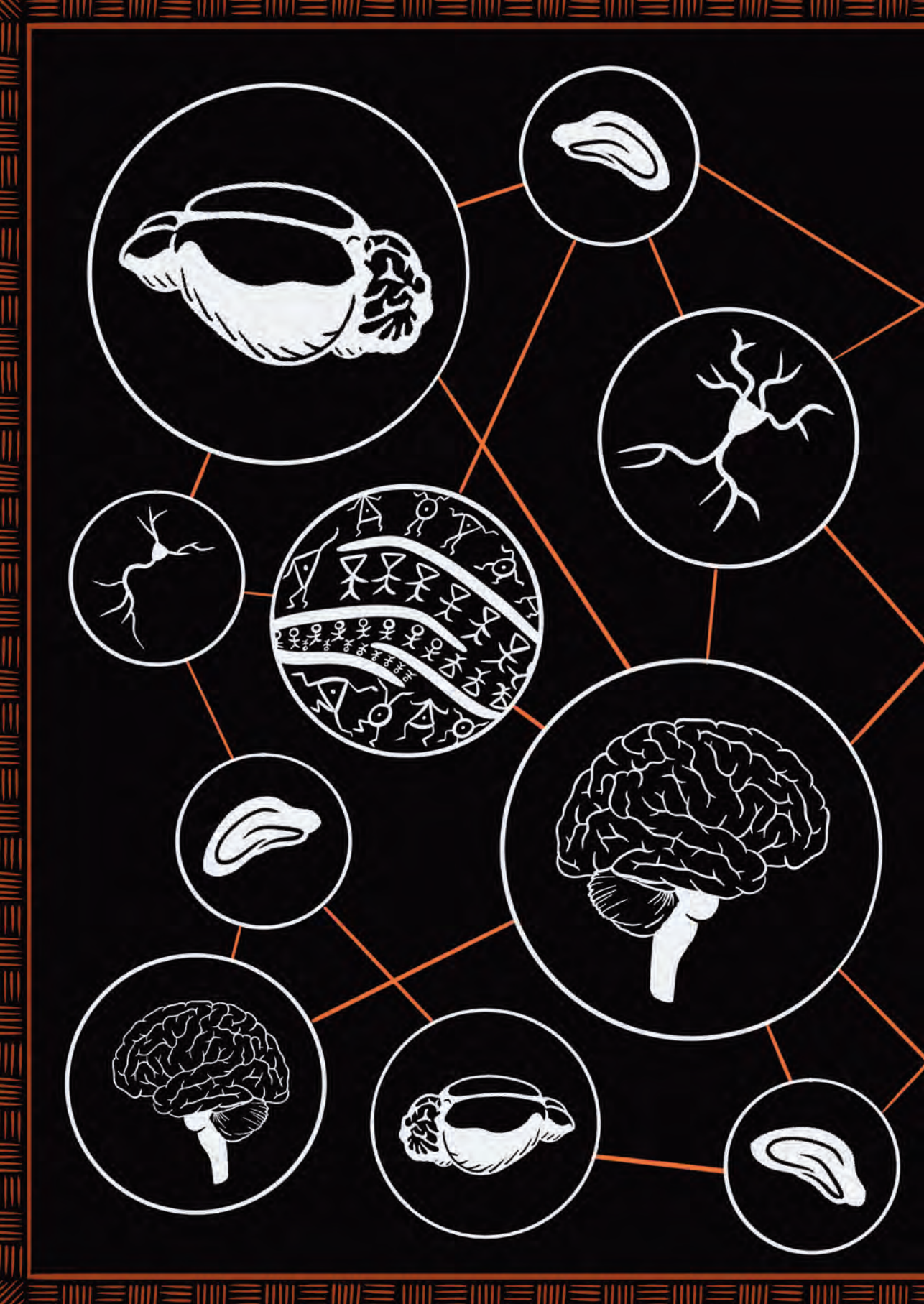
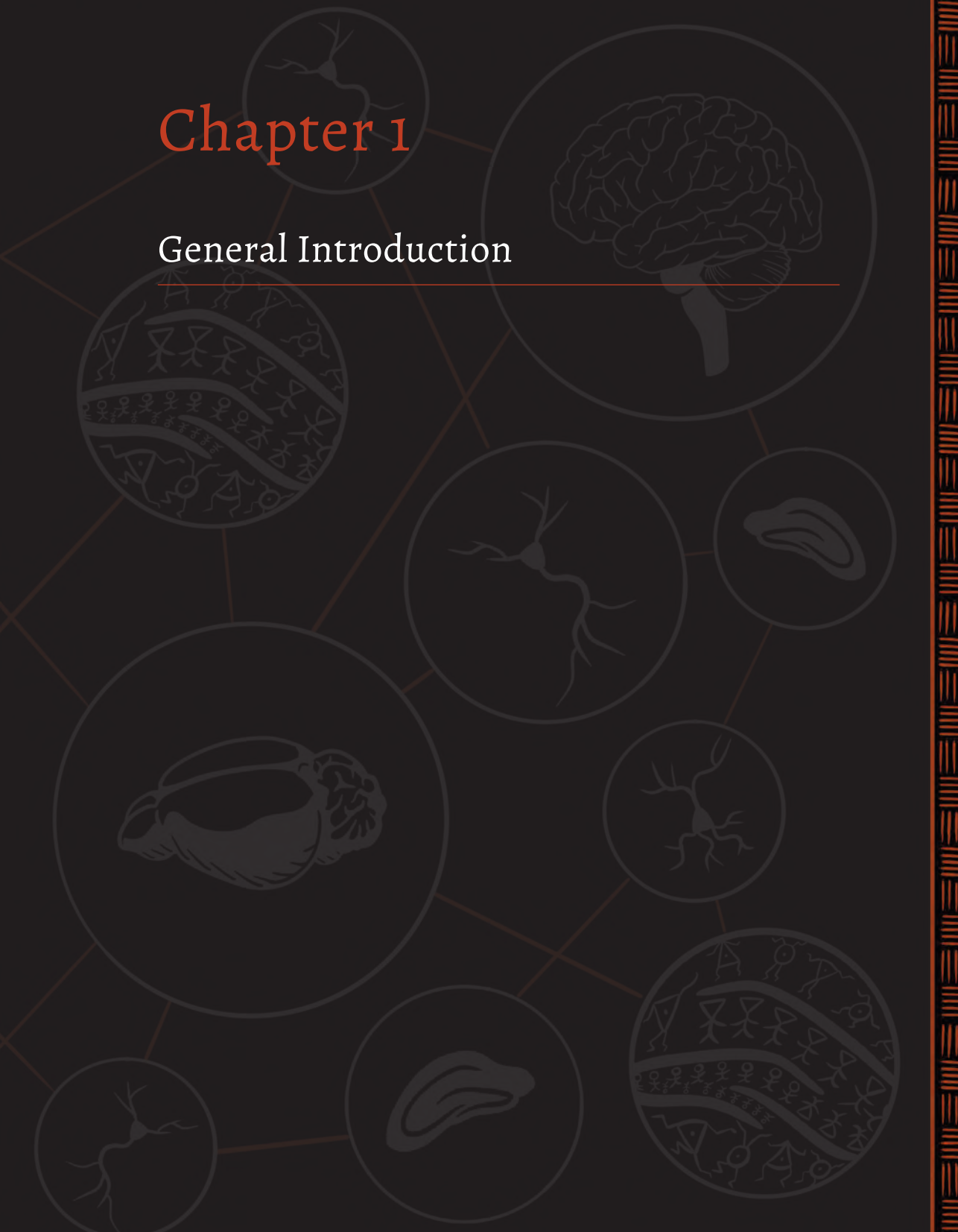


Chapter 1

General Introduction



One of the most treasured assets of human cognition lies in the ability to preserve acquired knowledge for the long term in the form of memories. This ability facilitates successful transmission of knowledge across generations, constituting a fundamental element in the survival of humans as a species. The overarching concept of "memory" encompasses the myriad experiences that we initially encode in our day to day lives, spanning various sensory modalities. Through repetitive exposures and the passage of time, these experiences get consolidated into more elaborate memory networks for long term storage. These networks, in turn, play a critical role in helping us recall specific events or information, thereby influencing our decision-making processes in the future. Historically, these long term memories are further broadly classified into two broad subtypes – *implicit/procedural memory* and *explicit/declarative memory* (Squire 1986) where the former refers to memories acquired through classical conditioning, learning of motor skills etc. whereas the latter deals more with memories acquired by conscious recall of information and includes facts, events and episodes experienced in day to day life. Delving deeper into the intricacies of the underlying neural mechanisms, 'memory' is not referred to as a standalone unitary experience localized to a single brain region. Instead, it pertains to the coordinated activity of multiple brain regions working in tandem to process diverse forms of information, also referred to as memory systems. In this thesis, I focus on the memory systems underlying the processing of declarative memories and in trying to understand how do the key brain regions involved coordinate with each other to consolidate these memories. In this regard, I further look at the potential role of sleep in regulating the consolidation processes.

Memory stages

Memory processing occurs in different stages. The first step where we acquire information inputs through the various sensory modalities is referred to as *encoding* wherein the memory trace is still quite labile and vulnerable to interference. The hippocampus plays a key role in integrating inputs from the different sensory streams and the encoding of these memories (Tulving and Markowitsch 1998). Subsequently, the memory trace goes through several stages of processing to transform into a more stable trace for the long term, which is referred to as *consolidation*. Continuous dialog between the hippocampus and neocortex dominated by sharp wave ripple complexes in the hippocampus and slow oscillations and spindles in the neocortex (Buzsáki et al. 1992; Steriade, Nuñez, and Amzica 1993; Maingret et al. 2016; Bramham et al. 2010) during offline states plays a key role in this step. The next steps of accessing these consolidated memories is referred to as *retrieval*. At a neural level, retrieval involves the full or partial

reactivation of networks that were active during the initial encoding of the specific memory. This is often triggered by reintroducing cues present during encoding. This process of recall has been shown to be dependent on the hippocampus when the memories are still in the early stages of consolidation. However, at later stages, when the memories are stabilized into long term networks in a more abstract form, recall of these memories starts to become more hippocampal independent (Wiltgen and Tanaka 2013; Tayler et al. 2013). In the final step of memory processing, when we integrate new information into previously learned memory networks, the memory enters back into a labile state, undergoing *reconsolidation* before restabilizing with the newly acquired information. Altogether these are the fundamental steps in memory processing.

With the increasing availability of tools that let us manipulate the workings of the brain, we are now able to identify the contributions of different brain regions at each of these stages with high temporal and spatial precision. Neuronal ensembles underlying a particular behavior as we know is dispersed across multiple brain regions and not localized to a single one. Using simple behavior models, researchers have now been able to identify key brain regions and track the neuronal ensembles as it evolves across each memory stage. Using gain/loss of function models, specific brain networks have been causally linked to play a key role at a particular stage of learning (Josselyn and Tonegawa 2020). However the question that still remains relatively unanswered is how does a memory network evolve as one partakes in more complex behavior. Using observational techniques, we attempt at disentangling the neural mechanisms underlying processing of simple and semantic-like memories and how would these processes be influenced by sleep and neuromodulators. Over chapters 2, 4 and 5 we address this question at the level of BOLD signal changes in human models, gene and subsequent protein expression changes and finally population activity of neurons in rodent models. In the next sections, we continue with reviewing the fundamentals of what we know about the processes underlying cellular and systems consolidation so far and the influence of sleep on memory, which are the central components of this thesis. Further we briefly elaborate upon what we know about how neuromodulators potentially influence these consolidation processes and end with summarizing the goals of this thesis.

Memory systems

Historically, two memory systems have been identified with clear anatomical and functional dissociations – declarative and procedural memory systems. The pioneering study of patient Henry Molaison, known as Patient HM (Scoville and Milner 1957)

represented a seminal investigation into memory formation and which brain regions are crucially involved. He suffered from epilepsy and as a cure, underwent a bilateral medial temporal lobectomy (including the hippocampus and most of the amygdala and entorhinal cortex) as a result of which he developed severe anterograde amnesia, rendering him unable to form new memories but still having intact memories from events prior to the surgery. This case prompted decades of research into memory systems, leading to the postulation of the medial temporal lobe memory system (Squire and Zola-Morgan 1991). This system including the hippocampus, parahippocampal cortices and amygdala has been shown to be crucial for processing of declarative memories. Procedural memories, including route learning, motor skill learning and habit formation, on the other hand, were shown to be dependent on the striatum (Squire and Zola 1996). The dominance of specific brain regions within each of these memory systems varies depends on the type of memory being processed. The hippocampal memory system plays a key role in encoding of episodic and spatial memories (O'Keefe and Nadel 1978; Eichenbaum et al. 1996). There are instances however when both memory systems work together in processing different aspects of a single memory trace. Especially, with regard to spatial navigation, the dorsal striatum has been shown to work in parallel with hippocampus and plays a key role in route learning and goal directed behaviors (Packard and McGaugh 1996). I will elaborate further upon this topic later in this chapter when I discuss processing of spatial memories. The individual contribution of each of these memory systems in the context of processing of spatial memories is also investigated in further detail in Chapter 2.

The declarative memories can further be divided to episodic and semantic memories. Episodic memories refer to our recollections of specific events with related details such as time and place and other contextual information. Semantic memories on the other hand consists of general knowledge of facts and concepts abstracted across multiple episodes (Tulving 1972). Information encoded in the short term is constantly relayed to the cortical structures for long term storage and stabilization of these memory traces. This process of transitioning these short term memories into long term semantic networks is termed as systems consolidation (Frankland and Bontempi 2005). It's a slow and gradual process that can last from days to weeks and is the central topic that we attempt to investigate in greater detail in this thesis. Using the Object Space Task (Genzel et al. 2019), in Chapters 4 and 5, I am able to model both of these memories in rodents and further attempt at disentangling the neural mechanisms underlying the processing of each.

In the next sections, I will delve deeper into the existing theories of memory consolidation and the contribution of sleep in this process.

Complementary learning systems / Systems consolidation

Our waking brain is remarkably good at continuously processing multitudes of information, forming new memories and revisiting old ones. When we look back, there are some life experiences, potentially dated years ago, that we still remember in great detail while other experiences exist as more abstract representations. I still remember in great detail, the first time ever that I experienced snow in the first year of coming to the Netherlands. The smell of gingerbread cookies, visuals of the white landscape all around, sounds of snowballs being hurled at one another, all tied in together to make an episodic memory trace. When looking into the underlying neural mechanism, sensory association cortices help in encoding the information across different sensory modalities and then the hippocampus is thought to be crucially involved in binding this information into a coherent memory trace. Multiple lines of evidence have hence reinforced this claim of hippocampus being important for rapidly encoding new memories in the short term, also referred to as the *fast learner* (Rudy and Sutherland 1989; Cohen and Eichenbaum 1993). Over the years, however, I encountered several snowfall episodes and have now formed a gist memory of how a typical snowy day would look like. Over time and repeated exposures, these episodic memory traces are thought to slowly become independent of the hippocampus and integrate for the long term with pre-existing memories into the neocortical regions, forming a semantic memory network. The prefrontal cortex (PFC) is thought to be one of the crucial hubs in this process and is referred to as the *slow learner*. This process of consolidating memories for long term storage is referred to as systems consolidation and is thought to occur for most day to day memories (Frankland and Bontempi 2005). One question pops up however in this regard as to what happens to memories that are completely unique and don't fit into any pre-existing memory network. For example, why do I remember my first snow encounter in such great detail but not the ones after? It is theorized that these unique memories serve as distinct novel experiences and are potentially retained in the hippocampus for a longer term with all the episodic details (Duszkiewicz et al. 2019). As is going to be explained in more detail in the later sections, neuromodulators such as dopamine and nor-adrenaline potentially influence the consolidation of novel memories. Depending on the salience of novelty, hippocampus has been shown to receive dopamine inputs from Ventral Tegmental Area (VTA) and Locus Coeruleus (LC), which prime the synapses surrounding the novelty event, further enhancing its consolidation (Duszkiewicz et al. 2019; Lisman and Grace 2005; Wang, Redondo, and Morris 2010; Takeuchi et al. 2016). In chapter 3, I elaborate further upon how neuromodulators influence these offline consolidation processes and proceed with testing some of the claims in chapters 4 and 5.

This framework of hippocampus being a fast learner and the PFC being a slow learner was first introduced as a part of the Complementary Learning Systems theory. The theory states that the complementary nature of the fast encoding of new memories by the hippocampus and slow integration of these new memories into pre-existing memory networks by the neocortex is the underlying mechanism of how we are able to perform complex behavior tasks with minimal interference (McClelland, McNaughton, and O'Reilly 1995; O'Reilly et al. 2014).

Subfields of the hippocampal system, namely the Dentate Gyrus (DG), CA3 and CA1 regions play distinct and complementary roles in memory consolidation (Rolls 2016). The DG is thought to play a critical role in pattern separation, allowing similar experiences to be stored as distinct memories with minimal overlap in representations. It gates memories associated with specific contexts and through crosstalk with CA3, optimizes retrieval of context specific representations. The DG further communicates with the CA3 via the mossy fiber pathway, providing input to the proximal apical dendrites of CA3 pyramidal cells (Henze, Urban, and Barrionuevo 2000). The CA3 region then plays a critical role in pattern completion where it binds fragmented inputs from the DG into a cohesive episodic representation of the memory trace (Guzman et al. 2016; Neunuebel and Knierim 2014). Together, the DG-CA3 circuit plays a critical role in extracting distinct temporal and spatial pattern relationships amongst events for rapid short term encoding (Leutgeb et al. 2007; McHugh et al. 2007; Coelho et al. 2024) and the CA1 region serves as a comparator, integrating processed information from CA3 and entorhinal cortex and projecting the information to the neocortex to strengthen the connections and consolidate these memories for the long term.

Furthermore it introduces an interleaved system of learning wherein we learn both new and old information simultaneously and having the complementary brain systems would help in temporally separating these memory traces, preserving the integrity of both old and new information. Having this system in place is hypothesized to prevent catastrophic interference (McCloskey and Cohen 1989) from taking place in biological systems. Being in the Netherlands, for example, when we park our bikes in a huge parking lot, we are still able to remember the different locations where we park each day without any interference. However, I don't have a vivid episodic memory of where I parked my bike a week ago. But if now, on a particular day, I witness a huge accident next to my parking spot, I will potentially retain the memory of the spot with all the details for a much longer time period. Studies have shown that occurrence of such "flashbulb memories" which are completely unique standalone experiences prime the consolidation of memories surrounding that experience (Brown and Kulik

1977). In Chapters 5 and 6, we model a behavior training paradigm in mice and rats which enables us to assess for semantic-like memories and test the theory posited by Complementary Learning Systems and if that holds true in a biological system. Further we raise a question on would interference potentially occur if a conflicting memory episode were followed by a novelty event. A previous study (Genzel et al. 2017) showed using a watermaze, that training followed by novelty shows a higher memory expression during test but was more prone to interference effects. However training followed by sleep led to a more long lasting stable expression of memory during test which was resistant to interference. These findings further emphasize the critical role of sleep in offline consolidation processes and warrants further investigation.

Memory consolidation during sleep

Sleep is a naturally occurring state of rest characterized by immobility, reduced sensory responsiveness and a relative lack of consciousness and has been shown to occur universally across all species. It plays a crucial role in regulating several vital physiological functions including development, energy conservation, metabolic clearance, brain homeostasis and cognition (Siegel 2008; Zielinski, McKenna, and McCarley 2016). As shown in Figure 1.1, the state of sleep is classically defined by electrical signals recordings from muscles and brain - Electromyogram (EMG) and Electroencephalogram (EEG) and consists of two well defined sub states – *non-rapid eye movement (NREM or NonREM)* and *rapid eye movement (REM)*. Humans are monophasic – i.e. have one sleep phase per day and sleep roughly for 6-8 hours a day. The period of sleep consists of several sleep cycles with one cycle lasting for typically ~90 mins and alternates between NREM and REM sleep. The NREM stage can be further classified into N1, N2 and N3, which is associated with increasing depth of sleep and slower EEG activity (Kishi et al. 2011; Dement and Kleitman 1957). Rodents on the other hand are nocturnal creatures and polyphasic and sleep multiple times during the light hours of the day, with short NREM-REM cycles in each period interrupted by wakefulness.

Research from both human and animal models has shown the active beneficial role of sleep in memory processing for both declarative and procedural memory tasks (Sara 2017; Stickgold 2013). However the underlying mechanisms still remain unclear.

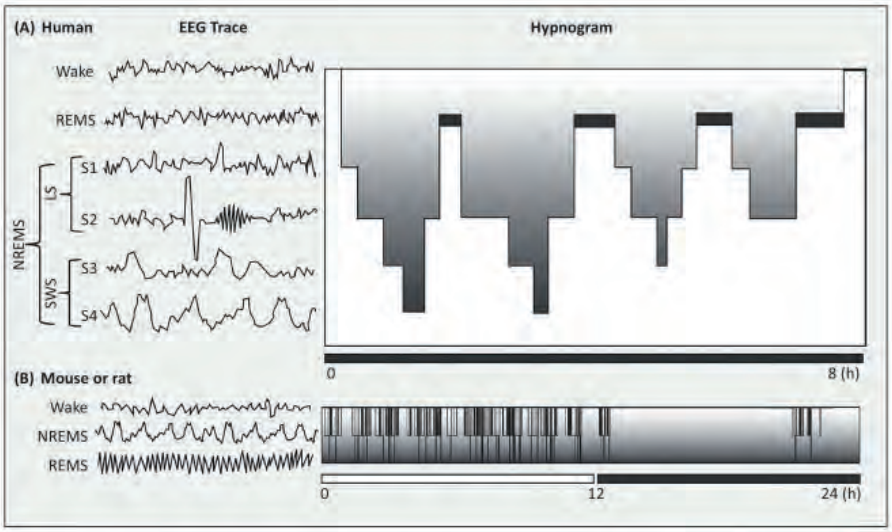


Figure 1.1 Sleep stages in humans and rodents. **(A)** EEG traces of different sleep stages detected in humans (left) accompanied by a hypnogram (right). NonREM sleep here is further subdivided into light sleep (LS) and Slow Wave Sleep (SWS) with each stage being further split into S1-2 and S3-4 respectively. LS is typically characterized by slow oscillations and spindles and SWS is characterized by large delta waves. Humans are monophasic and typically have one sleep phase per day during the night, lasting ~8 hours. This is represented in the hypnogram. **(B)** EEG traces of different sleep stages detected in rodents (left) accompanied by a hypnogram (right). Rodents are polyphasic nocturnal creatures, so they sleep mostly during the light period of the day, as can be seen in the hypnogram. During sleep, they alternate between several short NonREM-REM cycles interleaved with wake phases. Figure adapted from (Genzel et al. 2014)

The hippocampus has been shown to be the primary region responsible for rapid encoding of episodic memories and short-term storage. According to the systems consolidation model, over time, these hippocampal memories get integrated into the neocortex for long term storage and eventually become independent of the hippocampus (Frankland and Bontempi 2005; Buzsáki 2015; Ekstrom and Ranganath 2018). Broadly, this model could then be divided into two phases – encoding phase which happens across multiple brain regions followed by the consolidation phase where sleep, especially NREM sleep has been shown to play a crucial role. A landmark study by Wilson and McNaughton (1994) showed a possible mechanism on how this might occur. This was one of the first studies to introduce the phenomenon of *neural reactivation* in the hippocampus by which cell sequences active during encoding are replayed at an accelerated rate over high frequency burst oscillations during NREM sleep. These high frequency oscillations are identified as *sharp wave ripples* (SWR) which are characteristic oscillations (90-200 Hz) known to occur in the CA1 region of the dorsal hippocampus during NREM or during rest periods when the brain is disengaged from

the environment (Buzsáki et al. 1992). These SWR events have been implicated to play a crucial role in the memory consolidation phase. However the SWR events on their own are restricted to only replaying events encoded during wake. To be able to consolidate the memories, there needs to be a coordinated action between the hippocampus and the neocortical structures in response to the sharp wave ripples. There are two major neocortical events known to occur during NREM – *slow oscillations (1-4 Hz)* and *spindles (9-20 Hz)* which function in close coordination with the SWRs in the hippocampus. This coordinated crosstalk has been shown to be crucial for memory consolidation processes (Siapas and Wilson 1998; Ji and Wilson 2007; Peyrache, Battaglia, and Destexhe 2011; Poe, Walsh, and Bjorness 2010). In addition to these correlational studies, other studies were also able to show disruption of SWR events during sleep after learning led to an impaired memory performance the next day (Girardeau et al. 2009; Roux et al. 2017) or optogenetically increasing the coupling between spindles, slow oscillations and SWRs led to a better memory performance the next day (Maingret et al. 2016). Optogenetic prolongation of SWR events also seemed improve memory performance, indicating that potentially longer SWR events might replay more information from the maze which might aid in future planning (Fernández-Ruiz et al. 2019). It is important to note here that the longer SWR events detected occurred during wake. The functions and properties of these SWR events can hence vary depending on the brain state in which they occur. This is further elaborated upon in Chapter 3.

When looking at consolidation of encoded memories, sharp wave ripple events in the hippocampus have been shown to be pretty crucial for offline consolidation of memories (Buzsáki 2015). These events are thought to be initiated in the CA3 region of the hippocampus followed by synchronous high frequency burst firing of neurons in the CA1 region and have been shown to occur also during quiet wake and rest periods in addition to NREM following performing a task (Jadhav et al. 2012; Roumis and Frank 2015). In chapter 3, I attempt at further elaborating on the different types of SWR events and how could be underlying different learning events. It was speculated by Donald Hebb that memories could be encoded and strengthened by repetitive and persistent firing of cells active within that memory. LTP at synapses between CA3-CA1 cells have also been shown to underlie learning behaviors (Bliss and Collingridge 1993; Martin and Morris 2002). It could thus be speculated that NMDAR dependent synaptic plasticity underlies the storage of memories for subsequent reactivation (O'Neill et al. 2010). This has further also been shown in another study that timing of CA3 and CA1 place cells firing in relation to SWR onset is critical for induction of LTP and tuning of plasticity processes for subsequent reactivation (Sadowski, Jones, and Mellor 2016). Lastly, neurotransmitters like dopamine, acetylcholine and noradrenaline can also have on influence on memory reactivations and offline consolidation events. These

neurotransmitters are known to be well regulated in sleep wake cycles and also have innervations in the hippocampus, hence potentially influence the reactivation of specific events (Marrosu et al. 1995; Eschenko and Sara 2008). We elaborate further on this in chapter 3, explaining the role of each neurotransmitter in this context and in the end bringing together all the findings to shed light on potential molecular mechanisms underlying memory reactivations and SWR events.

Apart from its critical role in offline consolidation processes, the hippocampus has also been hypothesized to serve as the cognitive map of the brain and play a crucial role in spatial navigation (O'Keefe et al. 1998; O'Keefe and Dostrovsky 1971). It is thus not surprising that most of the hallmark studies investigating the hippocampus's role in offline consolidation mechanisms have utilized spatial learning tasks as their model system. Apart from humans, it serves as a core memory trait for other organisms like rodents as well, where navigating around one's surroundings is key to survival. It has hence been relatively easy to model spatial learning tasks for rodents and test for fundamental neural mechanisms. In the next section, we elaborate more upon which memory systems underlie spatial memory processing.

Spatial memories

The cognitive ability of an organism to be able to map one's surroundings within a greater environment and navigate to target locations is crucial to its daily functioning and has been a topic of interest amongst researchers for decades. We use spatial memories as a model system in this thesis to try and understand the underlying fundamental neural mechanisms of memory processing. Edward Tolman was one of the first behaviorists to study this cognitive process in more detail using rats as model systems wherein he investigated their navigation patterns in different maze environments and the strategies they use to find rewards within the maze. He introduced the concept of a "cognitive map" which is henceforth defined as the internal mental representation of the surrounding environment which an individual always refers to when trying to recall specific locations relative to the surroundings (Tolman 1948; Tolman and Honzik 1930). The cognitive map serves as a mental framework within which spatial memories can be encoded, organized and retrieved. This map is a dynamic representation of the environment which keeps evolving as an individual navigates through different locations and builds new spatial memories. The hippocampus has been shown to play a crucial role in integrating one's spatial memories within their cognitive map and continuously updating it with new information (Buzsáki and Moser 2013; Donato, Xu Schwartzlose, and Viana Mendes 2023).

When navigating through an environment, there are two main navigation strategies that are thought to be used – *place learning* and *response learning*. Place learning relies on a spatial cognitive map containing an internal representation of relationship between distal cues, independent of the location of the navigator and is thought to be dependent on the hippocampus (Morris et al. 1982; Gahnstrom and Spiers 2020; Packard and McGaugh 1996). The latter in contrast is more dependent on the relationship between the location of the cues and that of the navigator, potentially involving fixed routes to get to targets and is thought to be dependent on the striatum (Packard and McGaugh 1996; Brasted et al. 1997). When navigating in the real world, one uses information from both learning frames to form a cohesive representation of the environment and the position of the navigator within it (Andersen et al. 1997). However to be able to assess the workings of each strategy in greater detail, one can experimentally bias the use of one strategy over the other with different training paradigms (Figure 1.2). This could be incorporated in maze experiments for example by providing variable or stationary starting to get to the target every trial, which is otherwise referred to as an *allocentric* or *egocentric training* respectively (Figure 1.2b). When trained under allocentric conditions, the navigator would have more bias towards using the place learning strategy to re-route every trial and use the distal and proximal cues to orient themselves to find the correct goal location. Under egocentric training, a response learning strategy can be used since the start location is fixed, so one can take the same route to get to the goal location independent of the location of cues. However, when trained with a fixed start location, one can potentially use both strategies to navigate to the goal. For true egocentric training, the start location must be moved along with the target while maintaining a constant relative location to one another, such that there is always a fixed route between the two. Using these different training paradigms would enable us to investigate the underlying hippocampal and striatal memory circuits in more detail and their subsequent consolidation processes. (Genzel 2020; de Bruin et al. 2001). Several studies have been carried out in both rodents and humans to assess the involvement of hippocampus and striatum in each learning strategy and results indicate a dynamic and complex interaction between these systems for efficient solving of tasks (Gasser et al. 2020; Furman et al. 2014).

In the second chapter, we aim to further investigate the underlying neural mechanisms of the consolidation of these different aspects of spatial memory processing. We adopt a translational approach to test in rats and human models using the watermaze task, which has been previously used to test allocentric and egocentric navigation (Morris 1981; Schoenfeld et al. 2017).

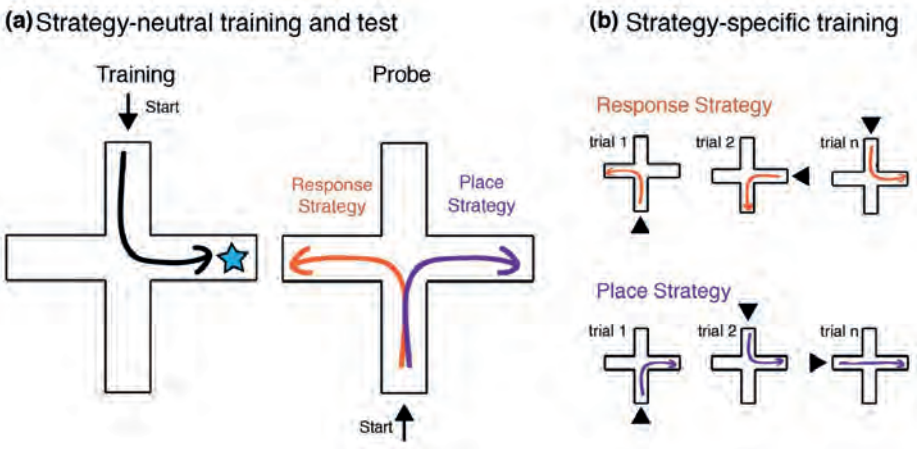


Figure 1. Depiction of navigation strategies using Plusmaze Task as an example. (a) If a rodent is always introduced to the maze from a fixed start location, then it can use either place or response learning to find the treat. To test which learning strategy is used, the memory can be tested with a probe trial and the rodent can be introduced to the maze from the opposite arm. If hippocampal dependent place learning strategy was used during training, then it potentially uses the external environment to orient and turn towards the right (correct) arm. If striatum dependent response learning strategy was used during training, then it will turn left and head to the wrong arm. **(b)** Training paradigm can be adapted to enforce the use of a specific strategy. Upper panel depicts the use of response learning strategy where the relationship between start and reward location (always left turn from start location) is kept constant across all trials. Lower panel depicts the use of allocentric learning strategy where the start location is different every trial, but reward location is fixed, so the rodent has to reorient itself every trial. Figure adapted from (Genzel 2020).

For the other research aims in this thesis, we wanted to use a behavior model that would enable us to study both simple memories and in addition, also complex semantic-like memories. The watermaze, although being an ideal behavior paradigm for spatial learning, was not a suitable model for our research questions, because one cannot distinguish between simple and semantic learning using this task. When rats get to the platform after multiple trials of training in the maze, it is not possible to tell whether the rats used information from each of the trials or only the last training trial to find the platform. To be able to better distinguish these differences in behavior, previously in the lab, we established a spatial object exploration task, called the Object Space Task (Genzel et al. 2019) for both rats and mice, where we were able to show that rodents are able to abstract information across multiple trials and form a cumulative memory. Further this task exploits the natural tendency of rats to explore novel environments, hence we get as close as possible to looking at naturalistic rodent behavior and then studying the underlying neural mechanisms. Due to these reasons, we use this as a behavior model in chapters 4, 5 and 6 for all experiments.

Next, I would like to take a step back and elaborate a bit on the cellular basis of memory and what we know so far about cellular consolidation processes.

The synapse as building block of memory

Synaptic transmission

The fundamental unit of information processing in the brain is communication between neurons. Across all species, this ability of the neurons to transmit information rapidly and precisely is key to our ability to process information around us, feel, build memory networks, make decisions and perform actions. Communication between neurons happens through specialized junctions called synapses with neurotransmitters being released as a chemical signal. The neuron that releases the neurotransmitter is called the presynaptic neuron. These presynaptic neurons contain specialized pocket like structures called synaptic vesicles at their axon terminals which is filled with neurotransmitters. The onset of an action potential leads to a Ca2+ influx which subsequently leads to the release of neurotransmitters from these synaptic vesicles into the synaptic cleft between two neurons. These neurotransmitter molecules then traverse the synaptic cleft and are taken up by neurotransmitter receptors in the postsynaptic neuron, thereby producing a post synaptic signal (Katz 1969; Südhof 2013). This process is referred to as synaptic transmission and occurs in a remarkably fast and precise manner.

The neurotransmitter receptors can be further divided into two major classes – ligand-gated ion channel (LGIC) receptors and G-protein-coupled receptors (GPCRs). LGIC receptors, also referred to as ionotropic receptors consist of a group of transmembrane ion channel proteins which when bound by the “ligand” such as neurotransmitters, become permeable to influx of ions such as Na+, K+, Ca2+ in the post synaptic targets. When bound to an excitatory receptor such as glutamate, the channels attached to the receptor open allowing an influx of Na+ ions, leading to depolarization of the cell. When bound to an inhibitory receptor such as GABA, on the other hand, the Na+ ion channels are closed and the K+ ion channels remain open, leading to the efflux of the K+ ions, thereby reversing the membrane potential of the cell, causing a hyperpolarization response (Kandel et al. 2000). The LGIC receptor signal transmission is quite rapid, leading to a strong response from the post synaptic neurons and takes a total of 1-2 milliseconds.

GPCR signaling, on the other hand, is often termed as neuromodulatory due to its relatively slow and long-lasting effects. GPCRs consist of proteins which

are specialized in binding to neurotransmitter molecules, causing a cascade of biochemical reactions which can influence a variety of cellular functions. When bound to a neurotransmitter, the G-receptors separate into multiple subunits which can further bind to "effector" proteins to alter cellular physiology and gene expression.

Long term potentiation (LTP)

Glutamate is an excitatory neurotransmitter, which accounts for the majority of rapid excitatory neurotransmission and has been shown to be critically involved in a wide variety of brain functions, especially with regard to learning and memory. The signaling involves the activation of three subtypes of LGIC receptors – AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate and NMDA (N-methyl-d-aspartic acid). At most excitatory synapses, glutamate is released from presynaptic vesicles which then binds to AMPA type receptors resulting in a rapid depolarization and firing of an action potential. The working of NMDA receptors follows a more complex mechanism. When the glutamatergic synapses are activated repeatedly, the repeated activation of AMPA receptors depolarizes the post synaptic neurons enough to unblock the channels and allow the ion current to flow through the NMDA receptors. The ion pores of these receptors are especially permeable to calcium ions and trigger a variety of signaling cascades. Strong NMDA receptor activation triggers molecular events which recruit additional AMPA receptors at synapses, thereby increasing synaptic transmission. This is termed as *long term potentiation (LTP)* and has been shown to be the fundamental mechanism underlying learning and memory processing in the brain (Kandel et al. 2000; Feldman 2009). Especially in the hippocampus, NMDA signaling has been shown to be critical for the ability to encode memories (Morris 1989; Morris 2013) and it has further been validated *in vitro* and in vivo that LTP can be induced by high frequency stimulation of presynaptic axon terminals in hippocampal synapses which mimic the effects induced by learning (Bliss and Collingridge 1993; Whitlock et al. 2006). In addition, it is also possible to induce LTP or LTD according to the spike-timing-dependent-plasticity (STDP) rule by coordinating the temporal order and timing of stimulating presynaptic and postsynaptic neurons. Overall strengthening the connectivity of neurons active within a certain environment could lead to efficient encoding of memories (Bi and Poo 1998; Hebb 2005).

Early LTP

As mentioned above, LTP has been shown to be a critical cellular event underlying the successful encoding of a memory trace. It can be divided into two phases – *early LTP* and *late LTP*. The early phase refers to the induction of LTP which is triggered by a high frequency stimulation in the presynaptic terminals (Bliss and Collingridge 1993),

sufficient enough to release glutamate into the synapse, leading to an influx of the Na+ ions and activation of the NMDA receptors in the post synaptic neurons. Depending on the temporal coordination between the pre and postsynaptic neurons, and the amount of resulting Ca2+ influx, the induction can lead to LTP or LTD (Malenka and Bear 2004; Nevian and Sakmann 2006). The activation of the NMDA receptors further triggers the activation of calcium-calmodulin-dependent kinase II (CaMKII) which has been shown to play a critical role in the induction of LTP, engaged in increasing spine density and related functional changes in the post synapse (Lisman, Yasuda, and Raghavachari 2012). This is followed by an activity dependent exocytosis of AMPA receptors containing GluR1 subunits, which get incorporated into the dendrites of post synapses for subsequent cascades of plasticity related changes. This step of AMPAR trafficking has been shown to be critical for long lasting transmission of LTP (Collingridge, Isaac, and Wang 2004; Malinow and Malenka 2002)

Overall, the early LTP phase typically has been shown to last for 1-3 hours following induction and depending on the strength of the stimulus, persists longer into the late phase, triggering a cascade of signaling pathways which can potentially lead to long lasting structural and functional changes in the post synapses. From a behavioral perspective, this would underlie the formation of a short-term memory, within hours after an encoding experience.

Late LTP

Late-phase long-term potentiation (L-LTP) is a sustained enhancement of synaptic strength that underlies long-term memory formation. Unlike early-LTP, which relies on post-translational modifications, L-LTP requires new protein synthesis to stabilize and maintain synaptic changes. This process involves the expression of immediate early genes (IEGs) and subsequent protein synthesis, leading to structural and functional modifications at synapses in response to learning. Studies have shown that protein synthesis inhibitors block the formation of persistent memories while leaving short-term memories intact, suggesting that stable, long-term memory formation depends on gene activation occurring shortly after the experience. Notably, delayed *c-Fos* expression, occurring 24 hours after training, has been shown to be essential for the persistence of long-term memory (LYNCH 2004; Katche et al. 2010). The induction of IEGs serves as a molecular gateway between synaptic activity and the protein synthesis required for L-LTP. Upon activation, they encode transcription factors and other proteins that regulate the expression of downstream genes involved in synaptic remodeling. This cascade ultimately leads to the synthesis of proteins that strengthen synaptic connections, such as receptors, scaffolding proteins, and signalling molecules (Abraham, Dragunow, and Tate 1991).

Functional relevance of IEGs

Following the stabilization of Ca2+ influx triggered NMDAR-LTP, several key transcription factors get phosphorylated, activating an intracellular cascade of reactions, including the synthesis of key proteins known to be crucially involved in regulating synaptic plasticity, learning and memory. A specialized group of these proteins are called Immediate Early Genes (IEGs) which are transiently activated and expressed across different brain regions in response to extracellular and physiological stimuli. Over the years, reports have shown them to be crucially involved in processes underlying synaptic potentiation and memory consolidation (Okuno 2011; Lanahan and Worley 1998; Yap and Greenberg 2018; Nambu et al. 2022). They have also been shown to be differentially regulated across cortical and hippocampal regions depending on sleep/wakefulness states (Seibt and Frank 2019; Ribeiro et al. 2007; Delorme, Kodoth, and Aton 2019) What remains fairly unknown are the critical factors underlying their proper functioning – timing of their expression, how long do they need to be transcriptionally active and roles of other molecules ensuring their stable expression. The synaptic tagging and capture hypothesis (Frey and Morris 1997) postulates how weak potentiation during phases of early LTP can further be converted into strong potentiation and late LTP by triggering the activity of plasticity related proteins (PRPs) at the synapses involved. With the help of calcium dependent kinases, these PRPs capture the weak potentials at the cell body or dendrites and stabilize them for long term potentiation (Redondo et al. 2010; Frey and Morris 1997). This could explain the underlying mechanism behind the abnormalities in IEG KO models, since most IEGs function as PRPs in the second wave of protein synthesis, lack of expression leads to deficits in long term potentiation. Although there is a lot of overlap in function, there still exists a significant heterogeneity in the individual mechanisms underlying the working mechanisms and expression patterns of different IEGs. The candidate genes that we focus on are – *Arc (Activity Regulated Cytoskeletal Associated Protein)* and *c-Fos*.

All IEGs are roughly divided into two categories – those which act as "regulatory transcription factors", otherwise called as RTF genes, meaning expression of these genes can further trigger the expression of more downstream genes. The expression pattern of genes belonging to this category, like c-Fos, can be relatively less specific and has been shown to be triggered by more robust plasticity changes in the brain (Herdegen and Leah 1998; Tischmeyer and Grimm 1999). The second category consists of genes which are called "effectors" which have a more direct role in modulating cellular function, Arc being one such effector protein. Expression patterns of these genes are known to be more specific and triggered by activity changes in the brain.

Box 1. Immediate Early Genes – Arc and c-Fos – mechanisms of action

Arc is a postsynaptic protein which was first discovered to be localized in dendrites, with its expression induced by seizures and learning experiences (Link et al. 1995; Lyford et al. 1995). Following behavioral stimuli or high-frequency seizures, Arc mRNA is transcribed in the nucleus and subsequently transported to dendritic compartments, where it undergoes local translation (Steward et al. 1998). This targeted expression enables Arc to play a crucial role in synaptic plasticity by modulating both long-term potentiation (LTP) and long-term depression (LTD), thereby maintaining the delicate balance of excitatory and inhibitory activity in the brain. It supports the maintenance of LTP by regulating actin cytoskeletal dynamics within dendritic spines, promoting long lasting structural changes. Arc is also rapidly transcribed in response to synaptic activity and transported to active synapses, where it gets locally translated (Chen et al. 2023). The synthesis of Arc is overall necessary for induction and consolidation of LTP, regulated by expansion of f-actin. BDNF additionally potentially plays a key role in triggering Arc transcription and Arc mediated LTP (Messaoudi et al. 2007). It's role in LTD is primarily associated with the downregulation of AMPA receptors. It facilitates AMPAR endocytosis by interacting with endocytic proteins such as dynamin and endophilin (Chowdhury et al. 2006), thereby weakening excitatory synapses and contributing to synaptic homeostasis. Increased Arc expression has been shown to reduce AMPAR-mediated excitatory transmission by selectively removing GluR2/3 subunits (Shepherd et al. 2006; Rial Verde et al. 2006). It can further also contribute to synaptic downscaling by altering spine morphology. It has been shown to specifically reduce surface GluR1 internalization at thin spines and overall increasing the proportion of thin spines, mediating AMPAR endocytosis (Peebles et al. 2010). Another interesting theory termed "inverse synaptic tagging" postulates that Arc preferentially binds to inactive synapses via CaMKIIβ which is not bound to calmodulin in an attempt to downregulate AMPAR activity and further weaken these synapses, ultimately increasing the signal from the activated synapses (Okuno et al. 2012). c-Fos is a transcription factor which shows active transcriptional activity in response to extracellular signals. The promoter region of cfos contain multiple binding sites which act as regulatory elements, essential for the functioning of the entire transcript. These elements include – the sis-inducible element (SIE), serum response element (SRE), fos AP-1 site and calcium/cAMP response element (CRE) (Robertson et al. 1995; Smeyne et al. 1992). CREB, the activity dependent transcriptional regulator serves as a key molecule in binding with CRE and mediating its activity. It serves as a crucial regulator of IEG expression and plays a key role in mechanisms underlying cognitive functions and neural plasticity (Bito and Takemoto-Kimura 2003; Alcino J. Silva et al. 1998). It was the first IEG to be identified whose expression seemed to be rapidly induced across multiple brain regions following pharmacologically induced seizures or physiological demands (Morgan et al. 1987; Sagar, Sharp, and Curran 1988). It is shown to be triggered following learning experiences with expression in both dorsal and ventral hippocampal region, parahippocampal cortices and prelimbic and cingulate cortices.

In spite of these differences, both Arc and c-Fos have been reliably used as markers of synaptic plasticity and learning. Comparative studies have revealed these genes to have a similar expression profile, but expression of Arc has been shown to be more responsive to changes in task demands (Guzowski et al. 2001). In Chapters 5 and 6, we look in more detail at the expression profile of these genes in the hippocampal and cortical regions following specific behavioral timepoints at both protein and RNA level. Several studies have been conducted so far which show an increase in expression following novelty and spatial exploration (Vazdarjanova and Guzowski 2004; Guzowski et al. 1999; Jenkins et al. 2004). Building up on these findings, we are interested in looking at how the expression changes as one participates in more complex behaviors, building up a semantic memory network.

Influence of neuromodulators in synaptic plasticity and memory consolidation

Neuromodulators have been shown to play a critical in mediating several functions ranging from stress effects, neural development, circadian rhythm regulation, emotional regulation of behavior, long term synaptic plasticity, brain oscillations during sleep and wakefulness and influencing learning and memory in the long term (Bazzari and Parri 2019; Wang and Pereira 2016). Glutamatergic transmission, especially the NMDAR-dependent cellular cascades has been established as playing a key role in regulating the induction and maintenance of LTP (Morris 2013; Redondo et al. 2010; Herring and Nicoll 2016). However, glutamatergic signaling mediated by GPCRs also play a crucial and diverse role in regulating several cognitive functions, including regulation of key protein synthesis cascades, essential in persistence of LTP and LTD (Wang et al. 2016; Pfeiffer and Huber 2006; Horwood et al. 2006). Molecules including BDNF and metabotropic glutamate receptors (mGluRs) play a critical role as mediators in both early and late phases of plasticity. Working through diverse mechanisms, like voltage gated calcium channels and activation of protein kinases which mediate trafficking and insertion of AMPA receptors into synapses, they can exhibit effects in maintaining the balance between LTP and LTD, resulting in influencing the processing of memories in the long term. Different sub units of mGluRs have been shown to be critically involved in either LTP or LTD induction, overall complementing each other in maintaining synaptic homeostasis (Bramham 2008; Rosenberg, Gerber, and Ster 2016; Wang, Rowan, and Anwyl 1997).

Dopamine is one such neuromodulator which has been shown to play a critical role in several domains ranging from motor control, limbic functions, brain reward circuitry

and adaptive behaviors. Dopamine receptors in the brain can be further classified into two types – D1 and D2 receptors, of which D1 is coupled to the excitatory G protein complexes which stimulate the expression of adenylyl cyclase and cAMP production, triggering LTP whereas D2 is coupled to the inhibitory G protein complex, which leads to reduced cAMP production, triggering LTD (Nieoullon 2002; Missale et al. 1998). Keeping a balance between excitatory and inhibitory transmission, it thus aids in regulating synaptic plasticity and gene expression.

The Ventral Tegmental Area (VTA) and Substantia nigra pars compacta (SNc) serve as the primary hubs of dopamine release, with extensive projections throughout the brain. While the hippocampus is known to receive dopaminergic input from the VTA, recent studies (Duszkiewicz et al. 2019; Takeuchi et al. 2016) have have also identified the Locus Coeruleus (LC) as a source of dopamine innervation. Traditionally regarded as the brain's primary norepinephrine center, emerging evidence suggests that the LC plays a modulatory role in dopaminergic signaling. Studies indicate that LC neurons co-release dopamine in regions such as the hippocampus and prefrontal cortex, enhancing spatial learning and memory via dopamine D1/D5 receptors(Kempadoo et al. 2016; Yamasaki and Takeuchi 2017). The VTA predominantly innervates the ventral hippocampus, while the LC projects to the dorsal hippocampus.

As discussed in earlier sections, these two dopaminergic pathways appear to be modulated by different types of novelty events. "Common novelty" events, which share a commonality with everyday memories primarily engage the VTA-HPC loop, whereas "Distinct novelty" events characterized by flashbulb memories (Brown and Kulik 1977) which are unique standalone experiences trigger the LC-HPC loop. These distinct novelty experiences potentially enhance the consolidation of events surrounding the experience but the precise underlying mechanisms warrants further investigation (Lisman and Grace 2005; Duszkiewicz et al. 2019).

In Chapter 3, I further elaborate on the role of dopamine in aiding in decision making tasks and mediating reward related navigation. Further in chapter 5, using novelty as behavioral interventions, we try to mimic the modulation of the LC-HPC pathway and look at its influence on memory processing and offline consolidation.

Apart from dopamine, there are several other neuromodulators in the brain like Acetylcholine and noradrenaline which also act as crucial mediators in several functions including long lasting persistence of synaptic potentiation, attention, arousal, learning and memory (Bazzari and Parri 2019). In chapter 3, I go into more detail in explaining the roles of each of these neuromodulators and how do they potentially influence

offline consolidation processes. Lastly, when talking about neuromodulators, the endocannabinoid system has also been shown to play a crucial role in modulating synaptic transmission and regulating the balance between LTP and LTD.

The endocannabinoid system is a neuromodulatory system that plays a crucial role in development of the Central Nervous System (CNS), synaptic plasticity, immunity, appetite and several other functions. It is primarily mediated by CB1 and CB2 receptors, endogenous cannabinoids (endocannabinoids) and enzymes required for synthesis and degradation of the endocannabinoids (Lu and Mackie 2016). Both CB1 and CB2 receptors belong to the group of GPCRs and are known to exist primarily in central and peripheral neurons, and exert diverse effects on activation including changes in synaptic plasticity, cellular physiology etc. CB2 receptors are shown to be present mostly in microglial cells and in the peripheral nervous system and play an important role in regulating immune system and neurodevelopmental aspects. CB1 receptors on the other hand are abundantly expressed throughout the CNS, especially in regions including the hippocampus, amygdala, basal ganglia, striatum and cerebellum and thus can influence various cognitive processes including sleep-wake cycle, learning and memory and emotional and arousal states (Howlett et al. 2002). In this thesis, we focus primarily on effects exerted by the CB1 receptors.

Multiple lines of evidence have shown CB1 receptors to regulate neurotransmitter release and maintain a state of homeostasis in the nervous system. They can have inhibitory effects on the release of various excitatory and inhibitory neurotransmitters such as Glutamate, GABA, acetylcholine etc. (Pertwee and Ross 2002; Szabo and Schlicker 2005). The endocannabinoids have been shown to serve as retrograde synaptic messengers wherein their biosynthesis and release is triggered by increase in levels of intracellular calcium by other neurotransmitters. Subsequently these endocannabinoids bind to the presynaptic CB1 receptors and inhibit further release of neurotransmitters such as glutamate and GABA (Kreitzer 2005; Vaughan and Christie 2005).

The most prominent endocannabinoids identified are – arachidonoyl ethonalamide (anandamide), 2-2-arachidonoyl glycerol (2-AG). Soon after phytocannabinoids from the extracts of Cannabis Sativa were also extracted - Δ^9-Tetrahydrocannabinol (THC) and cannabidiol (CBD) (Mechoulam, Fride, and Di Marzo 1998; Devane et al. 1992). Subsequent experiments showed anandamide to behave like a partial cannabinoid receptor agonist with a greater affinity for CB1 receptors. On characterization, effects exerted by binding of THC to CB1 receptors had a partial overlap with anandamide with respect to serving as a receptor agonist but relatively weaker effects (Pertwee 2008;

Howlett et al. 2002). Cannabidiol (CBD) in contrast lacks most of the psychoactive effects exerted by THC and exhibits a much lower affinity for CB1 and CB2 receptors. Several lines of experiments showed CBD to act as an antagonist to CB1 receptor at low concentrations (Pertwee et al. 2002) and further was also showed to be a high potency antagonist of CB receptor agonists in the mouse brain (Thomas et al. 2007).

Over the past decades, several lines of work have been conducted in investigating the effects of endocannabinoids on memory and offline processing of memories. Administration of anandamide was shown to disrupt sharp wave ripples in the hippocampus, known to be crucial markers of memory consolidation (Sun et al. 2012). This disruption effect was however abolished by administration of CB1 receptor antagonist. On similar lines, injection of CB1 receptor agonists in hippocampal cultures also disrupted SWR events (Maier et al. 2012). These could potentially indicate that endogenous cannabinoids or CB1 receptor agonists have impairing effects on memory consolidation processes, however other studies indicate that the mechanisms underlying the effects might be more complicated. In chapter 4, we wanted to look deeper into these mechanisms by studying the effect of CBD, a CB1 receptor antagonist, on simple and semantic-like memories and offline consolidation processes like occurrence of SWR events and hippocampal – prefrontal cortex interactions. A study looking at the effect of injection of anandamide and CB1 receptor antagonist in dorsal CA1 in hippocampus of rats prior to or after training and test in Inhibitory Avoidance Task showed that – infusion of CB1 antagonist disrupts or facilitates memory consolidation depending on timing of infusion – encoding or consolidation (De Oliveira Alvares et al. 2008). Chronic administration of CBD on the other hand, seemed to ameliorate disease phenotype and improve memory and alleviates anxiety symptoms (Kreilaus et al. 2022). Looking further at its effects in rats suffering from Alzheimer's, indicated chronic CBD administration to have a potential therapeutic effect - – improves cognition, novel object recognition memory and other spatial memory tasks and reducing anxiety behaviors (Watt et al. 2020; Cheng et al. 2014). Based on these few studies, we hypothesized CBD, being an antagonist, to have a positive influence on memory performance and consolidation.

Aims of thesis

The overall aim of the thesis is to investigate the workings of different memory systems, how they interact with another as the memories evolve through each stage of encoding, consolidation and retrieval and how are these processes influenced by sleep. Further, we would like to gain a deeper understanding of how novelty

events might influence these consolidation processes and in the process also better characterize the role of other neuromodulators such as endocannabinoids in memory processing. We would like to study these processes using behavioral tasks like the watermaze and a novel paradigm that enables us to distinguish between simple and semantic-like memories in both mouse and rat models.

In **Chapter 2**, we adopt a translational approach using humans and rat models to look at how sleep might differentially influence different aspects of spatial memory processing, in particular, allocentric and egocentric learning and subsequent systems consolidation. In rats, we test this using the watermaze to train under allocentric and egocentric conditions followed by sleep or sleep deprivation and then look at the retrieval induced changes in expression of IEGs Arc and c-Fos. Further, for the human analog, we develop a virtual watermaze and then look at BOLD signal changes using fMRI during training and test with either a nap or wake in between. Finally, we compare behavior and neural findings from both experiments and draw conclusions on the mechanisms of sleep that are conserved across both species and the differences in each model.

In **Chapter 3**, we provide a comprehensive overview of the literature on how offline consolidation processes are influenced by the action of different neuromodulators. As mentioned in the sections above, SWR events have been shown to serve as crucial markers of memory consolidation. In this review, we first describe the different types of SWRs that are observed across different studies looking at memory consolidation and how are they influenced by neuromodulators such as dopamine, nor-adrenaline and acetylcholine, which are known to be modulated by sleep. Finally, we summarize key findings in the literature in these aspects and indicate potential underlying mechanisms of action in memory processing.

In **Chapter 4**, we look into the influence of endocannabinoids, another important neuromodulator, in sleep and memory processing. In particular, we look at the effect of Cannabidiol (CBD), a CB1 receptor antagonist on simple and semantic-like memories using the Object Space Task and then combine with freely moving electrophysiological recordings to look at LFP activity from the hippocampus and prefrontal cortex during the rest periods interleaved between trials and after training. Further on, we look into the sleep characteristics and focus on characterizing key oscillatory events, such as SWRs, delta oscillations and cortical spindles, known to occur during NREM sleep and summarize the key findings with respect to behavioral and neural findings and the differences observed with CBD administration.

In **Chapter 5**, we use mouse models to better characterize the stages of semantic learning using a modified version of the Object Space Task. Using the task, we first establish a semantic network of the task in mice and then test their memory performance when confronted with a single (interference) trial containing conflicting information. Does the semantic memory network still stay intact or does it get overwritten by the new information? To better understand the memory processing dynamics, we pair the interference trial with a distinct novelty experience in an attempt to enhance the release of neuromodulators and thus the consolidation of this particular trial. To look at the underlying neural changes at each stage of learning, we look at IEG expression changes, in particular Arc and c-Fos across different brain regions including the hippocampal regions and other cortical structures known to play a role in spatial memory processing during different behavioral timepoints of the task. To this end, we use slice immunohistochemistry to track changes in expression. Finally. we summarize findings from behavior and protein expression and shed light on potential mechanisms underlying memory consolidation.

Finally **Chapter 6** consists of a general discussion, wherein findings from previous chapters are integrated and discussed in the context in existing literature. We shed some light on how neuromodulators influence offline consolidation processes and some novel insights on nuanced differences in consolidation processes between simple and complex memories.

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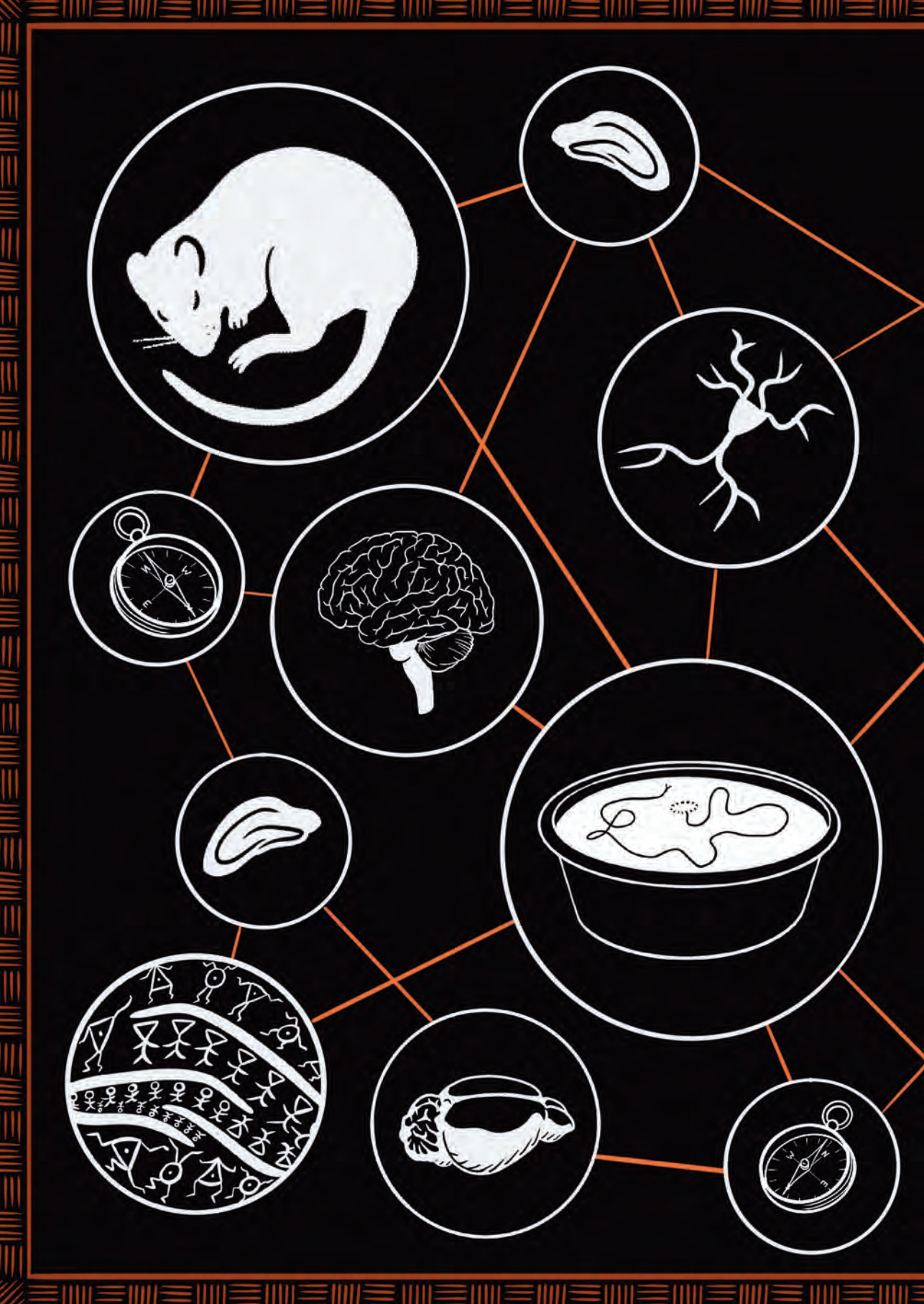

Chapter 2

Sleep leads to brain-wide neural changes independent of allocentric and egocentric spatial training in humans and rats

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A.S. performed the human experiments and wrote the first draft of the manuscript; L.v.R. helped with the analysis of the fMRI data; J.R. and J.J. performed the qPCR analysis in rodents; R.S. developed and adapted the human testing environment; and L.G. designed the project, supervised all experiments and analysis, and cowrote the manuscript. All authors contributed to the final revisions of the manuscript.

Abstract

Sleep is important for memory consolidation and systems consolidation in particular, which is thought to occur during sleep. Whilst there has been a significant amount of research regarding the effect of sleep on behaviour and certain mechanisms during sleep, evidence that sleep leads to consolidation across the system has been lacking until now. We investigated the role of sleep in the consolidation of spatial memory in both rats and humans using a watermaze task involving allocentric- and egocentric-based training. Analysis of immediate early gene expression in rodents, combined with functional MR imaging in humans, elucidated similar behavioural and neural effects in both species. Sleep had a beneficial effect on behaviour in both rat and humans. Interestingly, sleep led to changes across multiple brain regions at the time of retrieval in both species and in both training conditions. Thus, we provide cross-species evidence for system-level memory consolidation occurring during sleep.

Introduction

The ability to reliably navigate to known desired locations requires the integration of spatial information from different reference frames followed by consolidation of this information to build long-term spatial maps of the surrounding environment. It has been proposed that this consolidation occurs during sleep (Navarro-Lobato and Genzel 2019; Genzel et al. 2014; Girardeau and Zugaro 2011). Two navigation strategies are thought to be used to locate a target in space: a place learning strategy and a response learning strategy. Place learning, which relies on the development of a spatial cognitive map containing an internal representation of relationships between distal cues, is known to be dependent on the hippocampus (Kesner, Farnsworth, and DiMattia 1989; Eichenbaum, Stewart, and Morris 1990; Chersi and Burgess 2015). In contrast, response learning, which relies on the location of the navigator and may involve repeated use of relatively fixed motor movements to locate the target, is known to be dependent on the striatum (Packard and McGaugh 1996; Gasser et al. 2020b). During real world navigation, information from both reference frames are integrated to form a cohesive representation of the environment and the position of the navigator within (Andersen et al. 1997). However, one can experimentally bias the use of one strategy over the other by adopting specific training paradigms (de Bruin et al. 2001; Genzel 2020; Broadbent et al. 2020). Typically, this is done by including variable (allocentric training) versus stationary starting locations (egocentric training) within a maze, which will then bias in favour of place vs. response strategies, respectively. Using allocentric and egocentric training paradigms enable the investigation of specific initial memory circuits involving the hippocampus and striatum, and their associated consolidation processes.

Sleep is important for experiences to be consolidated to form long-term memories. It optimizes the consolidation of newly acquired information and has been proposed to reorganize brain circuits at both synaptic and systems levels (Navarro-Lobato and Genzel 2019; Genzel et al. 2014; Seibt and Frank 2019; Benington and Frank 2003). In particular, it has been proposed that memory consolidation processes associated with the hippocampus are dependent on sleep (Buzsáki 2015; Wilson and McNaughton 1994; Wang et al. 2024). At a systems level, the hippocampus is thought to be initially involved in memory encoding by binding different information stored in different cortical modules into a coherent trace; over time, connections between these cortical modules strengthen and the memory thus becomes hippocampal independent (Frankland and Bontempi 2005; Squire et al. 2015). One critical mechanism underlying this process is thought to be repeated memory reactivations during NonREM sleep, which then lead to progressive strengthening of the cortico-cortical connections, and

thus consolidation across the system (Navarro-Lobato and Genzel 2019; Girardeau and Zugaro 2011; Genzel et al. 2014; Maingret, Girardeau, Todorova, et al. 2016). These memory reactivations during sleep occur mainly during hippocampal sharp wave ripples (Girardeau and Zugaro 2011) and this may be the reason why the hippocampus plays a special role in sleep-related memory consolidation (Wilson and McNaughton 1994; Wang et al. 2024). Considering the position of the hippocampus as a crucial hub for spatial navigation and offline consolidation processes, it may thus be proposed that, in a spatial context, learning under allocentric training conditions would benefit more from sleep compared to learning under egocentric training conditions.

In this study, we adopted a translational approach to investigate differential effects of sleep on allocentric and egocentric memory representations in rats and humans. We used the watermaze (Morris 1981), which has been a well-established paradigm used to study different aspects of spatial navigation, especially contrasting allocentric and egocentric training (de Bruin et al. 2001; Ferguson, Livingstone-Lee, and Skelton 2019; Harvey et al. 2008). Further, a human analog of the maze has been developed and there is comparable performance in behaviour across both species (Schoenfeld et al. 2017; Müller et al. 2018b). Using the watermaze, we tested for the influence of sleep on allocentric and egocentric spatial memory training in both rats and humans and investigated the underlying neural signatures using immediate early gene expression analysis in rats, and functional MRI in humans. We hypothesized an improvement in memory performance after sleep, especially when trained under allocentric conditions, and that sleep would lead to a brain-wide consolidation process; we report a main effect of sleep. Interestingly, in both rats and humans, a sleep but not wake state led to brain-wide changes of neural activity at test after both allocentric and egocentric training.

Results

Memory performance in rats and humans

Both rats and humans were trained in the watermaze using a one-session paradigm to test spatial memory (Genzel et al. 2017). For rats, the task consisted of eight training trials followed 20 hours later with a (no-platform) probe trial to test for long-term memory performance. Rats were divided into two training groups – allocentric and egocentric – the main difference being that that the former started each trial from a different position while the latter always started from the same point in the maze (Fig. 1A). After training, these two groups were further divided into a sleep group (allowed to sleep in assigned sleep cages) and a sleep-deprived group (sleep deprived

in their home cages for six hours after training by gentle handling, Fig. 1B) (Genzel et al. 2017). To assess memory performance, time spent in the target zone in relation to total time during the test trial was calculated. Analogous to the rat paradigm, a virtual watermaze environment was used for humans (Schoenfeld et al. 2017). The environment setting consisted of two islands – cued and hidden – to enable subsequent functional MRI (fMRI) analysis. The cued island was a brown island with no distal landmarks and contained only a visible flag (cue) next to a treasure box (Fig. S1). The location of the flag was changed every trial and participants were instructed to scan the area to find the target. The hidden island was a green island surrounded by four landmarks and a hidden treasure box, which was the target location (analogous to the platform in the watermaze). The box was hidden in a fixed location in a small indentation on the virtual island surface such that it would only be visible to the participants when they were close to it. The overall task was run as a block design with eight alternating trials of cued and hidden island trials, resulting in a total of 16 trials (blocks for MRI analysis) that allowed us to isolate memory-specific effects excluding for general visual input and movement through the virtual world in the subsequent fMRI analysis. Each trial was self-paced and ended with the participant marking the target location. Participants could freely navigate through both islands with a joystick and their objective was to find the treasure box in each one and press a button on the joystick when they were in close proximity to the box. For the encounter with the hidden island, participants were randomly allotted to either of the training conditions, allocentric or egocentric, with either the same or changing starting position – and had to learn the location of the hidden box over trials (Fig. 1A). This training and later test sessions were conducted in the MRI scanner. After the session, participants were further grouped into the sleep (nap with polysomnography for up to two hours 83.17±3.3 min mean±SEM, range 33.5-113.5 min, for sleep stage analysis see Fig. S2) or wake group. During the wake period, participants watched a neutral, non-emotional movie with an experimenter in the same room to monitor sleep/wake status and was instructed to gently wake the participant if they fell asleep. Following the sleep/wake intervention, participants were taken back to the scanner and tested in the watermaze environment (Fig. 1B). In contrast to the rats, for which the probe trial consisted of a single trial, participants completed all eight trials again in each island to enable the correct contrast in the fMRI analysis. However, in this session, in each trial they were instructed to mark the location of the treasure box in the hidden island to the best of their knowledge without the box being present. In humans, memory performance was measured as latency to reach the location.

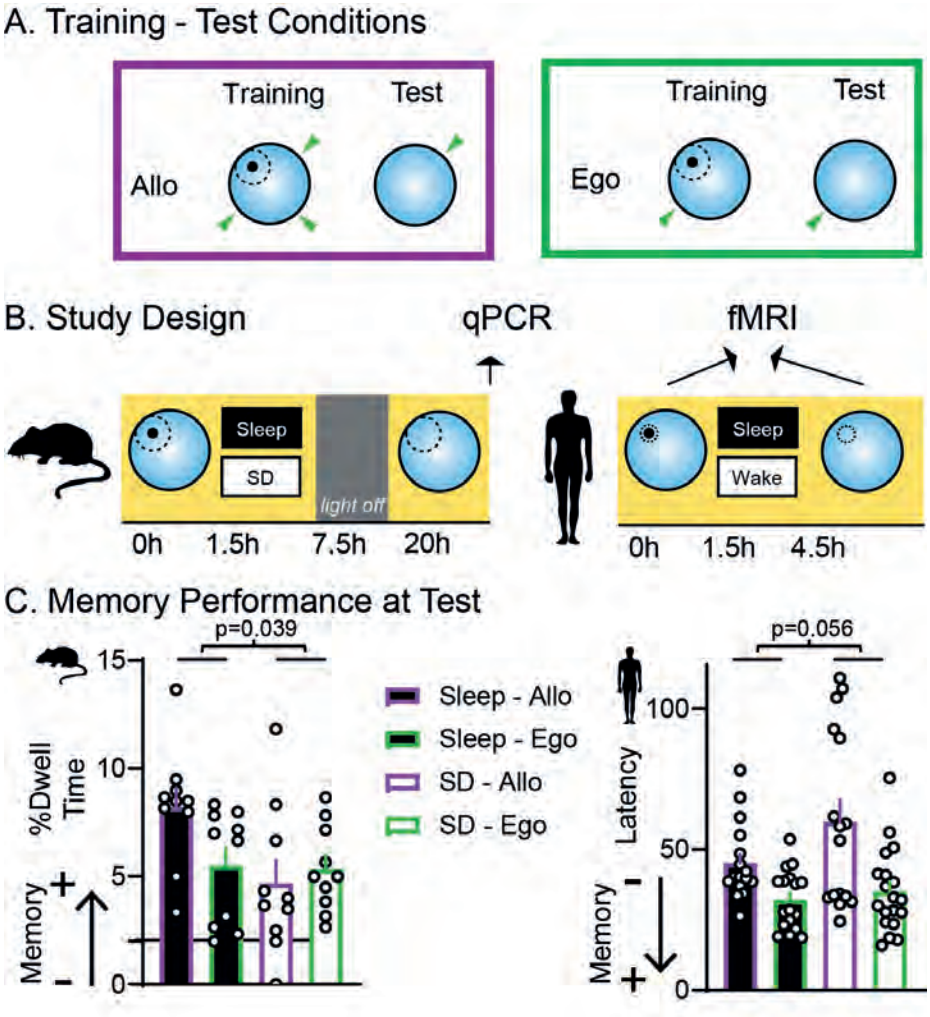

Figure 1. Study Design and Memory Performance. (A) The training-test conditions. The left panel shows the allocentric condition during which both rats and humans started from different locations on every training trial. In contrast, during the egocentric condition the starting location remained the same for every training trial (right panel). **(B)** Shows the study design for rats (left) and humans (right). Rats and humans were divided into two groups based on the training condition (allo/ego) and underwent a single day training protocol in the watermaze with eight training trials. After training, each group was subdivided into two additional groups: sleep (allowed to sleep individually in sleep cages with video monitoring) and sleep deprived (SD) group (sleep deprived for six hours after training by gentle handling in the home cage) in rats and sleep (a nap with polysomnography for up to two hours, for sleep stages see Fig. S2) and wake (watched a neutral movie for two hours) in humans. Overall, there were four groups each of rats and humans: sleep-allocentric (n=10), sleep-egocentric (n=10), SD-allocentric (n=10), SD-egocentric (n=10) in rats and sleep-allocentric (n=17), sleep-egocentric (n=16), wake-allocentric (n=16), wake-egocentric (n=19) in humans. Sleep/SD in rats ended when the light-on period switched to the light-off period. Rats from all groups were tested the next day at the onset of the light-on period with a probe trial in the watermaze (no platform present). 30 min after the test trial, rats were sacrificed and brain regions were collected

(prefrontal cortex, hippocampus and striatum) for qPCR analyses on retrieval-induced expression of different immediate early genes. Humans from all groups were tested after the sleep/wake session in the watermaze environment with fMRI. **(C)** Behavioural results of the rats (left) and humans (right). All rats performed above chance level (chance level was 2% based on zone area vs. pool area) and those animals that slept after training showed significantly better performance in dwell time (higher values indicate better memory) than awake animals, particularly for those trained under allocentric training conditions. Those trained under egocentric training conditions, showed similar performance levels for both sleep and sleep-deprived groups. Human participants, who took a nap between training and test, showed better memory performance compared to those that stayed awake (lower values indicate better memory). Those trained under egocentric conditions showed better memory performance compared to those trained under allocentric conditions. The purple bar contours used for the allocentric condition and the green contours for the egocentric condition. The black and white filled bars for both colors correspond to sleep and sleep deprived (SD) groups, respectively. Error bars are SEM.

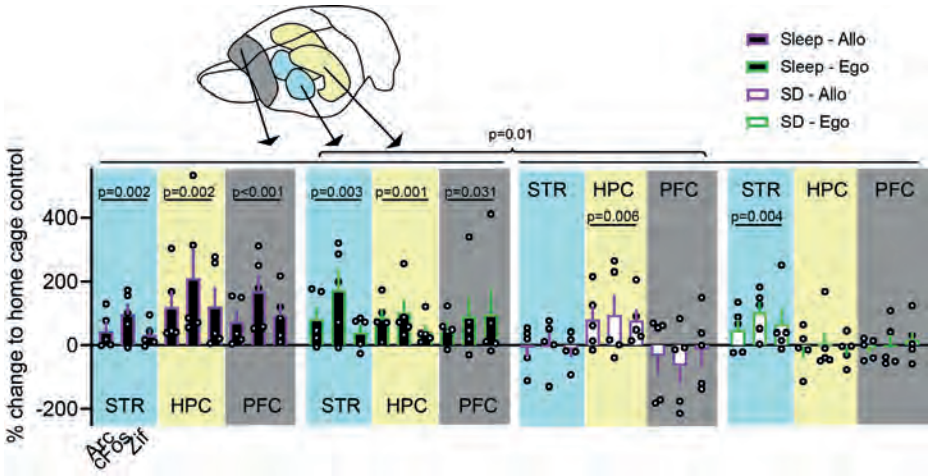

Figure 2. Memory Retrieval in Rats. Gene expression profile for immediate early genes *Arc, cFos* and *Zif268* (represented as % change in relation to home cage controls) across different brain regions for all groups of rats. Prefrontal cortex (PFC) in grey, striatum (STR) in blue, hippocampus (HPC) in yellow. There was an overall effect of sleep (p=0.01), with sleep groups showing higher gene expression levels. Further, one sample t-test to 0 showed that the sleep groups had higher gene expression in all three brain areas than the home cage. The p-values for the t-test corresponding to each brain region are displayed in the panel above their respective graphs. Under sleep deprivation, the allocentric group had higher gene expression only in the hippocampus and the egocentric group had higher only in the striatum in comparison to home cage (non-significant p>0.22, significant effects shown above, data for each brain area). Purple bar contours are used for the allocentric condition and the green contours for the egocentric condition. The black and white filled bars for both colors correspond to sleep and sleep deprived (SD) group, respectively. Error bars are SEM.

At test, rats performed above chance across both sleep and sleep deprived groups for both allocentric and egocentric training conditions (Fig 1C, left panel). However, there was a general effect of sleep and an interaction of sleep and training condition on performance (univariate ANOVA sleep/sleep deprivation $F_{1,39} = 4.6$, $p = 0.039$, allo/ego $F_{1,39} = 1.4$, $p = 0.244$, interaction $F_{1,39} = 3.8$, $p = 0.058$). Human participants were generally better in the egocentric condition than in the allocentric condition, and as in rats, there was a general effect of sleep on performance (univariate ANOVA allo/ego $F_{1,69} = 17.2$, $p < 0.001$, sleep/wake $F_{1,69} = 3.8$, $p = 0.056$, interaction $F_{1,69} = 1.6$, $p = 0.2$). In rats, the latency to reach the platform position at test showed a similar pattern to the dwell time analysis and human latency results, however, this was not statistically significant (Fig. S3, univariate ANOVA all $p>0.2$). In summary, there was an effect of sleep on behaviour in both rats and humans.

Retrieval-induced IEG expression analysis in rats

After establishing the behavioural effect of sleep, we next tested the neural effects of sleep. For this, in rodents, we measured the retrieval-induced expression of immediate early genes. More specifically, we measure expression of *Arc, cFos* and *Zif268* in the prefrontal cortex, striatum and hippocampus. Immediate early genes expression can be used as an index for neuronal activation (Fleischmann et al. 2003; Jones et al. 2001; Korb and Finkbeiner 2011; Genzel et al. 2017). In a full model including gene and brain area as within-subject factors, and sleep and training type as between-subject factors, there was a significant effect of sleep and gene and an interaction between training type (allo/ego) and brain area and an interaction between gene and brain area (repeated measure ANOVA sleep $F_{1,16} = 8.5$, $p = 0.01$; training type X brain area $F_{2,33} = 4.1$, $p = 0.026$; gene $F_{2,32} = 4.7$, $p = 0.016$; gene X sleep $F_{2,32} = 3.1$, $p = 0.061$; gene X brain area $F_{2.6,42.3} = 3.7$, $p = 0.023$; other $F<1.9$ $p>0.13$; Fig. 2). An interesting pattern emerged in which sleep led to an increase in gene expression in all brain areas (prefrontal cortex, hippocampus and striatum) upon retrieval, whereas after sleep deprivation, there was only increased gene expression in the hippocampus in the allocentric group and in the striatum for the egocentric group (one-sample t-test to 0 indicated change to home cage, all significant $p<0.032$ see Fig.2, all non-significant $p>0.22$). Thus, brain-wide activation for task-solving was seen only after sleep, and was independent of allocentric or egocentric training conditions. In contrast, if animals were sleep deprived after training, task-solving was associated only with those brain areas known to be necessary for each training type: striatum for egocentric and hippocampus for allocentric.

Effect of sleep on brain activity in humans

Next, to assess the neural correlates of sleep on spatial memory under allocentric and egocentric training in humans, we analysed the MRI BOLD images acquired during the training and test session. In both sessions, participants completed eight training trials to fixed treasure in the hidden island under either allocentric or egocentric conditions as well as eight trials to navigate to a visible flag in the cued island, where the position of the flag changed from one trial to the next. Thus, the first-level contrast was between the hidden and cued islands to enable isolation of memory encoding and retrieval specific effects while controlling for general task properties, such as joystick movement and visual input. Only the first 30 seconds of each trial were included in the analysis, to control for the fact that each trial length was different due to the self-pacing of the trial, and that there was a difference in average latency at test over the groups (mean 42.5 s; range 15.8-110.6 s). However, when the whole-trial periods were included, the general pattern of results were unaffected (see Fig. S5-6). Only when participants slept between training and test were significant changes seen in BOLD activity, with increased activity in the medial and lateral frontal cortices, anterior and posterior parietal cortices, the visual cortex, cerebellum and a decreased activity in the medial prefrontal cortex, precuneus and hippocampus (Fig. 3 shows the contrast between training and test for sleep both training groups, for sleep allocentric and sleep egocentric separately see Fig. S4, 6). All results were collected at uncorrected $p<0.005$ and then corrected at the cluster level to control for multiple comparisons with $p<0.05$ FWE (GLM full-factorial model with within-subject factor training-test and between-subject factor allo-ego and sleep-wake). It is noticeable that brain areas that had an increase due to sleep belong to the executive control network, which is related to goal-directed behaviour (Gruber and Goschke 2004) and spatial memory (Maguire et al. 1998). In contrast, brain areas that had a decrease belong to the default mode network, which is associated with spatial memory (Spiers and Maguire 2007; Doeller, Barry, and Burgess 2010; Navarro-Lobato and Genzel 2020; Brodt et al. 2016; Cowan et al. 2020).

The same contrasts for the wake subjects were empty (both for each training group separately as well as for the combined wake group). However, when signal change at the peak voxel for each cluster was extracted for each group separately, similar changes to those in the sleep groups were observed for the wake egocentric group, even although they were not significant in a whole-brain analysis (also not observed when uncorrected $p<0.005$). Additional contrasts were run to determine further differences between allocentric and egocentric changes across wake. Following allocentric training (but not egocentric), the same brain areas that had higher activation between training and test during sleep were also more active at test

between participants who slept and those that did not (allo sleep > allo wake at test, Fig. S7). Furthermore, there was no significant interaction of sleep versus wake and training to test changes when including both egocentric and allocentric or if only egocentric participants were included. However, this same interaction (sleep/wake and training/test) was significant if only allocentric participants were included, with areas belonging to the executive control network showing increases in the sleep but not wake group (allo sleep training < allo sleep test and allo wake training > allo wake test, Fig. S8).

In summary, we observed a change in the whole brain that was independent of training after sleep, which was not present after being kept awake. More specifically, we observed a shift in brain activation after sleep with higher activity in regions belonging the executive control network and lower activity in regions belonging to the default mode network, including the hippocampus. Similar changes were observed in the egocentric wake group, but these were much weaker and were not statistically significant. Further, these changes were absent in the allocentric wake group.

Functional Connectivity changes in humans

The brain areas that had significant changes in activity are known to be part of brain networks; areas that had increased activity belong to the executive control network and areas that had decreased activity are associated with the default mode network. Thus, we next investigated functional connectivity changes during task execution by conducting a Psychophysiological Interaction (PPI) analysis. Led by the activity analysis, we focused on the medial frontal cortex as region of interest (ROI -2 -10 48). This ROI is a key hub of the executive control network, related to goal-directed behaviour (Spreng et al. 2010). As with the activity analysis, we only included the first 30 seconds of each trial and all results were collected at uncorrected $p<0.005$ and then corrected at the cluster level to control for multiple comparisons with $p<0.05$ FWE (GLM full-factorial model with within-subject factor training-test and between-subject factors allo-ego and sleep-wake). At test, in contrast to training, the medial frontal cortex was functionally less connected to areas known to be part of in the default mode network but again, only for those participants that slept and not those that stayed awake (inferior parietal cortex, precuneus, prefrontal cortex, hippocampus, Fig. 4). Furthermore, for both sleep and wake groups, the cerebellum was decoupled from the medial frontal cortex (for wake contrast see Fig. S9). As with the activity analysis, we extracted the change in functional connectivity for the peak voxel in each cluster for each group separately.

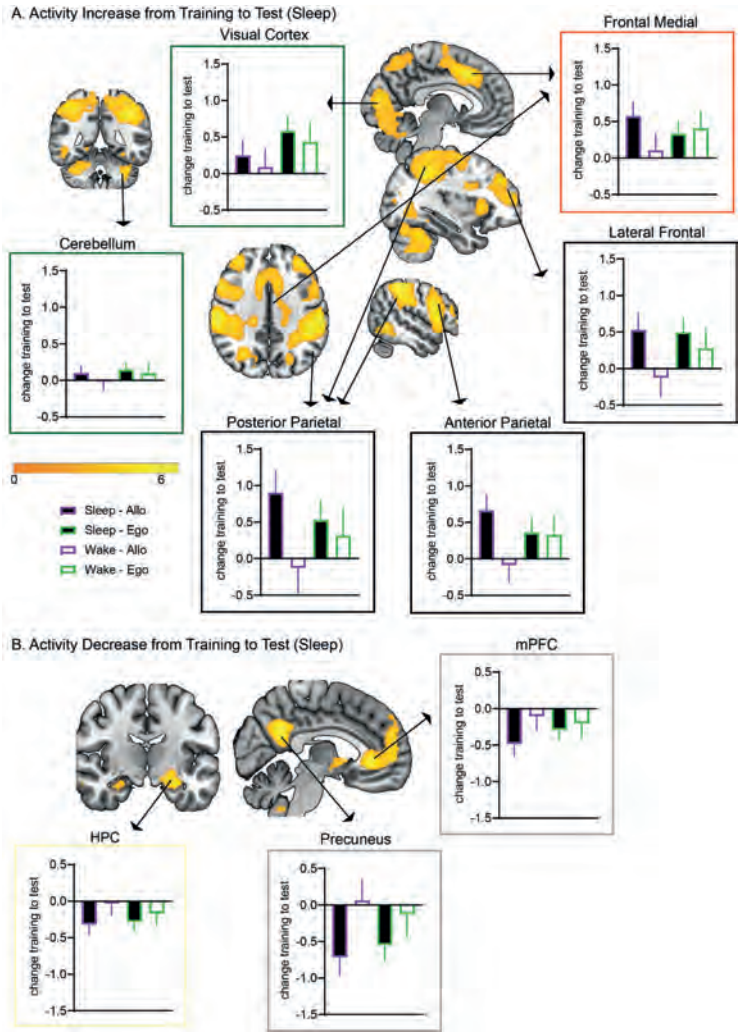

Figure 3. fMRI Activity Analysis in Humans. (A) Brain maps showing changes in activity from both sleep groups (test more so than training, p<0.05 FWE-cluster corrected). Bar graphs are extracted for each subgroup for the peak voxel of each cluster. An increase in activity was observed in the medial and lateral frontal cortex, anterior and posterior parietal cortex. Increases were also observed in a part of the visual cortex and cerebellum. This activity increase was similar across both training conditions over sleep (maps for each group see Fig. S4, 5, 7, 8). In contrast, after wake the whole-brain analysis maps were empty. However, the peak voxel activity extraction indicates that egocentric wake did show similar changes to sleep, even if the smaller change and larger variability in this group precluded statistical significance on the whole-brain level. **(B)** Brain maps show the changes from both sleep groups (test less than training, p<0.05 FWE-cluster corrected) and bar graphs are extracted for each subgroup for the peak voxel of each cluster. A decrease in activity was observed in the medial prefrontal cortex (mPFC), precuneus and hippocampus (HPC). The purple bar contours used for the allocentric condition and the green contours for the egocentric condition. The black and white filled bars for both colors correspond to sleep and sleep deprived (SD) group, respectively. Error bars are 95% confidence interval. For detailed statics on each cluster, see Tables S2-3.

The decreased functional connectivity, and thus decoupling, between the main hub of the executive network and default mode network parallels the finding in the activity analysis where increased activity was observed in the former and decreased activity was observed in the latter network, in the sleep but not wake group.

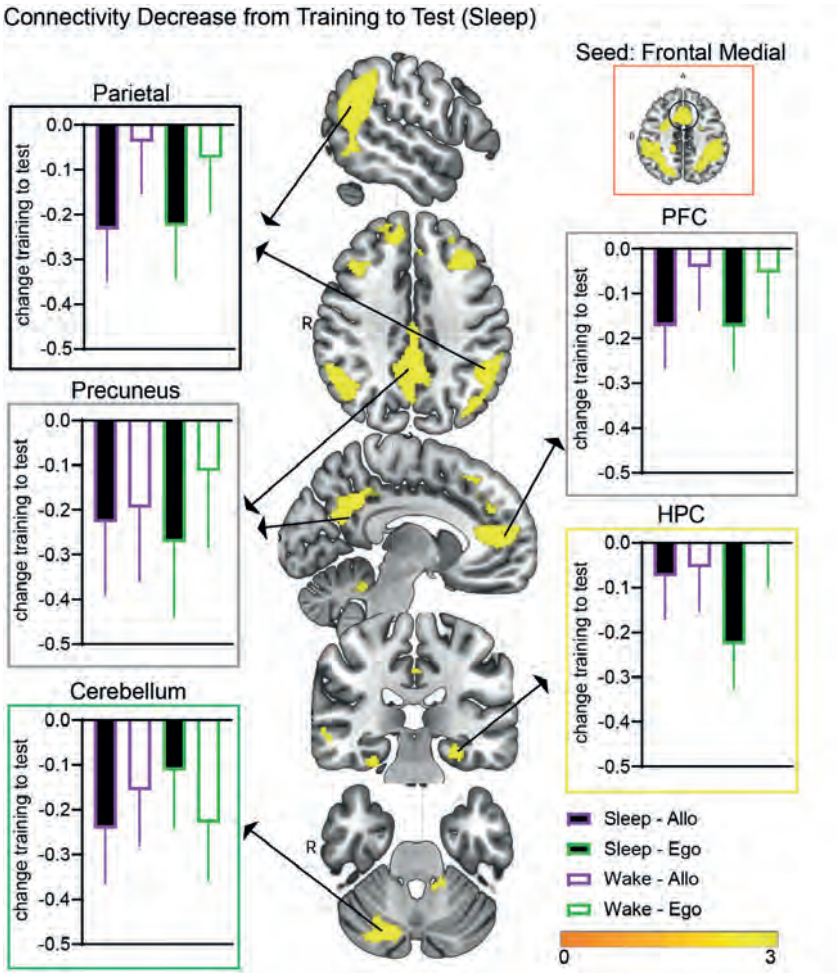

Figure 4. fMRI Connectivity Analysis (PPI) in Humans with Medial Frontal ROI. Brain maps showing changes from both sleep groups (test less than training, $p<0.05$ FWE-cluster corrected). Bar graphs are extracted for each subgroup for the peak voxel of each cluster. There was a significant decrease in connectivity in the frontal medial cortex with the prefrontal cortex (PFC), hippocampus (HPC), the precuneus and the inferior parietal cortex from training to test (see Table S4). These regions are known to be part of the default mode network. Additionally, there was also a significant decrease in functional connectivity with the cerebellum for both sleep and wake groups (wake contrast in Fig. S11). Purple bar contours are used for the allocentric condition and the green contours for the egocentric condition. Black and white filled bars for both colors correspond to the sleep and sleep deprived (SD) groups, respectively. Error bars are 95% confidence interval.

Discussion

In this study, we investigated the effects of sleep and no sleep on allocentric and egocentric memory representations in rats and humans using the watermaze. Overall, memory was intact – the subject/participant knew where the goal was – independent of training condition or if subject/participant slept or stayed awake between training and test. However, in both rats and humans, sleep led to better memory performance compared to sleep deprivation (of note, in humans p=0.056). This effect of sleep was numerically larger in the allocentric training group, however, the interaction between sleep and condition was only marginally significant in rats (p=0.058) and not significant in humans.

To investigate effects on brain activity, two different methods were used in the different species: in rats, we measured retrieval-induced immediate early gene expression in the hippocampus, striatum and prefrontal cortex in comparison to home cage controls. In contrast, in humans, we compared the MRI BOLD signal at training and test. These analyses showed, in rats and humans, a change in activity across multiple brain regions that was observed after sleep for both allocentric and egocentric training conditions, which was weakly present in the wake group.

More specifically, after sleep in rats there was an increase in retrieval-induced gene expression in all three tested brain areas (hippocampus, striatum, prefrontal cortex), for both allocentric and egocentric training conditions. However, there was a trend of increased gene expression in the hippocampus and prefrontal cortex for the allocentric condition, whereas in the egocentric condition, the striatum showed the highest expression.

In contrast, when rats were sleep deprived after training, task-solving was associated with increased gene expression only in those brain areas known to be necessary for each training type: the striatum for egocentric and the hippocampus for allocentric strategies.

In humans, fMRI analyses only showed statistically significant changes between training and test in participants that slept and not those who stayed awake. Significant increases were observed in activation in areas associated with the executive control network (such as the superior posterior parietal cortices and frontal medial cortex), and significant decreases in areas associated with the default mode network (hippocampus, medial prefrontal cortex, precuneus). Extracting activity changes for the peak voxel in each cluster for each group revealed that there was

similar changes in the egocentric wake condition as in both sleep groups; however, these changes were neither visible nor significant in the whole-brain analysis (also uncorrected $p<0.005$). Furthermore, only in the allocentric training groups was there a significant difference between wake and sleep at test in addition to a significant interaction between wake/sleep and training/test. Functional connectivity analyses during the task with PPI revealed a functional decoupling of the frontal medial cortex – the key hub of the executive control network – with areas of the default mode network.

Thus, across both species we observed brain-wide changes following sleep, for both allocentric and egocentric training conditions. In contrast, being awake led to more pronounced differential effects across training conditions.

Differences between species

With these results, it is important to consider the inherent, unavoidable between-species differences that are present, firstly in behaviour and secondly, in the source of the neural activation measure and baseline contrast.

With regard to behaviour, species will naturally differ in their awareness of the principle that they were being tested. Humans were aware that the testing session would not have a treasure reward at the goal and they needed to mark where they expected the goal to be. In contrast, rats would simply be searching for a place to rest within the pool. After not finding the platform in the correct location during the test, they would naturally search the rest of the pool in addition to repeatedly returning to the former platform location. To facilitate analysis, the tests themselves also differed between species. In humans, we ran eight test trials to allow for fMRI analysis. In rats, we only had one test trial. In rats, repeated test trials would have led to extinction and therefore, less goal-searching behaviour. This was not an issue in humans since they were aware of the nature of the test and therefore, did not expect the goal to be visible.

The method of measuring neural activity also differed between species. In rats, we measured retrieval-induced changes in gene expression of immediate early genes in the hippocampus, striatum and prefrontal cortex in contrast to home cage controls. Immediate early genes are expressed more in those cells that are especially active at a given moment and can thus be used to test for activity related to memory retrieval (Genzel et al. 2017). The rat data then highlights which brain areas were more active during memory retrieval in comparison to behaviours in home cage controls and therefore, these areas are also generally associated with task-solving. We chose a

neutral wake control condition (home cage), because possible alternative control conditions such as swimming in the watermaze without a platform can result in alterations in IEG expression in association with stress or with incidental learning about the environment through exploration (Shires and Aggleton 2008; Ons, Marti, and Armario 2004; Guzowski et al. 1999), and these confounding factors can hinder interpretation of results (Barry, Coogan, and Commins 2016). For humans, we measured BOLD responses both during encoding and retrieval phases of task and the results focus on relative changes in regions active during each of the sessions. However, the prime measure is relative since we first create contrasts of regions active during hidden versus cued island and then on the second level changes from first session to the second one. Further, whilst the BOLD signal is also known to measure brain activity, it is based on blood oxygenation changes and is thus a more indirect measure than the gene expression used in rats. The main difference in the results between species would be that for rats, the expression levels are compared to those in home cages whereas for humans, the comparison is memory specific (cued vs. hidden island) and between sessions (within subject).

It is thought that the role of sleep in systems consolidation processes and underlying mechanisms are fairly conserved across both rodent and human even although they have not been directly compared until now. With regard to the watermaze, several human analogue virtual maze environments have been developed to study mechanisms underlying spatial navigation and, more recently, to better understand the role of allocentric and egocentric learning strategies (Schoenfeld, Foreman, and Leplow 2014; Schoenfeld et al. 2017; Rodriguez 2010; Müller et al. 2018a). With these limitations in mind, our discussion here will focus on a rough comparison of differences observed across training types and sleep groups and focus less on direct between-species comparisons.

Sleep and egocentric vs. allocentric training

Sleep is crucial for offline consolidation processes and strengthening memories. The proposed key underlying mechanism is neural reactivation wherein neural activity present during encoding reemerges during NonREM sleep (Girardeau and Zugaro 2011; Genzel et al. 2014). These reactivation events have been shown to occur mainly during hippocampal high frequency burst oscillations, referred to as sharp wave ripples (Girardeau and Zugaro 2011; Dupret et al. 2010). Furthermore, these offline consolidation processes are thought to involve a dialog between the hippocampus and cortex via hippocampal sharp wave ripples in combination with neocortical slow oscillations and sleep spindles, which should stabilize labile memory traces in the cortex leading to systems consolidation (Squire et al. 2015; Maingret, Girardeau,

Todorova, et al. 2016; Navarro-Lobato and Genzel 2019; Girardeau and Zugaro 2011; Genzel 2020; Schabus et al. 2007; Schreiner, Lehmann, and Rasch 2015). Considering the crucial role of the hippocampus in spatial navigation and allocentric learning (O'Keefe and Nadel 1978), it has thus been proposed that memories encoded with allocentric training, known to depend on the hippocampus, would benefit more from sleep (Wilson and McNaughton 1994; Guzowski et al. 2001). In contrast, egocentric learning is dependent on the striatum and thus consolidation of these memories should be less dependent on sleep (Packard and McGaugh 1996; Genzel 2020). Several studies do provide evidence for this dissociation (Albouy et al. 2015; Viczko et al. 2018; Hagewoud et al. 2010). Hagewoud and colleagues (2010) reported that depriving rodents of sleep after learning a plusmaze led to a shift from a preferred place learning strategy, preferred by animals that were allowed to sleep, to a response learning; this was accompanied by a respective shift from hippocampal to striatal levels of retrieval-induced pCREB. In humans, allocentric vs. egocentric strategies have only been investigated using a motor-sequence task, the finger tapping sequence task, which was designed in a way that one could test both allocentric and egocentric memory representations of a learned motor sequence (Albouy et al. 2015; Viczko et al. 2018). After training on a sequential finger tapping sequence, participants were tested on their ability to recall motor or spatial representations of the sequence with the same hand, but with the hand bottom up and keypad turned upside down. The egocentric or motor representation corresponded to the internal features and tested for movement-based learning (e.g. left little to index finger tapping transition would remain left little to index finger tapping transition). The allocentric or spatial representation corresponded to the global features and tested for spatial-based learning (e.g. left little to index finger tapping transition would change to left index to little finger tapping transition, so same spatial sequence requiring subjects to produce different sequence of finger movement). These studies showed an improvement in performance for the allocentric strategy following sleep, in contrast to the egocentric memory expression, which was maintained after sleep deprivation (Albouy et al. 2015; Viczko et al. 2018).

Whilst similar effects were numerically visible in the behaviour in our study where allocentric training groups had larger differences between sleep and wake, statistical analysis did not confirm this dissociation between sleep and training strategy. In both rats and humans there was a main effect of sleep ($p=0.056$ in humans). In rats, the interaction between sleep and training type did show a marginal significant effect ($p=0.058$), but in humans this interaction was not significant.

Interestingly, the neural effects did not confirm this dissociation either. In both rats and humans, a brain-wide change was seen across sleep but not wake, which was the same in both training conditions. Thus, whilst the prediction from the theory was that only hippocampal dependent memories would benefit from sleep, perhaps the different memory systems may be more interrelated than previously thought, especially during sleep. The ventral striatum has been proposed to integrate inputs from the hippocampus, prefrontal cortex and related subcortical structures to construct outcome predictions and stimulate goal-directed behaviour (Pennartz et al. 2011; Pezzulo et al. 2014). Further, while memory reactivations are most known for hippocampal systems, Pennartz and colleagues have shown memory reactivations in the striatum, which were in close temporal association with hippocampal ripples (Pennartz et al. 2011; Lansink et al. 2009; Pennartz et al. 2004). Other recent studies also indicate that the interaction between the place and response learning memory systems is a lot more complex than the notion of having a hippocampus-independent response memory and striatum-independent place learning system (Ferbinteanu 2020; Gasser et al. 2020a). Therefore, perhaps it is less surprising that we see brain-wide neural changes after sleep in both allocentric and egocentric training conditions. These findings fit well with the proposed role of sleep for systems consolidation and thus, perhaps consolidation of memories is independent of learning strategy and this allows flexibility and adaptability for future use (Navarro-Lobato and Genzel 2019; Girardeau and Zugaro 2011; Genzel 2020; Maingret, Girardeau, Todorova, et al. 2016). One point to be considered here is that, in theory, rats and humans could use the allocentric strategy to solve the maze even in the egocentric training condition since the goal was always at the same location with respect to the cues. However, if this did occur, then the differences we see in the wake groups would not be explained.

Immediate early gene expression results in rodents

We know from rodent research that during learning, neuronal populations in different brain regions are recruited, leading to a learning-specific upregulation of plasticity markers such as immediate early genes (IEG) in these regions. One can use the same markers at retrieval to measure which brain areas are involved in this process (Genzel et al. 2017; Fleischmann et al. 2003; Jones et al. 2001; Korb and Finkbeiner 2011). Here, we found an increase of retrieval-induced expression of IEGs in rats in the prefrontal cortex in addition to an increase in the hippocampus and striatum for both training conditions following sleep. These findings fit well with the proposed role of prefrontal cortex in offline consolidation processes, during which salient information across multiple episodes is thought to be abstracted to build semantic memory networks (Frankland and Bontempi 2005; Navarro-Lobato and Genzel 2019; Maingret, Girardeau, Toderova, et al. 2016). Additionally, we also observed an

increase in expression in the hippocampus and striatum in both training conditions for the sleep groups. This is in line with several previous reports (Feldman, Shapiro, and Nalbantoglu 2010; Guzowski et al. 2001), which have shown increased levels of IEG expression in the hippocampus following successful memory performance in the watermaze. Guzowski and colleagues (2001) mapped IEG expression in rats trained in either an allocentric or egocentric condition and found similar expression profiles in the hippocampus for both conditions. There is also evidence supporting the potential use of both strategies to some extent for navigating efficiently in the maze (Harvey et al. 2008).

For the sleep deprived groups, we only saw a localized IEG increase in the hippocampus and striatum when trained under allocentric and egocentric conditions, respectively. These results fit well with the original finding that place learning is known to be dependent on the hippocampus (Kesner, Farnsworth, and DiMattia 1989). In contrast, response learning is known to be dependent on the striatum (Packard and McGaugh 1996). Therefore, after sleep deprivation, brain areas that are necessary for each training type should still show activation even if no additional recruitment of other brain areas can take place, such as after sleep. These results also fit well with Hagewoud and colleague's (2010) study, in which sleep deprivation led to a shift from place learning to a response learning strategy, which was accompanied by a shift from hippocampal to striatal levels of retrieval-induced pCREB.

Lastly, regarding the results above, it must be noted here that we extracted the entire hippocampal tissue to test for gene expression. However, it is well known that different subregions of the hippocampus, primarily the dorsal and ventral hippocampus, contribute to different functions. For example, the dorsal region is involved in spatial memory processes (Moser et al. 1995; O'Keefe 1976) and the ventral region is more involved in stress regulation and emotional memory processes (Kjelstrup et al. 2002). It could thus be speculated that our gene expression findings in the hippocampus would be more pronounced if we focused on only the dorsal part of the hippocampus. Across multiple studies, the role of dorsal hippocampus in spatial navigation and map-based learning has been extensively investigated, so one may expect a stronger effect of sleep on gene expression if only this specific subregion was analysed.

fMRI results in humans: executive control network

Our fMRI results show a dynamic interaction between areas of the executive control network and the default mode network over sleep across both training conditions. We observed an increase in activity in areas belonging to the executive control network

and a decrease over sleep in areas belonging to the default mode network, which includes the hippocampus.

The executive control network is active during attention-demanding visuo-spatial tasks, goal-directed behaviours and navigation and the parietal cortex is particularly implicated in playing a critical role in sensorimotor integration and activities of higher cognitive function (Gilmore, Nelson, and McDermott 2015). The posterior parietal cortex is of particular interest with respect to spatial navigation tasks and calculating route-centric information with respect to a target location. Using information from different sensory inputs, it produces an egocentric frame of the local environment where the target is located, and provides appropriate motor coordinates required for making directed movements (Andersen et al. 1997; Spreng et al. 2010). Multiple studies in primate and rat models have also indicated a role of the posterior parietal cortex in encoding route progression during navigation under both allocentric and egocentric conditions and adapting to external environments and maintaining an internal cognitive map of self-position in space (McNaughton et al. 1994; Driscoll et al. 2017). This is also the case when tests are in virtual environments (Harvey, Coen, and Tank 2012). The posterior parietal cortex serves as a cortical integration site for hippocampally-generated allocentric spatial information and egocentric spatial orientation to permit goal-directed navigation (Calton and Taube 2009; Nitz 2012; Whitlock et al. 2008b; Khodagholy, Gelinas, and Buzsáki 2017). Furthermore, memory reactivations during sleep have been observed in the parietal cortex in addition to the prefrontal cortex (Wilber et al. 2017; Peyrache et al. 2009) and both brain areas show high frequency oscillations during NonREM sleep cooccurring with hippocampal ripples (Khodagholy, Gelinas, and Buzsáki 2017). Consistent with these findings, we observed an increase in activation in the posterior parietal cortices across both training conditions after sleep.

fMRI results in humans: default mode network

In regards to the changes observed in areas of the default mode network, much evidence has pointed to the importance of the hippocampus and medial temporal lobe structures including the prefrontal cortex and precuneus (also known as the retrosplenial cortex (van Heukelum et al. 2020)) in spatial navigation in both human and rodent models (Maguire et al. 1998; Peigneux et al. 2004; Epstein 2008; Whitlock et al. 2008a). Furthermore, the default mode network is functionally modulated by sleep, with persistent functional connectivity during light sleep, and sleep spindles in particular, and a gradual decoupling with deep sleep (Horovitz et al. 2009; Larson-Prior et al. 2009; Andrade et al. 2011; Spoormaker et al. 2010; Schabus et al. 2007). Thus, this network could potentially play a role in offline consolidation processes

coordinating the systems-wide consolidation process during sleep (Spreng et al. 2013; Genzel 2020; Brodt et al. 2016; Cowan et al. 2020; Navarro-Lobato and Genzel 2020). Recent evidence in rodents show the co-occurrence of cortical high-frequency oscillations in default mode network areas including the posterior parietal cortex with hippocampal ripples, during NonREM sleep (Khodagholy, Gelinas, and Buzsáki 2017). This may be the mechanism by which memories are consolidated from the initial hippocampal storage to downstream areas, such as the posterior parietal cortex, via cortical default mode network areas (Genzel 2020). This may also be the mechanism underlying our findings, where we see a shift in activity with a decrease in activity in regions belonging to the default mode network, which is perhaps an intermediate storage, and an increase in the goal-directed network, including the parietal cortex, over sleep.

Conclusion

In summary, across both species and training conditions, we observed brain-wide changes at the time of retrieval following sleep, which were not present after sleep deprivation. This fits to the main effect we found of sleep on behaviour. Thus, we provide cross-species evidence for the proposed function of sleep for brain-wide consolidation of memories proposed by Marr (1970).

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Materials and Methods

Subjects

Rats

Adult male Lister-hooded rats (Charles River, United Kingdom), aged 8-10 weeks with an average weight of 250-300 g at the start of experiments, were used. Rats were housed in groups of four per cage on a delayed day-night cycle (10 A.M. – 10 P.M. light on) and had free access to food and water at all times. After arrival, rats were habituated to the housing environment for at least a week and then handled across 3 days for at least 5 minutes each day before watermaze habituation. A total of 45 rats were used from which 25 were used for qPCR experiments. All experimental procedures were in accordance with national (Animals [Scientific Procedures] Act, 1986) and international (European Communities Council Directive of 24 November 1986[86/609/EEC]) legislation governing the maintenance of laboratory animals and their use in scientific experiments. The minimal number of rats for the necessary statistical power was used, with random assignment to groups, and minimal suffering was ensured for all experimental procedures. The experiments were approved by the UK home office under project license 60/4566 and by the University of Edinburgh Division of the National Veterinary Service Experimental Request Forms.

Humans

Seventy-seven neurologically healthy, right-handed male participants (age range = 18-30 years, mean = 24) were recruited for the study. Because male rats were used, only male humans were chosen for this study. Participants were recruited through the Radboud Research Participation System. All provided written informed consent prior to the start of the experiment and were paid for their participation. This study was approved by the local ethics committee (CMO Arnhem-Nijmegen, Radboud University Medical Center) under the general ethics approval ("Imaging Human Cognition", CMO 2014/288), and the experiment was conducted in compliance with these guidelines. Exclusion criteria for the participants were (i) taking sleep medications, (ii) taking regular naps and (iii) being involved in professional gaming activities. Participants were screened for these criteria before the start of the experiment. Additionally, alertness levels and sleep quality were assessed using the Stanford Sleepiness Scale and the Pittsburgh Sleep Quality Index, respectively, during the experiment session. Group values for these measures are tabulated in Table S1. Eight subjects were excluded from the experiment due to technical issues during training or test: For five participants, the joystick was incorrectly calibrated, and for the remaining three, there were technical problems including abrupt crashing of the task environment program during the scan.

Watermaze Task - Rats

For both habituation and training, procedures were adopted from previous work (Genzel et al. 2017). Prior to the start of the main experimental session, rats were first habituated to a visual-cue version of the watermaze (diameter = 2 m) for three days, with four trials per day. The task was to find the submerged platform in the water maze, indicated by a visual cue placed on top of the platform (diameter = 12 cm), while curtains surrounding the pool hid any extramaze cues. On reaching the platform, rats had to wait on the platform for 30 seconds before being picked up for the next trial. After habituation, rats were familiar with the procedure before training on the task began.

A one-session training design was adopted for this experiment as used previously (Genzel et al. 2017). The session consisted of eight training trials followed 20 hours later by a probe trial. Rats were divided into two training groups – allocentric and egocentric. In the allocentric group, rats were placed in the watermaze from a different start quadrant on each trial and they had to reorient themselves to locate the platform. In the egocentric group, rats were placed in the watermaze from same start location each trial. For all trials, rats were introduced to the maze facing the watermaze wall. The goal location always remained the same with respect to the distal cues for each rat; only the start location differed depending on the allocentric/egocentric condition. Platform locations and start positions were counterbalanced across animals. In both training conditions, if a rat did not reach the platform by 120 seconds, they were guided to the location. Distal extra maze cues were present to help rats orient themselves in the watermaze. After the session, rats were randomly allocated to one of the two sleep conditions by either allowing them to sleep in allotted sleep cages or sleep deprivation by gentle handling in their home cages for 6 hours after training. Gentle handling included handling the rats occasionally, gently tapping on the cage or removing the cover as soon as the animal started showing signs of tiredness (Genzel et al. 2017). Memory performance was tested with a single probe trial 20 hours later, where rats were placed in the watermaze for 60 seconds with no platform present before being picked up from their current location. Swim paths were tracked using automated software (Watermaze, Watermaze Software, Edinburgh, UK (Spooner et al. 1994)). After sleep deprivation and before the test, rats were returned to their home cages and could potentially have slept. This was done to ensure that any behavioural or molecular effects seen were not confounded by fatigue or tiredness effects due to sleep deprivation, or by direct effects of sleep deprivation on immediate early gene expression. Further, previous experiments have shown that sleep within 6 hours after watermaze training is more important for memory consolidation than subsequent sleep, which cannot compensate for earlier

sleep (Genzel et al. 2017; Smith and Rose 1997). For the probe trial analysis, time spent in the zone around the platform location was divided by the probe trial time (60 s), meaning that, with a zone radius of 14 cm, chance level of rat spending time in this zone was 2% with zone area/pool area. Experiments were timed such that sleep/sleep deprivation ended at lights off (thus the transition to the active period) and the test was conducted at lights on. This way, each rat had 12 hours to recover from the intervention before being tested, but sleep rebound was minimized by using the active period. After the test trial, rats were sacrificed and brain regions (prefrontal cortex, striatum and hippocampus) were extracted for qPCR analysis. In total, 45 rats were used: 20 trained under allocentric and 20 under egocentric conditions. Ten rats from each condition were allocated to either sleep or sleep deprivation, resulting in n=10 per subgroup. In addition, 5 rats from each subgroup were randomly selected as representatives for qPCR analyses. Further, there were 5 home-cage rats that did not undergo behavioural training and were used as home-cage controls in the qPCR analysis.

RT qPCR Analysis - Rats

Analysis of qPCR was based on our previous work (Genzel et al. 2017). Briefly, rats were sacrificed 30 minutes after the probe test. The home cage controls were also sacrificed at the same time. We chose a neutral wake control condition (home cage), because possible alternative control conditions such as swimming in the watermaze without a platform can lead to alterations in IEG expression in association with stress or with incidental learning about the environment through exploration (Shires and Aggleton 2008; Ons, Marti, and Armario 2004; Guzowski et al. 1999), and these confounding factors can hinder interpretation of results (Barry, Coogan, and Commins 2016). Furthermore, for present purposes, the critical results are the comparisons between training and sleep groups. Immediately after brain extraction, the bilateral medial prefrontal cortex, striatum and hippocampi were dissected and flash frozen with liquid nitrogen and stored at -80 °C for later processing. We focused on these brain regions since we previously showed that the hippocampus and prefrontal cortex were involved in sleep-related memory consolidation in this task (Genzel et al. 2017) and they are known to be involved in allocentric training paradigms (Kesner, Farnsworth, and DiMattia 1989). We added the striatum because egocentric training is known to depend on the brain area (Packard and McGaugh 1996). Briefly, samples were homogenized and RNA was obtained via phenol-chloroform extraction according to the manufacturer's instructions. Next, cDNA was synthesized in vitro with use of random hexamers. Subsequently, a RT-qPCR and comparative Ct quantitation was performed in experimental duplicates for *cFos, Arc, Zif268*, and 18S ribosomal RNA as the internal control on a StepOnePlus (Applied Biosystems, Carlsbad, United

States) PCR machine. Plates were counterbalanced and amplification thresholds set manually (StepOne Software Version 2.3, Life Technologies). The amplified product size was verified using gel electrophoresis and amplification checked for primer-dimer formation and nonefficient DNase treatment. Data was normalized to the internal control 18S (also known as Rn18s, coding for ribosomal RNA47), and subsequently "fold change" and then "percentage change" to home cage control or other control was calculated. Percentage (%) change was used for statistical analysis and graphical presentation because fold change cannot be used for statistics and percentage change gives a more intuitive sense of effect sizes.

Study Design - Humans

The entire experimental session lasted for a maximum of 6 hours and was split into three sub-sessions: (1), an fMRI session in which participants were trained on the task, (2) a 2.5-3 hour interval involving either taking a nap with EEG or watching a neutral movie, followed by (3), a second fMRI session in which participants were tested. An EEG was performed to confirm that each participant slept. The session started at noon with the participants filling in screening questionnaires and rating their alertness levels on the Stanford Sleepiness Scale. After having fulfilled all inclusion criteria, participants started the first fMRI session. This began with a T1-weighted anatomical scan, followed by a Resting State scan where participants were asked to fixate on a cross projected on a screen. Next, they performed 8 blocks of the training sets (allocentric/egocentric groups both alternating hidden/cued blocks). The duration of this scan varied across participants and ended when they successfully completed all blocks. Following the task, the Resting State scan was repeated and then the fMRI session ended. At the end of the first fMRI session, participants were randomly allocated to either of the two conditions – wake (watched a neutral movie for 2 hours with an experimenter present to monitor that the participant stayed awake throughout) or sleep (a 1.5-2-hour nap with Polysomnography). At the end of the movie/nap, participants were asked to rate their awareness levels again on the Stanford Sleepiness Scale and then they started the second fMRI session. As with the first round, this session started with a Resting State Scan followed by 8 blocks of the test sets (allocentric/ egocentric both alternating hidden/cued blocks). The duration of this scan also varied across participants and ended when they successfully completed all blocks. Following the task, the Resting State scan was repeated and then the second fMRI session ended. This marked the end of the full experimental session. Participants from the sleep condition were asked to come another day for a second session where they had to take a short nap with Polysomnography (data not shown here).

Virtual Watermaze (VWM) Task - Humans

Humans were also trained in a watermaze environment, analogous to the rat task, to test spatial abilities. For this purpose, we employed a virtual watermaze (Schoenfeld, Foreman, and Leplow 2014; Schoenfeld et al. 2017), which consisted of a virtual island surrounded by four landmarks (distal cues) - a bridge, a sailboat, a wind turbine and a lighthouse (see Fig. S1). There was a hidden treasure box on the island, which was marked as the target location (equivalent to the platform in the water maze). The box was hidden in a fixed location in a small indentation on the virtual island surface such that it was only visible to the participants when they were close to it. This island is henceforth referred to as the "hidden island". The setup also consisted of another island that did not have any distal cues for orientation except for a visible colorful flag (cue) next to a treasure box, which was visible from a distance. The position of this flag changed each trial. This island is henceforth referred to as the "cued island". The overall task design was a block design with 8 alternating blocks of cued and hidden island resulting in a total of 16 trials. Each trial was self-paced and ended with the participant marking the target location. There was a 15 second interval between the end of one trial and the start of the next, during which the subject could turn around in the maze and orient themselves. The participants were allowed to freely navigate both islands with a joystick and their objective was to find the treasure box in each one and press a button on the joystick when they were in close proximity to the box. They would first encounter the cued island and had to find the visible flag. This island was used as a control to control for motor and visual input as well as isolate memory effects in the fMRI analysis. For the encounter with the hidden island, the participants were randomly allotted to either of the training conditions – allocentric or egocentric. The participants were not aware of these two possible conditions. In the allocentric group, they would have a different start location every trial and would have to reorient themselves each time to find the target location, thereby promoting place navigation. In the egocentric group, they would have a fixed start location every trial, hence could rely on a repeated fixed movement to get to the target location in addition to using the visible cues. The main objective of the participants in both conditions was to learn the fixed location of the target box across all the trials. Finishing all 16 trials would mark the successful completion of the training set. For the test set, the island setup remained the same with one modification – the treasure box was removed from the hidden island and the participants were instructed to mark the location to the best of their knowledge, where they recalled the box to be located.

Polysomnographic Recordings

For the nap condition in the afternoon, polysomnographic recordings were obtained with a 250-Hz sampling frequency, a 0.3-Hz high-pass filter, and a 35-Hz low-pass

filter (BrainAmp, Brain Products, Gilching, Germany). Thirty-two scalp electrodes were prepared including Fz, F3, F4, Cz, C3, C4, Pz, P3, P4, Oz, O1, O2 electrode sites and referenced to the left mastoid. Additionally, horizontal and vertical eye movements (EOG), electromyogram (EMG) on the chin and electrocardiogram (ECG) were recorded. Sleep scoring was performed by an experimenter blind to the conditions, based on EOG, EMG and the following channels – F3, F4, C3, C4, O1, O2 using 30 second epochs. Visual scoring of the recordings were conducted following the current, widely used American Academy of Sleep Medicine scoring rules (AASM) (Berry et al. 2012) with requirements including slow waves to occupy at least 20% of a 30 second epoch in order to be classified as Stage 3. All scoring was performed using the SpiSOP tool (https://www.spisop.org; RRID: SCR_015673).

Statistical analysis

Repeated measure ANOVAs were run in SPSS Statistics 25 (IBM, USA) for immediate early gene expression and multivariate analysis for the sleep analysis. Univariate ANOVAs were run for the behavioural analysis. For immediate early gene analysis, within-subject factors were brain area and gene. For sleep analysis, the different sleep stages were included as different variables in the multivariate analysis. For all analyses, between-subject factors were training (allocentric/egocentric) and sleep/wake. If sphericity was not given, Greenhouse-Geisser was used. Tests were calculated with an alpha of 0.05, but for each result exact p-values are reported. Results with p=0.056 and 0.058 were still viewed as significant due to a priori hypothesis and to avoid a type II error.

fMRI acquisition

For fMRI, functional images were acquired using ascending slice acquisition with a T2*-weighted gradient-echo multiband echo-planar imaging sequence (Prisma 3T,Siemens,Erlangen, Germany; 66 axial slices; volume repetition time (TR), 1000 ms; echo time (TE), 34 ms; 60° flip angle; slice thickness, 2 mm; field of view (FOV) 210 mm; voxel size 2x2x2 mm). Anatomical images were acquired using a T1-weighted MP-RAGE sequence (192 sagittal slices; volume TR, 2300 ms; TE, 3.03 ms; 8° flip angle, slice thickness, 1 mm; FOV, 256 mm; voxel size 1x1x1 mm).

fMRI data processing

Image preprocessing and statistical analysis was performed using SPM8 software (www.fil.ion.ucl.ac.uk/spm; Wellcome Trust Centre for Neuroimaging, London, United Kingdom). All functional contrast images went through the standard preprocessing steps. Images were realigned, slice-time corrected, spatially normalized and transformed into a common space, as defined by the SPM2 Montreal Neurological

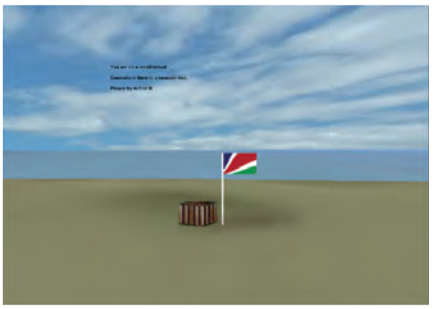
Institute (MNI) T1 template. The preprocessed datasets were then analysed using the general linear model and statistical parametric mapping (Friston et al. 1994). The first five volumes from every dataset after preprocessing were discarded to remove non-steady state effects. For statistical analyses, relevant contrast parameter images were generated for each participant and then subjected to a second-level GLM full factorial analysis with nonsphericity correction for correlated repeated measures. For the first-level analyses, individual contrast images for each participant were produced by comparing task-dependent activation (hidden > cued) for each session separately, with six movement parameters as regressors of no interest. Since the time taken to complete the task was not uniform across participants, we performed the first level analysis on the activity in the first 30 seconds of every trial. Analyses were also done for the activity corresponding to the entire task length and is shown in the supplementary section. For the second-level analyses, these contrast images were included in a full factorial model with between subject factors – Training (allo/ego) and Condition (sleep/wake) and within subject factor – session (training/test). In the whole-brain search, all results were collected at $p<0.005$ uncorrected and then corrected at the cluster level to control for multiple comparisons ($p<0.05$ FWE-cluster). To further elaborate, cluster level correction is one of the methods used to control for multiple comparisons (Woo, Krishnan, and Wager 2014). It takes advantage of the fact that the individual voxels in the dataset are not independent of each other, instead spatially adjacent voxels are likely to be functionally linked. So, instead of testing each voxel individually, we tested clusters of voxels for significance using a cluster defining threshold of $p < 0.05$.

For the functional connectivity analyses between training and test sessions, we chose the medial frontal cortex (coordinates 2 10 48) as the region of interest to capture network activity. The coordinates of the region were taken from the activity analysis where we observed an increase in activity in this region after sleep. This region has been shown to be a part of the executive control network and related to goal-directed navigation (Spreng et al. 2010). A psycho-physiological interaction (PPI) analysis was performed. In general, with this analysis method, we were interested in investigating task specific changes in connectivity between different brain regions with respect to a seed region of interest during the behavioural task (O'Reilly et al. 2012). In short, this method identifies regions in the brain that show the same modulation of the BOLD signal during the task as the seed regions and therefore these regions are assumed to be functionally connected. The psychological variable consisted of the activity of task blocks (hidden island) in the first 30 seconds of each training block convoluted to the hemodynamic response. The physiological factor was the time course of a spheroid volume of interest (VOI) located in the medial frontal cortex (2, 10, 48) with a 6-mm radius. The VOI time course was extracted for each individual and

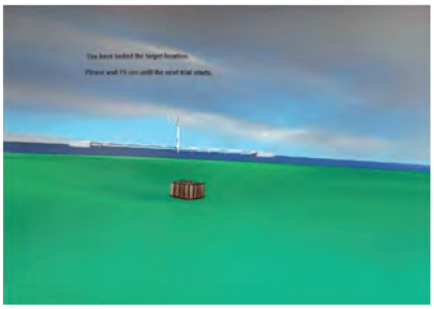
adjusted for head movement. With the PPI toolbox (SPM8) the interaction value (PPI) of both factors was calculated. The PPI, VOI time course and task timing was then included in a general linear model with the 6 movement parameters as regressors of no interest. For each participant, individual contrast images with the PPI activation were calculated. These contrast images were then included in a full factorial design model with the same factors as used for activity analysis. In the whole brain search, all results were collected at $p<0.005$ and then corrected at the cluster level to control for multiple comparisons ($p<0.05$ FWE-cluster). All data and analysis scripts will be available on the Donders Repository.

Supplementary Material

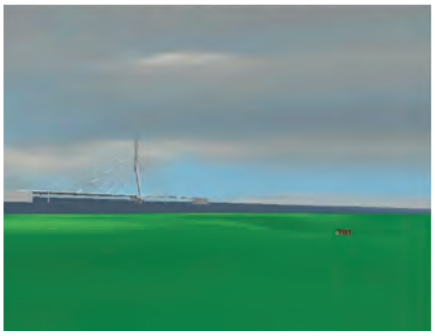
A. Cued Island



B1. Hidden island



B2. Hidden island (far away from the target location)



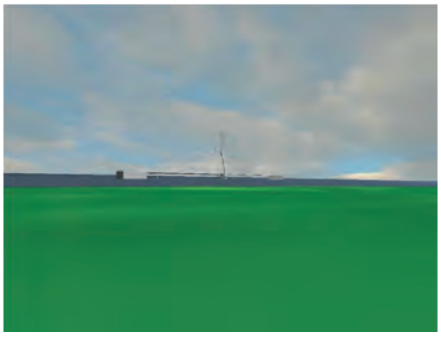


Fig. S1. Virtual Watermaze. (A) The left panel displays a representation of the cued island. A plain brown island with no surrounding distal cues. The flag in picture is visible to the navigator from a distance and keeps changing it position each trial. (B) The right panel displays the target quadrant of the hidden island. A green island surrounded by four landmarks (distal cues) like the bridge as shown in the picture. The location of the box is fixed on this island and is located on an indentation in the virtual surface and only visible from close distance. The panel below displays the target quadrant as it looks from a distance. The box is only visible when the subject is close to the target location.

Table S1. Demographic Data. Table S1 presents the background information of the human participants and their level of arousal before the first and second MRI sessions respectively. The last column shows the significance values as obtained after running an Independent-Samples Kruskal-Wallis Test (since both PSQI and SSS are ordinal measure) for both PSQI and SSS values across 4 groups. No statistical differences could be seen for the PSQI values across all groups. The arousal levels before the first MRI session (SSS-1) also showed no statistical differences across all groups. A significant difference could be seen for SSS-2 values between the sleep and wake groups, with arousal levels higher for the subjects in the sleep group. However, in both cases the average was between level 2 and 3.

	Sleep-Allocentric M SEM	**Sleep-Egocentric M SEM**	**Wake-Allocentric M SEM**	**Wake-Egocentric M SEM**	**Significance Value**
Age (Years)	23.06 0.74	21.90.56	24.8 0.81	23.31 0.75	
PSQI	4.53 0.19	4.00 0.6	3.8 0.15	4.68 0.21	$H_{(3)} = 2.2$ $P = 0.530$
SSS-1	2.08 0.18	2.00 0.68	2.46 0.23	2.150.19	$H_{(3)} = 2.74$ $P = 0.433$
SSS-2	2.170.19	2.11 0.6	2.56 0.15	2.89 0.21	$H_{(3)} = 9.25$ $P = 0.026$

PSQI – Pittsburgh Sleep Quality Index; SSS – Stanford Sleepiness Scale

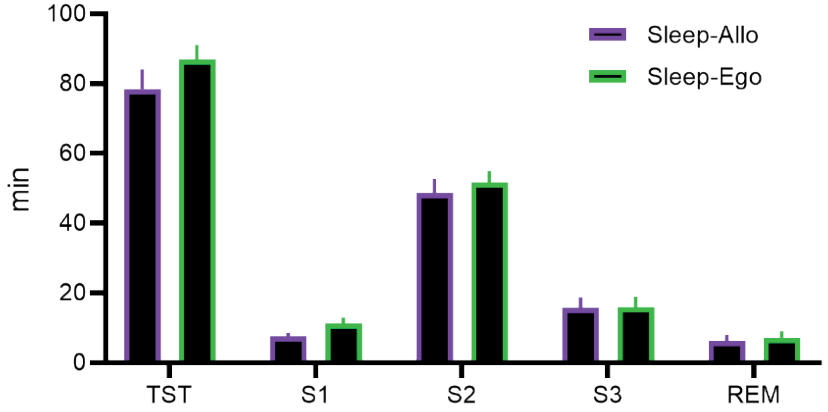


Fig. S2. Human Sleep Stage Results. Shown is the amount of sleep during the nap for each training group. There was no significant difference between the two groups (multivariate analysis Allo vs Ego $F_{5,30}$=0.9 p=0.52). Error bars indicate SEM values.

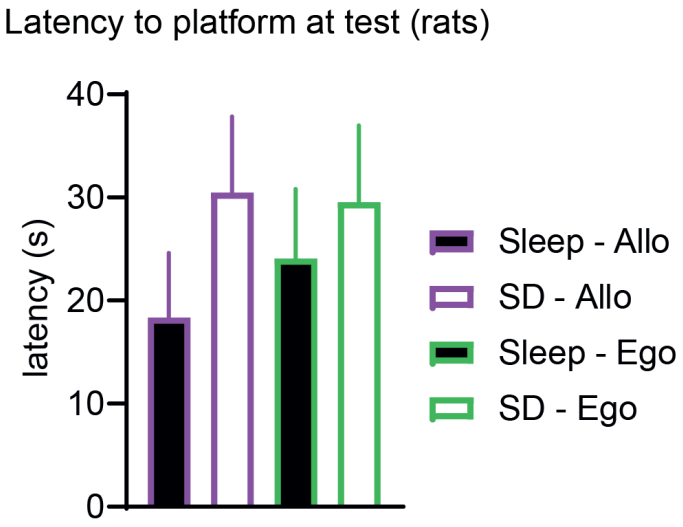


Fig. S3. Latency to reach platform at test for rats. The latencies showed a similar pattern as dwell times in rats and latencies in humans, however it was not significant (all p > 0.2). Error bars indicate SEM values.

Table S2. Activity Analysis. Increase in activity from training to test (sleep), $p_{FWE\text{-}cluster}$<0.05, see Figure 3A

Regions	$p_{FWE\text{-}corr}$	Cluster size k	Peak Voxel T Z	MNI coordinates x y z
Training > Test				
Posterior Parietal Cortex and Visual Cortex	0.000	47173	6.70 6.20	-60 -28 42
Medial Cortex Frontal	0.003	1932	5.59 5.28	44 48 12

L – left; R – right; FWE – Family wise Error

Table S3. Activity Analysis. Decrease in activity from training to test (sleep), $p_{FWE\text{-}cluster}$<0.05, see Figure 3B

Regions	$p_{FWE\text{-}corr}$	Cluster size k	Peak Voxel T Z	MNI coordinates x y z
Training < Test				
Precuneus	0.013	1432	6.30 5.88	-2 -54 30
Medial Prefrontal Cortex	0.001	2526	5.75 5.42	2 34 -2
Hippocampus L	0.049	1019	5.29 5.02	-30 -16 -20

L – left; FWE – Family wise Error

Table S4. PPI Analysis. Connectivity decrease from Training to test (sleep), $p_{FWE\text{-}cluster}$<0.05, see Figure 4

Regions	$p_{FWE\text{-}corr}$	Cluster size k	Peak Voxel T Z	MNI coordinates x y z
Training > Test				
Posterior Parietal Cortex L	0.000	4948	4.86 4.65	-56 -50 30
Posterior Parietal Cortex R	0.000	4617	4.82 4.61	42 -64 46
Anterior Parietal Cortex R	0.038	934	4.19 4.05	36 20 38
Medial Prefrontal Cortex	0.000	4163	4.17 4.03	12 42 14
Cerebellum	0.043	901	3.80 3.69	38 -52 -46
Precuneus	0.000	2428	3.75 3.65	6 -62 34

L – left; R – right; FWE – Family wise Error

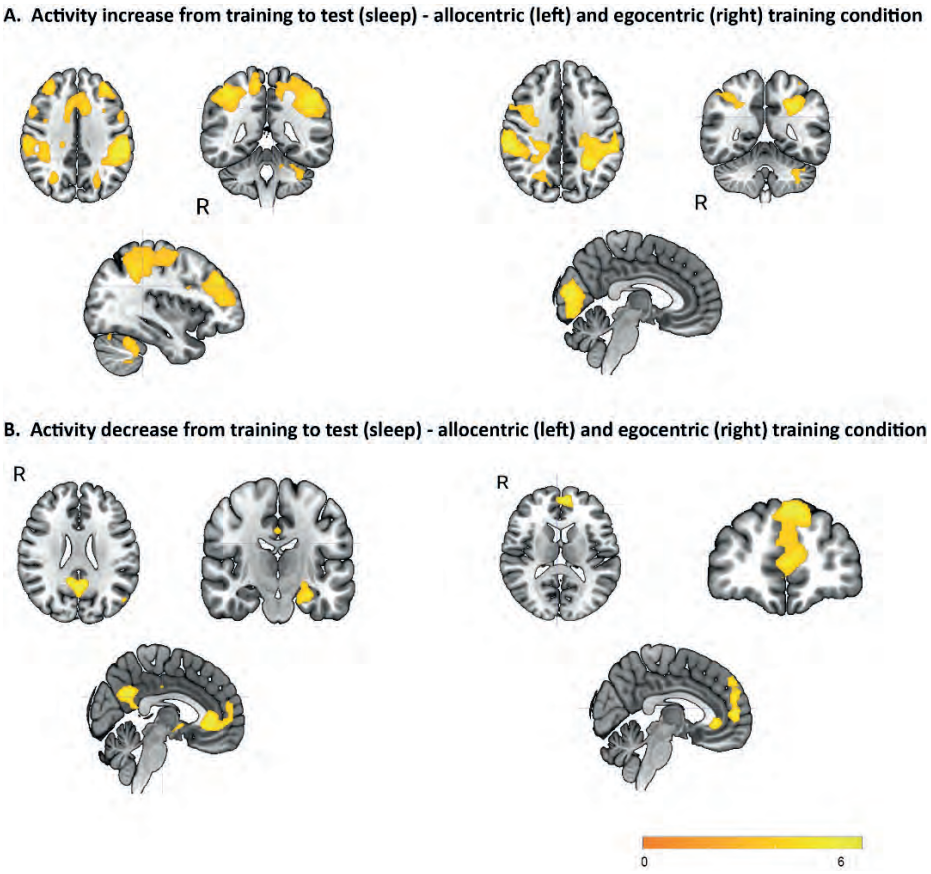


Fig. S4. fMRI activity analysis in first 30 seconds of each training and test trial – Allocentric and Egocentric. Brain maps show the changes from the sleep group consisting of participants who were trained under allocentric condition (left) and egocentric condition (right). (A) Regions with increased activation from training to test (test > training, $p<0.05$ FWE-cluster corrected). An increase in activity was observed in the medial and lateral frontal cortex, posterior parietal cortex for the allocentric condition and anterior and posterior parietal cortex, part of the visual cortex and cerebellum for the egocentric condition. (B) Regions with decreased activation from training to test (test < training, $p<0.05$ FWE-cluster corrected). A decrease in activity was observed in the medial prefrontal cortex, precuneus and hippocampus for the allocentric condition and in the medial prefrontal cortex and precuneus for the egocentric condition. All results were collected at uncorrected $p < 0.005$ and then corrected on the cluster level to control for multiple comparisons with $p<0.05$ FWE.

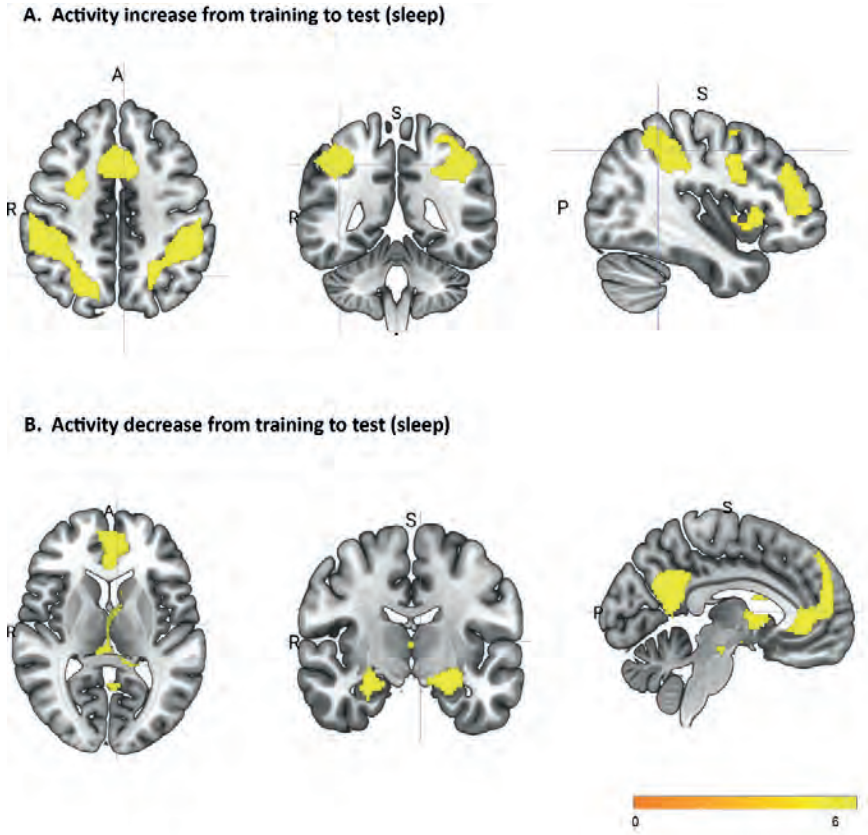


Fig. S5. fMRI activity analysis for the entire length of each training and test trial. Brain maps show the changes from the sleep group consisting of participants from both training conditions. (A) Regions with increased activation from training to test (test > training, p<0.05 FWE-cluster corrected). An increase in activity was observed in the medial and lateral frontal cortex, anterior and posterior parietal cortex. (B) Regions with decreased activation from training to test (test < training, p<0.05 FWE-cluster corrected). A decrease in activity was observed in the medial prefrontal cortex, precuneus and hippocampus. All results were collected at uncorrected p < 0.005 and then corrected on the cluster level to control for multiple comparisons with p<0.05 FWE.

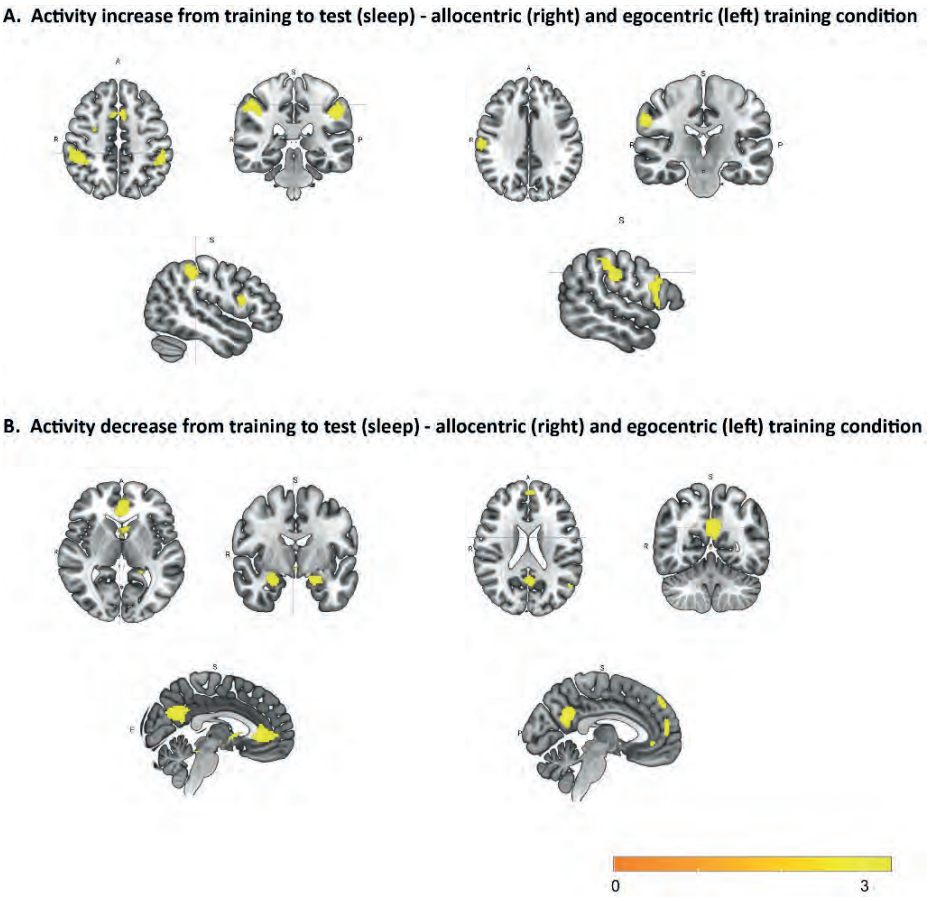


Fig. S6. fMRI activity analysis for the entire length of each training and test trial – Allocentric and Egocentric. Brain maps show the changes from the sleep group consisting of participants who were trained under allocentric condition. (A) Regions with increased activation from training to test (test > training, $p<0.05$ FWE-cluster corrected). An increase in activity was observed in the medial frontal cortex, posterior parietal cortex for the allocentric condition and in the anterior and posterior parietal cortex for the egocentric condition. (B) Regions with decreased activation from training to test (test < training, $p<0.05$ FWE-cluster corrected). A decrease in activity was observed in the medial prefrontal cortex, precuneus and hippocampus for the allocentric condition and in the medial prefrontal cortex and precuneus for the egocentric condition. All results were collected at uncorrected $p < 0.005$ and then corrected on the cluster level to control for multiple comparisons with $p<0.05$ FWE.

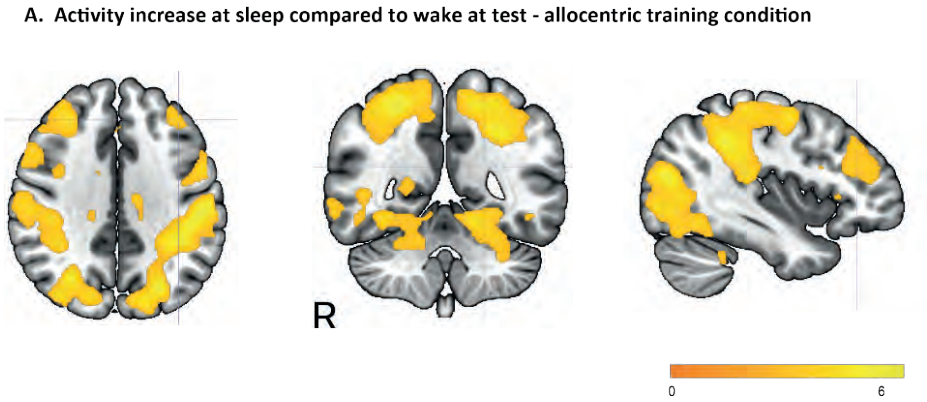


Fig. S7. fMRI activity analysis for sleep vs wake groups at test trial – allocentric. Brain maps show changes in brain activity between subjects in sleep and wake groups for the test trial when trained under allocentric condition (sleep > wake, $p<0.05$ FWE-cluster corrected). An increase was observed in the medial and lateral frontal cortex, posterior parietal cortex. All results were collected at uncorrected $p < 0.005$ and then corrected on the cluster level to control for multiple comparisons with $p < 0.05$ FWE.

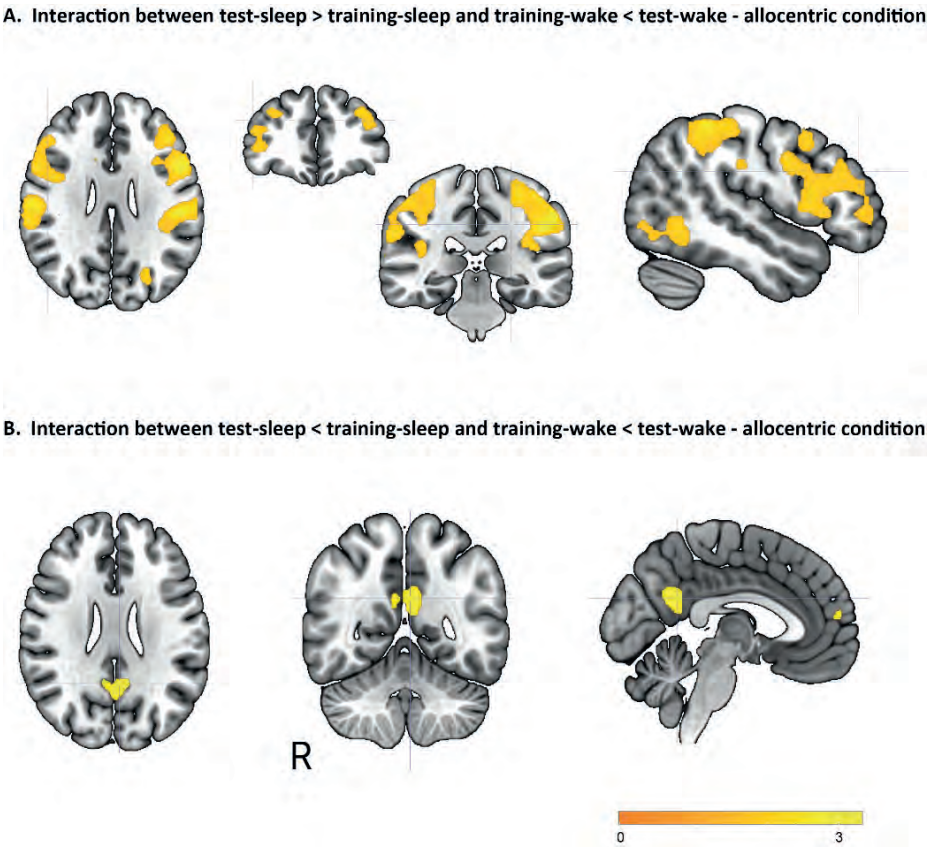


Fig. S8. fMRI activity analysis for the interaction between sleep and wake groups at training and test – only allocentric. (A) Regions significant for test-S > training -S and training-W < test-W, $p<0.05$ FWE cluster corrected (B) The opposite contrast with test-S < training -S and training-W > test-W, results shown for uncorrected $p<0.005$. The clusters were not significant when whole brain cluster corrected.

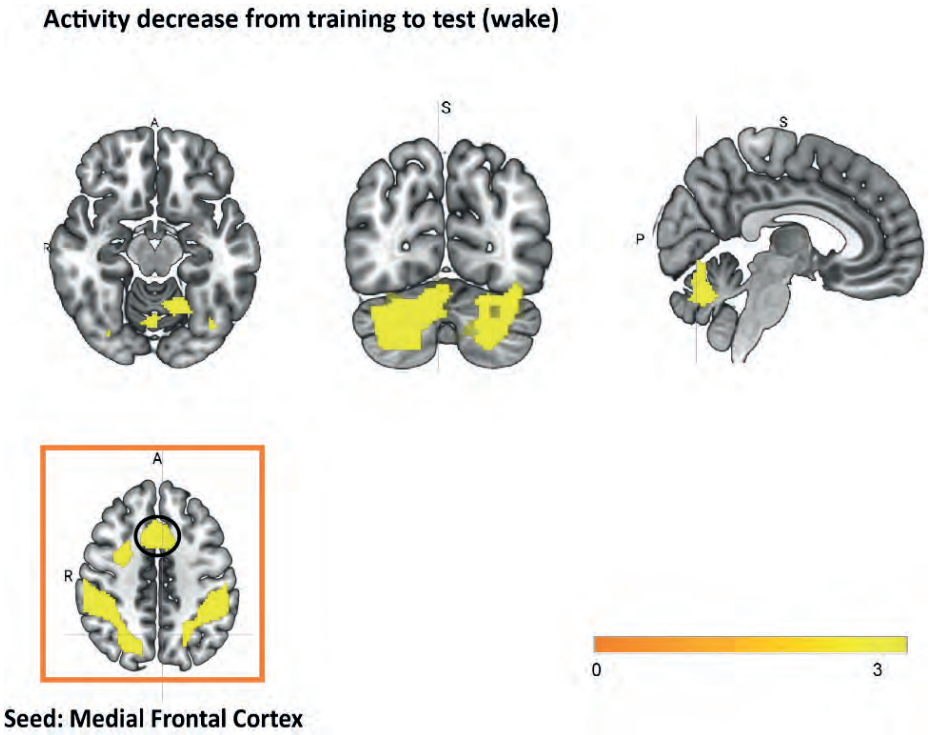
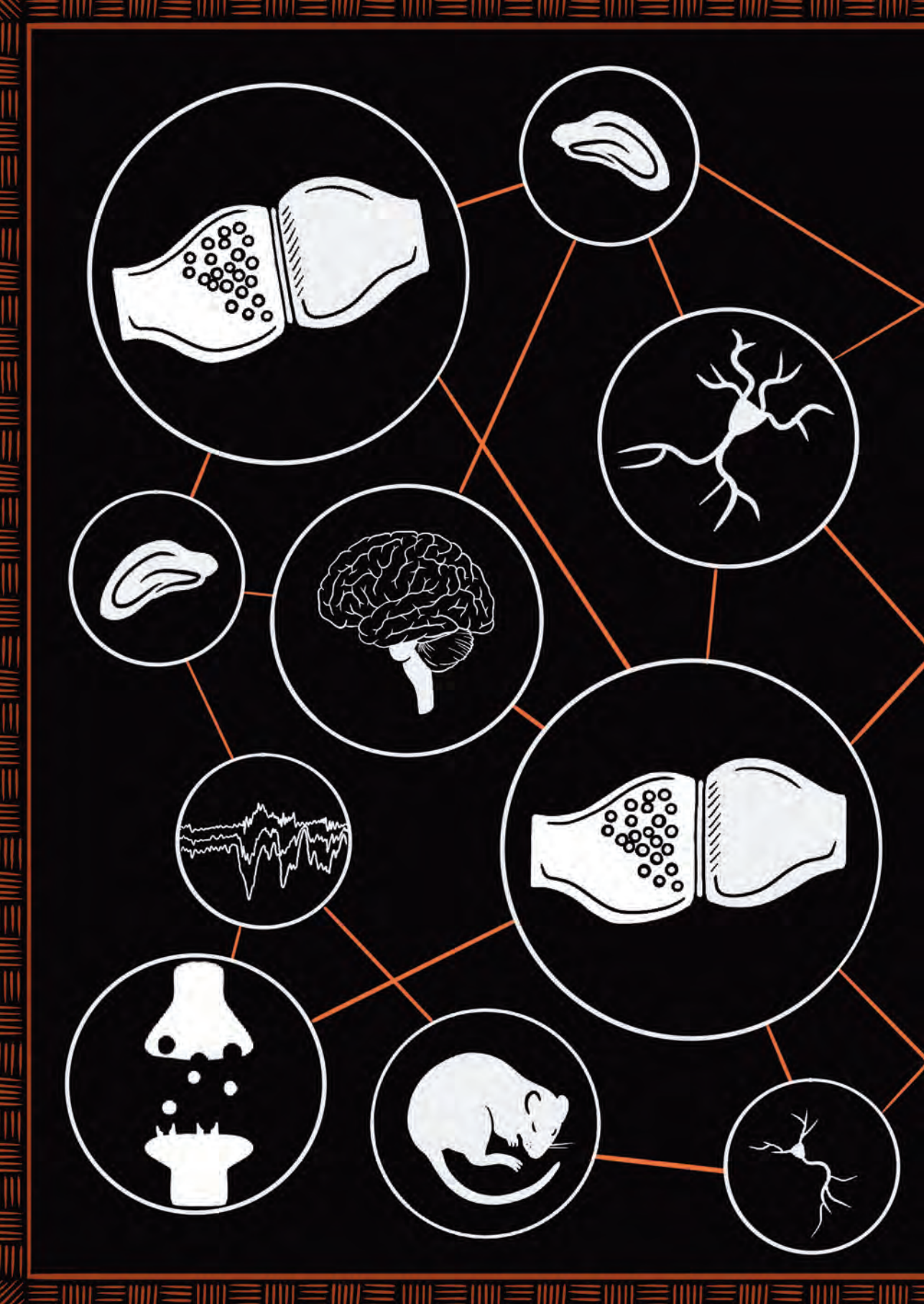


Fig. S9. fMRI Connectivity Analysis (PPI) with Medial Frontal Cortex as ROI. Brain maps show changes from both wake groups (test<training, p<0.05 FWE-cluster corrected). The frontal medial cortex showed a significant decrease in functional connectivity with the cerebellum for both wake groups. All results were collected at uncorrected p < 0.005 and then corrected on the cluster level to control for multiple comparisons with p<0.05 FWE.



Chapter 3

Memory Reactivations and consolidation: considering neuromodulators across wake and sleep

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Abstract

Memory reactivations occur during different states and behaviours and they may serve a multitude of functions. In this review we summarize differences in content, participating brain regions and neuromodulatory milieu of these reactivations. Further, we highlight how such differences could lead to the same phenomena of memory reactivation having different possible functions and outcomes such as memory retrieval, cellular consolidation and systems consolidation.

Introduction

Memory consolidation refers to the process wherein temporary, labile memory traces are stabilized to create long lasting memories. This is a dynamic process and involves processing initially on a cellular level: a cascade of cellular pathways leading to changes in synaptic strength within local circuits, also known as cellular/synaptic consolidation (Bailey, Kandel, and Harris 2015). In parallel to synaptic consolidation, processing then also occurs on a systems level, involving crosstalk between multiple brain regions and synaptic consolidation in those regions. This process is referred to as systems consolidation.

For the declarative memory system, the hippocampus is thought to be initially involved in encoding and the fusing of different sensory modalities into cohesive traces. Over time, these memory traces are thought to be integrated into neocortical regions for long term storage (Squire et al. 2015). Sleep is known to play an important role in this process and repeated reactivations (see glossary) of the new memories during this offline period are thought to be a critical contributing mechanism (Navarro-Lobato and Genzel 2018; Squire et al. 2015; Genzel et al. 2014; Diekelmann and Born 2010; Buzsáki 2015; Buzsaki 1989; Wilson and McNaughton 1994). Majority of these reactivations have been shown to occur during high frequency burst oscillations, referred to as sharp wave ripples (SWRs, 150-300 Hz, see also Fig. 1 and 2) in the hippocampus (Buzsáki 2015). These SWRs occur during NREM sleep and quiet rest periods. Since reactivations can also be seen in wake, the question remains why reactivations during sleep should play such a special role. One factor often not considered is the difference in neurotransmitter levels such as acetylcholine, dopamine, and noradrenaline across the different states. These neuromodulatory changes as well as the reactivation content (e.g. participating neurons) and participating structures in such events, could lead to different outcomes, e.g. both synaptic potentiation as well as down-regulation are possible (Norimoto et al. 2018; Sadowski, Jones, and Mellor 2011). Further, associations to other oscillations as spindles or slow oscillations can also be critical (Khodagholy, Gelinas, and Buzsáki 2017; Maingret et al. 2016). In this review we will highlight reactivation findings across different states and how neuromodulatory factors could influence the outcome and function of reactivations, focussing on the declarative memory system (for review on procedural memory consolidation please see (King et al. 2017)). Due to the invasive nature of the necessary methods, most studies investigating memory reactivation are performed in rodents (mice, rats), which we will focus on for this review.

State-dependent sharp-wave-ripples and reactivations

Both SWRs and reactivations can be observed in wake and sleep, but their properties and content can change across the states (Buzsáki 2015). For example the directionality of reactivations (faithful to the original experience, or in reverse neuronal order) and more importantly cross-brain reverberations are different in wake and sleep (Fig.1). Since hippocampal neurons fire in sequences during behaviour, the directionality of reactivations can be determined. Across many studies (Diba and Buzsáki 2007; Foster and Wilson 2006) both backward and forward reactivations have been observed, depending on different conditions. While the animal is running a linear track task and consumes the food at the reward site, reactivations are more likely to start at the current position and run backwards along the taken path (Ambrose, Pfeiffer, and Foster 2016). This type of reactivation occurs during consummatory sharp-wave-ripples (cSWR), has been shown to reinforce the current sequence and place-cell stability to enable later retrieval/reactivation (Roux et al. 2017) and is modulated by size of reward (Ambrose, Pfeiffer, and Foster 2016; Michon et al. 2019). After the task, when the animal is about to fall asleep (restingSWR or rSWR) or is in NREM sleep (sleepSWR or sSWR), reactivations also occur during sharp-wave-ripples and are again forward (Joo and Frank 2018). Hippocampal-cortical reactivations during NREM sleep are less faithful to the original experience, more "messy" (Tang et al. 2017), which may be critical for gist extraction during systems consolidation (Battaglia, Borensztajn, and Bod 2012). Gist extraction describes the process of extracting the important details across multiple experiences. Further, only in sleep does the anterior cingulate cortex show activity before the hippocampal ripple (Wang and Ikemoto 2016), indicative of a cortical-hippocampal-cortical loop specifically during sleep (Rothschild, Eban, and Frank 2017). Finally, only in sleep are hippocampal SWRs coordinated with other high-frequency oscillations (80-200 Hz) in default-mode-network structures (Khodagholy, Gelinas, and Buzsáki 2017) as well as with slow oscillations (0.5-2 Hz) and sleep spindles oscillations (9-16 Hz); a coordination which is critical for systems consolidation to occur (Maingret et al. 2016) (Fig.2). In general NREM light-sleep shows more cross-brain connectivity than wake (Spoormaker et al. 2010; Samann et al. 2011), which would be conducive for systems consolidation (Navarro-Lobato and Genzel 2018; Genzel et al. 2014). After sleep when the animal is placed back in the maze, reactivations are again forward and thought to enable memory retrieval and planning (Pfeiffer and Foster 2013; Jadhav et al. 2012; Wu et al. 2017) (exploratory sharp-wave-ripples, eSWR).

In sum, reactivation content and spread will change from wake to sleep to enable a multitude of functions the SWR can produce: initial strengthening of memory traces, systems consolidation, memory retrieval, and planning.

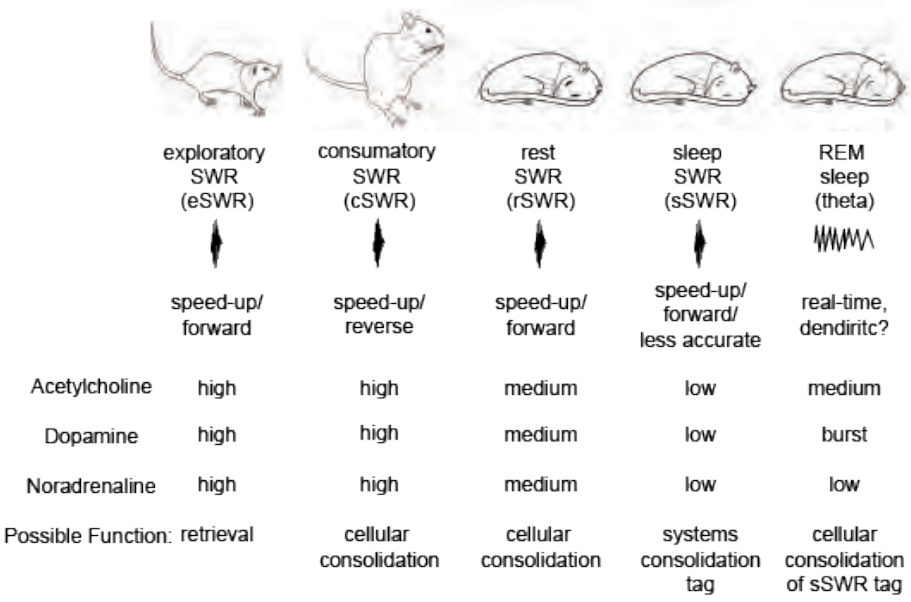

Fig. 1 Reactivation events: Across different behaviours and states reactivation events can be observed. These behaviours and states differ in tonic levels of neuromodulators. However, it is less clear if the neuromodulators show selective, phasic activations during reactivation events. During pause moments while running a task, rats will show reactivations (mostly forward) co-occurring with hippocampal ripples and these reactivations are thought to enable memory retrieval and planning (Jadhav et al. 2012; Pfeiffer and Foster 2013). During reward consummation rodents show reverse reactivations that are sensitive to reward size and stabilize place-cell sequences (Ambrose, Pfeiffer, and Foster 2016; Roux et al. 2017). After performing a task reactivations can be observed during quiet rest as well as subsequent NonREM sleep. Reactivations during NonREM SWR are less faithful of the original experience (Peyrache et al. 2009; Tang et al. 2017), which may be critical for gist extraction and systems consolidation (Battaglia, Borensztajn, and Bod 2012; Girardeau et al. 2009). Reactivations may also be found during REM theta, but these reactivations are perhaps just dendritic and due to the changed neuromodulatory milieu may be enacting the cellular consolidation during previous reactivations in NonREM (Li et al. 2017; Yang et al. 2014)

REM sleep and reactivations

Reactivations in wake and NREM sleep have been extensively characterized and tend to occur with SWR, however it remains less clear if reactivations occur in REM sleep when no ripples are present.

Overall, neural dynamics in REM are more similar to wake than those in NREM. Reactivations in NREM are 5-10 times faster than the original experience (Euston, Tatsuno, and McNaughton 2007), dynamics that are also captured in the head-direction system (Peyrache et al. 2015) (see glossary). However in REM, the head direction system shows again wake-like speed (Peyrache et al. 2015). Loui and Wilson presented the only evidence so far for REM-reactivations in rodents and their findings

did confirm the wake-like dynamics of REM (Louie and Wilson 2001). However, they had to use unconventional methods to be able to detect replay: (1) taking a template from REM sleep and searching for it in run and (2) only including perfect, stereotyped runs in a very familiar task in the analysis. Poe et al also investigated reactivations during REM sleep, but only of individual cells active in previous environments (in contrast to cell-pairs or groups of cells). Neurons active in familiar experiences were active again in the theta trough in REM sleep (Poe et al. 2000), while neurons from novel experiences were active in the theta peak. The authors proposed that this could lead to synaptic weakening and strengthening respectively (Pavlides et al. 1988). Interestingly, hypothalamic neurons that synthesize the neuropeptide melanin-concentrating hormone are claimed to be only active during REM sleep. However, using ca-imaging Blanco-Centurion et al could show that a sub-set of these neurons are also active during exploratory behaviour in novel environments and this sub-set seems to reactivate during REM sleep (Blanco-Centurion et al. 2019). However, also here the story may be more complex: depending on which subclass of neurons are activated and at which time point the manipulation occurs, both strengthening as well as weakening of memories can occur (Kosse and Burdakov 2019; Izawa et al. 2019).

One issue with detecting reactivations in REM, may be the method used. Reactivation studies most commonly use single-unit data, which captures the activity of the cell body. However, recently Rossato et al showed the phenomena of silent learning, during which even in absence of activity in the cell body, memories can be encoded with dendrite activity (Rossato et al. 2018). This silent learning may be occurring during sleep: during sleep spindles as well as REM sleep dendritic activity can become dissociated from the cell body (Seibt et al. 2017; Li et al. 2017; Yang et al. 2014; Aime et al. 2022). Interestingly, in humans using EEG-based decoding techniques reactivations can be found as easily in REM as in NREM sleep and also during sleep spindles (Schonauer et al. 2017; Cairney et al. 2018). EEG, in contrast to intracranial unit-recordings, is most likely capturing dendritic activity. If in REM sleep and during sleep spindles dendritic activity is dissociated from the cell-body and reactivations are occurring on the dendritic level, it would explain why reactivations are more easily found in human data using EEG in contrast to unit data in rodents. Accordingly, Yang et al have shown cell-body reactivations of motor-neurons in NREM but dendritic calcium spikes during REM that together enable the selective pruning and maintenance new synapses (Li et al. 2017; Yang et al. 2014).

Thus, evidence for REM reactivations is still sparse, which may be due to dissociations in dendritic and cell body activity.

Acetylcholine and reactivations

Acetylcholine activity facilitates learning in animals and humans (Deutsch 1971), and is a neuromodulator of physiological importance in the brain. Critically, acetylcholine levels show large differences across the sleep-wave cycle: high during wake and REM sleep but low during NREM (Marrosu et al. 1995; Teles-Grilo Ruivo et al. 2017). These different baseline levels could influence the differential outcome of memory reactivations across states.

Various experimental manipulations have now shown cholinergic activity to facilitate LTP in hippocampus in both in vitro (Blitzer, Gil, and Landau 1990) and in vivo models (Galey, Destrade, and Jaffard 1994). Leung et al (Leung et al. 2003) showed basal dendritic LTP in CA1 could be modulated depending on the behavioural state of the animal. LTP was largest when the high frequency tetanus was delivered during walking compared to quiet wakefulness, NREM or REM; and this enhancement was mediated by septohippocampal cholinergic inputs. A similar study (Brazhnik, Muller, and Fox 2003) has shown active exploration to be accompanied by large cholinergic input to hippocampus and muscarinic cholinergic blockade disrupted the place fields. This suggests behavioural modulation of basal-dendritic LTP by cholinergic inputs could be of functional significance. The septum also generates theta that is modulated by the behavioural state and movement speed and is critical for synaptic potentiation.

It is additionally well known that acetylcholine is regulated across the sleep-wake cycle, with high levels in wake and REM and low levels during NREM (Marrosu et al. 1995; Teles-Grilo Ruivo et al. 2017). Occurrence of SWR have been shown to be initiated when cholinergic drive to hippocampus is reduced (Buzsáki and Vanderwolf 1983). And optogenetic stimulation of cholinergic medial septal neurons suppressed SWRs in awake and anesthetized animals (Vandecasteele et al. 2014). Levels of acetylcholine also change the input/output properties of the hippocampal circuit: with high levels of acetylcholine during wake and REM and low levels during NREM. These changes may switch the hippocampus from a “recording” mode during wake, to an “output” mode during NREM and back to “recording” mode in REM (Schall, Kerber, and Dickson 2008).

If acetylcholine is permissive to LTP and in the hippocampus levels are lower during NREM than other behavioural states, this could affect the outcome of reactivations. Reactivations during e/c/rSWR and REM could then lead to synaptic strengthening, while in contrast reactivation in NREM (sSWR) would lead to no change or even synaptic weakening (Norimoto et al. 2018) (Fig.1). The synaptic strengthening by

cSWR would be necessary for later sleep reactivation to occur. O'Neill et al (O'Neill, Senior, and Csicsvari 2006) demonstrated exactly this, cells with similar place fields and correlated firing showed stronger coactivation during cSWRs and then subsequent sSWRs during NREM and both c and sSWR predict next day's memory performance (Dupret et al. 2010).

Dopamine and reactivations

When considering the influence of dopamine on reactivations, one needs to differentiate the effect of dopamine during learning on later reactivations as well as the level of dopamine during the reactivation itself. The hippocampus contains innervations from dopamine mesencephalic neurons from ventral tegmental area (VTA), substantia nigra and the locus coeruleus (LC) (Gasbarri et al. 1994; Takeuchi et al. 2016). When encountering novel environments or learning new goals, dopamine has been shown to play a role in stabilizing memory traces. Blockade of D1/D5 receptors during encoding impairs persistence of memory (Bethus, Tse, and Morris 2010; Rossato et al. 2009) and blockade during the exploration of novel environments impairs the stability of new spatial maps, thereby preventing novelty associated synaptic plasticity (Li et al. 2003).

It has been proposed that the degree of novelty is signalled by dopamine release from both the VTA and LC, which will also influence what happens to the memory after learning (Duszkiewicz et al. 2019). 'Common novelty' (new events that fit into our previous knowledge) would be signalled by increased activity in the VTA and lead to increased memory reactivations and thus systems consolidation from a hippocampal to cortical hub during NREM sleep (McNamara et al. 2014). In contrast, 'distinct novelty' (e.g. flashbulb memories of unique events that don't fit into any pre-existing memory networks and are known to last longer in a more vivid form) would lead to increased LC firing and cellular hippocampal consolidation via novelty (Takeuchi et al. 2016). This novelty-related, post-learning consolidation process is sleep independent (Genzel et al. 2017).

But how do dopamine neurons behave during the reactivations themselves? Gomperts et al (Gomperts, Kloosterman, and Wilson 2015) demonstrated higher coordination between reward responsive VTA neurons with SWRs in quiet wake state (rSWR), which are diminished in subsequent sSWR reactivations. This suggests distinct mechanistic contributions of VTA to reactivations during rest periods and subsequent sleep states. Interestingly, VTA dopamine neurons, which are quiet during NREM, show again burst firing and release of dopamine during REM sleep (Dahan et al. 2007)(Lena et al. 2005).

Thus, dopamine during learning may influence what type and where memory consolidation will occur afterwards but seems to be less involved in the reactivations themselves.

Noradrenaline and reactivations

As with dopamine, the effect on noradrenaline on reactivations has to be separated into learning and consolidation. Noradrenergic fibers originate mainly in the locus coeruleus (LC) and project widely through the forebrain, with dense innervations in the hippocampus, amygdala, and thalamus (Sara 2009). This is also known as the key arousal system and LC is necessary for novelty mediated sleep deprivation (Carter et al. 2010; Hayat et al. 2019). The hippocampal neurons express β-adrenergic receptors, which play an important role in induction of LTP and LTD (Lemon et al. 2009b; Harley 1991; O'Dell et al. 2015b). Several studies have shown LC stimulation to promote retrieval of spatial and associative memories, mediated by β-adrenergic receptors (Devauges and Sara 1991; O'Dell et al. 2015a; Lemon et al. 2009a). In addition to retrieval, the LC-norepinephrine system has also been shown to promote consolidation and reconsolidation after reactivation of memories containing different emotional valences (Przybyslawski and Sara 1997). Thus basic levels of noradrenalin are needed to effect synaptic changes, both decreasing as well as increasing synaptic strength.

In regards to noradrenaline and sleep, the relations are even more complex. General noradrenaline level is highest in wake, lower in NREM and lowest in REM sleep. Thus, in contrast to dopamine and acetylcholine, noradrenaline levels are very different in wake and REM sleep. Interestingly, activating LC neurons will inhibit both REM sleep and sleep spindles (Khanday et al. 2016; Swift et al. 2018). Further, persistent LC activity during REM sleep in insomnia in human subjects leads to more thought-like dreams and failure to decrease emotional reactivity across sleep (Wassing et al. 2019). Eschenko et al (Eschenko and Sara 2008) recorded activity from LC neurons during sleep in rats and found an increase in firing rate during NREM sleep 2 hours after a learning episode. They also found the LC unit activity to be time locked to cortical up-state of the slow oscillations during the sleep state, which is when SWRs and reactivations occur (Eschenko et al. 2012). To test if this association is important for systems consolidation, Novitskaya et al activated the LC time-locked to SWRs (Novitskaya et al. 2016). Surprisingly, this led to suppression of synchronous SWR events and inhibited systems consolidation.

Thus, overall the absence of noradrenaline during sleep seem to be permissive to sleep (spindles, ripples, REM) as well as memory consolidation during sleep.

Sharp-wave-ripple sub types

Differences in neurotransmitters, brain state and cross-brain connectivity all influence the content and outcome of memory reactivations, highlighting that perhaps we should not see SWRs as one single phenomena but instead consider sub-types. On the point, SWR related activity has been shown to be able to both upregulate (Sadowski, Jones, and Mellor 2016) and downregulate synaptic strength (Norimoto et al. 2018).

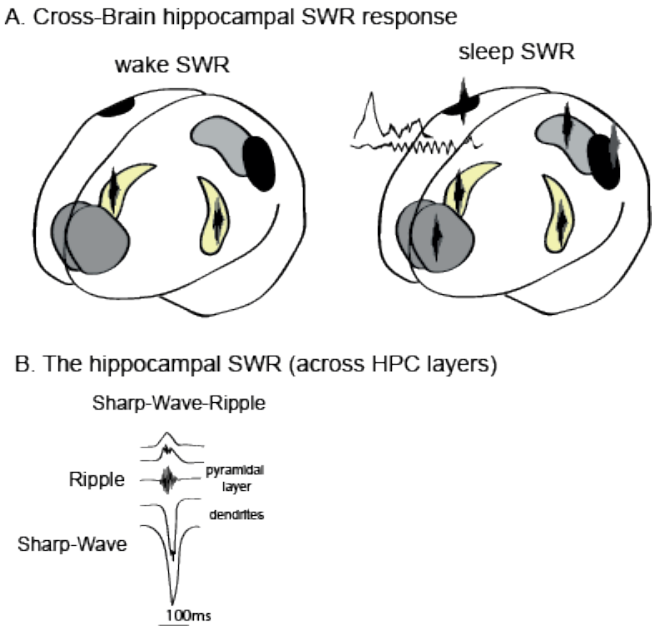

Fig. 3 Characterization of SWRs and differences in wake and sleep: Panel A shows the differences in SWR during sleep and wake. Sleep will modulate the association of hippocampal SWRs with other oscillations. In wake and also during sleep SWR will occur in the hippocampus alone. However, especially after a learning event hippocampal SWRs during sleep can be accompanied by co-occurring ripples in cortical areas belonging to the default-mode network (Khodagholy, Gelinas, and Buzsáki 2017) as well as being associated to slow oscillations and spindles in the cortex (Maingret et al. 2016).

Panel B shows a more detailed characterization of the two components of the Sharp Wave Ripple (SWR) in the hippocampus – the sharp wave and ripple. These are hippocampal events occurring as a result of interaction between pyramidal and granule neurons with interneurons in the circuit. Bursts of pyramidal neuron populations in CA3 region leads to depolarization of pyramidal neurons in CA1 region, giving rise to extracellular negative waves, which are called sharp waves. However, discharge of the pyramidal neurons in CA3, also activates the GABAergic interneurons. The combination of firing of neurons in CA1 and inhibition from the activated interneurons results in high frequency oscillations, which are the ripples, occurring with the sharp waves but in the pyramidal cell layer. Thus the sharp-wave and ripple components of the SWR occur on different electrodes. These SWRs are influenced by activity inputs from the dentate gyrus and entorhinal cortex and also by neocortical inputs, making them distinctly different from the cortical ripples, which due to the anatomical difference will contain a ripple but not necessarily a sharp-wave.

Some SWR sub-types can be identified by the frequency and shape of the individual sharp-wave and ripple components (Reichinnek et al. 2010; Nguyen et al. 2009). And, by combining electrophysiology with functional MRI such sub-types could further be characterized. SWR triggered fMRI maps showed that ripples aligned to the positive peak of their sharp-waves have enhanced neocortical metabolic up-regulation. In contrast, ripples occurring at the trough of their sharp-waves are linked to weaker neocortical activity and lack subcortical down-regulation. This indicates differentiated involvement of neuromodulatory pathways in the SWR phenomenon mediated by long-range interactions (Ramirez-Villegas, Logothetis, and Besserve 2015).

However, up until now such sub-type analysis have only been performed within homogenous data-sets: either only including anaesthesia or even just recording from a slice. It would be critical to perform such a sub-type analysis including wake as well as sleep and both after different types of behaviours.

Box 1. Definitions of frequently used terms

Memory consolidation: Dynamic process after learning wherein temporary, labile memory traces are stabilized to be more long lasting

Cellular/synaptic consolidation: Activation of a cascade of cellular pathways on learning, leading to changes in synaptic strength within local circuits. Usually occurs on a time scale of minutes to hours after learning.

Systems consolidation: Building up on initial, synaptic consolidation, processing of the memories now happen on a systems level, involving crosstalk between different brain regions. For declarative memory systems (the main focus in this review), hippocampus is thought to initially encode memory traces from different modalities, fusing them into a cohesive trace and over time and dialogue with the neocortex, with gradual changes, is integrated into the neocortical system along with pre-existing networks for long term storage.

Reactivations: Patterns of co-firing of groups of neurons that were active during encoding/active behaviour, during sleep, rest periods, subsequent active periods.

Replay: Specific type of sequential reactivations that occur during high frequency burst oscillations referred to as Sharp Wave Ripples (SWRs) in the hippocampus during NREM sleep, rest, and quiet wakefulness periods. These replay events are high

frequency (150-300 Hz) time compressed (50-100 ms) patterns of groups of neurons which fired during active behaviour.

Individual hubs: primary regions of activity, each having its specialized function and contributing to the greater functioning of the network.

Hippocampal hub: Includes both dorsal and ventral hippocampus

Head direction system: The head-direction system is thought to be the internal compass of the brain, consisting of a particular subset of neurons, which increasingly fire when a rodent's head is pointing in a specific direction.

Cross brain connectivity: Interaction on a global level across different brain regions, in this case, for instance, the interaction between hippocampus and neocortical structures

Sleep stages: Rodents show polyphasic sleep with several NREM and REM cycles interrupted by wake phases. Below mentioned are the key features that characterize each sleep stage:

Wake – characterized by desynchronized brain activity in the cortex and theta oscillations (4-8 Hz) in the hippocampus when the rodent is engaged in a task

NREM – this stage is considered to be the key stage for active memory consolidation and is dominated by sharp wave ripples (SWRs) in the hippocampus, spindles (9-16 Hz) and slow wave activity (delta waves, 1-4Hz) and nested slow oscillations (0.5-1 Hz) in the cortex. Overall it is characterized by oscillations occurring on both global and local scales. It can be further characterized into light NREM sleep and deep sleep. Light NREM is thought to be dominated by the SWRs, slow oscillations and spindles whereas deep sleep is characterized by delta waves.

REM – paradoxical sleep stage characterized by rapid eye movement, theta oscillations (5-10 Hz) in hippocampus and desynchronized activity in neocortex, accompanied by muscle atonia.

Conclusion

Reactivations can be measured in different behavioural states from active exploration to sleep. Most likely these reactivations fulfil a multitude of functions depending on the neuromodulatory milieu and participating regions. When executing a task, reactivations can occur during behavioural pauses as well as consummatory behaviour; due to high levels of acetylcholine, dopamine, and noradrenaline during wake, these reactivation could lead to cellular consolidation and synaptic strengthening. In contrast are reactivations occurring during NREM SWRs. Decreased levels of all neurotransmitters combined with associations to cortical high-frequency oscillations, sleep spindles and slow oscillation, could result in no change in synaptic strength; instead these reactivations would set "tags" in the cortex for later cellular consolidation to occur during REM sleep reactivations (Li et al. 2017; Yang et al. 2014; Seibt and Frank 2019). What the biological substrate of these "tags" would be remains unknown. Of note, de Lavilleon et al has shown that synaptic strengthening by pairing the activity of a place-cell with a positive stimulus is possible during NREM sleep (de Lavilléon et al. 2015).

The hippocampal SWR can induce tight network coherence for ~50-100ms spanning much of the brain (Kaplan et al. 2016; Logothetis et al. 2012; Khodagholy, Gelinas, and Buzsáki 2017). This global network coherence during sleep would be the ideal state for global network adaptions with both increases and decreases of individual network hub activity. Accordingly, SWR related activity has been shown to be able to both upregulate (Sadowski, Jones, and Mellor 2016) and downregulate synaptic strength (Norimoto et al. 2018) on the cellular level. Thus the SWR with its reactivations maybe the functional cogwheel that enables the shift in the memory network and e.g. allows upregulation of cortical hubs and downregulation of the hippocampal hub during systems consolidation.

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Highlights

(**)Norimoto, H., Makino, K., Gao, M., Shikano, Y., Okamoto, K., Ishikawa, T., Sasaki, T., Hioki, H., Fujisawa, S., and Ikegaya, Y., Hippocampal ripples down-regulate synapses. Science, 2018. **8**(10).

By combing in vivo and in vitro experiemnts, the authors show that reactivations during ripples can lead to synaptic weakening.

(**)Sadowski, J.H., Jones, M.W., and Mellor, J.R., Ripples make waves: binding structured activity and plasticity in hippocampal networks. Neural Plast, 2011. **2011**: p. 960389.

By artifically reinstating reactivations in a hippocampal slice the authors could show that ripple associated reactivations can lead to synaptic strengthening.

(**)Khodagholy, D., Gelinas, J.N., and Buzsáki, G., Learning-enhanced coupling between ripple oscillations in association cortices and hippocampus. Science, 2017. **358**(6361): p. 369-372.

The authors provide the first evidance for ripples occuring across the default mode network areas if learning preceeds sleep.

(**)Li, W., Ma, L., Yang, G., and Gan, W.B., REM sleep selectively prunes and maintains new synapses in development and learning. Nat Neurosci, 2017. **20**(3): p. 427-437.

With two-photon imaging the authors show a dissociation between cell-body and dendric firing in REM that is associated with motor memory consolidation.

(**)Yang, G., Wan Lai, C.S., Cicgen, J., Ma, L., Li, W., and Gan, W.B., Sleep promotes branch-specific formation of dendritic spines after learning. Science, 2014. **344**(6188): p. 1174-78.

With two-photon imaging the authors show memory reactivation in NonREM sleep in the motor cortex that lead to long term consolidation.

(*)Buzsaki, G., Hippocampal sharp wave-ripple: A cognitive biomarker for episodic memory and planning. Hippocampus, 2015. **25**(10): p. 1073-188.

This is the most comprehensive and thourough review on sharp wave ripples and their properties.

(*)Kaplan, R., Adhikari, M.H., Hindriks, R., Mantini, D., Murayama, Y., Logothetis, N.K., and Deco, G., Hippocampal Sharp-Wave Ripples Influence Selective Activation of the Default Mode Network. Curr Biol, 2016. **26**(5): p. 686-91.

By combining EEG and fMRI the authors could show different sharp wave ripple subtypes and their differential brain activation

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The authors could show the coordination of ripples, slow oscillation and sleep spindles are causally important for memory consolidation with a closed-loop paradigm.

Chapter 4

CBD lengthens sleep but shortens ripples and leads to intact simple but worse cumulative memory

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A.S. supervised and performed experiments as well as data analysis, A.A.Z. supervised and performed data analysis, K.A. and P.O. analysed the electrophysiology data, A.A., J.v.d.M., and I.N.L. helped with experiments and supervision, A.R. supported electrophysiology data analysis, L.G. designed and supervised the project. A.S., L.G., and A.A.Z. wrote the first draft of the manuscript.

Abstract

Cannabidiol (CBD) is on the rise as over-the-counter medication to treat sleep disturbances, anxiety, pain and epilepsy due to its action on the excitatory/inhibitory balance in the brain. However, it remains unclear if CBD also leads to adverse effects on memory via changes of sleep macro- and microarchitecture. To investigate the effect of CBD on sleep and memory consolidation, we performed two experiments using the object space task testing for both simple and cumulative memory in rats. We show that oral CBD administration extended the sleep period but changed the properties of rest and NonREM sleep oscillations (delta, spindle, ripples). Specifically, CBD also led to less long (>100 ms) ripples and, consequently, worse cumulative memory consolidation. In contrast, simple memories were not affected. In sum, we can confirm the beneficial effect of CBD on sleep; however, this comes with changes in oscillations that negatively impact memory consolidation.

Introduction

Cannabidiol (CBD) has become a popular remedy for many ailments and shows promising effects treating chronic pain, anxiety and sleep disturbances (Blessing et al. 2015; Urits et al. 2020). Furthermore, due to the action on inhibition in the brain, CBD is potentially a novel therapeutic agent for restoring excitation/inhibition balance in models of epilepsy (Rosenberg et al. 2023) and autism, which stem from dysregulation of inhibitory networks (Khan et al. 2018). CBD as well as CB1-receptor agonist have been shown to promote sleep in multiple, pre-clinical and clinical studies (Coronado-Álvarez et al. 2021; Murillo-Rodríguez et al. 2018; Murillo-Rodríguez, Machado, et al. 2021; Murillo-Rodríguez et al. 2016; Murillo-Rodríguez, Millán-Aldaco, et al. 2021; Shannon et al. 2019). For example, in patients with neuropsychiatric disorders, CBD administration improved sleep quality in more than half of the patients up to a month after administration and alleviated anxiety symptoms (Shannon et al. 2019). Interestingly, the effect of CBD on sleep is dose-dependent with high doses increasing sleep duration (Carlini and Cunha 1981) and the opposite effect is observed with low doses (Nicholson et al. 2004). CBD may also have different effects in aged population in contrast to young adults and adolescence (Murillo-Rodríguez et al. 2020). Despite the rising use of CBD by various patients as an over-the-counter remedy, very little is known about how CBD affects the microstructure and memory functions of sleep, which could lead to side-effects as seen with other sleep-modulating medications (Mednick et al. 2013; Plante et al. 2015; Wamsley et al. 2013).

Sleep is thought to play a critical role in the consolidation of memories, both for simple memories – such as the association of two items – as well as the more complex extraction of regularities across different experiences. Non-Rapid-Eye-Movement (NonREM) sleep has been proposed as a critical stage in offline memory processing (Frankland and Bontempi 2005; Genzel et al. 2014a). The reactivation of cells active during encoding has been shown to occur during quiet wake and NonREM sleep and is potentially crucial to memory consolidation (Girardeau et al. 2009; Aleman-Zapata, Morris, and Genzel 2022; Wilson and McNaughton 1994; Gridchyn et al. 2020). These reactivation events occur during hippocampal high frequency burst oscillations (100-250 Hz) referred to as ripples (Buzsáki 2015a). Interestingly, it had been shown that administration of CB1-agonist disrupts ripples in the hippocampus (Sun et al. 2012; Maier et al. 2012; Robbe and Buzsáki 2009; Robbe et al. 2006). However, to be able to consolidate memories during sleep, a coordinated action between the hippocampus and the neocortex around the ripples is needed. Two major neocortical events – delta waves (0.5-4 Hz) and spindles (9-20 Hz) – can occur in close coordination with the ripple, and this

crosstalk between brain regions is crucial for offline memory processing (Maingret et al. 2016; Peyrache, Battaglia, and Destexhe 2011; Qin et al. 1997; Siapas and Wilson 1998). The characteristics and coordination of these microstructural sleep events are influenced by the depth of sleep as well as most sleep-inducing medications (Plante et al. 2015; Wamsley et al. 2013); thus, there are likely effects of CBD on spindles and delta waves similar to the effect on ripples that could negatively impact their function in memory consolidation.

CBD can act as an antagonist of the CB1-receptors (Pertwee 2008; Pertwee and Ross 2002; Thomas et al. 2007). These receptors are present on hippocampal cholecystokinin (CCK) and parvalbumin (PV) positive interneurons (Katona et al. 1999). CB1-receptors are found in the presynaptic axon terminals of the GABAergic interneurons, most prominently in the CA1-CA3 subfields (Egertová and Elphick 2000; Tsou et al. 1999). They act like retrograde messengers, wherein their activation in the presynaptic targets is triggered by depolarization in the post-synaptic targets (Katona et al. 1999). CB1-receptors potentially regulate the suppression of GABA-mediated transmission following the depolarization of hippocampal pyramidal neurons (Pitler and Alger 1992; Wilson and Nicoll 2001), which is a key player in the occurrence of ripples. The ripple component is initiated by the activation of pyramidal neurons in the CA1, in response to input from CA3 (English et al. 2017; Buzsáki 2015a; Stark et al. 2015); however, the oscillation is maintained by interneuron activity (Stark et al. 2014b). Thus, changes in ripples after CBD administration could stem from the change in the E/I balance.

To test the effect of CBD on sleep and sleep-related memory consolidation, we performed two experiments using the Object Space Task (Genzel et al. 2019) in young, adult rats. This task enables measuring both simple and cumulative memory, where the former should be more resistant, whereas the latter should be more sensitive to manipulation effects. In the first experiment, we tested the effect of acute, oral CBD administration – mimicking human CBD intake (Deiana et al. 2012b) – before learning and consolidation on memory expression the next day. For the second experiment, we conducted in fewer animal's recordings of natural sleep and sleep oscillations after learning with electrophysiological implants targeting the hippocampus and the prefrontal cortex to elucidate how CBD affects the sleep macro- and microstructure. Finally, we returned to the animals from the behavioural experiment and recorded sleep-like states in anesthesia to confirm findings from the second experiment. Oral administration of CBD increased NonREM sleep at the end of the sleep period. Furthermore, it changed sleep microstructure – leading to less long ripples – that resulted in worse cumulative but intact simple memories tested the following day.

Results

CBD decreases cumulative memory expression the next day but leaves simple memory intact

Our first aim was to establish a behavioural effect of CBD administration targeting memory consolidation. For this, the Object Space Task (Fig. 1A) was combined with oral administration of CBD, in a similar dose as commonly used in oral administration to humans. Previous studies have shown oral administration of CBD to be effective in crossing the blood brain barrier and plasma concentrations are shown to reach their maximum around 4-6 hours after oral administration (Deiana et al. 2012b; Hložek et al. 2017).Thus, CBD and vehicle-control was given 1h before training start (1h after light on, Fig. 1B), such that the peak of CBD in the brain occurred after training during the main sleep period. Of note, all experiments were run counterbalanced and blinded. The experimenter was not aware of the current drug treatment of the rat.

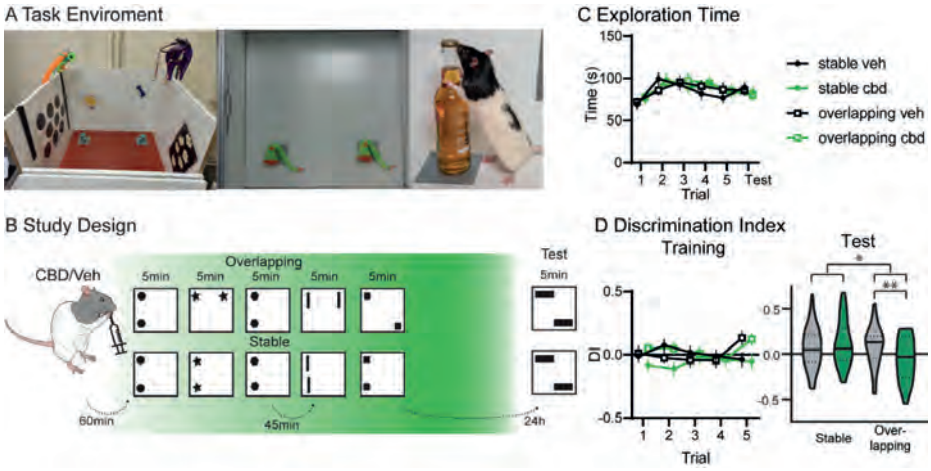

Fig.1 Behaviour: A. Shown is the object exploration box (side view, top view and with animal). B. Study Design. Rats received orally either vehicle or CBD (cross-over) and 60min later were trained in the OS task with either Overlapping (one stable, one moving object location) or Stable (two stable object locations) condition (5min trials, inter-trial-interval 45min) and tested 24h later. Green shading in background indicates expected CBD concentration, since previous studies have shown CBD to reach maximum levels in the brain around 4-6 hours after oral administration (Deiana et al. 2012b; Hložek et al. 2017).C. The exploration time remained stable for both conditions and treatment. D. There was no difference in discrimination index (DI) during training for treatment, but as expected in the fifth trial both showed positive DIs in overlapping. At test, there was a significant condition X treatment interaction ($p=0.031$, t-test overlapping Veh and CBD $t_{72}=2.74$ $p=0.0078$). *$p<0.05$ $n=37$.

Rats were trained in either the Stable or Overlapping condition, which test simple and cumulative memory respectively. In Stable, in each training trial identical object pairs (each trial different ones) are presented in the same two locations in the exploration box over 5 training trials. At the test (24h after training), two new objects are again used and presented with one on a usual location but the other one placed in one of the other two corners. Neophilic animals will explore the object in the novel location more, independent of whether they just remembered the last experience or all five training trials (leading to positive Discrimination Index, DI). Thus, the Stable condition tests for simple location memory. In contrast, in Overlapping, already during training only one location remains stable, while the second object will be in one of the three other corners. Now, the test trial has the same configuration as the final training trial. Therefore, only if the rats created a cumulative memory, abstracted over multiple training trials, will they show a positive discrimination index at test. All animals underwent both conditions and both treatments, therefore four rounds of training-test, in a cross-over design. Treatment/condition sequences were counterbalanced across rats and object locations.

While CBD was administered before training, due to the slow uptake the main effect should be after training in the sleep period. In confirmation that CBD did not affect encoding behaviours, both exploration time (Fig. 1C) and exploration preferences during training did not show a treatment effect (discrimination index, Fig. 1D). As expected, in Overlapping a positive discrimination index was already seen in trial 5, due to the moving object-location during training. At test, there was a significant condition X treatment interaction (rmANOVA condition $F_{1,36}$=5.6 p=0.023, treatment $F_{1,36}$=1.2 p=0.28, interaction $F_{1,36}$=5.1 p=0.031 only including animals that showed memory in the vehicle condition. However, the same interaction effect was seen when all animals were included $F_{1,43}$=3.0 p=0.089). CBD administration led to disrupted cumulative memory expression (Overlapping condition, p<0.01) but intact simple memory (Stable condition).

In sum, using a potentially translatable approach with oral administration of CBD, we showed a significant effect on behaviour, where CBD led to intact simple but worse cumulative memory.

CBD extends natural NonREM sleep

Our second aim was to characterize the effects of CBD on sleep and sleep oscillations. For this, new rats (n=4) were implanted with a wire-drive targeting the prelimbic cortex and hippocampus, which allowed us to acquire wake and sleep data in the task (5 times 5min trials) and sleep box (4 X 45min min after trials 1-4 and for 3h after trial 5,

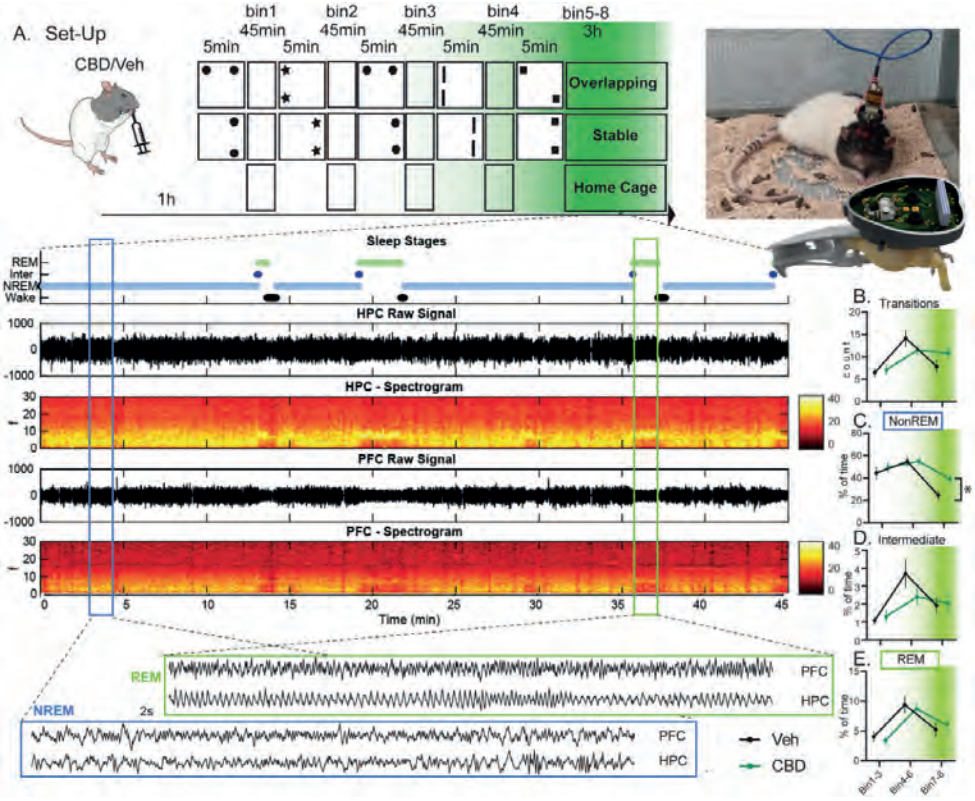

Fig.2 Electrophysiological experiments: A. Rats were implanted with a wire-drive (picture on the right). On study days they received CBD or Vehicle (Veh) orally and one hour later started the OS training as in Fig.1 and spent the time between trials in the sleep recording box (4X45min bin 1-4, 3h after final trial bin 5-8). In addition to the conditions Overlapping and Stable, animals also spent one study day always in the Home Cage during the usual training periods (circadian, no-learning control). Below manually scored sleep stages as well as raw signal and spectrograms arising from the hippocampus (HPC) and prelimbic cortex (PFC) are shown for an example 45min bin. Finally, for each NONREM (NONREM) and REM state a 2s example of raw LFP is presented. Next separated for Bin 1-3, 4-6 and 7-8 the count of state-transition (B) as well as percent time spent in NONREM (C), Intermediate (D) and REM (E). Only NONREM showed a significant effect with more sleep in the final bins in rats treated with CBD (Transitions: time $F_{2,43}$=17.2 p<0.0001, treat $F_{1,22}$=0.04 p=0.8, interaction $F_{2,43}$=3.7 p=0.03; NONREM: time $F_{2,43}$=17.3 p<0.0001, treat $F_{1,22}$=2.2 p=0.15, interaction $F_{2,43}$=1.5 p=0.23; Intermediate time $F_{2,43}$=12.5 p<0.0001, treat $F_{1,22}$=0.3 p=0.6, interaction $F_{2,43}$=2.6 p=0.08; REM time $F_{2,43}$=30.0 p<0.0001, treat $F_{1,22}$=0.04 p=0.9, interaction $F_{2,43}$=0.5 p=0.6; effect size NONREM final bin 1.01). For A-E green shading implies expected effective CBD levels. Orthogonal comparisons *p<0.05 n=4.

total 6h) for each study day (study design Fig.2A, histology Fig. S2). The recordings were divided into 5 min task and 45 min sleep-box bins (between each trial and 4 bins after the 5th trial). Raw signal and spectrogram for one such 45min is presented in Fig.2A. The signal was manually scored for sleep stages NonREM, Intermediate and REM. Fitting to the idea that CBD promotes sleep, rats treated with CBD had more NonREM sleep but no changes in Intermediate and REM sleep nor the number of sleep stage transitions (Fig.2B-E). However, the effect on NonREM sleep was modest, it only became apparent in the last two sleep bins, when control rats showed a decline in amount of NonREM sleep. Of note, this is also the time period where the maximum level of CBD should have reached the brain. In sum, CBD led to more NonREM sleep at the end of the sleep period.

CBD makes NonREM oscillations smaller

Next, we detected specific NonREM oscillations that are implied to be critical for memory consolidation (Genzel et al. 2014a). Delta and spindle oscillations in the prelimbic cortex and ripple oscillations in the hippocampal CA1 area were detected during NonREM sleep periods and in addition ripples during quiet wake in the sleep box. Deltas and NonREM ripples were much more numerous than spindles and quiet wake ripples (Fig. 3A). To account for these differences, the counts of oscillations were normalized by their overall average and then, as for the sleep stages, separated for the different sleep bins. After CBD there were more deltas, spindles and NonREM ripples in the final bins (Fig. 3B); however, this was due to the increased amount of NonREM sleep, since there was no change in rates (Fig. S3) and also no change in quiet wake ripples. CBD did change the properties of each type of oscillation. Deltas and NonREM ripples became slower (decreased intrinsic frequency), while spindles and quiet wake ripples became faster (Fig.3C). All four oscillations became smaller in amplitude (Fig.3D).

In sum, more NonREM sleep in CBD led to more NonREM oscillations in the final two bins of the day. However, CBD changed the frequencies and decreased the amplitudes of these as well as quiet wake ripple oscillations.

CBD decreases the number of long ripples

Memories are reactivated during sleep and these reactivations preferably take place during hippocampal ripples (Girardeau and Zugaro 2011). Recently, it has been shown that extending short ripples during a task led to better working memory due to longer reactivation events (Fernández-Ruiz et al. 2019). Thus, long ripples during sleep could be especially important for memory consolidation of extended experience or more complex information. Therefore, next we split detected NonREM ripples into

short (≤100ms) and long (>100ms) events (Fig.3E). Most events were short and these presented the same time-course as all ripples, with increases after CBD in the final sleep bins (Fig.3F). In contrast, long ripples occurred less already early in the day after CBD administration (Fig.3G). CBD decreased the amplitude of both short and long ripples (Fig.3H). Learning has previously been shown to be accompanied by an increase in magnitude of ripple events including amplitude and duration (Eschenko et al. 2008). Interestingly, while short ripples showed significant phase coupling with slow oscillations in both VEH and CBD (both $p<0.0001$), long ripples were only coupled to the slow oscillation after VEH administration and not CBD ($p<0.05$, $p=0.9$, Fig.3I).

Plotting the hippocampal and cortical power spectrum during short and long ripples (in comparison to random selected baseline signal) revealed a frequency independent increase in power for all events to baseline. However, in vehicles long in comparison to short ripples had an even higher power, especially in the prelimbic cortex (Fig.3J). This was not evident in CBD. The power spectrum can be used to calculate E/I balances as well as overall activity levels (Gao, Peterson, and Voytek 2017; Bódizs et al. 2021; Favaro et al. 2023; Gao 2016; Horváth et al. 2022). Specifically, from lines fitted to the double logged (power and frequency) power spectrum the offsets and slopes can be derived (Fig.3K). The offsets reflect the overall activity (higher offset → higher activity), while the slopes reflect the E/I balance (higher slope → higher E/I ratio). In vehicles, offsets (activity) in both brain areas increased from baseline to short and from short to long ripples, but the increase was less after CBD. Interestingly, slopes were generally higher in the hippocampus than in the cortex (higher E/I balance) and CBD increased these even more (especially in the hippocampus). After vehicle slopes decreased for ripple events (lower E/I balance). While this was similar in CBD, due to the overall higher baseline, slopes remained higher during ripples.

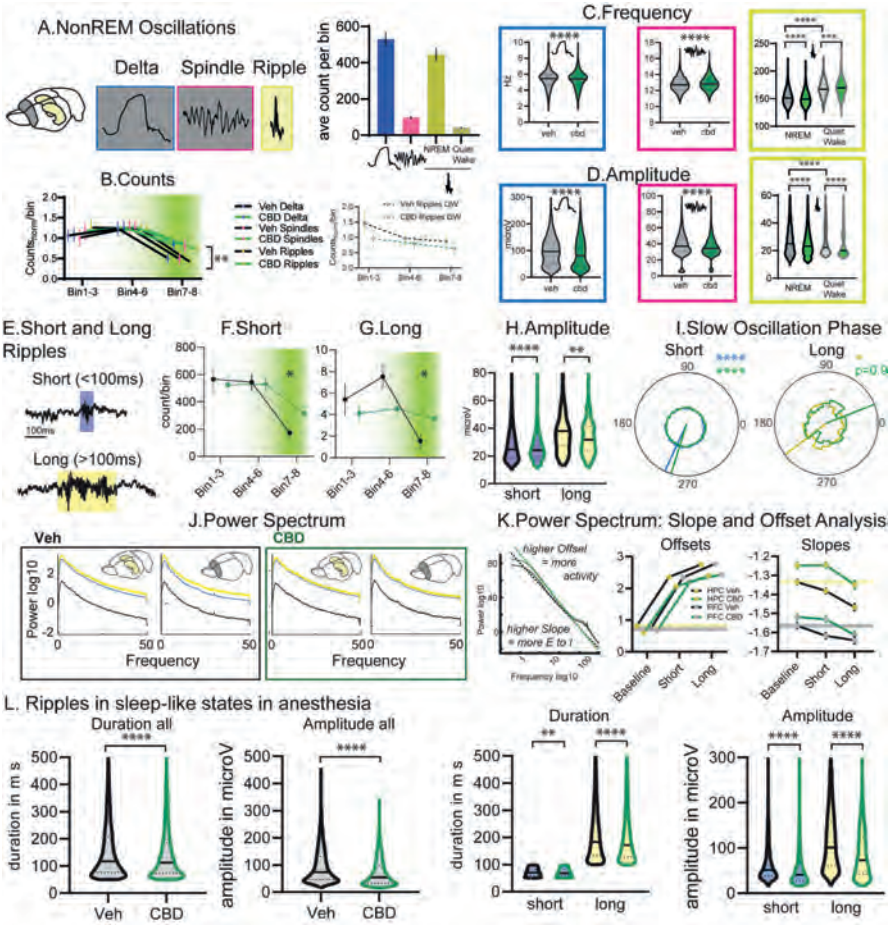

Fig.3 NonREM Oscillations: A. Cortical delta and spindles (both NonREM), and hippocampal ripples (NonREM and quiet wake in the sleep box) were detected. On the right, average counts per bin (types $F_{3,273}$=121.6 p<0.0001). B. Counts for all types normalized by their average. There was a significant treatment and time interaction (treat $F_{1,30}$=5.2 p=0.029, bin $F_{2,40}$=30.9 p<0.001, treat X bin $F_{2,60}$=4.7 p=0.012 all other p>0.75). This was not seen for quiet wake ripples (p>0.6). From left to right for delta, spindle and ripple the intrinsic frequency (C) and amplitude (D) for all events (bin1-8). Delta and NonREM ripples were slower and smaller, spindles faster and smaller (all K-S D, p<0.0001). Interestingly, quiet wake ripples were smaller but faster. E. Examples of short and long ripples. Counts for F. short and G. long ripples. H. Amplitudes. I. Slow oscillation phase, all short ripples were locked but for long ripples only in vehicles significant locking was seen. J. Power spectrum for vehicle and CBD, always left for hippocampus and right for prelimbic cortex. Of note, in Veh there was more power for long than short ripples especially in the prelimbic cortex. This was not seen in CBD. (full model without baseline long/short $F_{1,2792}$=86.4 p<0.001, HPC/PFC $F_{1,2792}$=302.9 p<0.001, treat $F_{1,2792}$=31.8 p<0.001, long/shortXHPC/PFC $F_{1,2792}$=8.2 p=0.004, long/shortXtreat $F_{1,2792}$=11.1 p<0.001 other interactions p>0.5; only vehicle long/shortXHPC/PFC $F_{1,1596}$=7.3 p=0.007 only CBD long/shortXHPC/PFC $F_{1,1196}$=2.1 p=0.14) K. Slope and Offset Analysis from left to right: how slopes and offsets are calculated, offset and slopes for ripple types and random selected baseline. Lines indicate vehicle baseline levels. Offsets: events $F_{2,3588}$=1592 p<0.0001, treatment $F_{1,3588}$=63.15 p<0.0001, events X treatment $F_{2,3588}$=4.5 p=0.011, brain area X treatment $F_{1,3588}$=5.0

p=0.026. Slopes: event $F_{2, 3588}$=24.6 p<0.0001, brain area $F_{1, 3588}$=397.5 p<0.0001, treatment $F_{1, 3588}$=47.3 p<0.0001, brain area X type $F_{1, 3588}$=6.2 p=0.0127 other p>0.27). L. Ripples detected in sleep-like states in anesthesia (n=9 and 10 for Veh and CBD respectively). After CBD ripples were shorter and smaller and even after split into short and long ripples this effect remained the same for both types (Duration short K-S p=0.0013 all other K-S D p<0.0001), ** p<0.01, **** p<0.0001

To replicate the finding that CBD led to shorter ripples in more animals, we performed acute experiments where animals from the behavioural experiment on their final day received either CBD or Veh, were subjected to urethane anesthesia and were implanted 32-channel silicone probes targeting the hippocampus. Ripples could be detected in the NonREM-like state and we replicated known changes (Buzsáki 2015a; Amzica and Steriade 1998; Steriade and Amzica 1998; Contreras and Steriade 1996) of ripples under urethane (slower and bigger Fig. S4). However, we also confirm that as in natural sleep after CBD administration ripples became shorter and smaller in comparison to vehicle (Fig. 3L). Also, after splitting into short and long ripples, the same effect was seen for both types.

In sum, after CBD there were fewer long ripples and these were less locked to the slow oscillation phase. During especially long ripples, CBD showed less activity in the cortex. In general, CBD led to higher E/I ratio that could have led to the changes in ripple events.

Only in Vehicle did cumulative-learning lead to more long ripples after delta waves

After establishing general effects of CBD on sleep, we next investigated the different memory training conditions in more detail. For this purpose, we analysed NonREM oscillatory coupling, which is known to be a reliable learning marker (Maingret et al. 2016). However, many different types of Delta-Spindle-Ripple couplings have been reported and implicated to be important for memory consolidation. These couplings can be interactions between two oscillations such as delta followed by spindle (Molle et al. 2002), delta followed by ripple (Peyrache et al. 2009), ripple followed by delta (Maingret et al. 2016), and spindles with a ripple in their troughs (Sirota et al. 2003), but also three-oscillation interactions such as delta followed by spindle with a ripple in the trough (Diekelmann and Born 2010), delta followed by ripple then spindle (Genzel et al. 2014b), ripple followed by delta and then spindle (Maingret et al. 2016). Until now, experiments tend to report only on one type of coupling, making it difficult to compare results and create a framework for potential different or common functions.

First, we focused on cortical events that can also be measured in human EEG experiments. Interestingly, the largest condition effect was seen for Delta-Spindle coupling (D-S), where only in the simple learning condition (Stable) there was an increase in D-S events that was not seen in single spindle events and was only weakly present for single delta events (Fig. 4A).

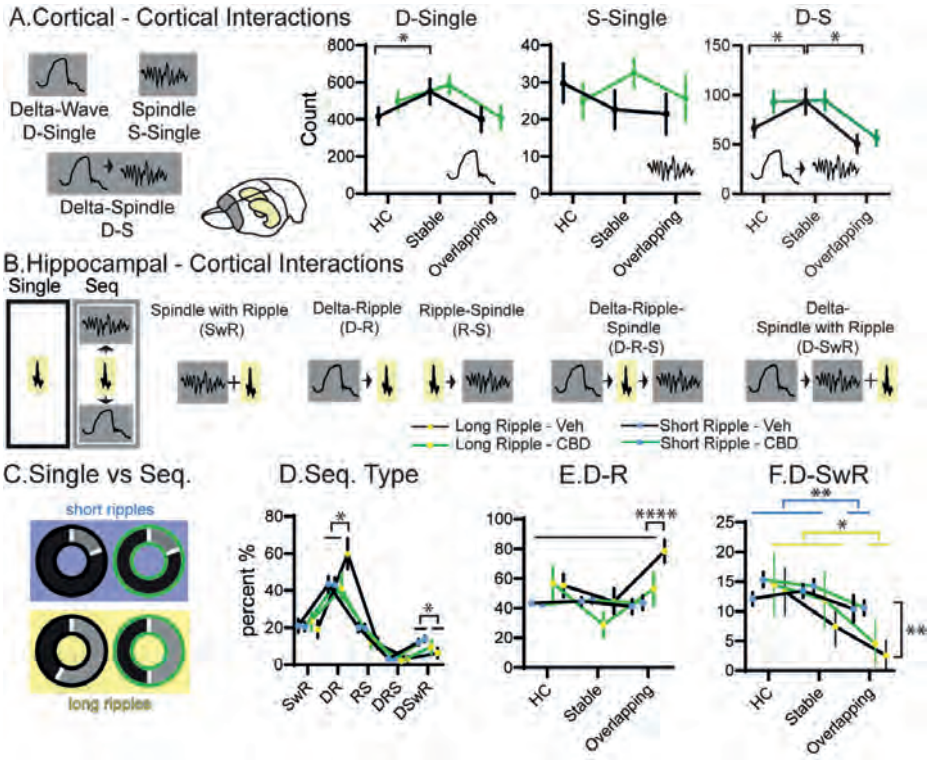

Fig.4 Oscillation interactions: A. Cortical interactions. Oscillations can be single delta (D) or spindle (S) waves, or coupled delta-spindle events (D-S). Counts of events shown for three different conditions home cage (HC), Stable and Overlapping (each rmANOVA with condition and treatment, D-single condition $F_{2,62}$=3.9 p=0.026 other p>0.2, S-Single all p>0.13, D-S condition $F_{2,62}$=6.2 p=0.0034, treatment $F_{2,62}$=3.4 p=0.076, interaction p=0.29, orthogonal comparison run only for condition collapsing treatment). B. shows different types of oscillatory coupling. C. Fraction of short (top blue background) and long (bottom yellow background) that are part of sequences (grey) or occur alone (black). Left vehicle (black edge), right CBD (green edge). There was a significant effect (chi-square 534.2 p<0.0001). D. Fraction of coupled long and short ripples spread over the different interaction types (sequence types $F_{4,160}$=77 p<0.0001, sequence types X ripple types $F_{4,160}$=2.3 p=0.06, other P>0.16). E. as in D fraction of events that are D-R but split for the different conditions (condition $F_{2,204}$=5.2 p=0.006, ripple type $F_{1,204}$=7.2 p=0.0081, treatment $F_{1,204}$=3.1 p=0.08, condition X ripple type $F_{2,204}$=7.4 p=0.008, ripple type X treatment $F_{1,204}$=3.9 p=0.05, other p>0.2), F. same for D-SwR (condition $F_{2,204}$=6.5 p=0.0018, ripple type $F_{1,204}$=5.5 p=0.019 other p>0.18). Only in overlapping an increase was seen in vehicles but not CBD. Orthogonal comparisons * p<0.05, ****<0.0001 n=4.

Next, we analysed different possible combinations of hippocampal-cortical interactions (Fig.4B): Delta-Ripple (D-R), Delta-Ripple-Spindle (D-R-S), Ripple-Spindle (R-S), Spindle with Ripple (SwR) and Delta-Spindle with Ripple (D-SwR). Overall, long ripples were more likely to occur in one of the sequences than alone. In contrast, short ripples were much less likely to occur in a sequence. After CBD, these differences remained, however, now the fraction of long ripples in sequences decreased (Fig.4C). When splitting for sequence type, it became apparent that in vehicles, but not CBD, long ripples occurred more than short ripples as D-R events. In contrast, for both CBD and vehicle short ripples occurred more as D-SwR events than long ripples (Fig.4D). Finally, since long ripples should preferentially be associated to longer reactivations and these in turn would be associated to previous knowledge, we next split the recordings for the different conditions. The increase of long ripples occurring as D-R events shown in panel D was due to a selective increase of these events after the Overlapping in comparison to the Home Cage or simple learning control (Stable) for vehicles and not CBD (Fig. 4E). This condition-selective CBD effect mirrors the behavioural finding that only in Overlapping – testing complex, abstracted memory – animals showed worse performance with CBD. Interestingly, D-SwR events were less in overlapping for both short and long ripples (Fig. 4F).

In sum, long ripples are more likely to occur in sequences than short ripples. After learning a complex memory (Overlapping) a selective increase in the fraction of long ripples that occurred after delta waves was seen and accompanied by a decrease in D-SwR events. The increase in D-R was absent in CBD and potentially led to the behavioural deficit in this memory condition. After the simple memory condition (Stable) an increase in D-S events was seen in both CBD and veh.

Discussion

We investigated the effects of oral CBD intake on memory consolidation and sleep in rats. We could show that CBD extended natural sleep but also led to a higher Excitatory/Inhibitory (E/I) balance, smaller amplitude NonREM oscillations and less long ripples in NonREM sleep. We replicated the shortening of ripples after CBD in more animals recording sleep-like anesthesia states. Only after vehicle treatment, complex learning resulted in an increased fraction of long-ripples following delta-waves, a potential consolidation mechanism. In CBD, this condition-specific increase in oscillation-coupling was not seen and the rat's showed deficits in complex memory expression the next day. In contrast, simple learning led to an increase in delta-spindle coupling events in both vehicle and CBD, and following both treatments

intact memory expression for simple memory was seen the next day. Thus, while we can confirm the beneficial effect of CBD on sleep length, this effect was modest and accompanied by changes in E/I balance and NonREM oscillations that led to a deficit in complex memory consolidation.

CBD and E/I balance

CB1-receptors are found on the presynaptic terminals of interneurons, thus a main neuronal effect of systemically-administrated CBD, should be a change in E/I ratio. Due to this, CBD is currently being handled as a novel therapeutic agent restoring E/I balance in neural models of epilepsy and autism (Khan et al. 2018; Rosenberg et al. 2023) in addition to being used increasingly as sleep medication.

We know that activation of the CB1-receptors with synthetic agonists inhibits hippocampal GABAergic neurotransmission and dysregulates the distribution of interneurons (Hájos et al. 2000; Katona et al. 2000). What remains unclear is in which direction CBD administration changes the E/I ratio. Depending on the dose, CBD can have different effects as functional agonist or as antagonist of the CB1-receptor (Pertwee et al. 2002; Thomas et al. 2007). Thus, actions of CBD are complex and depending on the dose, it can increase or decrease inhibition. High doses increased sleep durations (Carlini and Cunha 1981) and the opposite effect was seen with low doses (Nicholson et al. 2004; Babson, Sottile, and Morabito 2017).

Here, we aimed to model an acute, high-dose intake, as would be recommended in humans to promote sleep (Deiana et al. 2012b). We could replicate the finding that high-dose administration increased total sleep time, even though the effect was modest and was seen as an extension of the NonREM sleep period at the end of the day. It has been previously shown that by analyzing the slopes of the power spectrum, one can determine E/I ratio (Donoghue et al. 2020; Gao, Peterson, and Voytek 2017; Bódizs et al. 2021; Favaro et al. 2023; Gao 2016; Horváth et al. 2022). Interestingly, during NonREM sleep periods CBD led to an increase in E/I ratio (Fig. 3K), likely by decreasing inhibition. This was visible in the prefrontal cortex but was even more evident in the hippocampus. Perhaps the change in E/I balance contributed to the extension of sleep. While a direct relationship between E/I balance and sleep is not yet known, we do know that interneuron activity is necessary for most sleep oscillations as we will expand on next.

CBD and NonREM oscillations

NonREM sleep is dominated by the transition of up- and down-states (states of general neuronal activity and silence, respectively). Large down-states can induce a

delta-wave in the LFP signal, which we can detect. Interestingly, two major classes of interneurons, the parvalbumin and the somatostatin positive cells, tightly control both up-to-down and down-to-up state transitions (Zucca et al. 2017). The activity of these interneurons is needed to initiate and maintain the down-state (Zucca et al. 2017). Further, sleep spindles are associated with local, cortical parvalbumin interneuron activity measured with calcium imaging (Niethard et al. 2018), which again is potentially necessary to maintain this oscillation. Finally, hippocampal ripples are initiated by excitatory, pyramidal cell activity but then maintained by activity of interneurons (Stark et al. 2014a). Thus, any effects of CBD on these sleep oscillations, are likely due to its effect on inhibition. Already previously it had been shown that administration of CB1-agonist disrupts ripples in the hippocampus (Sun et al. 2012; Maier et al. 2012; Robbe and Buzsáki 2009; Robbe et al. 2006).

Here, we could show that CBD affects ripples and other sleep oscillations as well. Deltas and NonREM ripples became slower, spindles and quiet wake ripples faster (Fig. 3 C/D). Importantly, all four oscillations became smaller in amplitude, likely due to decreased entrainment of excitatory neurons by the now attenuated interneurons. E/I ratio was changed more in the hippocampus than in the cortex during sleep and we also saw a more complex picture of CBD interactions with hippocampal NonREM ripples. During ripples normally E/I balance decreases, this decrease was seen less in CBD and, due to higher E/I baseline levels in CBD, event E/I ratio remained higher than the baseline of vehicles. A specific E/I balance is needed for ripple maintenance (English et al. 2017; Buzsáki 2015a; Stark et al. 2015), correspondingly we saw fewer long ripples in CBD. We confirmed this finding in more animals using sleep-like state recordings under anesthesia. Urethane-anesthesia is a well-known model for sleep (Amzica and Steriade 1998; Steriade and Amzica 1998; Contreras and Steriade 1996). Urethane does change sleep oscillations in comparison to their natural-sleep counterparts (Yagishita et al. 2020; Buzsáki 2015b) (Fig. S4), however there are no known interactions with CBD.

Interestingly, those long ripples that remained in CBD showed smaller cortical, wide-band responses in the spectrogram (Fig. 4J). Critically, in CBD the condition-specific increase in coupling of large down-states to long ripples (D-R sequences) after complex learning was absent (Fig. 4E). Likely leading to the memory deficit seen after CBD treatment in this condition. In contrast, while there were E/I differences in the cortex, these were smaller than the differences in the hippocampus. Correspondingly, there was no CBD effect in the count of cortical oscillation-coupling events (delta-spindle, Fig. 4A), that were associated to simple-learning, and CBD also did not lead to deficits in this memory condition.

In sum, by changing excitatory and inhibitory neuronal balance, CBD led to changes in hippocampal-cortical oscillations and coupling resulting in deficits consolidating complex learning events. In contrast, within-cortex coupling remained intact, which likely was why CBD treated rats did not show deficits in simple learning.

CBD and NonREM sleep

In our study CBD led to more NonREM sleep, but this effect was modest and only visible at the end of the recording period (few hours before light off). CBD has been proposed to be a treatment chronic pain, anxiety, epilepsy and sleep disturbances (Blessing et al. 2015; Urits et al. 2020; Khan et al. 2018). CBD as well as CB1-receptor agonist have been shown to promote sleep in multiple, pre-clinical studies (Coronado-Álvarez et al. 2021; Murillo-Rodríguez et al. 2018; Murillo-Rodríguez, Machado, et al. 2021; Murillo-Rodríguez et al. 2016; Murillo-Rodríguez, Millán-Aldaco, et al. 2021; Shannon et al. 2019). A clinical study in patients with neuropsychiatric disorders showed that CBD administration improved sleep quality in more than half of the patients up to a month after administration and alleviated anxiety symptoms (Shannon et al. 2019). However, the effect of CBD on sleep is dose-dependent with high doses increasing sleep duration (Carlini and Cunha 1981) and the opposite effect is observed with low doses (Nicholson et al. 2004). CBD may also have different effects in aged population in contrast to young adults and adolescence (Murillo-Rodríguez et al. 2020). A recent meta-analysis criticized that rigorous, controlled evidence for the therapeutic efficacy of CBD is lacking for many health conditions, but they also found that the utility of CBD to treat epilepsy is well supported (Sholler, Schoene, and Spindle 2020) while additional controlled trials are needed to elucidate the efficacy of CBD as a sleep aid given that findings in this area appear to be mixed (Carlini and Cunha 1981; Chagas et al. 2014; ScottShannon and JanetOpila-Lehman 2016; Sholler, Schoene, and Spindle 2020). However, in one study (Carlini and Cunha 1981) participants received oral placebo or CBD capsules (40, 80, or 160 mg); those receiving 160 mg CBD had a longer duration of sleep – similar to our finding – while all CBD doses decreased remembrance of dreams relative to placebo (Carlini and Cunha 1981).

Oscillation-Coupling for Memory Consolidation

At this point we do know that ripples, spindles and delta-waves in sleep are important for memory consolidation. Further, for some of their functions these oscillations are coupled to each other, as reported by many researchers. Surprisingly, no two studies seem to be in agreement, which coupling is the one to look for. Reported couplings can be interactions between two oscillations (Molle et al. 2002; Peyrache et al. 2009; Maingret et al. 2016; Sirota et al. 2003), but also three-oscillation interactions

(Diekelmann and Born 2010; Genzel et al. 2014b; Maingret et al. 2016). Experiments tend to report only one type of oscillation, and different types of memories as well as different coupling events are never directly compared.

Here, we systematically investigated all these couplings directly comparing a non-learning control (HC) to both a simple and complex memory condition. By contrasting these different conditions, we can extrapolate, which effects are driven by which type of learning or experience (Navarro-Lobato et al. 2023; Schut et al. 2020; Genzel et al. 2019). Changes induced by all OS conditions in comparison to Home Cage, should represent more general experience-dependent effects enabling simple memory consolidation or homeostasis (Navarro-Lobato et al. 2023; Schut et al. 2020; Genzel et al. 2019). Changes seen only after simple learning, would represent consolidation of simple memories, already reinforced during training (Navarro-Lobato et al. 2023; Schut et al. 2020; Genzel et al. 2019). In contrast, changes seen specifically after complex learning should represent semantic-like memory consolidation and the comparison and integration of new and old information (Navarro-Lobato et al. 2023; Schut et al. 2020; Genzel et al. 2019).

Interestingly, here we can show in vehicles that simple learning led to more Delta-Spindle events (Fig.4 A). In contrast, in complex learning the fraction of long-ripples following a delta-wave increased while the fraction of ripples occurring during a spindle after a delta wave (D-SwR) decreased (Fig. 4E/F). We had already previously observed that the increase of delta and spindles and their coupling was linked to simple experiences (Aleman-Zapata, Morris, and Genzel 2022), and this coupling is most often reported by researchers investigating human subjects with simple, associative memory paradigms such as paired-associative word-list or reinforced spatial learning (Bastian et al. 2022; Diekelmann and Born 2010). In contrast, ripples following delta are reported, for example, in experiments focused on prelimbic reactivations when animals learn complex rules (Peyrache et al. 2009). It would be tempting to speculate that Delta-Spindle and Spindle with ripple coupling would facilitate simple learning while Delta-Ripple would correspond to consolidation of complex memories relying during consolidation on the comparison to other previously encoded experiences. Our current results support this, since after CBD the condition-specific Delta-Ripple increase was absent but Delta-Spindle coupling remained the same, and correspondingly CBD only induced a memory-deficit in the complex and not simple memory condition.

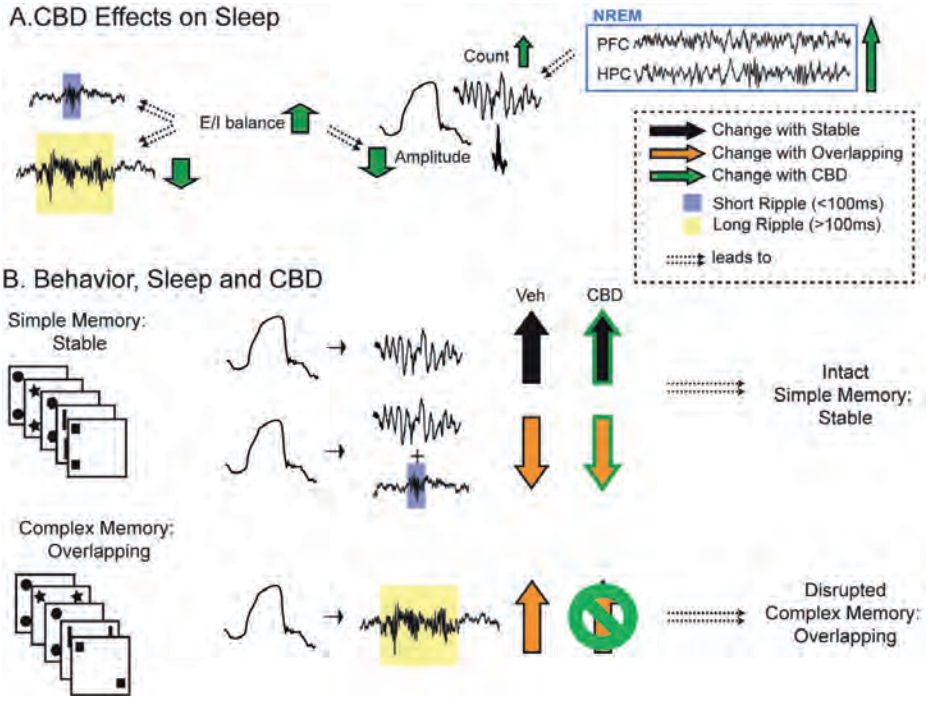

Fig.5 Summary: A. General CBD effects on sleep. CBD increases excitatory/inhibitory balance (E/I), which (potentially) leads to less long ripples and smaller NonREM oscillations. CBD also extends NonREM sleep and thus increases the number of NonREM oscillations at the end of the sleep period. **B.** Behaviour, Sleep and CBD effects. After simple memory training (condition Stable) there is an increase in Delta-Spindle events in vehicle (VEH), which is unchanged in CBD. After complex memory training (condition Overlapping) there is a higher fraction of long-ripples following delta waves, but a smaller fraction of both short and long ripples during spindles following delta waves. The former but not latter effect is abolished with CBD, leading to disrupted complex memory consolidation in CBD.

In conclusion, by combining oral CBD administration with the Object Space Task and electrophysiological recordings, we could show that CBD leads to 1) extension of NonREM sleep, 2) increased E/I balance, 3) smaller NonREM oscillations, and 4) fewer long ripples and less delta-long ripple coupling (Fig.5). The latter leads to 5) deficits in complex-memory consolidation but simple memory remains intact after CBD in young adult rats.

Limitations of the study

There are some limitations to be considered in this study. While most ripples occurred during sleep, some were during quiet rest and these also showed changes with CBD. Thus, we cannot conclusively determine if the effect of CBD on memory consolidation is solely due to sleep or also consolidation during rest. Here we examine the role of

an acute high dose CBD known to be beneficial for sleep. However, many users would use a chronic dosage which may show different interactions with sleep and memory.

We confirmed our ripple findings with recordings under anesthesia. While urethane anesthesia is a known model for sleep-like stages and oscillations, there are known differences that we also see here (Fig. S4). However, there was no interaction with CBD and we did replicate the findings shown in natural sleep recordings.

Finally, we investigate young adult rats (3-4 months at start of experiment). Due to potential differential effects of age of CBD (Murillo-Rodríguez et al. 2020; Murillo-Rodríguez, Millán-Aldaco, et al. 2021), these results may be specific to our targeted age group.

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Materials and methods

Study design

A total of 48 rats were used in this study: 44 rats were used for behavioural experiments and four rats were used for chronic electrophysiological recordings. All rats were first extensively handled for multiple days until they experienced minimal stress while working with the experimenters (see handling videos on www.genzellab.com). The first batch of 44 rats was first habituated to the oral feeding regime and the behavioural training box for a week. In the following week, they were orally administered with >98% trans-cannabidiol (CBD) or vehicle (VEH) (counterbalanced across animals) and trained in the Stable and Overlapping condition of the Object Space task (all conditions run cross-over within each animal) in smaller sub batches of 14-16 animals (maximum 8 in one day).

The 4 implanted rats underwent surgery for wire drive implantation. After surgery, they were allowed to recover for up to 2-3 weeks, during which the wire arrays were slowly lowered to reach the pyramidal layer in the CA1 region of the hippocampus and the rats were habituated to the behaviour training box and the oral feeding regime. Upon reaching the target, we started the behavioural experiments. Similar to the behaviour batch, these rats were orally administered with either CBD or VEH and then trained in the Object Space Task (all conditions cross-over within subject). Brain activity was recorded during exploration and rest periods.

Animals

Six- to eight-week-old male Lister Hooded rats weighing between 250-300g at the start of the experiment (Charles Rivers, Germany) were used in this study. They were pair-housed in conventional Eurostandard type IV cages (Techniplastic, UK) in a temperature-controlled (20 + 2 °C) room following a 12h light/dark cycle with water and food provided *ad libitum*. The first batch of 44 rats was kept under these conditions until the end of the experiment. The batch of chronic rats were single-housed after wire drive implantation and kept in these conditions until the end of the experiment. A total of 48 rats were used in this experiment: 44 in the behavioural experiment (3 sub batches of n=16, 14 and 14), and four for chronic electrophysiological recordings (two more served as implant-pilots but were not included in the study). The behavioural experiments and electrophysiological recordings were performed during the light period (between 9:00-18:00). All animal procedures were approved by the Central Commissie Dierproeven (CCD) and conducted according to the Experiments on Animals Act (protocol codes, 2016-014-024 and 2020-0020-006).

CBD

(-)-trans-Cannabidiol (CBD, > 98%) was obtained (https://www.cbdepot.eu/products/cannabidiolum-gmp) for all experiments. Rats were treated with either CBD (120 mg/kg in 300 µl flavoured olive oil, p.o.) or vehicle (300 µl flavoured olive oil, p.o.). Different flavouring agents, namely, vanilla, cinnamon, star anise and clove, were used to make multiple flavours of olive oil, which were then used to mask the taste of CBD, so that the rats would always be naïve to what they were fed. The use of all flavours was counterbalanced, such that each rat received all flavours for both CBD and VEH. CBD solution was always freshly prepared in one of the flavoured oils prior to oral administration. In order to prepare the solution, first the amount of CBD to be administered was determined based on the rat's weight and the compound was weighed accordingly. The flavoured oil was heated up to a temperature of 50-60°C and then the CBD compound was slowly dissolved into it. This process would take a few minutes until a clear solution was obtained, which is indistinguishable from vehicle oil. Individual syringes with 300 µl CBD or VEH were prepared for each rat. The experimenter who performed the oral administration was blinded to the treatment each rat received, and the drug was always administered an hour before the start of behavioural training. Further, the experimenters performing the behavioural training and electrophysiological recordings were blinded to the treatment of the animal as well. Previous studies have shown oral administration of CBD to be effective in crossing the blood brain barrier and plasma concentrations are shown to reach their maximum around 4-6 hours after oral administration, which is optimum time window to examine its effect on memory consolidation (Deiana et al. 2012a; Hložek et al. 2017).

Behaviour - Object Space Task

The Object Space Task was used in this study to assess the effects of CBD on simple and semantic-like memories (Genzel et al. 2019). This task is based on the tendency of rats to explore novel object locations in an open field arena across multiple trials. The animals were extensively handled by the experimenters in the first week to minimize their stress levels during the interactions. Next, they were habituated to the training box (75 x 75 cm) for five sessions over five days. On the first day, they spent 30 min in the training box with their cage mates. On the second and third days, each rat individually spent 10 mins exploring the training box. For the final 2 days, two objects (made from DUPLO blocks, not used in main experiment) were placed in the center of the box and the rats were allowed to explore for 10 mins each day.

A total of 44 rats were trained in the Stable and Overlapping conditions of the task and ran in smaller sub batches of 8. The condition sequence and object locations were

counterbalanced across all rats and the experimenter was blinded to the conditions and drug treatment. We followed a within-subject design, wherein every rat performed all conditions of the task with CBD and VEH.

For rats, running one round of one condition took two days – a training day and a test day 24 hours later. The rats were first orally administered CBD or VEH and then started with behavioural training an hour after drug administration. The training day consisted of five trials, 5 mins each with an inter-trial interval of ~45-50 mins. During each trial, the rat was placed in the training box to explore a pair of identical objects at fixed locations. The object pairs were changed from trial to trial. Additionally, to prepare for one session of training and test, the training box was equipped with 2D and 3D cues to create a unique spatial environment for the rats to orient themselves. This box setup remained the same for one session of training and test, and was changed as much as possible for the next session, in order to create a distinctly different environment each time. In the Stable condition, the positions of the objects remained the same across all trials. 24 hours later, during the test trial, one object was moved to a different position. This condition allowed us to assess simple memory in the rats. For training in the Overlapping condition, one position of the object remained constant across all trials whereas the other kept changing. However, the object positions in the final trial of the training day and the test day, 24 hours later, were kept the same. If the rat formed a cumulative memory of one object position constantly moving across multiple trials on the training day, he would spend more time exploring that position. This would let us assess semantic like memory in the rats. Implanted animals additionally had Home Cage days, where they would have the same daily regime as on a training day but instead of being placed in the training box for the 5 min training periods, the animal was kept awake in the home cage. Therefore, these days are non-learning controls that are controlled for general wake activity/sleep disturbances of training and time of day.

All behaviour sessions were recorded using a webcam placed above the training box. The object exploration times were scored online in real time during the trials using a scorer program developed in the lab for training and scoring (https://github.com/NeuroNetMem/score). Whenever a rat would sniff or climb on or interact with an object, it was scored as an exploring behaviour. Obsessive chewing or biting of an object was not considered as an exploratory behaviour, and extra care was taken to avoid using objects that would trigger these behaviours. The scoring data from the behaviour sessions was saved in an Excel sheet and was used for further analyses.

Bilateral wire drive implants

A customized lightweight wire drive implant was manufactured in collaboration with 3Dneuro (https://www.3dneuro.com/) to implant bilateral wire arrays in the prelimbic cortex and the CA1 region of the hippocampus. Customized individual wire arrays (Science Products, catalog no. NC7620F) were first built for each brain region. The wire arrays for the prelimbic cortex consisted of four wires of the same length glued to each other; for the hippocampus, the array consisted of four wires of different lengths with a 70° angle between them to enable recording from the pyramidal layer as well as from below and above the layer. These arrays were then glued to polyamide tubes which were glued to the 3D printed drive body. The drive body was designed according to the Rat Brain Atlas in Stereotaxic Coordinates (Büttner-Ennever 1997) to ensure the correct placement of arrays in the regions of interest. Additionally, a shuttle and screw were included in the drive body design for the hippocampal arrays, so that they could be turned down after implantation to reach the target layer. Finally, the drive body was designed to enable recording of each region from both hemispheres. Once all the arrays were loaded into the drive body, their free ends were connected to a customized 32 channel electrode interface board (EIB) using gold pins (Neuralynx). A single NPD dual-row 32 contact omnetics connector was attached to the EIB to connect the headstage for later recordings. The hippocampal wire arrays were flushed with the polyamide tubes before implantation. The bottom of the drive was deepened in 70% ethanol for at least 2 hours before implantation into the brain.

Drive implant surgery

Shortly before the start of surgery, all rats received a subcutaneous (sc) injection of carprofen (5mg/kg) to serve as an analgesic. After placing the rats into the stereotaxis, they received a subcutaneous injection of a mixture of 4 mg/kg lidocaine and 1 mg/kg bupivacaine in a 0.9% NaCl physiological serum locally at the skin surface above the skull as a local analgesic. Finally, they also received a 2 ml of 0.9% NaCl physiological serum subcutaneous injection at the start and end of surgery. Since this was a recovery surgery, utmost care was taken to maintain the most sterile conditions possible and the entire surgery was performed under isoflurane inhalation anesthesia. Additionally, the rats were administered a 10 mg/kg subcutaneous injection of Baytril antibiotic at the beginning of surgery to prevent postsurgical infections. Two pairs of holes were drilled bilaterally to reach the following targets – prelimbic cortex (AP +3.5mm and ML +-0.5mm) and hippocampus (AP -3.8mm and ML +- 2mm). Additionally, a ground screw (M1x3mm) was placed on the right hemisphere of the cerebellum. Three more M1x3mm supporting screws were placed and bound to the skull using Superbond C&B dental cement (Sun Medical, Japan). Upon drilling all holes above the target regions, the dura mater was carefully

removed, exposing the surface of the brain. Finally, the wire-drive was carefully positioned on the brain's surface, such that the wire arrays fit well in the drilled holes. On finalizing the position, the drive was attached to the skull and screws by simplex rapid dental cement (Kemdent, UK). The wire arrays for HPC were slowly lowered (~1.5mm DV from brain surface) to a layer close to the pyramidal layer in the CA1 region. The pyramidal layer was reached progressively in the subsequent days during signal checks in the rats' recovery period. With one out of the 4 rats, we were not able to reach the pyramidal layer. This rat was thus included only for analysis of cortical events and sleep architecture analyses.

In vivo electrophysiological recordings

The animals were closely monitored for a week following the drive implant surgery to ensure they had a good recovery. Their weights were monitored daily during this period to ensure a stable growth curve. The rats were allowed to recover for a couple of weeks following surgery before starting with recordings. Prior to the surgery, the rats were habituated to the sleep boxes, chocolate treats and the oral feeding regime, to minimize any stress post-surgery. During the recovery period, they were re-introduced to the sleeping boxes and their local field potential (LFP) activity was monitored during wake and rest periods to assess the placement of the wire arrays. The HPC arrays were further lowered in small steps during this period and the correct placement was confirmed with the LFP activity. Finally, after a week of recovery, the rats were also habituated to the Object Space box according to the habituation protocol described above.

Once all arrays were in the target regions, recordings were started while the rats were being trained in the Object Space task and during the in between rest periods. The feeding and training protocol that was followed here was similar to the one described above. Each rat had a session per experimental condition (Homecage, Stable, Overlapping) with prior administration of CBD or VEH. In the end, every rat ran each session, once with CBD and another with VEH. The sequence of conditions was counterbalanced for every rat and the experimenter scoring the behaviour was blinded to which drug treatment the rat was assigned to.

The LFP and accelerometer activities detected by the channels were amplified, filtered and digitized through a 32 channel headstage (IntanTechnology) connected through an Intan cable and a commutator into the Open Ephys acquisition box (Siegle et al. 2017). The signal was visualized using the open source Open Ephys GUI at a sampling rate of 1 Hz – 30 kHz.

Electrolytic lesions

After the final recording session, the animals received electrolytic lesions under isoflurane inhalation anesthesia 48h before perfusion to identify the electrode tips placement. To this end, a current of 8 µA for 10 s was applied in two wires per array using a stimulator.

Acute recordings

At the end of behaviour recordings, 30 rats were used to carry out acute electrophysiological recordings to monitor the effects of CBD on sleep like states under anesthesia using silicon probes. The rats would receive an oral administration of CBD or VEH (120 mg/kg) at ~10 am and 30 mins later would receive an i.p. injection of urethane anesthesia (1.4g/kg). After injection, we would wait for the next 30 mins for the anesthesia to set in and start with surgery at ~11 am. The aim was to start with surgery an hour after oral administration of CBD/VEH.

Stereotaxic surgery

Shortly before the start of surgery, all rats received an s.c injection of carprofen (5mg/kg) to serve as an analgesic. On setting the rat into the stererotax, they further received an s.c. injection of a mix of 4 mg/kg lidocaine and 1 mg/kg bupivacaine in a 0.9% NaCl physiological serum locally at the skin surface above the skull as a local analgesic. Lastly they also received a 2 ml of 0.9% NaCl physiological serum s.c. injection at the start and end of surgery. The target areas for the recordings were medial prefrontal cortex (mPFC) and hippocampus (HPC) with the following coordinates – AP = 3.5mm, ML = 0.5mm and DV = 2.6mm (from brain surface) for PFC and AP = -3.2mm, ML = 2mm and DV = 4.3mm (from brain surface) for HPC. All coordinates were calculated with respect to standard bregma and lambda coordinates (BÜTTNER-ENNEVER, 1997). Two craniotomies (2x1 mm and 1x1 mm for PFC and HPC, respectively) were drilled above the target areas on the right hemisphere and a hole in the left cerebellum for the ground screw. Finally, once the craniotomies were cleaned and the dura mater was clearly visible, the silicon probes were placed in position and lowered slowly to both targets. The tips of the probes were coated with DIL stain (catalog no. D282) before lowering to facilitate better visualization of the electrode damage in the target region during histology.

Recordings

The surgery setup was enclosed in a Faraday cage to prevent any electrical noise in the recordings. Both silicon probes (32 channels in each probe) were connected to an OpenEphys acquisition box (Siegle et al., 2017). The signal was visualized using the OpenEphys GUI and recorded at a sampling rate of 30kHz. Signals from both

brain regions were recorded for 6-7 hours and we checked every hour whether the craniotomies were hydrated and the temperature of the heatpad remained constant. At the end of the recordings, the animals were sacrificed via transcardial perfusion and the brains were extracted for histology analyses.

Histology

Brain processing

Rats were sacrificed via transcardial perfusion 48 hours after the electrolytic lesions. They were overdosed with 150 mg/kg sodium pentobarbitol ip prior to perfusion. They were perfused first with 100 ml of 0.1 M phosphate-buffered saline pH 7.4 (PBS) followed by 250 ml of 4% paraformaldehyde (PFA) made in 0.1 M PBS. After extracting the brains, they were kept overnight in PFA at 4°C. The brains were rinsed in 0.1 M PBS the next day (3 X 10 mins) and then kept in a solution of 30% sucrose, 0.02% NaN_3 in PBS for cryoprotection. Once the brains sank to the bottom of the vial, they were frozen in dry ice and stored for the long term in a -80°C freezer. For further processing, the brains were sectioned in a cryostat (SLEE medical, Germany) and 50 micron coronal sections of target regions were obtained and collected in 48-well plates containing 0.02 % NaN_3 in PBS and stored at 4°C.

Nissl Staining

Coronal sections containing the prelimbic cortex (PFC) and hippocampus (HPC) were sequentially mounted (in increasing AP coordinates) on gelatin-coated slides and incubated overnight at 37°C. The following day, the slides were further processed for Nissl staining. Slides were first hydrated in 0.1 M PBS and then in Milli Q water for 20 min each. Next, the slides were stained in 0.7% acetate Cresyl Violet for 20 min and dehydrated in an increasing ethanol gradient (water for 3 min, 70% ethanol for 20s, 96% ethanol + acetic acid for 45s, 100% ethanol for 5 min). Finally, in the last step, the slides were immersed in xylene for 15 min and then then mounted with DePeX mounting medium and covered with coverslip. Lesions from wire arrays were then observed under a bright field microscope (LEICA DM IRE2) and images were taken in 5X and 10X magnification.

Behavioural Data Analyses

The amount of time the rats spent exploring objects was scored in real time which would then get saved in an excel sheet. The total exploration time was calculated as the sum of time spent exploring both objects. Further on the discrimination index (DI) was calculated by subtracting the amount of time exploring the familiar location from the novel location and dividing by the total exploration time. DI > 0 indicated

a preference for the novel object location, which was used as a prime measure for memory performance. DI = 0 indicated no preference for either location and finally DI < 0 indicated a preference for the stable object location. Data was analyzed with rmANOVA (within subject factors treatment, condition).

Local field potential analysis

Here, we hereby describe the methods followed to process and analyze the local field potential data acquired during chronic and acute recordings. The scripts used to implement this can be found in the following github repository https://github.com/genzellab/cbd. All the recorded data is in the Donders repository (di.dcn.DAC_626830_0008_841). A downsampled version of the LFP data can be found at https://osf.io/fwshb/.

Scoring of sleep in chronic recordings

Chronic recordings from the rest periods across all study days were further classified into different sleep/wake states using a sleep scoring GUI called TheStateEditor in MATLAB developed by Dr. Andres Grosmark (Grosmark 2014). As the first step, one PFC and HPC channel and three accelerometer channels were selected per animal as input into the scorer interface. One could then visualize the frequency spectrograms and bandpass filtered LFP signal per brain region and the motion spectrogram during the recording period. Using this information, an experienced researcher scored the recording data in increments of 1 second epochs into one of the following states – wakefulness; NonREM; Intermediate; REM. The researcher scoring the datasets was blinded to condition and treatment for that respective study day. Absence of movements in certain periods in the motion spectrogram further aided in discriminating between sleep and wake periods. Desynchronized activity in PFC and HPC accompanied with movement in the motion spectrogram was classified as a wake state. NonREM sleep was classified when slow oscillations (0.5-4 Hz) were detected in the PFC channel accompanied by lack of movement in the motion spectrogram. REM sleep was classified when we detected desynchronized activity in the PFC channel and dominant periodic theta activity (6-8 Hz) in the HPC channel accompanied by lack of movement in the motion spectrogram. Intermediate sleep was defined as short transition periods between NonREM and REM states which were characterized by irregular theta activity in the HPC channel and high frequency desynchronized activity in the PFC channel accompanied by lack of movement in the motion spectrogram.

Scoring of sleep-like states in acute recordings

Sleep-like stages were classified from the recordings of anesthetized rats. Considering the absence of wakefulness during anesthesia, an automatic state classifier was designed to detect NonREM-like and REM-like states based on the spectral features of the cortical and hippocampal recordings. The spectral power of selected hippocampus and prefrontal cortex channels was estimated from 0 to 100 Hz, with a 0.5 Hz step, using a multitaper filter with a time-bandwidth product of 4 in 10-second-long epochs. Epochs with artifacts were detected using the isoutlier Matlab function on the absolute value of the epochs' amplitudes. Artifacts were removed and blanked with the mean of the signal. A Principal component analysis (PCA) was employed to identify spectral features of the epochs that characterized the two sleep-like states and explained most of the variance in the data. The following parameters were used as an input for the PCA: Slow oscillation power (0.1-1 Hz), delta power (1-3 Hz), theta power (3-6 Hz), low beta power (10-20 Hz), low gamma power (30-45 Hz), high gamma power (55-80 Hz), ripple power (90-300 Hz), theta-slow oscillation ratio and amplitude of the 10-second-long epoch. The frequency ranges used here and in the ripple detection were intentionally lower than those reported in the literature since urethane anesthesia has been shown to slow down the brain activity (Murillo-Rodriguez et al, 1998). The first and second principal components were kept, given that they explained most of the variance, and a K-means clustering algorithm with two partitions was computed in the PC1-PC2 state space to identify the epochs that belonged to the NonREM-like and the REM-like state clusters. The first PCA component (PC1) contained high weights for features like the epoch amplitude and slow oscillation power in both brain areas, which are expected to be high in the NonREM stage during natural sleep. Therefore, the cluster of epochs found containing higher values for PC1 was labelled as NonREM-like sleep, while the remaining cluster was labelled as REM-like sleep.

Sleep architecture analysis

Based on the manual scoring of the states, we computed the total sleep time (TST), wake, and total time spent in different sleep states (NonREM, REM and Intermediate) per session in MATLAB. Averages for these variables were further calculated across all sessions per rat and the mean and SEM was computed per treatment. Additionally, % of TST spent in NonREM, REM and intermediate was calculated in 45 min time bins for the 3-hour rest periods after the final trial. Furthermore, the distribution of bout duration per sleep stage was calculated for every session across different treatment groups. A bout was defined as a continuous period spent in a specific sleep state and only bouts longer than 4 seconds were considered for analysis. The state of switching from one sleep stage to another was defined as transitions. Using MATLAB,

we computed the number of bouts per sleep stage, the duration of the bouts, and transitions between sleep stages, for example, number of NonREM-REM and REM-NonREM transitions. This was computed per session for each rat and later averaged for CBD and VEH treatment groups across rats. The MATLAB scripts for this analysis can be found at https://github.com/genzellab/sleep_architecture.

Preprocessing of chronic recordings

A prelimbic cortex channel with large slow oscillations during NonREM sleep was selected per rat. A hippocampal channel from an electrode placed in the pyramidal layer was selected based on the quality of ripples visually detected on the channel. The channels were downsampled from 30 kHz to 2.5 kHz by first employing a 3rd order zero-phase Butterworth low pass filter with a cutoff of 1.25 kHz to prevent aliasing of the signal. Artifacts observed during NonREM bouts were subject to a blanking method, which consisted of applying an amplitude threshold determined visually. Artifacts crossing the threshold were "blanked" by replacing them with the mean value of the NonREM bout. For some types of artifacts, a preceding build-up period of one second was also blanked. The artifact removal was done after the signal had already been bandpass-filtered in the corresponding frequency range used for the detection of ripples, spindles or delta events. This prevented artifacts due to discontinuities.

*Preprocessing of acute recordings*The best channel in the hippocampal CA1 pyramidal layer was chosen per rat. The selection criteria consisted of selecting the channels in which ripples were visually more prominent. The channels were downsampled from 30 kHz to 600 Hz to be used for local field potential analysis. A 3rd order Butterworth low pass zero-phase filter with a cutoff of 300 Hz was used to prevent aliasing before downsampling the signal. The first 15 minutes of the recordings were discarded to control the brain signal instability after the probe implantation. Artifacts were detected and removed by applying thresholds to the sum of absolute values of the unfiltered recordings for the selected hippocampal and prefrontal channels. When an artifact was detected, a buildup of 0.5 seconds prior to the artifact and a washout period of 3.5 seconds after the artifact were removed and replaced with the mean value of the artifact-free signal. Before "blanking" the artifact, we bandpass-filtered the channels which would be used for the detection of ripples in the 90-200 Hz range. This was made to prevent the addition of spurious high frequency events which occur due to filtering signal discontinuities after the artifact blanking.

*Detection of hippocampal ripples in chronic recordings*To detect NonREM ripples the downsampled channels (2.5kHz) of the hippocampal pyramidal layer were loaded

into the MATLAB workspace and their NonREM bouts were extracted. Using a 3rd order zero-phase Butterworth bandpass filter, the NonREM epochs of HPC signal were filtered to a frequency range of 100-300 Hz. A custom MATLAB function was used for detecting the start, peak and end of the ripples by thresholding voltage peaks which lasted a minimum duration of 20 ms above the threshold. The start and end of the ripple were determined as half the value of the detection threshold. A closeness threshold of 20 ms was used to count ripples occurring within the proximity of each other as a single event. The detection threshold was determined by computing the standard deviations of concatenated NonREM bouts individually for pre and post sleep trials in a study day. These standard deviations were multiplied by a factor of 5 and an average was calculated to find a single detection threshold per study day. An offset of 5 μV was added to the threshold to reduce false positives. Only pre- and post-trial sleep periods with more than 3 minutes of NonREM were included. This process was repeated for all study days pertaining to all rats in both treatment groups. The detections were then grouped as long (>100ms) or short (≤100ms) ripples based on their duration, in accordance with Fernandez-Ruiz et al. 2019.To detect quiet wake ripples a similar procedure was followed, with the exception that we used signal bouts from the pre- and post-sleep periods manually labelled as wakefulness instead of NonREM.Moreover, we included thresholds for the resultant accelerometer signals, theta power (5-10 Hz) and high-amplitude artefacts to remove epochs that could lead to false positive detections due to movement or noise.

*Detection of hippocampal ripples in acute recordings*To detect hippocampal events, we used the bandpassed channels of hippocampus CA1 pyramidal layer (90-200 Hz). Both raw and filtered versions of the hippocampal channels were displayed in a graphical user interface, which displayed an amplitude threshold for ripples on the filtered CA1 pyramidal layer channel. Thresholds were adjusted for each rat after visually verifying the correct detection of ripples. The threshold values were set in terms of the floating-point number of standard deviations with respect to the mean of the filtered signals. All selected thresholds were close to five times the standard deviation of the filtered hippocampal signal. Once the thresholds were determined, we ran the detection of ripples by thresholding voltage peaks which lasted a minimum duration of 50 ms above the threshold. The start and end of the ripple were determined as half the value of the detection threshold. A closeness threshold of 80 ms was used to count ripples occurring within the proximity of each other as a single event. We only used the detections that occurred during the NonREM-like stage.

*Detection of cortical spindles and delta waves*A downsampled prelimbic cortex channel (2.5kHz) with large slow oscillations was selected and using a 3rd order zero-phase

Butterworth filter the signal was filtered to 9-20 Hz for detecting spindles and to 1-6Hz for detecting delta waves. The NonREM bouts were then extracted from the filtered signal and concatenated. The functions FindSpindles and FindDeltaWaves from the Freely Moving Animal (FMA) toolbox(http://fmatoolbox.sourceforge.net) (Zugaro) were modified to adapt the thresholds for optimal detections and were then used to detect the start, peak and end of spindles and delta waves, respectively. The optimal threshold was found for each rat by visually inspecting the detections and modifying the default parameters of the functions when needed. The results were saved as timestamps in seconds with respect to the concatenated NonREM signal. They were then used to find the timestamps with respect to the recorded signal. This process was repeated for pre- and post-trial sleep periods with more than 3 minutes of NonREM in study days pertaining to all rats in both treatment groups.

*Detection of oscillation sequences*The sequences between ripples, spindles and delta waves were counted in various combinations to study cortico-hippocampal coupling during NonREM sleep as done by (Maingret et al. 2016) Maingret et. al. 2016. The time difference between the peaks of these events was compared to a fixed duration to establish if there was a sequential relationship in the following combinations of oscillations: Delta-Spindle (D-S), Delta-Ripple (D-R), Ripple-Spindle (R-S), Delta-Ripple-Spindle (D-R-S), Delta-Spindle co-occurring with Ripple (D-SwR). For D-S a sequence was considered when the interevent interval was between 100-1300 ms, for D-R it was 50-400 ms and for R-S it was 2-1000 ms. To find D-R-S sequences, the results of D-R and R-S were compared to find ripples preceded by a delta wave and followed by a spindle. To find the D-SwR sequences, the results of D-S and spindles co-occurring with ripples (see next subsection) were matched to find spindles preceded by a delta and co-occurring with a ripple. The results were saved as counts of each sequence for each pre- and post-sleep trial. This analysis was then repeated using timestamps of long and short ripples separately.

*Co-occurrence between ripples and spindles.*The co-occurrence between ripples and spindles was computed by comparing the start and end timestamps of both events. To consider co-occurrence between a ripple and a spindle, either one of the following conditions had to be fulfilled: 1) A ripple had to start and end within the duration of the spindle. 2) One of the events had to start or end within the duration of the other. Given that more than one ripple could co-occur with the same spindle, we counted separately ripples co-occurring with spindles and spindles co-occurring with ripples. This analysis was then repeated using timestamps of long and short ripples separately.

*Slow oscillation phase analysis*The downsampled PFC signal (2.5kHz) was filtered to the 0.5-4 Hz range using a 3rd order Butterworth zero-phase bandpass filter and its Hilbert transform was computed to find the phase angle of slow wave oscillations in the range of 0° to 360°. The peaks of long and short ripples were then used to find the corresponding phase and this process was repeated for all study days pertaining to all rats.

Oscillations characteristics The traces of each detected event (ripples, spindles, delta waves) were extracted using the start and end timestamps obtained from the detectors. The traces of the events were filtered in their corresponding detection frequency band. Characteristics such as the amplitude and mean frequency were calculated for these filtered events using built-in and custom MATLAB functions. Namely, the amplitude of the events was calculated by computing the envelope of the filtered trace using a Hilbert transform. The absolute value of the result was taken and its maximum was found. The mean frequency of the filtered traces was computed using the meanfreq function of MATLAB.

Power spectra & power spectral density slope and offset analysis

Power spectra of HPC and PFC and slope/offset analysis of power spectral density (PSD) were computed for chronic recordings. They were computed for both VEH and CBD treatments. Baselines were randomly selected NonREM periods of 4 seconds. First, the Fieldtrip toolbox (Oostenveld et al., 2011)(Oostenveld et al. 2011) was used to compute PSD. PSD was computed from 0 to 100 Hz in steps of 0.25 Hz. A Hanning window taper was used with a length of 4 seconds and a 0.25-second overlap. Line noise of 50 Hz was removed using a Notch filter. To generate power spectra, the PSD values were computed as logarithms with a base of 10. To calculate the slope and offset, the 'Fitting Oscillations and One-Over-f (FOOOF)' Python toolbox was used (Donoghue et al., 2020)(Donoghue et al. 2020). The minimum peak height threshold was set to 0.05 (units of power – same as the input spectrum), and peak width limits were between 1 Hz and 8 Hz. The outputs were in logarithms with a base of 10.

The resulting values of slopes and offsets were analyzed using IBM SPSS Statistics (Version 29)(IBM-Corp 2022). For slopes and offsets, a two-way ANOVA was conducted to determine the effects of treatment (VEH & CBD) and the ripple type (random NonREM baseline, short & long ripples). Statistical significance was accepted at the $p < 0.05$ level for simple interactions and simple main effects. Post hoc comparisons using the Tukey HSD test were conducted to investigate interaction effects.

Supplementary Figures

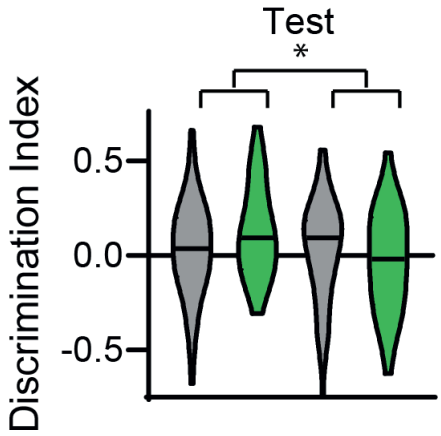

Fig. S1: Shown is the test data for all rats. The interaction CBD and Condition remained marginal significant (condition $F_{1,43}$=5.0 p=0.03 treatment $F_{1,43}$=0.22 p=0.6 interaction $F_{1,43}$=3.0 p=0.089).

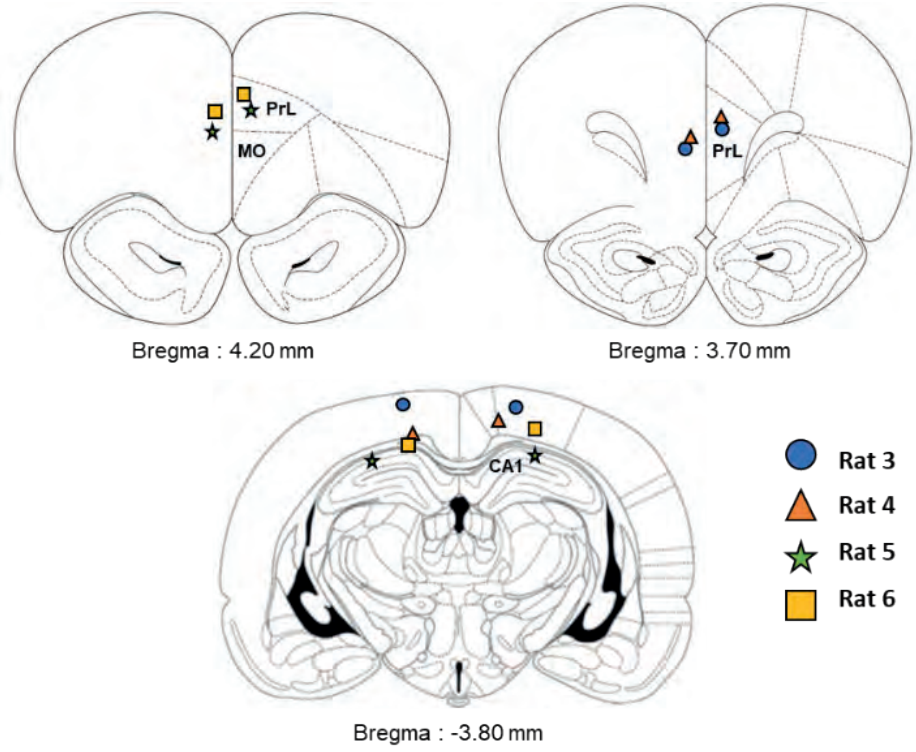

Fig. S2: Placement of electrodes after histological confirmation. Rat 3 did not reach the hippocampus and was only included for analysis of sleep stages and cortical events. All the other rats had at least one electrode with ripples.

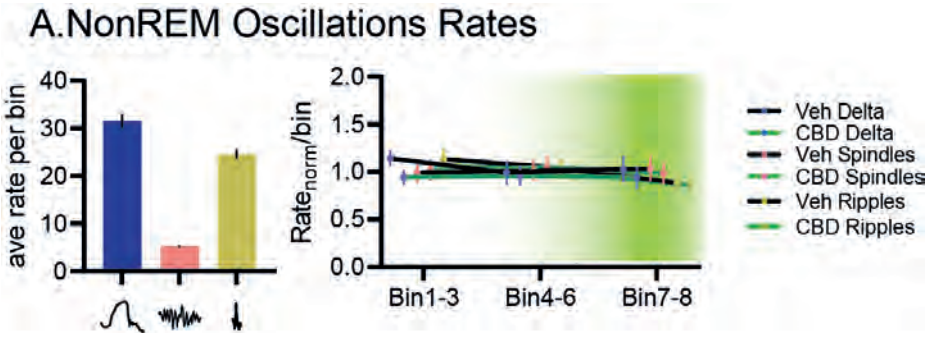

Fig. S3: Rates of NonREM oscillations

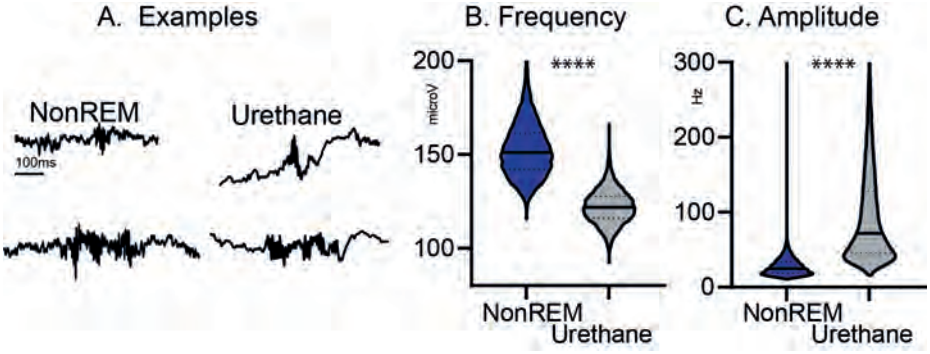

Fig. S4: NonREM and Urethane ripples. Urethane came with slower and larger ripples. **** p<0.0001 K-S

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Chapter 5

Tracking semantic-like memories in the rodent Default-Mode-Network

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AS performed and supervised the experiments, JS, LvdB, LO performed experiments, AAZ advised on data-analysis, AA and INL supported experiments and LG designed and supervised the project. AS and LG wrote the first draft of the article together.

Abstract

Memories are stored in engrams that can be visualized using immediate early gene (IEG) expression-based tools. Until now, these have been utilized to investigate episodic-like memories, and semantic-like memories are rarely – if ever – targeted. Here, we tracked IEG expression in a 2-week semantic-like memory paradigm in mice across the whole-brain spatial memory network. Absolute gene expression was driven by first experiences and later learning led to smaller changes. This highlights that multiple episodic memories are not consolidated after encoding to create semantic-memories, instead semantic-memories are already encoded differently. Increased c-Fos expression was seen in the prefrontal cortex for task congruent conditions and semantic-like memories were resistant to incongruent interference. However, post-training novelty enhanced interference by increasing gene expression during the consolidation period. Graph-theory analysis revealed the retrosplenial cortex to be the critical hub in all memory networks. Further, semantic-like memory was represented in the DMN characterized by prelimbic-centered network, while 1-Trial and Interference learning presented with a Ca1 and DG-centered network respectively.

Introduction

Individual memories of distinct events – known as episodic memories – are proposed to be stored in a fixed set of neurons, i.e., the engram (Semon 1921; Bruce 2001). A tool to visualize the engram is expression-mapping of immediate early genes (IEG) such as cFos and Arc. Recent sophisticated "capture" experiments have harnessed additional tools such as DREADDs and optogenetics in combination with IEG-driven promoters to enable detailed tracking of episodic-like memories over time as well as query the importance of sub-parts of the whole brain memory network (Josselyn and Tonegawa 2020; Josselyn, Kohler, and Frankland 2015; Ryan and Frankland 2022). These studies have let us gain unprecedented new insights into the composition and properties of the memory engram as well as necessity and sufficiency of specific parts within the engram, and thus, have brought our understanding of the neurobiology of memory a leap forward. However, they tend to be limited to investigating one-trial experiences. Usually memories of unique events, e.g., fear conditioning, are targeted or simple memories, e.g., one-trial object-location/recognition, both modelling episodic-like memory. However, most of cognition is not based on memories of unique events, instead decision-making will be influenced by accumulated knowledge known as semantic memories. In contrast to episodic-like memories, cumulative, semantic-like memories, that are abstracted from multiple events, are rarely – if ever – investigated in rodents using IEG-based tools to track how engram formation evolves over time and across multiple brain regions.

The encoding of new information relies on the hippocampus but the long-term storage of semantic memories is cortical and independent of the hippocampus (Marr 1971, 1970; Zola-Morgan, Squire, and Ramus 1994). This dual-system is proposed to protect previous memories from interference due to slow updating of the cortical memory network by the fast learning hippocampus during offline periods (McClelland, McNaughton, and O'Reilly 1995). This process of systems consolidation should take weeks to months, but has been shown to occur faster when learning new information that fits with pre-existing knowledge (Alonso et al. 2020; Tse et al. 2007; McClelland 2013).

However, as an alternate route to long-term memory very novel experiences can lead to enhancement of the initial cellular consolidation mechanisms, leading to a stronger one-trial memory (Duszkiewicz et al. 2019). This neuromodulatory, dopamine-dependent memory-enhancement – known as behavioural tagging – will also strengthen memories that occurred before or after the novelty experience (Takeuchi et al. 2016; Redondo et al. 2010; Wang, Redondo, and Morris 2010; Genzel

et al. 2017; Duszkiewicz et al. 2019). Novelty can therefore strengthen a weak memory and potentially enhance interference effects of one-trial events on existing knowledge (Genzel et al. 2017; Duszkiewicz et al. 2019).

In this project we track cumulative learning in a multi-day, semantic-like memory task mimicking accumulation of experiences to create knowledge from daily life experiences. We employed the semantic-like memory condition (Overlapping) of the Object Space Task (Genzel et al. 2019), where mice are exposed to new object-pairs in a predetermined statistical distribution across trials – one corner always had an object while the other corners had either 20 or 40% likelihood of containing an object. We combined this semantic-like task with interference – a trial with objects-positions violating previous presented statistical distributions – and post-interference novelty interventions as well as gene expression analysis of cFos and Arc. This allowed us to track how new memories are encoded and expressed in light of previous experiences.

Behaviourally, we show that by default semantic-like memories are expressed and resistant to interference. However, post-interference novelty boosts the interference effect on semantic-like memory. Interestingly, absolute gene expression was driven by novelty. The first trial of any experience – also within the OS task – led to the largest gene expression changes and later learning showed only a fraction of these plastic changes. cFos expression in the prefrontal cortex was sensitive to task-congruency and semantic-like memories triggered the expression of IEG-networks in structures known as part of the default mode network (DMN, prefrontal-retrosplenial cortex). One-trial experiences were consolidated (Arc) in hippocampal Ca1-retrosplenial-parietal network and incongruent interference in dentate gyrus-DMN-parietal network.

Results

Semantic-like Memories

To track how memories are encoded over multiple days, 118 mice were trained for 10 days on the Overlapping condition of the Object Space Task (Genzel et al. 2019). In this semantic-like memory task, animals undergo 5-minute trials in a 75 cm × 75 cm open field box, featuring 2D and 3D wall cues to aid orientation, along with identical objects placed in two of the four possible locations (Fig. 1A). There are 5 trials a day over multiple training days (full round 10 days), with each trial containing a new pair of objects and separated by ~15-25min (3-5 animals trained interleaved, Fig. 1B). Animals not included in later IEG experiments, were trained in multiple conditions

with changing orienting-cues to enable within-subject comparison after behavioural interventions (see Fig. S1, 2 and 3).

During training one location will always contain an object, while the second object has an 80% likelihood to appear in one of two corners (each corner 40% likelihood) and 20% likelihood to be placed in the fourth corner (physical locations counterbalanced across animals and switched for within-subject repeats, Fig. 1C). The discrimination index (DI) is calculated by subtracting exploration time of the stable location (100% likelihood) from the variable location (20-40% likelihood) divided by total exploration time (direction of DI calculation indicated in arrows in Fig. 1-3). Thus, a positive DI indicates that the animals are behaviourally expressing a semantic-like memory of the previously presented distribution due to the detection of subtle differences in how novel a location is. When plotted over time (Fig. 1C) the DI values fluctuate for the initial training days with low values in the first trials of each day. As the mice start forming a semantic memory, the values are stable in the final training days, when it remains stable also in the first trial of the day, replicating previous findings (Genzel et al. 2019).

On day 8 (D8), during the first trial, the mice are presented with the same object location pattern they encountered in the final training trial on D5. This setup allows us to evaluate whether they have formed a semantic-like memory based on their exploration behaviour, serving as a 3-day memory test (3d Test). Since the test configuration is identical to the last training trial, mice that remember only the most recent event should show no preference between locations, resulting in equal exploration times. However, if they have integrated information across multiple trials to form a cumulative memory, they should prefer the location that appeared less frequently (20% vs. 100% likelihood), leading to a positive discrimination index (DI) (Fig. 1D). This expected pattern was confirmed, as shown in Fig. 1E ($p < 0.0001$, one-sample t-test against chance).

Subsets of animals were sacrificed at different time points during training (5-7 animals per group, 90 min after D1-Trial 1, D1-Trial 5, D5-Trial5, and D8-Trial1/ Test, home cage control, Fig. 1B pink) and both cFos and Arc positive cells were identified with immunohistochemistry, automatically counted with Ilastik (Berg et al. 2019), and then normalized to area and subsequently home cage controls. Initially, we focused on the prefrontal cortex (PFC, prelimbic and anterior cingulate cortex) and hippocampus (HPC, Ca1 and Ca3, Fig. 1E) as areas known to be important for memory encoding and long-term storage and sensitive to novelty and interference manipulations (Genzel et al. 2017; Squire et al. 2015). PFC showed more positive

cells for both cFos and Arc after the first trial in comparison to all other time points (each orthogonal comparison $p<0.0001$, Fig. 1F, see figure legends for all detailed statistics). In contrast, in the hippocampus only cFos expression was higher than home cage and showed a linear decrease over time (linear contrast $p=0.008$).

In sum, we replicate the finding that mice express a semantic-like memory, abstracted across multiple trials. Interestingly, at the first training trial the highest number of IEG-positive cells were seen across training and time. In the prefrontal cortex a sharp drop is already present on the fifth trial of the same day in both genes (cFos and Arc); in contrast, in the hippocampus a more gradual linear decrease over time is seen in cFos and no change at all to home cage in Arc.

Interference

Next, we investigated how information, which is incongruent with previously learned regularities, is processed. For this, mice were exposed to either a congruent or incongruent object configuration for 15 min on day 9 (D9, Interference Day, for comparison of 5 and 15min interference see Fig. S1). For congruent, the same 100%-20% location paring as in the 3d Test was used, thus both last event and semantic-like memory would lead to a positive DI (Fig. 2A, B). For incongruent, no object was presented in the previously stable location (100% likelihood location), instead the objects were placed in a 40% and 20% likelihood location (Fig. 2A). In this case, the last event memory would relate to both locations being equally novel, since in the previous trial the other two locations were used. In contrast, the semantic-like memory relates to a positive DI, since the 20% likelihood location would still be more novel than the 40% likelihood location (Fig. 2B). Interestingly, the animals expressed in both conditions the statistical distributions of the semantic-like memory and always showed a positive DI during this trial ($p=0.0016$, Fig. 2C). Some animals (5-7 per group) were sacrificed 90 min after the trial and cFos and Arc expression was measured in PFC and HPC. cFos in PFC was sensitive to congruency and showed higher cell counts after the congruent in comparison to the incongruent condition (condition X BA $p=0.039$), fitting to previous results in humans using fMRI (van Kesteren et al. 2013; van Kesteren et al. 2010).

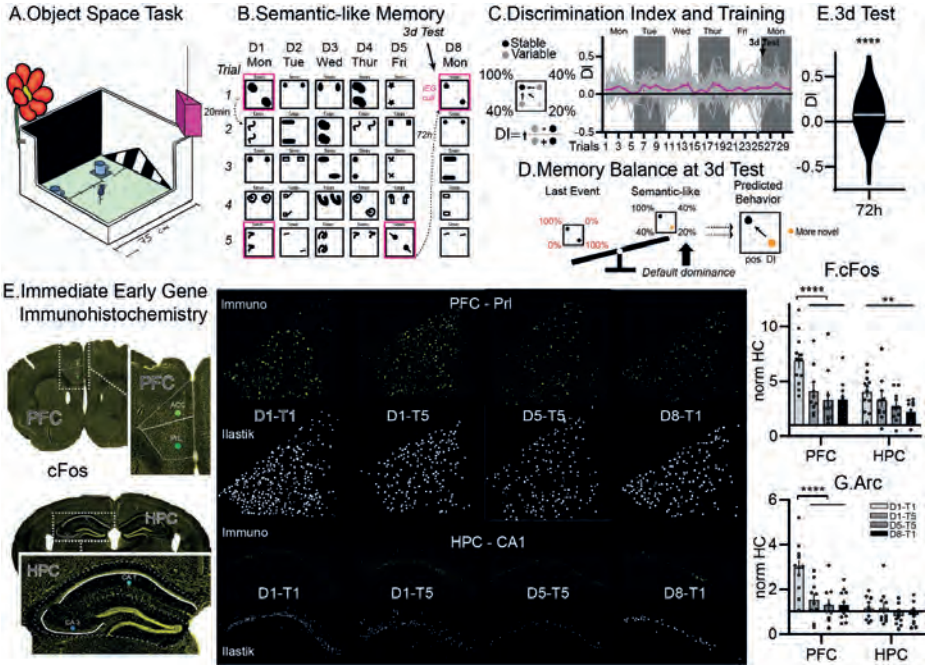

Fig.1 Semantic-like Memory: A. Schematic of behavioural setup of the training arena, equipped with 2D and 3D cues for orientation. **B.** Training schedule with 5x5min trials per day for 8 days (D1-8) including on D8 a 3-day (3d) test trial. **C.** Discrimination index (DI) is calculated by subtracting exploration time of the stable location from the variable location divided by the total exploration time; thus, a positive DI indicates expression of the statistical distribution of object-likelihood across training, i.e., semantic-like memory (100% for stable, 20/40% for variable, DI calculated in direction of arrow). **D.** Comic of memory balance at test. Statistical distribution for last event memory (left, red font) and for semantic-like memory (right, black font) with current object placement presented in the boxes as dots, in orange the more novel location considering each memory-distribution. On the right, the overall prediction which object location will be perceived as more novel, in this case considering that by default the semantic-like memory should dominate behaviour a positive DI is predicted (Genzel et al. 2019). The last event memory would result in a DI around 0 since no location is perceived as more novel. **E.** DI at 3-day test, animals express the semantic-like memory with a positive DI (one-sample t-test to 0 t_{284}=5.2 p<0.0002). **E.** Immunohistochemistry for cFos and Arc was performed and positive cells in the prefrontal cortex (PFC, prelimbic and ACC) and hippocampus (HPC, Ca1 and Ca3) were counted and normalized for area and then to home cage controls (norm HC in F and G). On the right examples for each time point. First for each ROI immunostaining (yellow) and then Ilastik identified positive cells (white). Already noticeable is a decrease in cFos-positive cells after the first trial. **F-G.** Gene expression for both brain areas and genes are shown for D1-Trial1, D1-Trial5, D5-Trial5, D8-Trial1 (light grey to black, rmANOVA Gene $F_{1,38}$=117.7 p<0.001, gene X trial $F_{3,38}$=3.3 p=0.03, BA $F_{1,38}$=30.7 p<0.001, BA X trial $F_{3,38}$=5.6 p=0.003, gene X BA $F_{1,38}$=6.1 p=0.018, trial $F_{3,38}$=7.2 p=0.001). For both cFos (**F)** and Arc (**G**) the first trial showed higher expression than the other time points in the prefrontal cortex (orthogonal comparison p<0.0001). In the hippocampus cFos showed a linear decrease across time (linear contrast p=0.008). **p<0.01, ****p<0.0001 error bars are SEM

It has been previously shown that distinct novelty events increase initial cellular consolidation in both PFC and HPC (Genzel et al. 2017) and can strengthen weak memories (Takeuchi et al. 2016; Wang, Redondo, and Morris 2010). Here, we tested if such a distinct novelty experience – 30 min exploration of a box filled with novel items and textures (Fig. 2D) – can enhance the effect of an incongruent interference trial on a later memory test. Initially, we established that novelty leads to increased gene expression, wherein we sacrificed animals 90 min after the novelty condition and compared it to animals that remained in the home cage after training (Fig. 2E). Interestingly, the increase in cells positive for cFos was larger in the PFC than HPC (condition X BA interaction p=0.0065) and no condition effect nor interaction was seen for Arc (all p>0.25) (Fig. 2F).

In sum, we show that cFos expression in the PFC is sensitive to congruency and post-interference novelty leads to increased cFos expression in the PFC.

Post-interference Test

After establishing that post-interference novelty led to an increase in activity in the PFC, we went on to test the behavioural interference effect on the original trained semantic-like memory during a 10 min test trial the next day (D10 Test). Specifically, there were three conditions (Fig. 3A): Test after incongruent trial with post-interference novelty (Incon+N), incongruent trial no novelty (Incon) and congruent trial with post-interference novelty (Con+N). The latter condition was included to ensure any interference effects were not due to the novelty experience per-se but instead specifically due to the strengthening of the memory for the incongruent interference trial. At test we returned to the 20%-100% likelihood pairing, thus in the incongruent conditions in relation to the last event memory the originally stable location (100% likelihood) is now more novel (since not presented in the previous trial). Therefore, if novelty strengthens the last event memory, the memory balance at test would shift and Incon+N would present with a negative DI (Fig. 3B). In contrast, in Incon without the novelty-induced-boost the semantic-like memory would still prevail (with resulting positive DI) and in Con+N the last event memory would not contradict the semantic-like memory (with resulting positive DI). At test (Fig. 3C), this pattern was expressed but only in the final 5 min of the trial (timebin X condition $F_{2,336}$=3.2 p=0.04, last 5 min condition $F_{2,168}$=5.0 p=0.008). A previous study (Vallianatou et al. 2021) in the lab showed that initial exploration patterns in mice include a random foraging component, which likely accounts for their behaviour during the first 5 mins of the test. In the second half, however, their exploration becomes more directed, with increased attention to the cues and surroundings. This was our rationale behind splitting the test trial duration. If novelty followed

incongruent interference, the discrimination index became negative (one-sample t-test to 0 t_{66}=2.2 p=0.03), indicating the animals expressed the last event memory. In contrast, if no novelty followed incongruent interference, discrimination index remained positive (one-sample t-test to 0 t_{65}=2.1 p=0.04) with animals continuing to express the semantic-like memory.

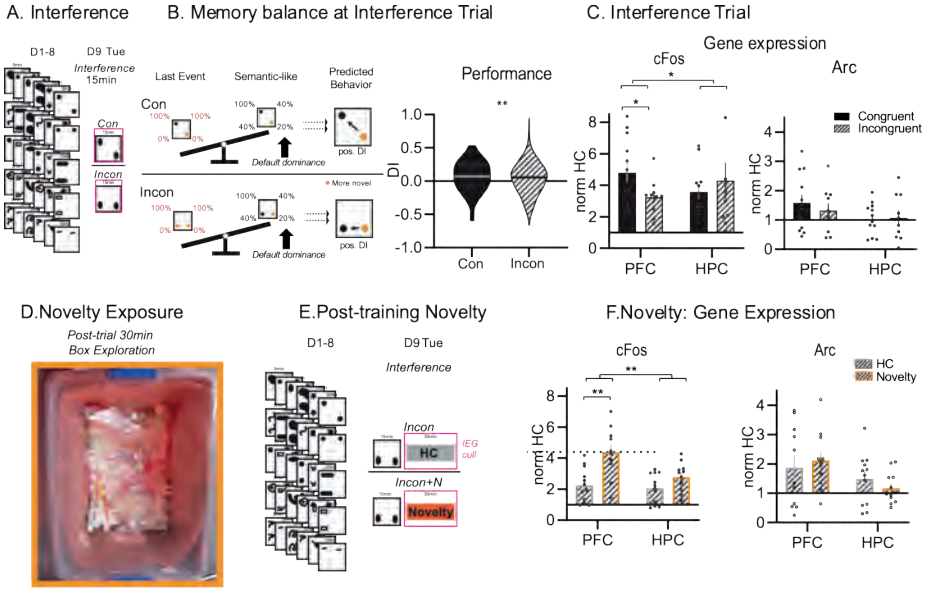

Fig.2 Interference: A. Study Design. After D1-8 training on semantic-like task, animals either were exposed to a congruent or incongruent (no object in 100%-likelihood location) object configuration for 15 min (D9 Interference). **B.** Shown is the predicted memory balance during the trial for both conditions. In each case one location would still remain more novel, if the semantic-like memory is again expressed dominantly. **C**. This was the case where there was no difference in the DI for both conditions (t-test t_{278}=0.51 p=0.6) and DI was above 0 during this interference trial (one-sample t-test t_{279}=3.18 p=0.0016). On the right gene cFos and Arc positive cells are shown for both conditions and brain areas (rmANOVA gene $F_{1,20}$=62.3 p<0.0001, BA x condition $F_{1,20}$=3.8 p=0.065, BA x condition x gene $F_{1,20}$=5.4 p=0.03, rmANOVA only cFos condition X BA $F_{1,20}$=4.9 p=0.039). **D.** Novelty box used for 30 min novelty exploration. **E.** Study design to test IEG-effect of post-interference novelty. **F.** cFos and Arc positive cell counts shown for novelty (orange frame) and control (ANOVA BA $F_{1,22}$=14.7 p=0.0009, gene $F_{1,22}$=35.5 p<0.0001, condition $F_{1,22}$=5.2 p=0.03, BA X condition $F_{1,22}$=6.1 p=0.022, gene X condition $F_{1,22}$=13.0 p=0.0015, ANOVA only cFos BA $F_{1,20}$=14.5 p=0.0011, condition $F_{1,22}$=12.4 p=0.0019, BA X condition $F_{1,22}$=9.2 p=0.0065, ANOVA only Arc BA $F_{1,20}$=6.5 p=0.019 other p>0.25). *p<0.05, **p<0.01

To examine if behavioural expression of the semantic-like memory before interference, predicted the impact of interference, we correlated the average DI of all training trials (D1-8) with the performance at test. Interestingly, for both time periods (first and last 5 min of test) and all conditions significant correlations were

seen (Fig. 3D, r=0.31-0.53, p=0.037-0.0001). Thus, if they expressed the semantic-like memory better before the interference, they showed smaller interference effects and still had a higher DI at test. Next, we sacrificed animals 90 min after the test and compared the conditions Incon+N, Incon, and Con (Fig. 3E). Again, cFos expression in PFC was sensitive to congruency and the test after both Incon conditions led to less cFos positive cells.

In sum, we show that semantic-like memories are resistant to interference. However, post-interference novelty strengthens the interference memory and results in a behavioural effect of interference at test.

Gene expression and novelty

Next, we aimed to characterize the wider spatial memory network and extracted cFos and Arc positive cell counts from additional regions of interest (ROIs) (Fig. 4A). Gene expression was measured in prelimbic and anterior cingulate cortex (Prl and ACC, together PFC), granular and agranular retrosplenial cortex (G and aG RsP), which together make up the rodent default-mode-network (DMN). In the hippocampus (HPC) we counted cells in the Ca1, Ca3 and dentate gyrus (DG). Finally, as potential downstream, cortical target for spatial memories, that the associative areas DMN and HPC connect to, we measured gene expression in the posterior parietal cortex (PPC). Together these areas represent the general memory network of the brain (DMN and HPC) that should connect to PPC due to the spatial nature of our task (Genzel 2020).

Initially, we plotted the absolute gene expression sorted by time in task (Fig. 4B). Interestingly, cFos but not Arc showed a distinct pattern across time in task. Thus next, we resorted the conditions in accordance to a novelty scale ranking how novel each condition should be for an animal, e.g., one trial events and novelty exposure (Fig. 2D) are on the high end of the novelty scale, congruent conditions on the low end and incongruent conditions in the middle (Fig. 4C). Interestingly, cFos gene expression was positively correlated with this novelty scale especially in the DMN areas ($p<0.01$).

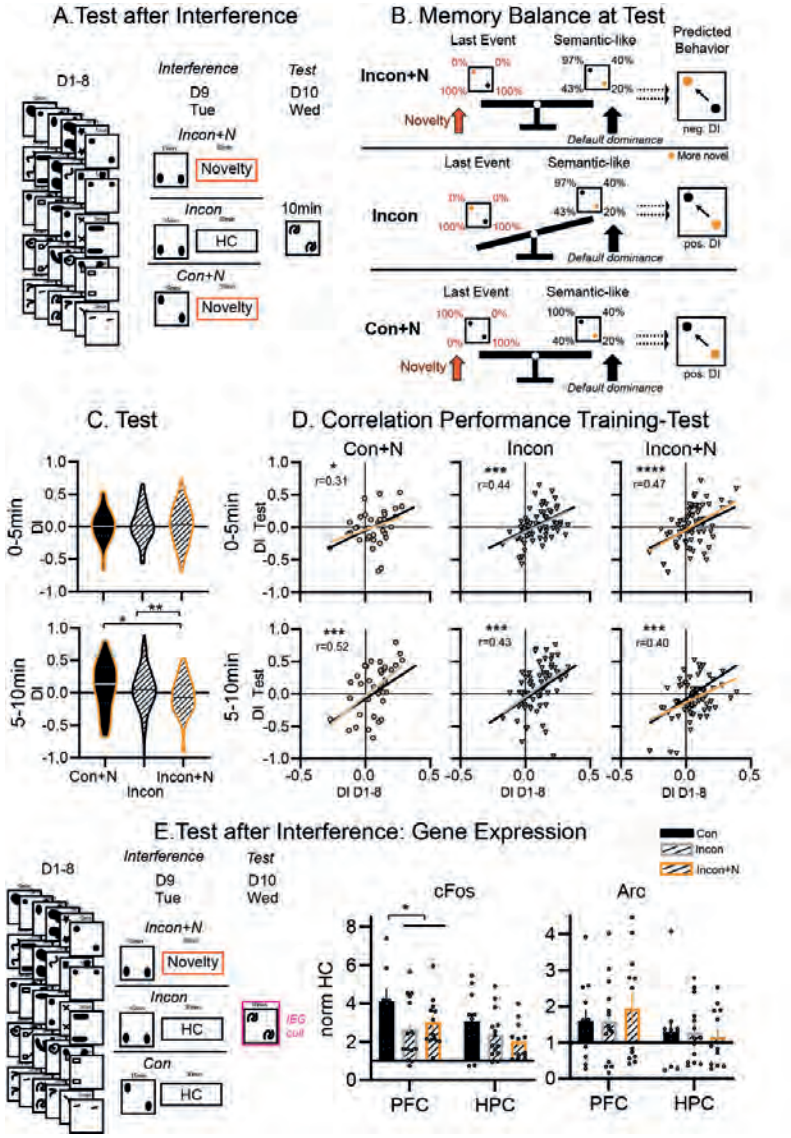

Fig. 3 D10 Test: A. Study Design testing for interference effects of an incongruent (Incon) or congruent (Con) trial on D9 with post-interference novelty (+N) or control on 10 min test day 10 (D10 test). **B.** Comic of predicted memory balance at test. In Incon the former stable location is now the more novel location considering the last event memory. If novelty boost the last event memory this would then lead to a negative DI at test in contrast to a positive DI for Incon and Con+N. **C.** Performance at test split for first and last 5 min of the trial (rmANOVA timebin X condition $F_{2,336}$=3.2 p=0.04, ANOVA first 5 min condition p=0.96, ANOVA last 5 min condition $F_{2,168}$=5.0 p=0.008, t-test to Incon+N for Con+N t_{103}=2.5 p=0.016 for Incon t_{131}=3.01 p=0.0031). **D**. Pearson correlations of average DI of D1-8 (expression of semantic-like memory) and Test DI split for condition and timebin. **E.** Study design to measure gene expression at test (rmANOVA gene $F_{2,35}$=35.0 p<0.001, gene X condition $F_{2,35}$=2.6 p=0.091, brain area $F_{1,35}$=13.6 p=0.001). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Next, the different conditions were combined to create functional groups. The 1-Trial learning group consisted of mice that were sacrificed after performing the first trial on Day 1 and those that ran a control condition where they were placed into a new open-field box on day 9. All congruent training conditions were combined to Semantic-like and all incongruent conditions to Interference functional group (Fig. 4D). Absolute cFos and Arc-positive, normalized cell counts were plotted for the different functional groups. 1-Trial learning led to higher cell counts especially in the DMN (group effect $p<0.001$, contrast per brain areas $p<0.05$, Fig. 5B). Interestingly, in CA1 we saw the opposite pattern for cFos and Arc. In cFos 1-Trial learning led to higher gene expression, for Arc it was Interference. To better understand the differential expression patterns of cFos and Arc in the CA1 region, we computed a cFos/Arc ratio and showed a linear effect across conditions ($p=0.001$, Fig. 5F).

In sum, we show that absolute cFos gene expression in the DMN is driven by novelty and first experiences.

Memory Networks

Next, we extracted memory networks for each functional group. For this, gene expression correlation maps were created, where gene expression of each brain area was correlated with expression in the other brain areas across all subjects for each condition (Fig. 5A). First, we thresholded for significant correlations (p-thresholds at 0.001, 0.0001 and 0.00001 derived from Semantic-like and interference group. Same r thresholds applied to 1-trial learning which had a smaller n) and displayed significant functional connections in glass brains (Fig. 5B top row). We next used a graph-theory approach to visualize and analyze the memory networks (using Cytoscape, graph Fig. 5B lower row).

As can be seen in the glass-brain networks, for cFos, 1-Trial learning presented with an Ca1-RSP-PPC network, Semantic-like learning with a whole brain network driven by PrL and Ca1 connectivity, and Interference with an HPC-RSP-PPC network (Fig. 5B). Contrasting the different groups highlighted that Semantic-like learning in contrast to the other two groups showed higher Prl and ACC connectivity to the rest of the brain ($p<0.001$). In contrast, Interference showed higher DG connectivity than the other two groups ($p<0.001$).

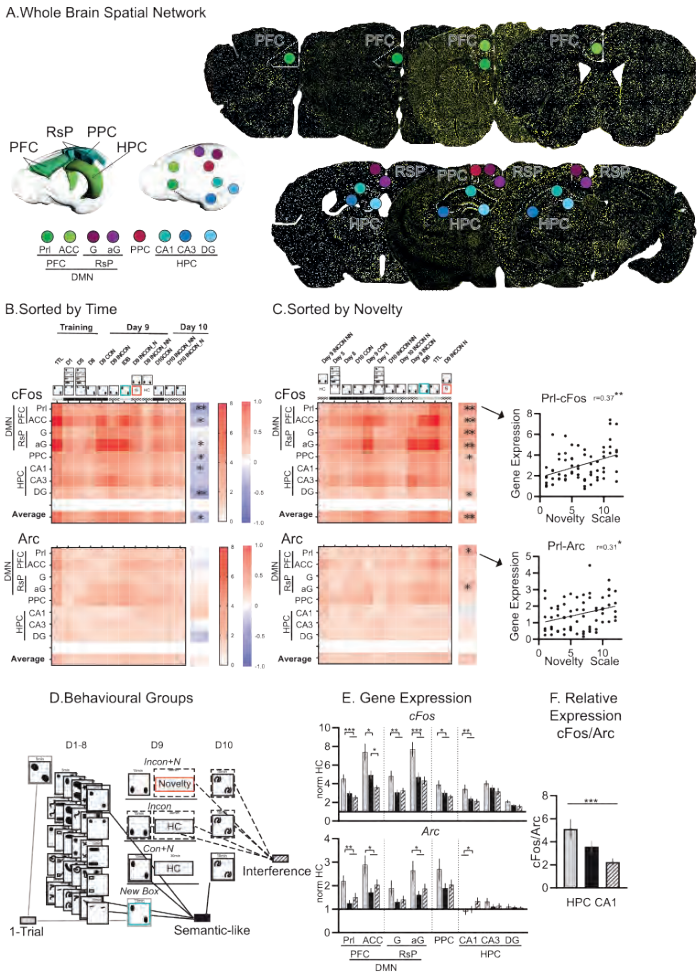

Fig. 4 Network Gene Expression: A. Shown is the spatial memory network with prefrontal cortex (PFC, prelimbic Prl, anterior cingulate cortex ACC), retrosplenial cortex (RsP, granular G and agranular aG) together they are the default mode network in rodents (DMN). Further, posterior parietal cortex (PPC) as downstream target for spatial memory and hippocampus (HPC consisting of Ca1, Ca2 and dentate gyrus DG). Target areas in example slices with staining for cFos. Gene expression maps sorted by **B**. time in task and **C.** sorted by how novel each condition is. Red scale for absolute gene expression values and blue-red scale for correlation coefficients. There was a negative correlation with time but there was a positive correlation with novelty for cFos. For Arc only Prl and Rsp aG showed a slight correlation with novelty. On the right correlations for PrL are shown in detail. *p<0.05, **p<0.01 **D.** We combined individual conditions to the functional groups 1-Trial, Semantic-like and Interference learning. **E.** Absolute gene expression per functional group confirmed that the first trial in a new environment leads to more cells expressing cFos and Arc in the cortex then other behavioural conditions (rmANOVA gene $F_{1,66}$=150.5 p<0.001, gene X group $F_{2,66}$=5.6 p=0.006, brain area $F_{7,462}$=69.3 p<0.001, brain area X group $F_{14,462}$=4.8 p<0.001, gene X brain area $F_{7,462}$=45.0 p<0.001, gene X brain area X group $F_{14,462}$=3.8 p<0.001, group $F_{2,66}$=7.1 p=0.002). **F.** cFos/Arc expression ratio for Ca1 showed a differential effect per functional group (ANOVA $F_{2,64}$=6.6 p=0.002, linear contrast p=0.001).

For Arc 1-Trial learning was associated again with a Ca1-RSP-PPC network, Semantic-like learning with DMN-PPC network and Interference with a DG-RSP-PPC network (Fig. 5B). Contrasting the groups revealed that both Semantic-like and Interference in comparison to 1-Trial learning showed higher Prl and ACC driven connectivity ($p<0.001$). Interestingly, Interference in comparison to the other two groups came with higher DG-driven connectivity ($p<0.001$) and 1-Trial learning with higher Ca1 connectivity ($p<0.001$). Thus, RSP was always central to the memory network and the groups differed by the additional nodes linked to RSP: PFC for Semantic-like and HPC for 1-trial and Interference, with Ca1 and DG respectively (see also Fig. S2).

Graph-theory analysis of the networks showed, that 1-Trial learning came with a smaller network (nr edges 10/9 cFos/Arc, ave nr of neighbours 3.33/3.2, characteristic path length 1.33/1.2, clustering coefficient 0.69/0.87, network density 0.67/0.8, network heterogeneity 0.37/0.23, network centralization 0.5/0.33, count of components 3/3) than Semantic-like learning (nr edges 23/10 cFos/Arc, ave nr of neighbours 5.75/3.6, characteristic path length 1.12/1.1, clustering coefficient 0.94/0.9, network density 0.98/0.9, network heterogeneity 0.26/0.14, network centralization 0.24/0.17, count of components 1/3). Interference showed a mixed network between (nr edges 15/14 cFos/Arc, ave nr of neighbours 4.7/3.5, characteristic path length 1.07/1.64, clustering coefficient 0.93/0.55, network density 0.93/0.5, network heterogeneity 0.10/0.45, network centralization 0.1/0.29, count of components 2/1). Interestingly, in 1-Trial learning (cFos and Arc) and Interference (only cFos) Prl and ACC was outside of the network, however in Semantic-like the hippocampus dropped out (only Arc).

To describe the network in more detail, we present number of network edges (Fig. 5C, count significant correlations), average edge-strength (Fig. 5D, average r across significant correlations), betweenness (Fig. 5E) and average shortest path (Fig. 5F) per node (brain area). In all measures 1-Trial learning presents with a posterior network often led by Ca1 (edge count, betweenness, shortest path), while Semantic-like learning comes with an anterior network led by Prl and Ca1 (edge count, betweenness, shortest path). In contrast, Interference is associated again with a posterior network led by DG (edge count, betweenness).

Finally, to link the strength of the expression of the semantic-like memory to these gene-expression networks, we extracted average absolute gene expression for the DMN and correlated this with the DI during training. Arc but not cFos expression in the DMN correlated with the average training DI during the basic training conditions (D1, D5, D8, $p=0.0022$).

In sum, we highlight different memory networks for different types of learning. 1-Trial learning presents with RSP-PPC and Ca1-RSP-PPC network for cFos and Arc respectively. Semantic-like learning shows a DMN that is driven by Prl connectivity. Interference presents with decoupling of the PFC – especially for cFos – instead DG drives connectivity – especially for Arc. For a general discussion on difference between genes, please see supplemental materials.

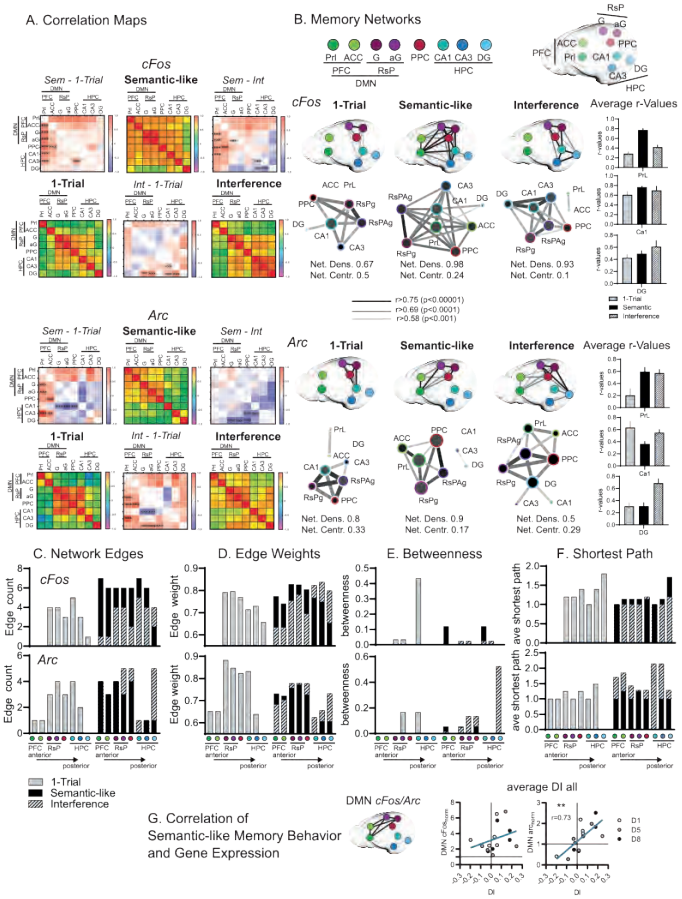

Fig. 5 Memory Networks: A. Correlation maps of gene expression per group (in rainbow) and difference maps (blue to red color map). **B.** In glass brains significant correlations are presented and highlight specific memory networks for the different functional groups. Below networks presented as graphs (Cytoscape). Edge color shading indicating strength, node size edge count, node color shade closeness, node border indicates brain area. **C.** Count of network edges per node (Friedman test 11.24 p=0.0013) **D.** Average edge weight per node. **E.** Betweenness per node (Friedman test 1.44 p=0.56) **F.** Average shortest path per node (Friedman test 3.19 p=0.22) **G.** Absolute gene expression across the DMN network was correlated for the semantic-like memory conditions with the average DI D1-8 (subgroups per sacrifice time point in white D1, grey D5 and black D8, cFos r=0.31 p=0.26, Arc r=0.73 p=0.0022). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Discussion

This study combined the Object Space Task (Genzel et al. 2019) with cFos and Arc gene expression measurements to track - for the first time - semantic-like memory and interference effects over two weeks of learning. The main findings were as follows: (1) In the Default-Mode-Network (DMN) novelty drove absolute gene expression, with highest positive cell counts seen after the first trial in an environment. (2) cFos expression in the PFC was sensitive to task congruency. (3) Post-interference novelty led to increased cFos expression in especially the PFC and enhanced the behavioural effect of interference at test. (4) Learning groups (1-Trial, Semantic-like, Interference) came with distinct gene-expression networks, that all shared the retrosplenial (RSP) cortex as central hub. 1-Trial learning was associated with a Ca1-RSP-PPC network, Semantic-like learning with a Prl-driven DM network (PFC-RSP) and Interference with a DG-driven RSP-PPC network. Thus, RSP was central to all memory networks and connected to different key hubs for each type of memory.

1-trial and semantic-like memory

In recent years a plethora of new tools designed to manipulate and interrogate the engram have emerged. These tools are usually tied to gene expression and have resulted in a multitude of insights on, for example, which activity in what parts of the network are necessary and sufficient for memory retrieval (Josselyn and Tonegawa 2020; Josselyn, Kohler, and Frankland 2015; Ryan and Frankland 2022). We are gaining unprecedented new insights on how engrams evolve (Roy et al. 2019; Roy et al. 2017; Ramirez et al. 2013; Ryan et al. 2015). These studies harness unique, one-trial memories for these investigations, mainly out of practical reason to ensure specificity of the manipulated memory, but also because they want to target episodic-like memory. However, the majority of our daily decisions will be based on knowledge accumulated over longer time and more experience and not just a single, past event. So far, this type of memories – known as semantic-memories – are rarely, if ever, investigated.

Our results highlight, that semantic-like memories are not just many individual episodic memories that are processed during consolidation after encoding to extract semantic-memories, as has been proposed so far (Morris 2006; Lewis and Durrant 2011). Instead, already at the time point of encoding multi-trial experiences show distinct mechanisms. In all measured brain areas, but especially in the DMN, fewer neurons show increased gene expression in multi-trial learning than in 1-trial learning. Further, the network of involved brain regions shifts from a Ca1-RSP-PPC network to a Prl-driven ACC-RSP-PPC network. This latter finding is not surprising,

since the shift from a hippocampal to cortical based network is long known to occur after systems consolidation (Frankland and Bontempi 2005). What is surprising and has not been shown before, is that it can be seen already in the first week of learning instead of after a longer consolidation period of many weeks as previously proposed (Squire et al. 2015).

Novelty drove absolute gene expression in the DMN and the first trial in the OS task led to the highest positive cell counts. Later trials only induced a fraction of the change. Already classic systems consolidation theory proposed that initial learning encodes in both the hippocampus and cortex, systems consolidation would be needed to strengthen within-cortical networks to suffice for memory retrieval (Squire et al. 2015). Our findings would fit this proposition and expand it, by proposing that after the first memory, later events in the same context would only lead to updating and smaller adaptations to the cortical network; thus, semantic memories are already encoded directly into the cortex. Potentially, these network refinements are via long-term depression (LTD) after the original first-experience was mapped with long-term potentiation (LTP) (Stacho and Manahan-Vaughan 2022). It has been proposed that LTP and LTD work together in aiding the creation of a cohesive memory trace of object place associations and the environment. LTP occurs whenever a rodent explores a novel spatial environment (Straube and Frey 2003), however, with repeated exposures, LTD has been shown to play a crucial role in encoding the finer details within the environment, such as the changing object place associations across trials (Kemp and Manahan-Vaughan 2004). The first exposure with LTP and many cells expressing IEG is likely associated to the global remapping described in the hippocampus, where introduction into a novel environment leads to a scrambling and shifting of hippocampal place fields (Kubie, Levy, and Fenton 2020). Interestingly, the first-big-than-retract principle is reminiscent how networks are created in development, an initially overconnected brain uses pruning to select the correct connections. It is also how slime molds solve mazes: first expansion to cover all connections and then retractions to a more efficient network once salient features are identified (Nakagaki, Yamada, and Tóth 2000).

DMN as semantic-memory network

Our findings highlight the DMN as semantic memory network. Our brain is organized into whole brain networks, which networks are utilized in certain behaviours will change over time and strategy (Genzel 2020). The DMN was first identified in human resting-state studies (Raichle 2015) and earned its name to the fact that it tended to be more active at rest than during different tasks. The structures that are part of the DMN (PFC, RSP and sometimes HPC) are long known to be involved in memory

storage and will likely always be active (as measured in BOLD signal) due to their role in memory encoding and retrieval of daily experiences. However, especially during rest with mind-wandering (or memory-wandering) and offline consolidation occurring during ripples, the network is active (Raichle 2015).

Here, network analysis identified the DMN as semantic-like memory network and absolute gene expression in the DMN was also associated with stronger behavioural expression of the semantic-like memory (measured in average DI, Fig. 1E). Interestingly, across all groups cFos expression in the DMN was driven by novelty, a finding that would have been more expected to occur in the hippocampus.

We proposed previously that initially the hippocampus would connect via the cortical DMN to downstream cortical structures, in case of spatial memories this would be the parietal cortex (Genzel 2020). Thus, the DMN would be a key player in the cortical memory network. Over time and after consolidation, the cortical DMN structures could support this binding role without the hippocampus. Our findings confirm this proposition. They further highlight a differential role of PFC and RSP.

Early in learning (1-Trial) and during incongruent memory updates (Interference) the hippocampus connects to the RSP that then facilitates the connection to the PFC (Ca1 and DG driven-connectivity respectively, each in a posterior-to-anterior network). In contrast, during normal semantic-like memory and congruent trials Prl becomes central to the network (anterior to posterior). Thus, in our data RSP was the cog-wheel within each memory network, connecting to different nodes in accordance to our behavioural manipulations (see also Fig. S2).

Interestingly, others have shown that optogenetic induced sleep reactivations in the RSP drive faster cortical consolidation via changes induced in ACC (de Sousa et al. 2019). Hippocampal ripples – associated to natural memory reactivations and critical for sleep-related consolidation effects (Aleman-Zapata, Morris, and Genzel 2022) – can cooccur with cortical ripples in the DMN via the RSP (Khodagholy, Gelinas, and Buzsáki 2017; Nitzan et al. 2020).

cFos expression in the PFC was sensitive to congruency during the interference and test trial. It has been proposed that the prefrontal cortex detects the context (Eichenbaum 2017) and congruency of current events with previous knowledge (van Kesteren et al. 2013; Wang, Tse, and Morris 2012; Tse et al. 2011; Richards et al. 2014). However, it was assumed that it would take many weeks of training and the presence of a schema, for this function to develop (Tse et al. 2007). In accordance,

previously, a Prl-driven IEG-network was shown for spatial, schema memories after many weeks of training (Takeuchi et al. 2022). Here, we see both the Prl-network and congruency-effect already in the second week of learning, before a schema with resulting expedited long-term memory and systems consolidation is established (Alonso et al. 2020). Thus, with continuous input, the cortex learns much faster than previously assumed and tracks statistical regularities in the environment. Previously, this has been suggested for human spatial learning (Brodt et al. 2018), but not yet shown in rodents.

Thus, our results would fit to the proposition that the PFC detects congruency, which leads to an anterior-to-posterior network. In contrast, in novel events – either one trial learning or interference – a hippocampal-driven, posterior-to-anterior network is observed (van Kesteren et al. 2013). Furthermore, the RSP would be the connecting hub for HPC to PFC consolidation, instead of the entorhinal cortex or the sparse direct pathway via ventral HPC.

Hippocampal Ca1 and DG

Interestingly, in our new learning conditions we saw hippocampal-driven gene-expression networks, but which hippocampal subfield was involved differed. 1-Trial learning was associated to Ca1-RSP-PPC network and Interference learning with DG-RSP-PPC network. These differences, while never compared like this directly, fit to proposed roles of each subfield.

Ca1 is the common target of studies investigating ripple-related memory reactivations and consolidation (Girardeau and Zugaro 2011; Buzsáki 2015). Further, we know that this hippocampal ripple-activity is necessary for the retention of 1-session learning (Aleman-Zapata, Morris, and Genzel 2022). The DG, on the other hand, is known for sparse coding and pattern-separation (Treves and Rolls 1994). Pattern-separation is likely to contribute to detection of incongruency and the selective consolidation thereof.

When introduced to explore a novel spatial environment, both Ca1 and DG have been shown to crucially involved in encoding and the underlying LTP pathways. Ca1 aids in processing the general environmental features and positional cues to build a map of the environment, while DG plays a more critical role in encoding of the finer features within (Stacho and Manahan-Vaughan 2022; Kemp and Manahan-Vaughan 2008). In this regard, LTD has been also shown to play a crucial role in tasks involving object place associations. Within the environment, when large orienting cues are used to facilitate exploration, the DG has been shown to be a crucial hub for LTD processes

(Kemp and Manahan-Vaughan 2008). Previous study also showed Arc expression to be differentially regulated in Ca1 and DG depending on the type of cues used within the environment (Hoang, Aliane, and Manahan-Vaughan 2018). Tracking the memory engrams across multiple days in mice undergoing training in the watermaze, a study showed cFos expression reduced significantly from Day 1 of training to the next day in the DG. Interestingly, not the same was observed for Ca1. (Lamothe-Molina et al. 2022). However, expression in Ca1 was not measured beyond 24 hours. ΔcFosB has been proposed to be a key regulator of experience dependent learning and gene expression in hippocampal tasks (Lamothe-Molina et al. 2022; Eagle et al. 2015). This could be one potential mechanism behind the decrease in cFos expression as we observe at later learning timepoints. Another study looking at object recognition behaviour in rats found a similar effect with cFos expression in the prefrontal cortex where the expression was reduced over multiple trial exposures (Bekinschtein et al. 2018), however, again no measurements beyond 24h hours were performed.

It would be tempting to speculate, that thus each subfield has a unique contribution in driving memory consolidation. Ca1 driving LTP, when no previous memory and thus cortical memory network is present, DG driving LTD, when selectively updating (or not) preexisting memory networks once information contradicting previously learned rules is presented.

Novelty Strengthens Memories

After the incongruent interference trial, some mice were exposed to a novelty experience in order to strengthen the memory of this trial and thereby induced behavioural effects thereof at test. This manipulation was successful, highlighting an increased complexity not previously considered in models of memory. The complementary learning systems theory proposes that the slow-learning cortex would be updated by the fast-learning hippocampus during offline periods to avoid “catastrophic interference”, where new information would overwrite existing knowledge (Marr 1971, 1970; McClelland, McNaughton, and O'Reilly 1995). Previously, we had provided first biological evidence for this proposition by showing that increasing plasticity in the cortex increased interference effects in the Object Space Task (Navarro-Lobato et al. 2023). Here, we again confirmed, that by default the semantic-memory is still expressed after interference, thus in the biological system “catastrophic interference” is avoided. However, we also show that one can tip the memory balance behaviourally and strengthen the last event memory with post-interference novelty, an effect not considered in the original theory.

We have previously already shown that post-training novelty strengthens memories via increased initial consolidation, which is independent of consolidation processes during sleep (Genzel et al. 2017). With a memory-competition design, we provided evidence that novelty also changed the quality of the memory and led to enhanced gene expression in the HPC as well as PFC. This stands in contrast to the current findings, where novelty showed stronger effects on gene expression in the PFC than in the HPC. Others have shown that novelty leads to increased dopamine release of locus coeruleus (LC) terminals and consequently higher expression of plasticity-related proteins that strengthen previously occurring memories due to neuronal coallocation (Takeuchi et al. 2016; Kempadoo et al. 2016; Chowdhury et al. 2022). These findings were all reported for the hippocampus, however, PFC data was not investigated. Neuronal coallocation has been proposed to be directed by time via CREB-induced neuronal excitability (Park et al. 2016). Perhaps this mechanism also is at play in the cortex and thus novelty induced increased gene expression may be biased to the memory-networks of the previous event. Thus, since in this experiment the previous event was cortical, we saw novelty effects there.

In sum, in this study we investigated the evolution of IEG changes while mice learned a semantic-like memory task over two weeks. We show that absolute gene expression is driven by novelty and first experiences. This initial memory network connects Ca1 to RSP and PPC. Continued semantic-like learning leads to smaller modifications and a memory network in the DMN, which is driven by Prl connectivity and predicts memory performance. Interference can be strengthened by post-interference novelty and leads to an DG-RSP-PPC memory network. The key conceptional finding, is that contrary to previous ideas, semantic-memories are not created via the post-processing of multiple episodic memories, instead incorporation into a semantic-memory network occurs already at encoding.

This study also establishes that one can target semantic-like memories with IEG-based methods, since they are established earlier and faster than previously assumed. Overall, the lack of significant markers of consolidation effects in the hippocampus with instead dominance of the cortical DMN, raises doubts on the identity of the hippocampus as the key memory structure for semantic-like memories. Instead of being the loci of long-term memory, it may be mainly contributing processing mechanisms such as scene-reconstruction and sequential binding, as has been suggested by others (Barry and Maguire 2019; Buzsáki and Tingley 2018).

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Materials and Methods

Study Design

A total of 118 mice were used in this study – 38 mice for initial behaviour experiments and 80 mice for gene expression experiments. Mice from all batches were first extensively handled for multiple days until they experienced minimal stress working with experimenters. The first batch of 38 mice ran a modified version of the Object Space Task (Genzel et al. 2019) in sub batches of eight and were sacrificed after. Having established the behaviour paradigm with this batch, the next batches ran the same task, however perfused mice at different timepoints of the task to check for expression of Immediate Early Genes – Arc and c-Fos across multiple brain regions. The second batch of 80 mice were run in smaller sub batches of 40 at a time. Expression level of Arc and c-Fos was later quantified across multiple regions of interest and clustering analyses was performed to detect expression patterns across different behavioural time points.

Animals

Six-eight week old male C57BL/6J mice weighing between 19-25g at the starting of the experiment (Charles Rivers, Germany) were used in this study. They were housed in groups of four in conventional mouse Eurostandard type II L cages (Techniplastic, UK) in a temperature-controlled (20 + 2 °C) room following a 12 h light/dark cycle with water and food provided *ad libitum*. All behavioural experiments were performed during the light period (between 9:00-18:00).

All animal procedures were approved by the Central Commissie Dierproeven (CCD) and conducted according to the Experiments on Animals Act (protocol codes, 2016-014-023 and 2016-0014-035).

Behaviour – Object Space Task in mice with interference and novelty

A modified version of the Object Space Task in mice was used for all behaviour experiments in this study. This task is based on the tendency of mice to explore novel object-locations in an open field arena across multiple trials, and we indirectly assess their memory performance by abstracting statistical similarities over their exploration patterns. The animals were extensively handled by the experimenters in the first week to minimize their stress levels during interactions. Next, they were habituated to the training box (75 X 75 cm) for five sessions over five days. On the first day, they spent 30 mins in the training box with their cage mates. On the second and third day, each mouse individually spent 10 mins exploring the training box. For the final 2 days, two

objects (made from DUPLO blocks, not used in main experiment) were placed in the center of the box and the mice were allowed to explore for 10 mins each day.

For the behaviour training paradigm, we used the *Overlapping* condition of the task with some modifications to include for other interventions – interference and novelty. One round of training and test lasted for 10 days and the experiment was run in a within subject condition, with all animals running all conditions, and a total of 38 mice were used for the behaviour experiments. To prepare for one session of training and test, the training box was prepared with 2D and 3D cues to create a unique spatial environment for the mice to orient themselves in. This box setup remained the same for one session of training and test and changed radically for the next session, in order to make a distinctly different environment each time. A typical training day consisted of 5 trials, 5 mins each with an inter trial interval of ~15-25 mins. During each of the trials, the mice were put into the training box to explore a pair of identical objects in fixed locations. The objects pairs changed from trial to trial. All mice underwent training in the overlapping condition for the first 5 days with 5 trials each day, with one object location remaining constant across all the training trials and days and one continuously changing. After 5 days of training, the mice were on a break for 2 days (Day 6 and 7) and underwent another day of training on Day 8. The first trial on Day 8 also served as a 72-hour long term memory test. On Day 9, they ran a single trial which served as an interference trial for some of the mice. For this trial, our aim was mimicking an interference in learning by introducing a different object location pattern from what they had seen so far. To do this, during the trial, we moved the object location which served as the stable one throughout all training days so far to a different location. For some of the mice, the single interference trial was followed by a 30 min exposure to a novelty box. The novelty box consisted of a collection of items of different colors, textures and shapes and the mice were allowed to explore this box for a period of 30 mins. On day 10, they ran a single test trial for 10 mins where they saw the same object location pattern as that on Day 8, prior to the interference trial. Our hypothesis was that if mice still had an intact original memory of the stable and moving object location from the training days, they would spend more time exploring the moving location. However, if the interference trial exposure disrupted this memory, then they would consider the stable location to be the moving one and spend more time exploring it. Further we hypothesized novelty box exposure to strengthen the interference trial memory.

All behaviour sessions were recorded with a webcam placed above the training box. The object exploration times were scored online in real time during the trials using a scorer program developed in the lab for training and scoring (https://github.com/

NeuroNetMem/score). Whenever a mouse would sniff or climb or interact with an object, it was scored as exploring behaviour. Obsessive chewing or biting an object did not count an exploratory behaviour and extra care was taken to avoid using objects which would trigger these behaviours. The scoring data from the behaviour sessions would get saved in an excel sheet which was used for further analyses.

Immediate Early Gene experiments

Building up on the behaviour findings, we next wanted to investigate further into the role of different brain regions at different timepoints of the task. 80 mice were used overall for this set of experiments, which were further run in sub cohorts of 40 mice each. All mice performed the behaviour task described above, however they were sacrificed at specific predefined timepoints during the training paradigm. They were always sacrificed 90 mins after the last experience, in order to be able to capture the gene expression during the last experience. More precisely, mice were sacrificed at the *end of training day 1 and 5*; after the *first trial on training day 8*, *after interference and interference + novelty/no novelty trials on day 9* and finally *after the retrieval test session following different interference groups*. The training and sacrifice schedule was counterbalanced across all mice, with each group consisting of 5-6 mice. In addition, 3 control groups were included with 6 mice each – *home cage controls* where mice were handled regularly but did not run any behaviour trials, *one trial learning* where mice were sacrificed 90 mins after running a single trial and *interference in a different box* where mice performed the interference trial in a training box radically different from the one they were training in.

Brain processing

At the end of the behavioural timepoints, the mice were first anesthetized via intraperitoneal injection of 150 ul sodium pentobarbital (Eutanax 200mg/ml, Fatro). Once there were well under the effect of the anesthesia, they were sacrificed via transcardial perfusion. With regard to the perfusion procedure, first an incision was made to the chest cavity of the mouse in order to expose the heart. Next a cut was made on the left atrium of the heart and 30 ml of 0.1M PBS (phosphate buffered saline) was injected via the right ventricle using a peristaltic pump to ensure a stable and continuous flow rate. The PBS injection was followed by 30 ml injection of 4% paraformaldehyde (PFA) in 0.4M PB (phosphate buffer). At the end of the procedure, the brains were extracted and left overnight in ~30 ml of 4% PFA at 4°C for post fixation of tissue. 24 hours later, these post fixated brains were washed 3 times in 0.1M PBS to get rid of residual amounts of PFA and then immersed in 30% sucrose (containing 0.02% sodium azide) in PBS and kept at 4°C until they sunk to the bottom of the jar. Once they sunk to the bottom, they were frozen under dry ice and stored

for the long term at -80°C. In the next step, the brains were sectioned into 30µm coronal sections at -20°C using a MEV cryostat (SLEE medical GmbH). The sections were sequentially (in an ascending order of anterior to posterior slices) stored in 12 well plates containing ~2 ml of 0.02% sodium azide in PBS and kept at 4°C. Prior to sectioning the brain, a superficial mark was made on the left hemisphere to be able to distinguish later between the two hemispheres.

Immunohistochemistry

The aim of the study was to check for Arc and c-Fos expression across different brain regions – Prelimbic Cortex (Prl), Anterior Cingulate Cortex (ACC), Retrosplenial Cortex granular and agranular (RsPg and RsPAg), Posterior Parietal Cortex (PPC) and hippocampal subfields – Hippocampus Ca1, Ca3 and Dentate Gyrus (DG). For this purpose, 7 sections were selected for each animal spanning the frontal and parietal regions which were subsequently processed for free floating immunofluorescence staining. The staining protocol was always run in sub cohorts of 6-7 mice belonging to different behavioural timepoint groups to minimize any group specific bias related to staining steps. The entire staining protocol consisted of seven days. On day 1, the selected sections were placed in 1 ml of 4% PFA and were allowed to incubate at room temperature (RT) under the fume hood for 24 hours. On day 2, the sections were removed from PFA and washed 3 times with 0.1M PBS, 10 mins each time on a shaker. Following the washes, the sections were incubated for one hour with a blocking buffer (2% Bovine Serum Albumin (BSA) and 0.3% Triton X-100 in 0.1M PBS) on a shaker at RT. At the end of the blocking incubation, sections were washed again (3 x 10min in 0.1M PBS) and then incubated in primary antibody. The sections were incubated in 1:1000 Guinea pig anti-c-Fos (Synaptic Systems, cat no. 226 308) and 1:500 Rabbit anti Arc (Synaptic Systems, cat no. 156 003) prepared in blocking buffer and left on a shaker at 4°C for 72 hours. On day 6, the sections were washed again (3 x 10min in 0.1M PBS) and then incubated for 2 hours at RT with the secondary antibody in the dark. The secondary antibodies were 1:1000 Alexa fluor 647 anti-Guinea Pig (Jackson ImmunoReseArch) and 1:1000 Alexa fluor 488 anti-rabbit (Abcam) prepared in blocking buffer. Due to light sensitivity with the secondary antibody, all steps including and following the incubation period were done in the dark. At the end of the incubation period, the sections were washed again (3 x 10min in 0.1M PBS) and then mounted on microscopic slides and left to dry. Once the slides were dehydrated, a drop of mounting medium containing DAPI was added to each section and allowed to incubate for 10 mins. Following the incubation period, the slide was covered with coverslip (1.5mm thickness) and stored in the dark at 4°C for imaging.

Microscopy

All samples were visualized under Leica Thunder Wide Field Fluorescence microscope and whole section images were acquired at 10X and 20X magnification to be further processed for cell counting analyses.

Behavioural Data Analyses

The amount of time the mice spent exploring objects was scored in real time which would then get saved in an excel sheet. The total exploration time was calculated as the sum of time spent exploring both objects. Further on the discrimination index (DI) was calculated by subtracting the amount of time exploring the familiar location from the novel location and dividing by the total exploration time.

*Discrimination index = (Novel location – Familiar location) / Total time exploring both locations*DI > 0 indicated a preference for the novel object location, which was used as a prime measure for memory performance. DI = 0 indicated no preference for either location and finally DI < 0 indicated a preference for the stable object location.

The behaviour experiments were conducted in a within – subject design, such that all mice performed all conditions in a counterbalanced manner. Taking this into account, for statistical analyses, the behavioural data from the "behaviour" group was fed into a repeated measure ANOVA model with within subject factors – interference and novelty. A p value of ≤ 0.05 was used as the threshold for test of statistical significance.

Quantification of gene expression

For further analyses, our aim was to quantify the expression of Arc+ and c-Fos+ cells in the following Regions of Interest (ROIs) – **Frontal regions** (Prelimbic cortex (PrL), Anterior Cingulate Cortex (ACC)), **Parietal regions** (Retrosplenial cortex – granular and agranular (RsPg and RsPAg), Posterior Parietal Cortex (PPC)) and **Hippocampus** (HPC-Ca1, HPC-Ca3 and HPC-DG). In the first step, we mapped the images acquired from the microscope with the mouse brain atlas (BÜTTNER-ENNEVER 1997) and cut these acquired images to the corresponding ROIs as per the atlas coordinates. This step was done using ImageJ. For the next steps of cell counting, we used Ilastik (Berg et al. 2019), a machine learning software which uses a wide range of algorithms to segment and classify objects from background. To prevent bias and errors from manual counting of cell, we set up a training pipeline in Ilastik to enable automatic detection of c-Fos+ and Arc+ cells from the ROIs.

Separate training pipelines were set up in Ilastik for detection of c-Fos+ and Arc+ cells. Two workflows, namely, *Autocontext* and *Pixel and Object Classification* workflows

were used consecutively to enable accurate identification of cells from the ROIs. For each workflow, a subset of ~30 ROI images were selected across all groups to initially train the classifier to detect cells and background. On getting a reliable detection of cells in the training set, the remaining ROI images could be fed to the classifier to use the same criteria to automatically detect cells from background for the rest of the images. To maximize the detection accuracy, the images were first processed in the Autocontext workflow. This step helps with pre-segmentation and refining of images, and removing background artefacts. At the end of this workflow, we get a binary image output of probability maps of cell and background clusters which is then used to train the Pixel and Object Classification workflow. At the end of the Pixel and Object Classification workflow, we get a final output which is a binary black and white image consisting of white dots corresponding to the detected cells in the ROIs. In addition, it generates a csv file with the counts of detected cells. The binary output images are compared to the raw ROIs to assess for the quality of detection before finalizing the counts. Finally, the areas of the different ROIs were computed using ImageJ and then divided by the corresponding cell counts to get density values per ROI for each animal. These density values for all animals belonging to the different task groups were normalized by the average of the home cage controls for further analyses steps. The raw and ROI data can be accessed from the Donders repository (di.dcn.DAC_626830_0004_821).

Supplementary Material

Supplementary text

cFos and Arc

The immediate early genes cFos and Arc are commonly used as markers of encoding, consolidation and retrieval in the brain and their expression patterns tend to correlate. However, at times cFos is described more as a marker for neuronal activity while Arc is described as consolidation marker. Both proteins have been shown to be necessary for LTP (Guzowski et al. 2000; Plath et al. 2006; Fleischmann et al. 2003). Interestingly, Arc also has a role in LTD (Shepherd et al. 2006). Most IEG-associated manipulation tools, are tied to either cFos or Arc expression, but these markers are often used interchangeably not considering their underlying dynamics. Recent research highlights that many assumptions of how these markers function, are not as simple as proposed (Lamothe-Molina et al. 2022). Combining electrophysiology with cFos activity measurements, it has been shown that hippocampal cFos positive cells in CA1 are highly context specific and code more for experience than place (Tanaka et al. 2018).

Here, we could see effects such as congruency detection and sensitivity to novelty mainly in cFos and not Arc. Further, in Ca1 we saw a dissociation between cFos and Arc across groups, where a linear decrease in the cFos/Arc ratio from 1-trial learning to semantic-like to interference was seen. In general, cFos was more sensitive and showed larger gene expression changes in comparison to home cage than Arc. Interestingly, while the memory-networks differed slightly for cFos and Arc, they largely overlapped. cFos networks aligned with areas of expected neuronal activity during memory encoding and retrieval for each learning group, while the Arc networks would correspond to known consolidation networks. Thus, the relationship of activity vs. consolidation marker for cFos vs. Arc respectively, would be supported by our results.

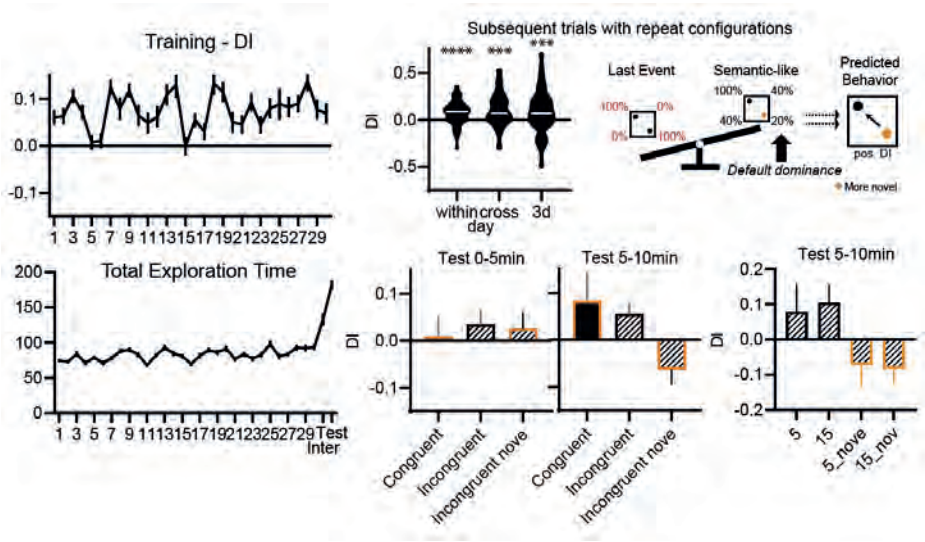

Fig. S1. Behaviour

Left: Training discrimination index and total exploration time per trial. Right: DI for trials where the previous trial had the same configuration within day (trials 2-4, 6-9…., average for each animal), cross day (trials 5, 10, 15, 20, 25, average for each animal) and 3d test (trial 30), * one-sample t-test to chance. Below: Test trial for the behavioural (non IEG) cohorts. The first two graphs show the performance at the test trial split into first and second 5 mins. Results show similar findings as that shown in Fig. 3C. The third graph shows performance at test trial for mice groups that had different interference trial durations (5 and 15 min).

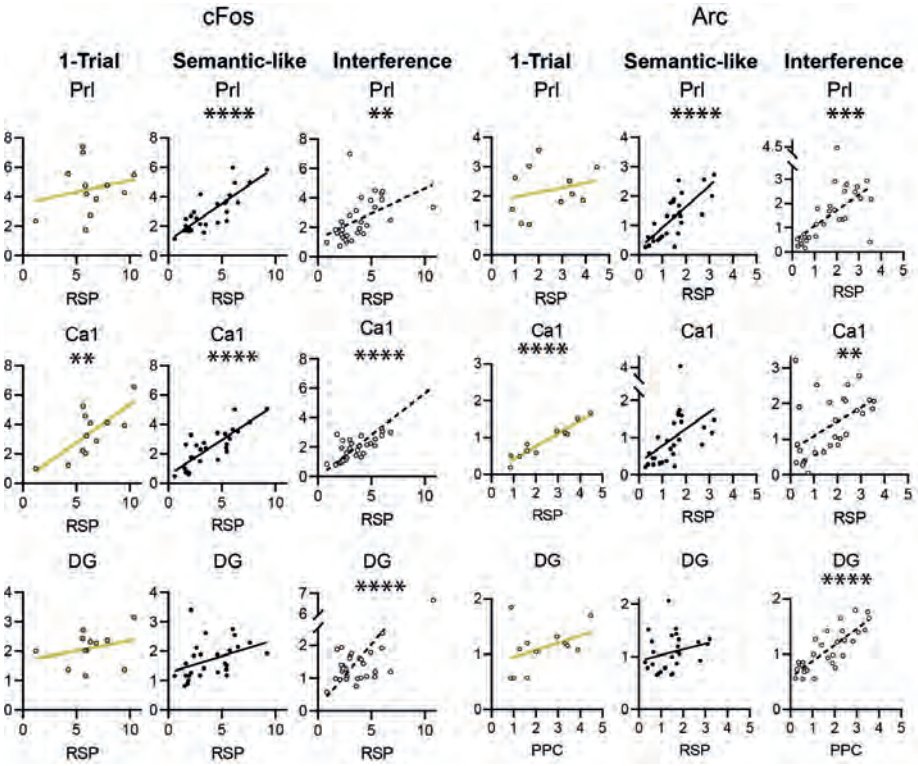

Fig. S2. RSP with Prl, Ca1, DG

Correlations per key brey areas: retrosplenial (RSP) as central cogwheel connected to prelimbic (Prl), hippocampal Ca1, dentate gyrus (DG) depending on memory condition. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

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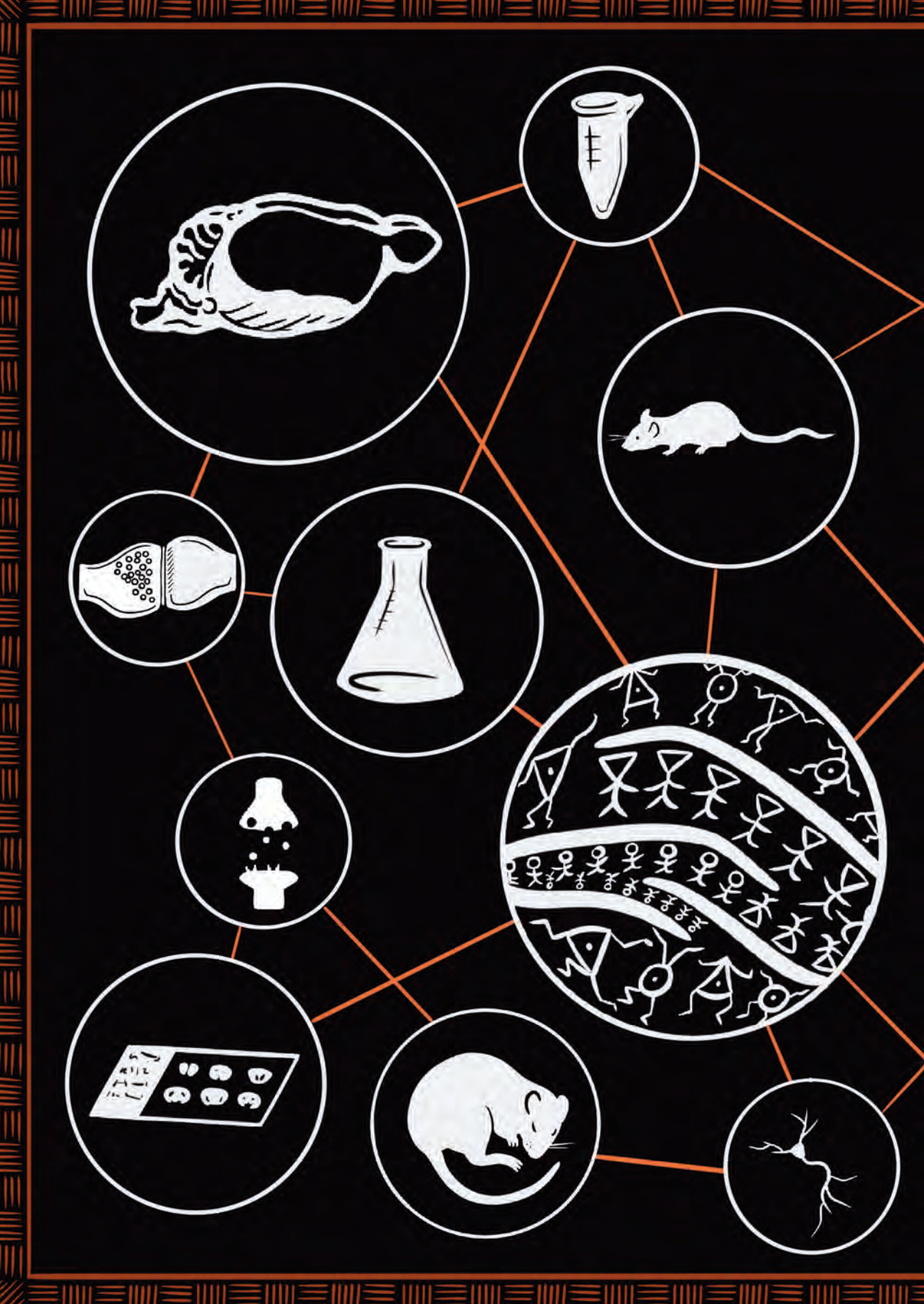

Chapter 6

General Discussion

"Before you sleep, read something that is exquisite, and worth remembering."
– Desiderius Erasmus, Dutch Philosopher

Speaking in colloquial terms, "sleeping on it" is the most common strategy we use when faced with scenarios of having had to process a lot of new information and use it to make decisions. Research from the past decades has provided us with several key mechanistic insights on how sleep potentially regulates memory consolidation processes. These regulation processes range from offline reactivation of experiences to synaptic downscaling and pruning of memories (Tononi and Cirelli 2014; Wilson and McNaughton 1994). Each of these mechanisms make a significant contribution in ensuring proper consolidation of memories and creating long lasting memory networks in the brain. However one caveat lays in the fact that a lot of these mechanisms have so far been modelled on simple memories that do not adequately reflect higher human cognition that we are interested in. It is generally thought the hippocampus plays the role of a "fast learner" in rapid encoding of information in the short term and the neocortex takes up the role of being a "slow learner" in integrating the new incoming information with existing knowledge networks for long term storage (Frankland and Bontempi 2005; McClelland, McNaughton, and O'Reilly 1995). Building upon this hypothesis, using human fMRI and EEG, rodent electrophysiology and molecular techniques, I aimed to investigate different behaviors and underlying hippocampal – cortical dynamics in light of simple and complex semantic memories. Furthermore, offline consolidation processes have been shown to be influenced by factors including previous knowledge, sleep, endocannabinoids, novelty events and complexity of information being processed (Tse et al. 2007; Duszkiewicz et al. 2019; Havekes and Abel 2017; Wilson and Nicoll 2002). In this thesis, my focus lies in investigating in greater detail how each of these factors influence consolidation processes at a behavioral and neural level and further gain an overall better understanding of the evolution of buildup of semantic memory networks in the brain.

The dichotomy of simple and complex learning is better understood when we examine how memory processing mechanisms evolve as the brain encounters increasingly complex information. With chapter 2, I am first able to show the conserved role of sleep in leading brain wide systems consolidation in both rat and human models. In chapter 3, I summarize how neural reactivations are influenced by neuromodulators. Knowledge I applied in Chapter 4, where I am able to show CBD induced differences in oscillatory patterns of activity during NREM sleep and its effects on learning. Lastly, in chapter 5, looking at gene expression patterns underlying different timepoints of a complex learning paradigm in mice, I am able to disentangle the

individual contributions of the hippocampus and the frontal and parietal cortical regions to the buildup of a semantic memory network of the task.

In summary, with the findings in my thesis, I propose a novel mechanistic insight on how semantic memories might be processed in the brain. Building up on the general role of sleep in memory processing, I am first able to show that the dependency of a memory trace on sleep for offline processing and long term stabilization increases based on the complexity of the memory. Secondly, contrary to the generally assumed role of the prefrontal cortex as a slow learner, the cortex is potentially equally involved at the encoding stage and adapts its workings according to the information that is being processed.

Learning and memory – definitions

Learning and memory are closely associated concepts that fundamentally shape how we perceive and navigate through our environment. Learning is defined as a continuous process of acquiring knowledge and/or skills over time through training, experience and repeated exposures to relevant stimuli while memory refers to the expression or stored trace of the acquired information in the brain (Squire 1986). Both go hand in hand with initial learning being more time consuming since one needs to encode the features of the context and the rules depending on the task at hand. The encoded features get stored as memory traces and are used subsequently for the next learning steps. To explain in a better way, the process of learning and the resulting formation of memories occurs in 3 stages – encoding (acquiring of new information), consolidation (transition of new information to more a stable pre-existing memory network) and retrieval (recall of previously encoded information using cues from the context of encoding) (McGaugh 2000). The question that next appears is how do the learning dynamics evolve from an initial stage to processing of more complex information over many different experiences? Which brain regions are more active when a naïve individual is starting to form simple memories and how does this evolve at a more advanced stage? Are complex memories formed using more simple memories as building blocks or are there other processing steps that are happening in parallel? Considering the fact that in a wake state, we are continuously processing information from our surroundings, it could be hypothesized that there are potentially multiple processes occurring simultaneously in the brain to integrate information from multiple sources and then transform them into more coherent memory networks that the organism can use for future decision making steps. These processes can range from – pruning of unnecessary information, prioritizing or enhanced consolidation

of events that are tagged with high salience, simple updating of pre-existing memory networks with new congruent information, reconsolidation of previous events with changing emotional valence or in the light of new conflicting information (Kitamura et al. 2017; Davis and Squire 1984; Duszkiewicz et al. 2019; Tse et al. 2007; McClelland, McNaughton, and O'Reilly 1995; Dudai 2006). In the aim of understanding these neural processes in more detail and the crucial hubs in the brain that orchestrate each of these actions, one needs to model specific behaviors in an organism where each of the factors contributing to learning can be disentangled. Rodents serve as an excellent model in this regard since they are naïve to knowledge in terms of the task at hand in the beginning, so the learning curve can be closely monitored and intervened with at different stages to better understand each contributing factor.

Within the scope of this thesis, I focus on declarative memory processing. In humans, these are further split into episodic and semantic memories wherein the former refers a to a vivid recollection of a single episode or event with all the details while the latter refers more to a gist memory formed by abstracting necessary information across multiple events (Squire 1986). Episodic memory has classically been known to be unique to humans (Tulving 1983) since it involves conscious recall of the spatiotemporal details around the episode, which is thought to be almost impossible in nonverbal organisms. However attempts have been made to model it in rodents using single trial object recognition tasks (Kart-Teke et al. 2006). However many of the behavior tasks and mazes developed for rodents involve repetitive trials, with each trial playing a role in reinforcing or updating the information to be learned. This would be then more analogous to semantic memories in humans. Depending on whether the same information is repeated across all trials or if there is information unique to each trial, we can further classify them as simple and complex memories.

Simple memories

Simple memories refer to behavior setups where rodents can perform the task with learning one element (e.g. rule, item, location) which remains stable across the sessions. Examples of such tasks range from those that test for simple object – location associative memory or a tone-shock memory (Antunes and Biala 2012; Curzon, Rustay, and Browman 2011). Rodents in these cases get subjected to one or multiple encoding trials with the same information being reinforced across them and a test trial assessing for memory retrieval at recent or remote timepoint. Simple memory in this thesis is tested using a couple of behavioral paradigms. The Stable Condition of the Object Space Task (Genzel et al. 2019) assesses simple object-

location memory by reinforcing the same object locations across multiple encoding trials followed by a retrieval test 24 hours later where one object is moved to a novel location. Rodents are said to have retained a simple memory if they spend more time exploring the novel object location. The watermaze is another task which also tests for spatial abilities of rodents to navigate in an environment to find the hidden platform below the water surface (Morris 1981). It allows researchers to test for different features of spatial processing – like allocentric or egocentric components. Both paradigms are defined as simple memory tasks because the same information is reinforced over multiple trials at encoding – hidden platform or object locations. However, with the watermaze, one can test for allocentric or egocentric components in navigation by using different or same start locations to introduce the rat into the maze. From the rat's perspective, it can solve the task using information from either just a single trial or multiple trials.

In my thesis, I tested simple memory processing in Chapter 2 and 4, with the watermaze and Stable condition of the Object Space Task respectively. In chapter 2, we see that rats are able to perform well in the watermaze for both sleep and sleep deprived groups. However sleep showed a boost in memory performance only when trained in the allocentric training condition. Memory performance when trained in egocentric condition reached a plateau phase and didn't improve further with sleep. In chapter 4, with the Stable condition of the Object Space Task, we looked at simple object location memory in rats and we see that their performance remains unaffected by CBD administration. Using the same task, I looked at the performance of rats in the Stable Condition following sleep deprivation during the encoding and immediate post training consolidation period (3 hours) and was able to show that they had an intact memory the following day at test (Samanta et al., in prep).

Regarding sleep related benefits, our findings align with previous studies looking at influence of sleep on allocentric and egocentric training representations which indicate that the allocentric representation benefits more from sleep (Albouy et al. 2013; Albouy et al. 2015; Nguyen et al. 2013; Peigneux et al. 2004). Given the role of hippocampus in spatial navigation (O'Keefe and Nadel 1978) and neural replay (Wilson and McNaughton 1994), it is assumed that hippocampal dependent memories would benefit more from offline consolidation processes. Human studies have previously also shown a positive correlation between the hippocampal activity observed during NREM sleep and the performance in navigating an environment and finding the correct goal the next day (Peigneux et al. 2004). In light of this context, our results align well with the previous findings where we see that rats who underwent allocentric training in the watermaze benefit more from sleep because these memories potentially get preferentially

consolidated by the hippocampus. When navigating our surroundings, we however constantly switch between strategies in a more dynamic way depending on the neural state and the cognitive demands of the task at hand. Both hippocampal and striatal systems work in cooperation and compensate for each other when one of the systems is impaired (Genzel 2020). When examining which strategy is more impacted by sleep deprivation, previous studies have tested rodents in simple learning paradigms like the T and Y maze. After sleep deprivation, the rodents tended to avoid the allocentric approach and instead relied more on an egocentric strategy (Hagewoud et al. 2010). In this case, the overall performance of the mice was not affected since the mice could use either of the strategies to find the food reward. The shift from an allocentric to an egocentric strategy has also been observed in humans during extended navigation periods through the same environment (Maier et al. 2024). Initially, navigation relies on a hippocampal-dependent strategy, but over time, individuals adopt fixed routes, transitioning to a simpler striatal-based strategy. These findings combined indicate that the hippocampal system is more dependent on sleep compared to the striatal system, potentially due to the higher amount of information detail encoded when adopting a hippocampal strategy which further extends to the need of more resources when consolidating the memory traces. Our results from Chapter 2 align with these previous findings and fit well into assumed roles of hippocampal and striatal systems in memory processing. Although the memory remains strong enough to perform above chance even in the sleep-deprived group, allocentric training involves encoding subtle environmental features across multiple trials as the rat reorients each time, engaging more molecular markers of plasticity during sleep, leading to better consolidation (Guzowski et al. 2001). In contrast, egocentric training focuses on route learning, where the rat takes the same path each trial to reach the goal. This performance likely plateaus, with less room for improvement, and consolidation is less sleep-dependent as memory processing in this case relies more on the striatum.In human counterpart, sleep overall improved memory performance independent of training. Virtual environment settings combined more mental navigation instead of physically moving to goal location potentially confounds the clear dissociation between training strategies (Kalantari et al. 2024). Also human subjects could have used allocentric strategies to perform the task even when training under egocentric condition.

Both the watermaze and the Object Space Task also share the feature of a training phase consisting of multiple trials where the same information is consistently reinforced. In the watermaze, this involves the fixed location of the hidden platform, while in the Object Space Task, it concerns the position of identical object pairs within the arena. This repeated reinforcement allows the rats to form a robust memory trace, resilient enough to withstand sleep deprivation. Findings reviewed in Chapter 3

show that hippocampal replay also takes place during brief periods of immobility while awake, right after a spatial experience (Foster and Wilson 2006; Carr, Jadhav, and Frank 2011). For simple memories, these consolidation processes during brief periods of inter trial intervals and quiet wake periods after training might suffice for the sleep deprivation group and explain the above chance performance. This would be especially true for our findings from the Object Space Task and also explain why the memory performance remained unaffected by CBD administration (Chapter 4) and sleep deprivation (Samanta et al., in prep). Potentially information from initial trials gets consolidated during quiet wake/brief rest periods happening during inter trial intervals and then this memory network simply gets strengthened during the subsequent trials. Due to lack of new information every trial, minimal updating of memory network is required and remains strong enough and resistant to effects by neuromodulators like CBD in this case. A study (Prince et al. 2014) looking at Object Place Recognition memory showed that consolidation of the memory trace was dependent on the onset of the sleep deprivation period after training. In this case, since the mice performed only one training trial, the memory potentially remained dependent on the hippocampus and hence was more sensitive to sleep deprivation effects. However, in our case, with repeated trials, the cortex was also potentially active in consolidating the information and strengthening the memory network, protecting it from sleep deprivation effects.

In conclusion, we see a dissociation between the nature of memories and their dependency on sleep for consolidation depending on the conditions during encoding and the content being processed. Simple memories, by definition, include those memories that involve straightforward associations and minimal need to abstract information from multiple sources, however there are differences depending on the brain regions being used at encoding. In consensus with previous findings, we show that striatal based memories including route learning and egocentric navigation are least dependent on sleep (Tucker et al. 2006). Object-location association memory classically is known to be dependent on the hippocampus (Milner, Johnsrude, and Crane 1997). However, the cognitive load of the memory is reduced with repetition of content being encoded and time and potentially gets consolidated into the cortex earlier on during quiet wake periods, becoming less dependent on sleep. Memories encoded under allocentric training in contrast show the highest level of dependency on sleep due to the involvement of hippocampus at encoding and potentially needing more resources to form a cohesive memory representation across multiple navigation frames. The dependency of memory consolidation on sleep hence varies with the complexity and nature of memories being processed.

Complex memories

In our day to day life experiences, we interact with a diverse range of inputs with continuously changing rules. Our brain is constantly adapting and matching these incoming inputs with pre-existing information and in the end guiding us in our future decisions. To be able to understand the neural underpinnings of how are we able to process and consolidate this information, we developed a behavior paradigm to model complex learning in rodents. The paradigm aims to design a task that subtly reinforces a fixed rule while introducing new information in each trial, assessing whether rodents can adapt to the task's demands.This aspect of changing rules has been tested previously using mazes like the radial arm maze and T maze wherein they show that rodents are capable of "unlearning" a previously learned path to get to the food reward and update the memory network with a newly learned location (Olton and Samuelson 1976; d'Isa, Comi, and Leocani 2021). This shows that rodents have the cognitive flexibility needed to deal with tasks with higher cognitive demands. In other tasks like the cheeseboard maze (Llano Lopez et al. 2010) and HexMaze task (Alonso et al. 2021), we see that rodents are also capable of building long term schema memories of complex environments as they navigate around it for food rewards and can easily update this network with newly changing paths to get to the food locations or other variables changing in the environment. In all of these tasks, there are features in the environment that remain constant which the rodents use to orient themselves and build a cognitive map. In a stepwise manner, their brains display cognitive flexibility, allowing them to pay attention to changing environmental variables and learn to interpret them effectively to successfully complete the task.

With the Object Space Task, we wanted to assess whether rodents intrinsically possess the ability to abstract information across multiple episodes and form a cumulative memory trace in the absence of external motivators and rewards. This refers to the Overlapping condition in the task with one object location fixed and one location changing across trials (Genzel et al. 2019). However, the object locations at test trial are identical to the last training trial, so enables us to assesses whether rodents abstract information across multiple trials to form a cumulative memory trace and thereby explore more the location that kept moving across all the training trials and hence perceived as more novel. In contrast to simple memories, processing of these memories needs a constant dialogue with encoded information from previous experiences to abstract the similarities/regularities, and in the long run, form a semantic memory network. This makes it a slower learning process compared to that involving simple memories. The resulting memory traces are also subjected to labile states since new information needs to be constantly integrated into pre-existing network after assessing for congruence or incongruence of information.

It has been previously established in the lab that both rats and mice are capable of forming cumulative memory traces and showing an increased preference for the moving location. With the results of chapter 5, we were able to show that mice had an intact semantic memory also at 72 hours. This semantic memory network was further shown to be resistant to interference from a single trial containing incongruent information. However when we increased the salience of the interference trial by tagging with a novelty exposure event, the old memory was overwritten. These findings were further replicated in rats (Samanta et al., in prep) where they showed an intact semantic memory at 72 hours. The semantic memory network in this case was impaired when the rats were sleep deprived after training. In addition, we also tested the robustness of the semantic memory network when subjected to a single trial containing incongruent information. In specific subgroups, the salience of the interference trial was either increased by tagging with a novelty exposure event after the trial or decreased by a 3 hour sleep deprivation session immediately after encoding. Similar to the results from chapter 5 in mice, the rats showed an intact semantic memory network resistant to interference effects under most condition except when they were sleep deprived and the salience of the interference episode was increased. With these results we are able to show empirical evidence in support of the theory posed by McClelland (McClelland, McNaughton, and O'Reilly 1995) regarding the hippocampus being a fast learner and prefrontal cortex being a slow learner which prevents the overwriting of old information.

Going top down from the level of looking at coordination between brain regions to cellular level changes, there are multiple mechanisms at play to coordinate successful consolidation of memories. The hippocampus is largely responsible for encoding of memories at the short term (Eichenbaum et al. 1996). Encoding of new memory induces LTP and subsequent plasticity related changes triggering the release of plasticity related proteins (PRPs) and other cascades of molecular reactions – ultimately strengthening the memory trace (Frey and Morris 1997; Redondo et al. 2010; Wang, Redondo, and Morris 2010). Consolidation of these memories further happens during sleep when they are replayed during SWR events in the hippocampus (Wilson and McNaughton 1994). It is stipulated that the memories are integrated into the cortex for long term but how fast is the transition remains unknown. This above mentioned mechanism has been shown to hold true for general consolidation of memories. However there are differential mechanisms depending on the content and type of memory that is being processed. The first time the rodents are exposed to a new environment, there is a lot of new information to encode, which initiates LTP and potentially a boost in expression of PRPs. This memory is then tagged and prioritized for replay during the rest/sleep period after the task and subsequently

consolidated for further recall (Redondo et al. 2010; Frey and Morris 1997; Seibt and Frank 2019). For simple memories, the next trials contain repeated information, so one might speculate that this serves to further reinforce the existing memory, and minimum amount of updating of the memory network. Over the next sections this will be discussed in further detail, but we hypothesize that for these simple memories, the baseline level of plasticity processes might suffice to successfully consolidate them for the long term. However, in the case of complex memories, the environmental settings and features across the trials largely remain the same but the rodents are exposed to new information every trial. So in contrast to processing of simple memories, this requires constant updating of the memory network, which necessitates a constant dialog between the hippocampus and cortex. In chapter 4, we see that with CBD administration, the rats become worse at complex memory processing. We also observed specific oscillatory effects mirroring the behavior findings, which potentially explains the different effects of CBD and sheds new light on neural mechanisms underlying processing of different types of memories.

In the next sections, I will discuss the neural mechanisms at the level of brain region coordination, cellular consolidation processes and electrophysiological correlates underlying memory consolidation processes and finally hypothesize a novel framework regarding buildup of semantic memory networks and how do they evolve with time.

Sleep leads to systems consolidation

Systems consolidation refers to the transforming and strengthening of labile memory traces from short term to long term storage. Sleep plays a crucial role in this process by contributing to functions ranging from synaptic downscaling, pruning of memory networks and replay of relevant information encoded during wake (Tononi and Cirelli 2014; Seibt and Frank 2019; Wilson and McNaughton 1994). Looking at the behavior results from Chapter 2, we see that in rats, sleep boosted memory performance in the watermaze with a slight bias for those trained under allocentric training condition. Mirroring these behavior findings, we observed an increase in IEG expression in the hippocampus and striatum respectively for allocentric and egocentric training conditions and additionally, an increase in expression in the PFC across both training groups. In contrast, we only observed localized increases in the hippocampus and striatum for the specific training type for the sleep deprived group. These results are in agreement with previous findings in literature using the watermaze which have shown a sleep induced increase in IEG expression in the hippocampal and striatal sub regions (Genzel et al. 2017; Gardner et al. 2016). This region specific increase in

expression has also been mirrored when rodents are seeing shifting their strategy from place to response learning when subjected to sleep deprivation (Hagewoud et al. 2010). In an unpublished study (Samanta et al., in prep) with rats being trained in the Overlapping condition of OS task, we also see an increase in c-Fos expression at retrieval solely for those that were allowed to sleep during the encoding and consolidation phase post training. This further mirrored the better memory performance over sleep compared to the sleep deprived group which showed minimal expression in the PFC and subcortical regions. Sleep deprivation has also been shown to adversely influence the synaptome architecture in the cortex and hippocampus , with modelling studies indicating that it affects the neural activity in these regions (Koukaroudi et al. 2024). In summary, our experimental evidence across both behavioral paradigms indicates that there is an increase in cross region connectivity during sleep, leading to long term consolidation of the encoded information which potentially is the underlying reason for successful memory retrieval in the task.

The behavior and molecular results conform to the theory of systems consolidation (Frankland and Bontempi 2005). Since we only looked at retrieval induced gene expression, we are unable to tell the contribution of these brain regions for certain at encoding and consolidation timepoints. However previous evidence has shown the critical involvement of hippocampus and striatum at encoding, so we would expect the same in our paradigm. It is interesting to speculate at which time period of memory processing step does the prefrontal cortex start getting critically involved. Recent evidence from human studies indicates that the PFC and other related cortical areas play an active role in abstracting meaningful learning statistics in real time as humans are engaged in semantic memory tasks that require inferring higher order relationships between task components (Jamali et al. 2024; Wittkuhn et al. 2024). Animal studies (Centofante et al. 2024; Leon et al. 2010) have shown the lack of PFC activation during consolidation to impair recall at both recent and remote timepoints. Further reports have also observed a differential involvement of PFC sub regions with the anterior cingulate cortex being more involved in recent recall and prelimbic cortex in more remote timepoints (Frankland et al. 2004; Takehara-Nishiuchi and McNaughton 2008). So one could potentially conclude that PFC serves as a key brain region during the consolidation period and sleep plays a critical role in this process. In addition to serving as a region for long term storage, the PFC could also potentially be helping with flexibly guiding the rats' behavior in the next trials using information from previous trials, especially in the allocentric training scenario (Euston, Gruber, and McNaughton 2012). Having said this, the PFC could then be potentially differentially recruited at later stages of encoding, more in allocentric training than egocentric training since there is more decision making involved with respect to

having to find a more novel path to the platform in contrast to taking the familiar route with egocentric training. Having tested only at a recent timepoint of 24 hours, the memory was still dependent on both hippocampus and striatum along with PFC. Analogously, processing of complex memories in the Overlapping condition of the OS task showed a greater dependency on sleep than when they were engaged in a more simple learning variant of the task. In both behavior paradigms, the rats are exposed to changing spatial and contextual information in every trial and they are required to use this information in real time to make well guided decisions to successfully perform in the task. They potentially use the brief sleep periods during the intertrial intervals to initiate a dialog between the HPC and PFC in consolidating the encoded information from each trial. As the rats undergo several trials, the PFC potentially drives the dialog by activating the context specific hippocampal representations, guiding the rats in making correct context dependent decisions (Place et al. 2016). In both cases, one could speculate that the memory is potentially independent of the subcortical regions at a remote time point, but there are mixed results in this regard. The overall recruitment of all the brain regions including HPC, STR and PFC depend on multiple factors including memory content and strength, presence of extramaze cues, type of training both at initial stages of when the memory is formed and when it is recalled. To conclude this section, with the findings discussed above, we reconfirm the claim that sleep plays a critical role in increasing cross region connectivity and in that regard initiating communication between the subcortical and cortical regions. Furthermore, we are able to show that the dependency on sleep for consolidation increases with the complexity of the task being executed.

Distributed roles of different brain regions in memory processing

Different brain regions are continuously working in coordination with each other in processing incoming information to come up with optimal decisions and translating them into actions. Depending on the task, certain brain regions become more active at specific stages, and as the task progresses, a distributed memory network forms, encoding various aspects of the task. The essential role of hippocampus in encoding of memories has been empirically shown by multiple studies (Shapiro and Eichenbaum 1999) but its role in memory recall seemingly depends on the content of the memory. Owing to its critical roles in memory reactivations (Wilson and McNaughton 1994), neurogenesis, (Aimone, Deng, and Gage 2011), pattern separation and completion (Leutgeb et al. 2007), and offline consolidation processes (McNaughton, Leonard, and Chen 1989; Qin et al. 1997), it still remains uncertain whether and at which time point

do the memories become independent of the hippocampus. Several converging lines of evidence strengthen the role of frontal regions including the prefrontal and anterior cingulate cortices in long term systems consolidation and retrieval of memories at both recent and remote time points in different tasks ranging from fear conditioning (Frankland et al. 2004), spatial tasks (Lopez et al. 2012) and paired – associate memories (Tse et al. 2007; Wang, Tse, and Morris 2012). The role of hippocampus in retrieval could be through partial or full reactivation of the context around the original encoded experience and sub hippocampal regions retrieving the original memory through pattern separation and completion. The PFC could potentially on the other hand be leading retrieval by "familiarity/congruence" by matching incoming information with pre-existing information and thereby activating the original engram and reconsolidating with the new information. Apart from the frontal cortices, other regions like the retrosplenial cortex have also been implicated in playing an important role in coordinating information transfer between the hippocampus and prefrontal cortex due to its anatomical position. It has been shown to play a crucial role in spatial orientation, planning, object location associations and memory consolidation processes (Kwapis et al. 2015; Vann, Aggleton, and Maguire 2009; Balcerek, Włodkowska, and Czajkowski 2021). In the next sections, I will elaborate further upon the communication dynamics between the hippocampus and cortex and explain the changes we see depending on the neural state and the information being processed.

Hippocampal – cortical circuit dynamics

As mentioned previously, communication between hippocampus and the neocortex is key to successful consolidation of memories. The mechanisms by which the hippocampus communicates with the prefrontal cortex is also well studied and plenty of experimental evidence exists in favor of close coordination between these regions during offline states being crucial for memory consolidation (Maingret et al. 2016; Qin et al. 1997). To briefly recapitulate, memories encoded during wake are reactivated in the hippocampus largely during NREM sleep in the form of high frequency burst oscillations called Sharp Wave Ripples (SWRs) occurring in the CA1 region of the hippocampus. Since its discovery, it has been identified as a crucial marker for memory consolidation (Wilson and McNaughton 1994; Buzsáki 2015). In addition to the SWRs, other oscillation markers including Slow Oscillations and cortical spindles, also predominantly observed during NREM have also been implicated in memory consolidation processes. Apart from these events occurring individually, closed coordination of these events occurring together is key to successful memory consolidation (McNaughton, Leonard, and Chen 1989; Qin et al.

1997). Specifically, the hippocampus works in close coordination with the frontal and parietal cortices in regulating these offline processes. However it still remains to be understood how are these communication dynamics influenced by learning and complexity of information to be processed.

The Default Mode Network (Raichle 2015), reflecting a coherent co-activation of these brain regions has recently been implicated in playing a key role in regulation of semantic memory processing. One theory is that the parietal cortex regions belonging to this network serves as a key intermediary region in gating information transfer between the hippocampus and prefrontal cortex (Kaefer et al. 2022; Genzel 2020). This will be further touched upon in the later sections when discussing the circuit dynamics underlying complex learning at a molecular level. Several rodent studies have shown learning induced co-occurrence of high frequency ripples in prefrontal and parietal cortices along with hippocampus (Khodagholy, Gelinas, and Buzsáki 2017; Aleman-Zapata, Morris, and Genzel 2022) which could be reflective of this process of integration of memory traces from hippocampus to more downstream cortical targets for long term storage. Our fMRI findings from Chapter 2 support this hypothesis where we also see an increase in activity of the Excecutive Control Network (Gilmore, Nelson, and McDermott 2015) which is known to be active when engaging in goal directed behaviors coupled with a decrease in activity of the Default Mode Network (Raichle 2015) over sleep. Resting state analysis of this dataset showed a positive correlation between the spindle – slow oscillation coupling with better memory performance across both training conditions. Furthermore, the coupling also correlated with a decrease in activity of the DMN at resting state (Bastian et al. 2022). Bringing these findings together, we hypothesize that initially the hippocampal regions serves as a dominant hub during the task encoding state and during the resting state, there is a decoupling in the network taking place wherein the encoded information is sent from the hippocampus to other cortical regions. This consolidation process seen solely over sleep could be the underlying reason of a better memory performance at retrieval. In the next sections, I will further reinforce the positive role of sleep in consolidation by discussing the changes in hippocampal and cortical oscillation dynamics when processing complex information.

NREM oscillations and memory consolidation

The close coordination of NREM oscillatory events occurring in the hippocampus and cortex are key to successful memory consolidation. The baseline consolidation dynamics underlying simple learning events include an ongoing process of neural

replay via SWRs in the hippocampus followed by slow oscillations and cortical spindle events in the cortex (Maingret et al. 2016; Qin et al. 1997). However during semantic learning, the complexity of information to be processed increases as new incoming information has to first be matched with pre-existing memory networks for overlap and then the important details consolidated for the long term . This necessitates an enhanced coordination between the hippocampus and prefrontal cortex, which potentially gets reflected with a change in the oscillation dynamics (Preston and Eichenbaum 2013; Aleman-Zapata, Morris, and Genzel 2022). Considering the critical role of SWRs in reactivation of memories, one would expect an increase in the length of reactivation events as the complexity of information to be processed increases. These reactivation events have to further be matched with the existing memory networks in the cortex and continuously updated in real time, hence there would potentially also be an increase in hippocampal-cortical coupling events (Peyrache et al. 2009; Aleman-Zapata, van der Meij, and Genzel 2022). Multiple studies have shown the incidence of long ripples leading to better memory in support of this hypothesis (Davidson, Kloosterman, and Wilson 2009; Fernández-Ruiz et al. 2019). Consistent with these findings, in Chapter 4, we see a decrease in the number of long ripples upon CBD administration, however the number of short ripples stay intact. This mirrors the behavior finding wherein CBD administration led to a worse performance in the rats solely in the Overlapping condition of the Object Space Task while performance in the Stable condition remained unaffected (Genzel et al. 2019). Additionally, we also observe long ripples to be occurring more in sequences compared to short ripples and an increased number of delta-ripple coupling events following training in the overlapping condition for the control animals. This was attenuated for the CBD group, further explaining the deficit in complex memory processing. On the other hand, for processing of simple semantic memories, short ripples suffice to reactivate the information and relay to the cortex. For the consolidation of this information, hippocampal-cortical coordination would only be required in the first stages to integrate the information into the cortical network, but for the later stages, it suffices for the cortex to simply strengthen the existing memory network, since it's the same information getting reinforced with every trial. This gets reflected in our results where we see an increased number of Delta-Spindle coupling events during Simple learning which stays intact upon CBD administration. This matches with previous findings from human studies where they also see an increase in this coupling event as a result of better performance in simple associative learning tasks (Cox, Hofman, and Talamini 2012; Mölle et al. 2002).

In summary, findings from this chapter provide us with a mechanistic insight into how the brain processes and consolidates both simple as well as intricate details

across multiple experiences into long term memory. Basic resources suffice for the consolidation of simple memories and the resulting memory trace is robust and more resilient to neuromodulatory effects. In contrast, consolidation of complex semantic memories requires a gradual build up of the knowledge network and hence more resources are recruited to ensure the formation of a strengthened long term network. However continuous updating of the network makes it more vulnerable to the interference and neuromodulatory effects. In the next sections, I will discuss the findings from Chapter 5 and provide a novel insight into the build up of semantic memory networks.

How Semantic Memories are formed

Individual memories or experiences are thought to be stored in fixed populations of neurons called neuronal ensembles or engrams (Josselyn and Tonegawa 2020). Engrams are defined as physical substrates of memory consisting of neurons that have been activated through encoding of a new experience, undergoing physical and chemical changes in the process and can be reactivated to retrieve information from the previously encoded experience. These engrams can be localized to a single brain region or distributed across multiple brain regions depending on which are the dominant hubs in the memory network. According to classic systems consolidation theory, an encoded memory trace is initially labile and bound to the hippocampus and over time (days or weeks), this memory trace is strengthened and integrated into the neocortex (Frankland and Bontempi 2005). Another competing theory states that when a memory is encoded, the hippocampus serves as an index of the activity and stores the abstract representation of the experience and the details of the encoded experience are stored across different cortical areas. When retrieving this memory, then the projections of the hippocampus to the neocortex containing the relevant information get reactivated to activate the relevant memory traces in the cortex containing the detailed information (Teyler and DiScenna 1986). On similar lines, the multiple trace theory states that for every experience encoded by the hippocampus, it will always be involved in the retrieval of that experience. Every time the memory trace is reactivated, it creates an additional trace in the hippocampus containing the contextual information from the recall. The neocortex then abstracts information from these traces independent of the context and creates a gist representation for the long term (Nadel and Moscovitch 1997). Finally, the complementary learning systems theory states that the hippocampus serves as a fast learner, rapidly encoding information in the short term and the neocortex is more of a slow learner which is slowly abstracting information across overlapping experiences and integrating

information into pre-existing memory networks (McClelland, McNaughton, and O'Reilly 1995). Having this dual memory system further helps with avoiding memory interference, i.e. overwriting of existing information with newly obtained information. We recently showed (Navarro Lobato et al. 2023) results in support of this theory, showing that artificially increasing the plasticity of the prefrontal cortex makes the brain vulnerable to memory interference and compromises its role in slow building of semantic memory networks.

Role of PFC in buildup of semantic memory network

The PFC plays a crucial role in organizing and structuring memories into distinct networks. Classically it has been shown to be more involved in later stages of memory consolidation, interacting with hippocampus to update the memory networks and guiding retrieval by assessing the specific contexts (Eichenbaum 2017; Preston and Eichenbaum 2013). As relevant as a role it is thought to play in semantic memory processing, however so far its functions has mainly been studied in relatively more simple memory tasks consisting of single or multiple training trials but testing for more episodic like memory. There is ample amount of evidence showing its necessity in retrieval of memories at recent and remote time points, confirming its role in storing information for the long term, but not much is known about how it adapts to constant influx of information and how plastic or rigid are the structures to adapt the pre-existing networks. Interestingly, in Chapter 5, looking at IEG expression as the mice undergo multiple trials in the Overlapping condition of the Object Space Task and are in the process of building a semantic memory network, we see a significant boost in c-Fos expression in the PFC after the first trial experience and then a gradual reduction in activity as they undergo more training trials. The expression in HPC follows a similar pattern. The first trial of object exploration in the training box filled with 2D and 3D cues potentially mimics a distinct novelty event for the mice and triggers the LC signaling pathway, leading to a vivid and long lasting memory of the trial. Contrary to what is assumed, the information then gets simultaneously encoded in both the Hippocampus and Prefrontal cortex (Duszkiewicz et al. 2019; Ott and Nieder 2019). In addition to the hippocampus enhancing consolidation of experience tagged by dopamine, the prefrontal cortex has also been shown to have dopaminergic innervations which aid the mice in flexibly guiding and decision making for the next steps (Seamans and Yang 2004). Having largely similar features in the environment in the subsequent trials, there is a reduction in the activity of the frontal cortex by proxy of reduced expression. The reduced amount of activity is potentially focused on abstracting the overlapping features across the multiple trials and building the

semantic network. One can see the reduced response of the hippocampus on Day 8 of learning, which confirms the claim that at that time period, the mice largely depend on the prefrontal cortex for retrieving the cumulative memory trace and hippocampus is not actively involved at this point. Similarly, we see a boost in expression in the PFC when mice run a single trial containing congruent information. According to its role in familiarity, the PFC is more active in integrating new information into existing networks, hence shows higher activity during congruent trials than incongruent ones. Behaviourally, the interference trial on its own fails to compete against the semantic memory network and the mice still show a strong memory of what they learnt prior to the trial. This is in contrast to recent findings in object recognition paradigms where the interfering information overwrites the original memory (Autore et al. 2023). However in this case, the interference memory was competing against a single trial memory which was still dependent on the hippocampus. Our results provide evidence to support the hypothesis of PFC being a slow learner, resulting in the buildup of a robust and resilient semantic memory network. Interestingly the activity of the PFC is also high when the mice encounter a distinct novel experience after an interference trial containing incongruent information. Similar to the novelty grading of the first trial, this novelty experience mimics more the "distinct novelty" which doesn't share any commonalities with surrounding experiences, instead boosts the salience of the memory encoded prior to it, which results in the interference trial overwriting the old information. With the network analysis, we see that the interference events are more led by the hippocampal regions, due to its nature of containing conflicting information and being primed for enhanced consolidation due to the dopamine projections from the LC triggered by the distinct novel experience. The novelty experience, potentially puts the PFC on a more rapid learning mode, rendering the semantic memory trace to a more labile state and it gets prone to interference. The exact mechanisms of whether this reconsolidates the original memory engram from semantic learning or recruits a new engram competing with the old one still remains unknown. Ongoing experiments are being currently run to check the degree of overlap in cFos expression of neurons between the first trial and the learning trial on day 8 using cFosTRAP2 mouse models (DeNardo et al. 2019) that allow for permanent tagging of neurons active during a specific time period. Upon injection of 4-hyroxytamoxifen after the first trial, we label the cells active during that period. They are then trained for the rest of the week and are tested for memory at 72 hours on Day 8 and sacrificed 90 mins after the trial. Under the congruent conditions, one would expect a large degree of overlap in the engram cells active at both time points, but it would be interesting to assess how this changes in the light of conflicting information or if there is a distinct change in environment.

Retrosplenial cortex serving as a bridge of communication between hippocampus and prefrontal cortex

The retrosplenial cortex (RSC) stands out as a central hub potentially regulating the information transfer between the hippocampus and frontal cortices depending on the behavioral challenge. Anatomically it is located in an important position and has reciprocal connections with both hippocampus and the prefrontal cortex (Vann et al. 2000). Further it has been implicated in previous studies to be critically involved in regulating spatial relationships between objects and locations (Vann and Aggleton 2002; Bar 2004). The task is dependent on mice exploring the objects within the box and building object location association memories by using the 2D and 3D cues, and potentially using a mix of allocentric and egocentric strategies to build a cognitive map of the general environmental features and the fixed object location. The retrosplenial cortex and hippocampus have been shown to work together in this regard where the hippocampus encodes the spatial inputs from different sources and the retrosplenial cortex helps in binding the allocentric and egocentric relationships together to build a coherent memory trace of the object location association (Stacho and Manahan-Vaughan 2022). This would explain the RSC and HPC being more closely connected to each other for the 1 trial and interference groups. Other studies looking at memory consolidation mechanisms have highlighted the role of the RSC as an intermediary region mediating the interactions between hippocampus and prefrontal cortex in consolidation of new memories. Further as mentioned in previous sections, occurrence of cortical ripples in the parietal regions potentially also contributes to replay of older memories and strengthening of the semantic memory network (Kaefer et al. 2022; Khodagholy, Gelinas, and Buzsáki 2017)

Buildup of semantic memory networks – my new framework

Having discussed the key findings from different chapters, we try to better understand the neural mechanisms underlying the buildup of semantic memory networks and the individual region contributions. Sleep is key to building of long term semantic networks. However, the dependency of a memory trace on offline consolidation processes is potentially dependent on the complexity of the information being processed. For simple learning, or learning events with the same information reinforced across multiple trials, minimal resources are needed for consolidation. The information gets rapidly encoded into the hippocampus and neocortical regions in the first stages and is integrated into the cortex for recall. The

timing of sleep becomes less crucial in this regard since the memory gets further strengthened within the cortex. Cortico-cortical interactions are enough to facilitate the integration of new information into the network, resulting in a simple semantic network that is resilient to all interference and neuromodulatory effects. However the underlying mechanisms might differ when the brain is met with the challenge of having to continuously process more complex information, abstracting gist representations and building an evolving semantic memory network.

Semantic memories are classically thought to be formed from an accumulation of various episodic memories and the memory consolidation mechanisms are thought to be similar in both cases. Previous theories state that the hippocampus is more involved in the short term encoding of new information and the PFC is more involved in later stages to integrate the information and guide decision making depending on specific contexts and ensuring successful retrieval of memories (Preston and Eichenbaum 2013). Building upon these assumed roles of HPC and PFC, it has also been hypothesized that semantic memory networks are formed through the repeated reactivation of shared information across related memories. The theory states that the hippocampus selectively reactivates overlapping elements across memories and the cortex is involved at a later stage in selectively strengthening these networks and creating a generalized "gist" representation, while the unique details of each memory are downscaled (Lewis and Durrant 2011). In a nutshell, the cortex is theorized to play a more rigid role and get involved only at a later stage of memory consolidation. Complex semantic memories, however, involve continuous updating of the cortical networks as opposed to simple memories and hence need more resources for consolidation. With the gene expression results from Chapter 5, we see that both the prefrontal cortex and hippocampus are equally active when mice perform their first trial of object exploration**,** with a significant increase in gene expression in the prefrontal cortex compared to the hippocampus for this time period. **In light of these findings, in contrast to the previously assumed roles of the PFC and hippocampus in encoding and consolidation processes, I propose that the cortex is equally or more engaged compared to the hippocampus during encoding of a new experience (Fig.1).** Empirical evidence in support of our theory suggesting PFC playing an active role at encoding has also been recently shown in other studies (Kitamura et al. 2017; DeNardo et al. 2019). The cortex then consolidates this encoded information on a much shorter time scale than assumed and builds a memory network. The subsequent trials contain features largely similar to the pre – existing networks and minimal resources are used to match the information and integrate new gist features. Pruning existing networks to eliminate redundant information while reinforcing relevant, abstracted knowledge may be an ongoing process in cortical regions. This mechanism helps

in continuously updating and strengthening networks over time, making them more resistant to interference. Over extended periods, the memory network may enter an inactive state during consolidation. But upon long term retrieval test, by the rule of "familiarity or congruence", the PFC gets more active with an increase in gene expression, making the network more accessible for retrieval. This claim would also align well with previous studies showing the critical role of PFC in matching incongruent vs congruent information and prioritizing replay of information that is congruent to the existing knowledge network (Euston, Tatsuno, and McNaughton 2007; Takehara-Nishiuchi and McNaughton 2008).

When faced with incongruent information, the hippocampus gets more active and leads the network. Sub regions of the hippocampus play differential roles in processing information from the environment. The dentate gyrus plays a critical role in distinguishing between different contexts and send relevant information to the CA1 for further processing (Coelho et al. 2024). When processing information in a novel environment, CA1 triggers an initial LTP response in encoding the spatial and contextual information. The Dentate Gyrus plays a complementary role in triggering an LTD response in consolidating context specific information, paying attention to details related to cues and other features (Kemp, Tischmeyer, and Manahan-Vaughan 2013; Kemp and Manahan-Vaughan 2008). Our results from the network analysis align with these assumed roles of hippocampal structures where we see a differential involvement of the CA1 in single trial events and Dentate Gyrus for interference events.

In a final step, we try to further understand the dynamics of hippocampal – cortical communication using novelty as one factor. Hippocampus is known to have dopaminergic innervations from both VTA and LC and either of these gets activated when we encounter a common or distinct novelty event and subsequently bias the consolidation of the episode surrounding the event (Duszkiewicz et al. 2019; Genzel et al. 2017). With findings from this chapter, we are able to disentangle the effects of "distinct novelty" that occurs when the mouse explores the box for the very first time and leads to a boost in activity and strengthening the synapses of that experience and an increase in expression of IEGs across both hippocampal and cortical regions. The subsequent trials would mimic more of the "common novelty" experience where new information is continuously semantically updated into the existing memory network that turns out to be resilient to interference effects. However, when we boost the salience of the interference episode by pairing with an artificial "distinct novelty" episode, it overwrites the old information and the original memory trace gets potentially reconsolidated with new information.

In summary, with my findings, I want to highlight the differences in memory consolidation mechanisms that can be seen when we look at a buildup of a complex semantic memory network. **Contrary to the assumed role of the Prefrontal Cortex as a slow learner, it shows a high level of plasticity, adapting its workings to the task demands. In the absence of previous knowledge, it first plays an active role in rapidly encoding information and building a base knowledge network. Over time, its role shifts to integrating new information into this network. Initially, the network experiences a distinct novelty-driven dopamine boost, but as learning progresses, it transitions to a more generalized novelty mode for incorporating new information.** Generally the process of semanticization of memories is assumed to take days, however with our results, we see this step happening in the hours after encoding of the first experience.

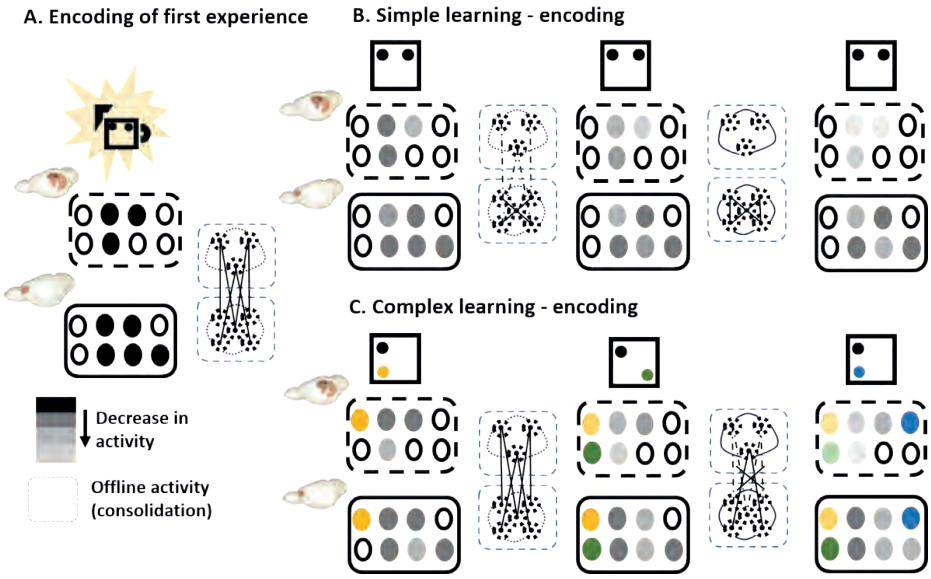

Figure 1. Model of consolidation for processing of simple and complex semantic memories. **(A)** Encoding of first experience. The left panel shows the activity of the HPC (top) and PFC (bottom) during the encoding of the first trial in the Object Space Task. Experience of the first trial acts as a *"distinct novelty"* event and is followed by a high activity in the HPC and PFC. The PFC is more active (depicted by more neurons recruited to the engram) compared to the HPC (based on gene expression findings from Chapter 5). This is followed by crosstalk between HPC and PFC (blue panels) during the intertrial interval after encoding. **(B)** Activity of the HPC and PFC during encoding of the Stable Condition of the Object Space Task. Same information (object locations) is reinforced during every trial. The overall activity of the hippocampal (top panel) and cortical engram (bottom panel) decreases with subsequent trials but the cortical engram remains more active with partial reactivation of the engram from first experience as it gets updated with every trial. The interleaved offline consolidation periods (blue panels) depict the communication between HPC and PFC. Due to the same information being reinforced during every trial, the crosstalk between HPC and PFC gradually decreases and the cortical networks within the PFC are strengthened (based on electrophysiological findings from Chapter 4). For encoding of the last trials, the memory is potentially independent of the hippocampus and relies more on the cortex (depicted by the second blue panel). **(C)** Activity of the HPC and PFC during encoding of the Overlapping Condition of the Object Space Task. New information (changing object location) encoded with every trial. This is depicted by different colored circles added to the hippocampal (top) and cortical (bottom) engrams. Decrease in overall activity of the hippocampal (top panel) and cortical (bottom panel) engram with subsequent trials but more active in comparison to the Simple learning condition. Due to new information being processed at every trial, there is more crosstalk between the HPC and PFC (blue panels). The activity of the HPC gradually decreases with every trial and the cortex more active in continuously updating and strengthening the memory network. The figure is based on the results from Chapter 4 and 5 and proposes a novel framework of memory consolidation where the Prefrontal Cortex is actively engaged at encoding along with the hippocampus and plays a more active role than assumed in building of semantic memory networks and updating them based on task requirements.

Future directions

Overarching questions from the thesis –

1. What are the differential roles of Anterior Cingulate cortex and Prefrontal cortex in consolidation of complex memory processes? Is there a redundancy in their activity?
2. How would lesions of the RSC influence consolidation processes? Would it affect more complex memory processing compared to simple memories?
3. What are the molecular underpinnings of different ripple types? Would occurrence of more long ripples correlate with higher expression of IEGs in specific brain regions?
4. When tracking a memory engram in a semantic learning paradigm, are the neurons recruited during encoding overlapping with those activated at retrieval? What happens for incongruent information? Is there reconsolidation with the original engram in the light of new information or does it recruit a different subset of neurons?
5. How would artificial silencing of the PFC during encoding of first trial vs later trials influence the memory performance of the mice in the task?
6. What are the role of interneurons in processing of semantic memories and how is the activity of these cell populations regulated in the cortex vs hippocampus

Conclusion

With findings from this thesis, I hope to have provided a more detailed outlook on the nuanced role of sleep in systems consolidation of memories. Further using results from both rodent and human studies, I provide a novel insight into how differentially modulated the memory consolidation mechanisms can be depending on the information being processed. Although still at a very nascent stage, we can use these findings to attempt at looking at different aspects of complex memory processing and better understand the intricacies of individual brain region contributions to a dynamic and continuously evolving memory network.

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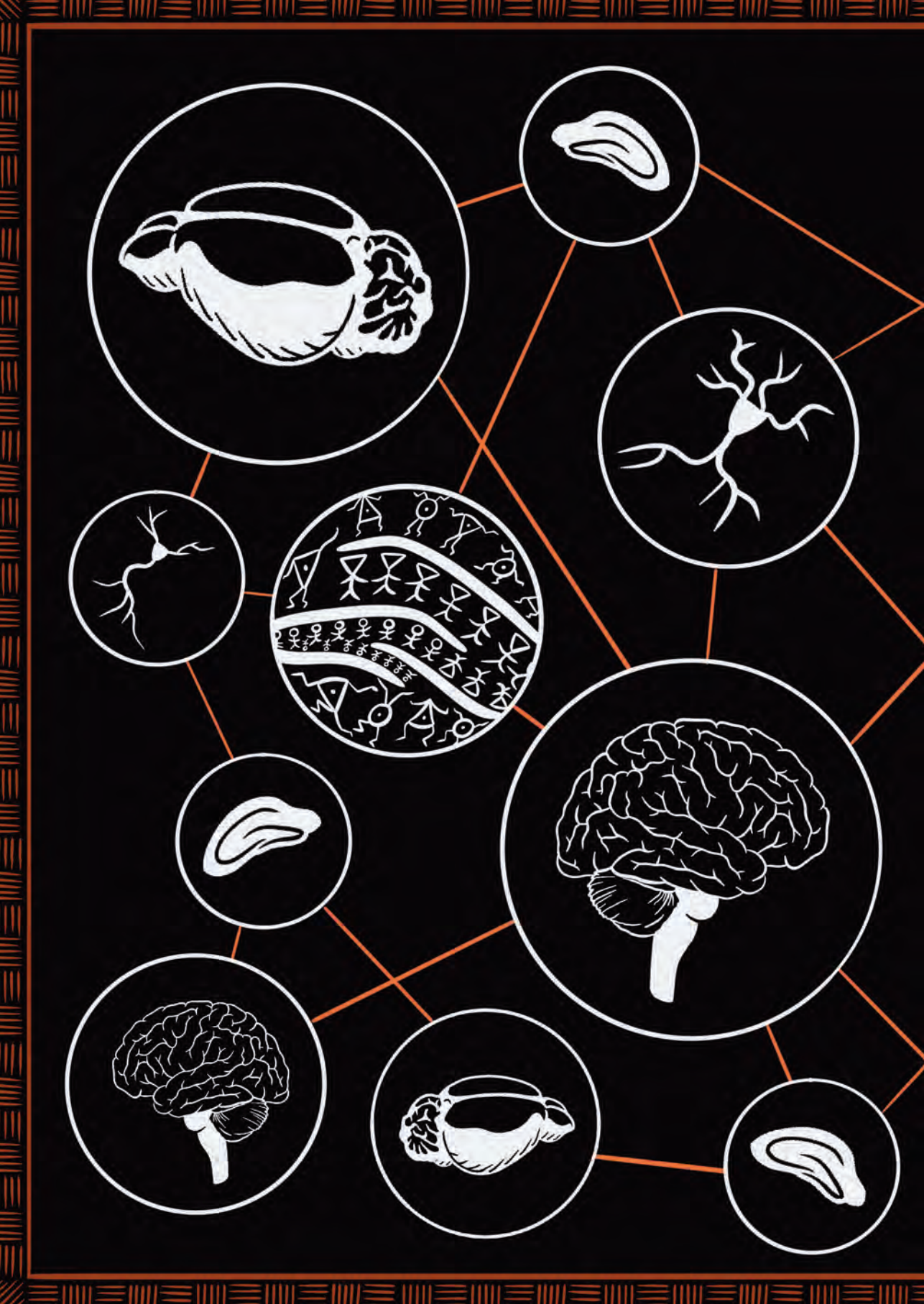

Appendices

Summary

This thesis explores the formation of semantic memory networks in the brain, with a focus on distinguishing between simple and complex learning and examining the distinct roles of sleep in consolidating each of these memory types. Utilizing a combination of imaging, electrophysiology, and molecular techniques, it investigates various factors—including novelty, neuromodulation, and interference—and their influence on memory processing. In addition to looking at hippocampal – cortical dynamics, the role of individual brain regions at different stages of memory processing are also elucidated in detail. Finally, the empirical findings are collectively discussed to propose a novel framework for how the brain stores information long-term, leading to the development of semantic memory networks.

Chapter 2: Sleep Leads to Brain-Wide Neural Changes Independent of Allocentric and Egocentric Spatial Training in Humans and Rats

This chapter shows that sleep plays a conserved role in systems consolidation of memories across both human and rodent models. Behaviourally, sleep boosted memory performance in both species with rats being trained under allocentric training condition receiving a higher benefit. This was reflected with a brain wide increase in retrieval induced expression of Immediate Early Genes (IEGs) over sleep, while only localised increases pertaining to specific brain regions were observed for the sleep deprived group. In the human analogue, sleep led to a decoupling of the Default Mode Network from the Executive Control Network over sleep, reflecting an offline mode of consolidation taking place, integrating the memories from hippocampus to more downstream cortical target areas.

Chapter 3: Memory reactivations and consolidation: considering neuromodulators across wake and sleep

This is a review chapter that provides a comprehensive overview of how offline consolidation processes are influenced by neuromodulators. With a focus on SWR events, we first discuss the role of these events depending on whether they occur during wake, rest periods or sleep. Further on, we summarize how neurotransmitters like Acetylcholine and Dopamine influence the occurrence of SWR events, thereby regulating memory consolidation.

Chapter 4: CBD lengthens sleep but shortens ripples and leads to intact simple but worse cumulative memory

This is a chapter where we investigated the effect of oral administration of Cannanbidiol on sleep architecture and memory processing. Rats were trained in the Stable and Overlapping conditions of the Object Space Task after orally administering CBD. Memory in the Stable condition remained unaffected but CBD led to worsening of cumulative memory. Consumption of CBD extended the period of NREM sleep, while also altering the properties of characteristic NREM oscillations. Further it led to a reduced occurrence of long ripples (>100 ms) which have been implicated to play a role in consolidation of complex memories. In summary, CBD led to longer NREM sleep, but influenced the properties of ripples in a way that potentially could explain the worsening of complex memory.

Chapter 5: Tracking semantic like memories in the rodent Default Mode Network

In this chapter we looked at the individual contributions of brain regions including the frontal and parietal cortical regions and the hippocampus at different learning timepoints while the mice were engaged in a 2 week semantic-like behaviour paradigm. All mice showed an intact long term semantic memory at 72 hours after training, and this memory was shown to be resistant to interference until the salience of the interference trial was increased by exposure to a distinct novel environment immediately thereafter. The first trial in the training arena led to the biggest increase in IEG expression, after which there were only more subtle changes. Different memory networks were characterised to capture different aspects of the learning curve and distinct hubs in the brain were identified to be leading the memory processing within each network.

Chapter 6: General Discussion

This chapter includes a general discussion of the empirical findings from previous chapters in context of existing literature to propose that the dependency of a memory trace on sleep for consolidation depends on the complexity of memory. Further a novel framework of memory consolidation is proposed of how complex semantic memories might be processed in the brain.

Samenvatting

Deze dissertatie onderzoekt de vorming van semantische geheugennetwerken in de hersenen, met een focus op het onderscheid tussen eenvoudig en complex leren en het onderzoeken van de verschillende rollen van slaap in het consolideren van elk van deze geheugentypes. Door gebruik te maken van een combinatie op geheugenverwerking van beeldvorming, elektrofysiologie en moleculaire technieken wordt het effect van verschillende factoren onderzocht, waaronder nieuwheid, neuromodulatie en interferentie. Naast de hippocampus-cortex interactie wordt ook de rol van individuele hersengebieden in verschillende stadia van geheugenverwerking in detail belicht. Tot slot worden de empirische bevindingen te samen besproken om een nieuw raamwerk voor te stellen voor hoe de hersenen informatie op lange termijn opslaan, hetgeen leidt tot de ontwikkeling van semantische geheugennetwerken.

Hoofdstuk 2: Slaap leidt tot hersenbrede neurale veranderingen onafhankelijk van allocentrische en egocentrische ruimtelijke training bij mensen en ratten

Dit hoofdstuk laat zien dat slaap een behouden rol speelt in systeemconsolidatie van herinneringen in zowel menselijke als knaagdiermodellen. Gedragstechnisch gezien verbeterde slaap de geheugenprestaties voor mens en rat, waarbij ratten die getraind werden onder allocentrische trainingscondities meer voordeel hadden. Dit werd weerspiegeld in een hersenbrede toename van de expressie van Immediate Early Genes (IEGs) tijdens de slaap, terwijl alleen lokale toenames in specifieke hersengebieden werden waargenomen in de groep met slaaptekort. In de menselijke proefpersonen leidde slaap tot een ontkoppeling van het Default Mode Network van het Executive Control Network, wat een offline consolidatiemodus weerspiegelt die herinneringen integreert van de hippocampus naar meer stroomafwaartse corticale doelgebieden.

Hoofdstuk 3: Geheugen reactivatie en consolidatie: beschouwing van neuromodulatoren in waak- en slaapstand

Dit is een literatuuroverzicht dat uitgebreid bespreekt hoe offline consolidatieprocessen worden beïnvloed door neuromodulatoren. Met een focus op SWR- gebeurtenissen bespreken we eerst de rol van deze gebeurtenissen, afhankelijk van of ze plaatsvinden tijdens de waak-, rust- of slaapperiode. Verder vatten we samen hoe neurotransmitters zoals acetylcholine en dopamine het voorkomen van SWR-gebeurtenissen beïnvloeden en zo geheugenconsolidatie reguleren.

Hoofdstuk 4: CBD verlengt de slaap maar verkort de lengte van hoogfrequente oscillaties en leidt tot een intact eenvoudig maar slechter cumulatief geheugen

In dit hoofdstuk onderzochten we het effect van orale toediening van Cannabidiol (CBD) op de slaaparchitectuur en geheugenverwerking. Ratten werden getraind in de stabiele en overlappende condities van de Object Space Task na orale toediening van CBD. Het geheugen in de stabiele conditie bleef onaangetast, maar CBD leidde tot een verslechtering van het cumulatieve geheugen. Consumptie van CBD verlengde de periode van NREM slaap, terwijl het ook de eigenschappen van karakteristieke NREM oscillaties veranderde. Verder leidde het tot een verminderd optreden van lange hoogfrequente oscillaties (>100 ms), waarvan is aangetoond dat ze een rol spelen bij de consolidatie van complexe herinneringen. Samengevat leidde CBD tot een langere NREM-slaap, maar beïnvloedde het de eigenschappen van hoogfrequente oscillaties op een manier die mogelijk de verslechtering van het complexe geheugen zou kunnen verklaren.

Hoofdstuk 5: Het volgen van semantische herinneringen in het Default Mode Network van knaagdieren

In dit hoofdstuk hebben we gekeken naar de individuele bijdragen van hersengebieden, waaronder de frontale en pariëtale corticale gebieden en de hippocampus op verschillende tijdstippen tijdens het leerproces, terwijl de muizen bezig waren met een semantisch gedragstaak van 2 weken. Alle muizen vertoonden een intact semantisch langetermijngeheugen 72 uur na de training en dit geheugen bleek resistent tegen interferentie totdat de relevantie van de interferentietest werd verhoogd door blootstelling aan een aparte nieuwe omgeving onmiddellijk daarna. De eerste trial in de trainingsarena leidde tot de grootste toename in IEG expressie, waarna voor latere trials waren de verandering in expressie beduidend kleiner. Verschillende geheugennetwerken werden gekarakteriseerd om verschillende aspecten van de leercurve vast te leggen voorts werden er verschillende hubs in de hersenen geïdentificeerd die de geheugenverwerking binnen elk netwerk leidden.

Hoofdstuk 6: Algemene discussie

Dit hoofdstuk bevat een algemene bespreking van de empirische bevindingen uit voorgaande hoofdstukken in de context van bestaande literatuur om voor te stellen dat de afhankelijkheid van de sterkte van een geheugenpatroon van slaap voor consolidatie afhangt van de complexiteit van het geheugen. Verder wordt een nieuw raamwerk voor geheugenconsolidatie voorgesteld die beschrijft hoe complexe semantische herinneringen verwerkt zouden kunnen worden in de hersenen.

Acknowledgements

This thesis represents not just years of research, but also a collection of conversations, shared struggles and laughs, and unwavering support from those who have been a part of this journey. To all of you, I extend my deepest appreciation.

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Over the years, I have built a wonderful collection of semantic memories—brainstorming sessions that sparked new projects, long behaviour days in the lab with our beautiful rodents, surgery marathons, conference travels, Easter egg hunts and other lab traditions, discussing science over great food, Christmas parties, and so much more! These moments will stay with me for a lifetime.

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Everything I know about rodent electrophysiology is thanks to your patience and dedication in training me every step of the way. I'll always look back fondly on our late-night drive-building sessions, the thrill of finally reaching the hippocampus and seeing those beautiful ripples on the screen, our relentless ghost artifact hunts, and, of course, our inspiring scientific discussions. Through it all, from the highs to the tough moments, I'm grateful to have had you there—pushing each other forward and keeping the motivation alive.

Alejandra, Liz, Adrian, Abdel, my amazing partners in crime and pillars of support, I am truly grateful that I got to share my journey with you all. **Alejandra,** you are the soul of the lab! Thank you for the perfect cover and going down the memory lane together made me realize, just how many of those we shared together. It feels like a full circle to have started off as colleagues, which transformed into a beautiful friendship and getting the chance to celebrate the ending together. You inspire me every day to become the best version of myself, thank you. **Liz**, none of my molecular experiments would have been possible without you! A big big thank you for being my right hand in setting up the histology pipeline, keeping all of us sane through chaotic times and just being a great friend. **Adrian,** I will fondly remember all our times setting up computers and servers together, and you never losing patience in teaching me the zillion data analysis pipelines you established that were very instrumental to this thesis. **Abdel**, I am so happy to have crossed paths with you in this adventure! Thank you for all the coffee talks, life lessons, walks and always inspiring scientific discussions. I still hope we can achieve our dream of opening a lab together some day and celebrate it with the best of cakes.

A heartfelt thank you to the dedicated bachelor's and master's students I had the pleasure of supervising and mentoring over the past years. Many of my experiments would not have been possible without their hard work and support—whether it was assisting with behaviour, histology, or data analysis. Working with them not only made my research more manageable but also reinforced the idea that science is a true team effort. Guiding and mentoring young minds has been incredibly fulfilling, and it has been a privilege to play a small part in their journey toward a career in research. Special mentions to **Jonna** and **Laura** for their commitment and contributions towards establishing the molecular analyses pipeline which was instrumental to the completion of my projects and also laid the foundation to several other projects. A big thank you also to **Debbie** and **Nick**, the amazing technicians of the neurobiology department of DCN for their technical expertise and support in ensuring I had all the reagents and equipment in time to smoothly run my experiments. On a similar note, I would like to thank the General Instrumentation department for the microscopy

facilities and support. Lastly, I would like to sincerely thank our department secretaries, **Gea** and **Marie-Louise**, for their unwavering support in handling administrative tasks and navigating bureaucratic hurdles. Your help ensured that we could stay focused on our research while also reminding us to take our well-deserved holiday hours.

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Madhurya, my dear friend and roommate, I honestly couldn't have asked for a better person to share this PhD adventure with. From the very beginning, we stepped into this journey together, and I can't imagine what these years would have been like without you. Thank you for always being there—through the good, the bad, and the downright chaotic. For every coffee break that turned into a much-needed venting session, for our endless cooking experiments and feast-like dinners, epic concerts, for making sure I didn't completely drown in work on those insanely hectic lab days. You kept me sane when everything felt overwhelming, especially during the COVID days when we somehow managed to turn isolation into something bearable and actually fun for the most part. More than anything, thank you for being my constant source of support, for reminding me to take breaks, to laugh, and to keep pushing forward no matter what. I couldn't have done this without you, and I know that wherever life takes us next, this friendship will always remain. On this note, I would also like to extend a thank you to another dear friend, **Milan,** for being a great drive building partner and the best Dutch teacher that I could have asked for! Those weekly reading sessions of Harry Potter in Dutch not only got us through covid times but also was the coolest way one could possibly learn the language. Lastly a big shoutout to all my incredible friends from across the globe, **Thomas, Francisco, Stefanos, Sevgi, Ine, Carolina, Alejandro** for the brilliantly fun DnD sessions, barbecues, crazy parties and dinners.

An unexpected but equally special part of my PhD journey was studying natural behaviour in wild vervet monkeys in a game reserve in South Africa. I am very grateful to **Dr. Erica van de Waal** for this amazing opportunity. It was a very insightful and thrilling experience to look at evolution of natural behaviours and gain insights into research conducted outside of the lab environment. A big thank you to some close

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I would also like to thank a very special friend, **Oscar,** who has been instrumental in keeping me sane and motivated throughout the years. Despite the distance, our journey has been special and I will be forever grateful for your love and support throughout all times – exciting, good and bad. Thank you for being my primary rock whenever my world turned upside down, for constantly assuring me that I could always rely on you and for your infinite patience in bearing with my clumsy gaming skills.

Lastly, I would like to thank my dear family – **Mom, Dad** and my **little brother**. I dedicate this thesis to you all! Thank you for always believing in me and having relentless faith that I will succeed in whatever challenge I take. Despite being miles away, you have always been my pillars of strength, my biggest cheerleaders, and the steady rock I needed to keep going. Somehow, even from afar, you always had the intuition to sense when I needed a lift and pulled me through, reminding me that I was never alone. As I am finally ending this chapter, I am very happy to make you proud. I couldn't have done this without you, and for that, I am forever grateful.

Curriculum Vitae

Anumita Samanta was born in Indore, Madhya Pradesh, India on July 10, 1991. She graduated with a bachelors degree in engineering in the field of Biotechnology from Vellore Institute of Technology, Tamil Nadu, India in 2013. During the bachelors, she was selected into a summer internship program at the Center for Cellular and Molecular Biology, Hyderabad where she got the opportunity to work in a lab looking at neural mechanisms underlying depression in rodent models, which served as an introductory platform to the field of research in neuroscience. She continued working in the same institute as a junior research fellow until end 2015. Her research focused on establishing maternal separation (post-natal) and Prenatal Restraint Stress as depression models and studying the underlying metabolic changes happening in the brain with respect to the neuronal and astroglial activity in different brain regions using 1H-[13C] NMR spectroscopy.

From February 2016 - 2018, Anumita did a research masters in Cognitive Neuroscience with a specialization in the Plasticity and Memory Track. For her masters thesis internship, Anumita started working under the supervision of Dr. Lisa Genzel where she was introduced to the over arching topics of the thesis. Her masters thesis was primarily focused on data acquisition and behavioural analysis of the human imaging datasets that are a part of Chapter 2 from this thesis

In March 2018, Anumita joined the Genzel Lab as a PhD candidate. During the course of her PhD, Anumita gained expertise in a range of experimental techniques – fMRI and EEG recordings in humans, performing behaviour tasks in rodent models, acute and chronic free moving electrophysiological recordings in rats and developing novel drive implants for multi site LFP recordings in rats. In addition, she established the lab pipeline for processing of molecular data – immunostaining, imaging, cell quantification algorithms and analyses. Research work conducted during this period has culminated in this thesis and additional unpublished work.

In 2023, Anumita spent her time working as a field assistant in South Africa in a field site organized by Dr. Erica van de Waal where she learnt how to work with vervet monkeys in the wild and perform behaviour experiments with them. Since February 2024, she has rejoined the Genzel Lab as a postdoctoral researcher.

List of Publications

Thesis publications as first author

Samanta A, Aleman-Zapata A, Agarwal K, Ozsezer P, Alonso A, Van der Meij J, Rayan A, Navarro-Lobato I, Genzel L (2023) CBD lengthens sleep but shortens ripples and leads to intact simple but worse cumulative memory. *iScience*, volume 26, Issue 11.

Samanta A, van Rongen LS, Rossato JI, Jacobse J, Schoenfeld R, Genzel L (2021) Sleep Leads to Brain-Wide Neural Changes Independent of Allocentric and Egocentric Spatial Training in Humans and Rats. *Cerebral Cortex*, Oct 1;31(11):4970-4985. doi: 10.1093/cercor/bhab135.

Samanta A, Alonso A, Genzel L (2020) Memory reactivations and consolidation: considering neuromodulators across wake and sleep. *Current Opinion in Physiology*, 15:120-127

Other publications as co-author

Aleman-Zapata A, Capitan Maidana M, Samanta A, Özsezer P, Agarwal K, Adam T, Rayan A, Genzel L (2025) Differential Contributions of CA3 and Entorhinal Cortex Inputs to Ripple Patterns in the Hippocampus Under Cannabidiol. *iScience*, in press, https://doi.org/10.1016/j.isci.2025.111782

Rayan A, Agarwal A, Samanta A, Severijnen E, Van der Meij J, Genzel L (2024) Sleep scoring in rodents: criteria, automatic approaches and outstanding issues. *European Journal of Neuroscience*, 59(4), 526-553, doi: 10.1111/ejn.15884

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Chakravarty S, Maitra S, Reddy R. G, Das T, Jhelum P, Kootar S, Samanta A, Kumar A. (2015) A novel natural product inspired scaffold with robust neurotrophic, neurogenic and neuroprotective action. *Scientific Reports*, Sep 21;5:14134. doi: 10.1038/srep14134

Research Data Management

This thesis research has been carried out under the institute research data management policy of the Donders Institute for Brain, Cognition and Behavior (as of 25.2.2020, https://www.ru.nl/publish/library/397/rdmpolicy_di_20190110.pdf). This research followed the applicable laws and ethical guidelines. Research Data Management was performed according to the FAIR principles. The information below details how this was achieved.

Ethical approval

This thesis is based on the results of human and animal studies, which were conducted in accordance with the European, Dutch and local regulations on the basis of the General ethic approval ('Imaging Human Cognition,' CMO2014/288) and DEC projects 2016-0014 and 2020-0020, which covers all imaging, behavior, molecular and electrophysiological experiments. The human experiments were approved by the local ethics committee (CMO Arnhem-Nijmegen, Radboud University Medical Center). For the animal studies, the local Animal Welfare Body approved the protocols 2016-0014-023, 2016-0014-024, 2016-0014-035 and 2020-0020-006.

Findability and Accessibility

The table below details where the data and research documentation for each chapter can be found on the Donders Repository (DR). All data archived as a Data Sharing Collection remain available for at least 10 years after termination of the studies.

Chapter	Data Acquisition Collection (DAC)	Research Documentation Collection (RDC)	Data Sharing Collection (DSC)
2	di.dccn.DAC_3013066.01_890	di.dccn.RDC_3013066.01_376	tbd
4	di.dccn.DAC_626830.0008_841	tbd	tbd
5	di.dccn.DAC_626830.0004_821	tbd	tbd

Interoperability and Reusability

For all data in these repositories long-lived file formats have been used, ensuring that data remains usable in the future. All data collections have been structured in a standardized way that is described in accompanying text files. The documentation includes specifications on: (i) Experimental setup, (ii) Data variables, (iii) Formatting of the raw data, (iv) Specification of version numbers for the software used.

Donders Graduate School

For a successful research Institute, it is vital to train the next generation of scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School in 2009. The mission of the Donders Graduate School is to guide our graduates to become skilled academics who are equipped for a wide range of professions. To achieve this, we do our utmost to ensure that our PhD candidates receive support and supervision of the highest quality.

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data scientists, web- or software developers, business owners, regulatory affairs specialists, engineers, managers, or IT architects. As such, the career paths of Donders PhD graduates span a broad range of sectors and professions, but the common factor is that they almost all have become successful professionals.

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