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SPÉCIALITÉ NEUROSCIENCES

Par Cecilia CASTELLI

**Etude du lien entre plasticité synaptique et adaptation
comportementale**

**Study of the link between synaptic plasticity and
behavioural adaptation**

Sous la direction de : Frédéric LANORE

Soutenue le 5 Avril 2023

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Résumé : La mémoire peut être définie comme la capacité de stocker des informations qui pourront être récupéré au besoin et facilite l'adaptation comportementale. Au niveau biologique, la mémorisation est un mécanisme complexe qui est soutenue par l'interaction coordonnée de plusieurs régions du cerveau et fait l'objet d'un déclin progressif avec le vieillissement. Comprendre les mécanismes qui soutiennent sa formation et son stockage est donc un prérequis pour développer des traitements adaptés aux troubles de la mémoire.

Différentes théories décrivent la consolidation de la mémoire d'un point de vue intégratif : 1) la théorie des engrammes postule l'existence d'assemblées neuronales stablement modifiées par l'expérience ; 2) la théorie de la plasticité synaptique et de la mémoire lie ces deux concepts d'une façon causative ; 3) la théorie de la consolidation systémique postule que les engrammes sont distribués à travers plusieurs aires cérébrales interconnectées. Dans cette dernière, déterminer où, quand et comment une modification particulière du système a lieu pendant la consolidation est encore une question en suspens. Pour chacune de ces théories, plusieurs lignes de recherche ont émergé et ont permis de déterminer plusieurs régions cérébrales clés. Tout d'abord l'hippocampe qui est considéré comme le site principal pour la consolidation des mémoires récentes, mais également le cortex préfrontal comme un site important pour la consolidation des mémoires passées, faisant de la communication entre ces deux aires un axe de recherche important. Le sommeil, qui est caractérisé par l'absence d'information externe additionnelle, favorise la consolidation des mémoires. La réactivation coordonnée des assemblées neuronales dans l'hippocampe et dans le néocortex grâce à des oscillations hippocampiques à haute fréquence appelé Sharp Wave-Ripples (SPW-Rs), considéré comme étant des événements caractéristiques des périodes de consolidation mnésique, favorise cette consolidation. Au niveau cellulaire, la potentialisation synaptique à long-terme (LTP) est le mécanisme qui sous-tend la formation des mémoires long-terme le plus étudié.

Durant ma thèse, j'ai étudié l'interaction entre l'hippocampe dorsal (dHPC) et deux aires néocorticales associatives dans un contexte d'apprentissage d'une règle d'alternation spatiale avec délai chez la souris. Mon objectif a été de répondre aux questions suivantes :

- 1) Le cortex préfrontal médian (mPFC) et le cortex pariétal postérieur (PPC) participent-ils à l'engramme qui soutiens l'acquisition de cette règle ? Leur contribution respective est-elle quantitativement ou qualitativement différente ?
- 2) L'activité de réseau (oscillations et réactivation neuronale coordonnées) de ces deux aires est modulées par le SPW-Rs de l'hippocampe ; cette modulation est-elle modifiée par la consolidation de la règle ?
- 3) La consolidation de la règle est-elle affectée par l'inhibition de la LTP au sein de ces aires néocorticales ?

Pour répondre à ces questions nous avons utilisé des souris implantées avec des électrodes dans le dHPC, mPFC et PPC pour enregistrer le Potentiel Local de Champ (LFP) et l'activité de décharge des neurones pendant la tâche et pendant trois heures de repos à la fin de chaque journée de test. Nous avons observé que, pendant la tâche, les neurones du PPC étaient plus particulièrement corrélés à des paramètres de navigation spatiale, tandis que les neurones du mPFC étaient préférentiellement activés par des attributs cognitifs. Nous avons également montré que la modulation positive des neurones néocorticaux par les SPW-Rs était augmentée après l'apprentissage de la règle dans le mPFC, mais pas dans le PPC. De plus, les neurones du mPFC les plus actifs pendant la tâche sont les neurones préférentiellement modulés par les SPW-Rs. Enfin, nos données préliminaires montrent que l'inhibition de la LTP au sein du mPFC pendant la période de sommeil suivant la première période d'apprentissage ne semble pas avoir d'effet sur la consolidation de la règle, mais suggèrent cependant que la modulation des neurones par les SPW-Rs pourrait en être affectée.

Mots clés : Mémoire, hippocampe, cortex associatifs, comportement, électrophysiologie

Title: Study of the link between synaptic plasticity and behavioural adaptation

Abstract: Memory can be defined as the ability to store information within the brain in a way that allows retrieval of such information and promotes behavioural adaptation. At the biological level, memorization is a complex mechanism which demands coordinated brain-wide interactions and is subject to progressive decline upon natural aging. It is a shared idea that understanding the mechanisms regulating formation and storage of memories could help in developing targeted approaches for mnemonic deficiency.

Different theories have been formulated to describe memory consolidation in an integrated system: 1) the engram theory postulates the existence of neuronal assemblies persistently modified by experience and memory consolidation; 2) the synaptic plasticity in memory theory postulates that these persistent modifications rely on synaptic plasticity mechanisms 3) the system consolidation theory postulates that engrams are, in fact, distributed across an interconnected network encompassing multiple brain areas. In each of these theories, memory consolidation is a multi-step process and the precise identification of whether, where, when and how a specific modification is produced inside this complex system is a hot topic in research. For each of these questions collected evidences have directed the focus toward precise lines of research. The hippocampus is considered to be main site of recent memory consolidation and the prefrontal cortex has progressively emerged as an important site for remote consolidation, making the communication between these two areas a main centre of interest. During sleep, the lack of additional experience favours memory consolidation and the coordinated reactivation of neuronal assemblies of both the hippocampus and neocortical areas during fast-oscillatory hippocampal events called Sharp Wave-Ripples (SPW-Rs) is now taken as the principal hallmark of memory consolidation. At the molecular level, long-term potentiation (LTP) is the most studied mechanism for the formation of long-lasting memories.

I studied the interplay between the dorsal hippocampus (dHPC) and two neocortical areas in the context of consolidation of the rule for successful completion of a Delayed Spatial Alternation (DSA) task. We aimed to answer the following questions:

- 1) Does the medial Prefrontal cortex (mPFC) and the Posterior Parietal cortex (PPC) participate in the engram sustaining acquisition of this rule? Is their participation equal or are they quantitatively or qualitatively differentially solicited?
- 2) Both areas display signs of hippocampal modulation, in the form of coherent oscillations and coordinated neuronal firing patterns during hippocampal SPW-Rs; how is this modulation modified by consolidation of this rule?
- 3) Does preventing LTP within the neocortex affect consolidation of this rule?

To answer these questions, we used mice implanted with single electrodes in the dHPC, mPFC and PPC to record Local Field Potential (LFP) and single neurons' activity both during the task and a three-hour long rest period at the end of each behavioural training day. We observed that, during behavioural training, PPC neurons were mostly engaged in navigation, while mPFC neurons engaged with more cognitive features of the task. Surprisingly, mPFC neurons, but not PPC neurons, exhibited an increase in positive modulation during hippocampal SPW-Rs following learning, with a high proportion of mPFC's neurons active during the behavioural protocol that were positively modulated around SPW-Rs' peaks during sleep. Last, preliminary data showed that prevention of LTP within the mPFC during the sleep period allocated to memory consolidation does not affect the behavioural performance on the following day, but might affect the modulation of mPFC's neurons around SPW-Rs' peak.

Keywords: Memory, hippocampus, associative cortices, behaviour, electrophysiology

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List of Abbreviation

ACC – Anterior Cingulate Cortex
AMPA - 2-Amino-3-hydroxy-5-Methyl-4-isoxazole-Propionic Acid
AP – Antero-Posterior
AP- - Acceptor Protein
AAV – Adeno-Associated Virus
CA – Cornu Ammonis
CaMKII – Calcium/Calmodulin II Kinase
CREB - cAMP Response Element Binding protein
DG – Dentate Gyrus
dHPC – dorsal Hippocampus
DSA – Delayed Spatial Alternation
DV – Dorso-Ventral
EC – Entorhinal Cortex
IgG – Immunoglobulin G
IL – InfraLimbic cortex
INs - InterNeurons
KI – Knock-In
LFP – Local Field Potential
LTD – Long-Term Depression
LTP – Long-Term Potentiation
ML – Medio-Lateral
mPFC – medial Prefrontal Cortex
PrCm - Medial PreCentral cortex
NMDA - N-Methyl-D-Aspartate
OFC – OrbitoFrontal Cortex
PL – PreLimbic cortex
PNs – Pyramidal Neurons
PPC – Posterior Parietal Cortex
PRPs – Plasticity-REleted Products
PSD – Post-Synaptic Density
REM – Rapid Eye Movement
RSC – RetroSplenic Cortex
SD – Standard Deviation
SEM – Standard Error Mean
SPM – Synaptic Plasticity and Memory
SPW-Rs – Sharp Wave-Ripples
STC – Synaptic Tagging and Capture
STDP – Spike Timing-Dependent Plasticity
SWS – Slow Wave Sleep
TeA – Temporal Associative cortex

Premise

“Memory” can be defined as the ability to store learned information within the brain in a way that allows retrieval of such information. In humans as well as in animals, memory processes promote behavioural adaptation to different environmental stimuli and are therefore pivotal for survival. Efficient storage of past positive or negative stimuli is, in fact, fundamental for their correct recognition upon a second encounter and a damage at this level may result in failure in recollecting food and shelter locations or in recognizing a potential death threat, with obvious consequences. At the biological level, memorization is a complex mechanism which interest most if not all brain regions, demands coordinated brain-wide interactions and is subject to progressive decline upon natural aging. With an increase in aging population worldwide and the increasing diffusion of many different cognitive disorders that impact memory in its different forms, memory has gained a status of hot topic in the scientific community.

It is a shared idea that understanding the mechanisms regulating formation and storage of different types of memories could help in developing targeted approaches for each specific type of mnemonic deficiency. However, due to its vastness and complexity, as any form of information acquisition might be, indeed, reduced to a memorization process, a comprehensive investigation of “memory” results in an elusive matter. Research tracks focus on single memory types or even single phases of the memorization process and many branches of research are still at a fundamental level. Therefore, a comprehensive discussion of “memory” in its entirety is beyond the scope of this work as it would take the focus far away from the main experimental question.

During my PhD I investigated the acquisition, storage and expression of a cognitive rule for reward retrieval in mice, focusing in particular on the mechanisms regulating the second phase: storage (or consolidation). In my introduction, I will start by presenting and defining the tri-phasic process that has been linked to memorization and give a brief introduction about the main memory forms identified in humans and how they rely on different memory systems, encompassing different brain regions. I will then discuss my behavioural model based on this knowledge. In the second part, I will introduce the brain areas that supposedly compose part of the memory system sustaining memory consolidation in my model and that were the object of my investigation. My aim is to highlight their individual roles but also to present them as a functional network. In the third part, I will discuss the main theories regarding memory consolidation, focusing on synaptic plasticity and the role of sharp wave-Ripples. I hope to give a concise, comprehensive and coherent summary of the different levels (from molecular to behavioural) of investigation required to produce a complete theory of memory consolidation.

INTRODUCTION

Chapter 1: Memory

1.1 Phases of Memory

In order to clarify some principles behind the classification of different types of memories, it is important to state that memorization is not a unitary process, but three distinct phases can be distinguished:

Encoding: it is the moment in which the item or experience that will later become the subject of a memory is first encountered, resulting in the activation of specific brain areas and the first formation of a memory trace. It is often equate to the concept of learning and, in the context of repetitive behavioural tasks designed for animals, it specifically defines the moment in which the animal starts to display a clearly recognizable goal-directed behaviour, resulting in performance's improvement.

Consolidation: it is the process of reinforcing of the memory trace to allow long-term storage of the item or experience. It has been linked to re-activation of neurons firstly activated during encoding, an event that has mainly been observed during the slow wave phase of sleep, however reactivation to a certain extent has been observed also in wake periods following encoding or during inter-trial intervals in repetitive tasks. The importance of sleep for memory consolidation evolutionary-wise might be justified by the need for an experience-free time-slot allocated to stabilization of memory traces for relevant experiences encountered during the day and concurrent suppression of competing traces of un-relevant experiences (Seibt and Frank, 2019). For certain types of memory, consolidation may involve the transfer of the memory trace from a primary site of storage to secondary site to assure long-term storage of the information (Frankland and Bontempi, 2005) e.g. in the case of declarative memory, the information is transferred from the Hippocampus (primary storage site) to the Neocortex (long-term storage site).

Retrieval: It is the ability to recall specific information upon the exposure to an appropriate trigger. Even though retrieval often occurs upon re-exposure to all or part of the same conditions encountered during encoding, the brain areas activated during this phase are not necessary the same and this is particularly true for those memory types presenting distinct primary and long-term storage sites (Kitamura et al., 2017). Other than retrieval, re-exposure to already encountered encoding conditions can lead to subsequent phases of reconsolidation of the memory trace.

1.2 Types of Memory

The hypothesis that “memory” is not a unitary concept originated in psychology. In this field, starting at the end of the 19th century and proceeding all throughout the first half of the 20th century, many researchers have formulated theories based on different observations and using different terms, but all could be ascribed to an ideal dichotomy between a conscious and an unconscious forms of memory (Squire and Zola-Morgan, 2015). From a biological point of view, the enquiry whether psychologically distinguishable types of memories also relied on distinct areas within the brain started in the wake of the tests performed on patient H. M. (Scoville and Milner, 1957). Patient H. M. had been subjected to bilateral medial-temporal lobe resection as a treatment for severe epileptic seizures, which resulted in extended damage to his ability to recall past event (retrograde amnesia) but also and foremost to form new memories (anterograde amnesia). Multiple memory tests were conducted on him and results showed that patient H. M. was able to learn new motor skills even in the complete absence of recollection of going through practice sessions: the medial temporal lobe was needed for conscious recollection of daily practicing with the test, but not for unconsciously acquisition and storage of the motor skill, which proceeded at a normal learning rate. Further research on other patients and animal models eventually led to the division of memory types into declarative and non-declarative (Squire and Zola-Morgan, 2015), a distinction that still holds and is presented first when approaching studies on memory. The distinction between declarative and non-declarative forms of memory is based on consciousness:

- *declarative memories* are consciously expressed (i.e. retrieved; oftentimes it also means that they are consciously encoded) through the process that it is commonly called recollection; hence, these forms of memory are also called noetic or explicit;
- *non-declarative memories* are, instead, expressed unconsciously through performance, implying that also encoding of the memory takes place in an unaware or semi-unaware subject; these forms of memory can also be called anoetic or implicit.

This definition based on consciousness is certainly relevant for humans, but jeopardize our ability to translate memory-related concepts formulated on humans to animals and vice-versa, as consciousness is a debated concept in its application to animals, mainly because the only recognized way to assess awareness in humans is by its vocalization (Clayton et al., 2003). Classification and schematization serve the purpose to identify distinct memory systems (i.e. specific brain regions and their mechanisms of network connection that sustain one or more specific forms of memories sharing a distinguishable and unique list of properties) implicated in the encoding, consolidation and retrieval of a specific form of memory in order to restrain the area of investigation when only a single form of memory is at the centre of the query. Given that a large part of scientific research, especially at the fundamental level, is still exerted in animals, having a translatable work-frame from animals to humans is of a certain importance in order to provide consistent data. Hence, in recent years, new models for memory systems classification were proposed, removing consciousness from the spotlight and focusing on other

characteristics. In a review, Henke (Henke, 2010) proposes a classification based on three processing modes:

- Number of learning trials required for memory formation, distinguishing between rapid encoding (one trial) and slow encoding (multiple repetitive trials);
- Cognitive complexity of the memory processing, meaning the number of processing modules solicited by the experience (e.g. a memory can be made up by a single sensory input or encompass multiple senses and being blended with conceptual, emotional and/or spatiotemporal information);
- Nature of the mental representation, dwelling in particular on compositionality (i.e. if the memory represents a unitized item or if it can be divided in more than one individual elements which is accessible on its own) and flexibility (i.e. if individual elements of the memory can be used to infer from a similar but different situation in a retrieval setting);

From these processing modes, Henke later distinguish three memory systems: fast encoding of flexible associations, slow encoding of rigid association and rapid encoding of single or unitized item. It is important to notice that in the classification system we are more familiar with, declarative and non-declarative memories do not represent two distinct memory systems, but are just terms that express one characteristic that can be dichotomically found in each individual system, thus removing the consciousness criterion from the classification does not really jeopardize the fundamental distinctions that had already been stated between different memory systems or invalidate the memory forms individuated in the past, but rather shifts the frame of their classification. That classification distinguishes five memory systems (Schacter, Daniel L. and Tulving, Endel, 1994):

- **Perceptual Representation System (PRS)**. This system belongs to the non-declarative group and process structural properties of objects (but also words, for humans) at a pre-semantic level, meaning that said properties are recognized unconsciously but cannot be verbalized or consciously structured. The main form of memory ascribed to this system is priming, which is the phenomenon guiding the preference for an object even in the absence of explicit recollection of having already encountered that object. Avoiding the consciousness criterion, priming is listed by Henke as being part of the system supporting rapid encoding of single or unitized items, as a single exposure to the object is sufficient to solicit this form of memory. In both classifications, this system relies on the para-hippocampal gyrus and neocortex and is independent from the hippocampus.
- **Procedural memory**. This is another system belonging to the non-declarative group and it actually serves as a super-structure that probably contains more specific but not yet enough explored subsystems. Procedural memory refers to all types of memories dealing with learnt procedures and automatic behaviour developed over multiple trials, but also progressively acquired motor and cognitive skills. Schacter and Tulving list simple associative conditioning as being one of the subsystems included in procedural memory, however the two concept are

often presented separately in more recent literature, even though it is not clear if the distinction is made at the level of memory forms or memory systems. For Henke, both forms of memory belong to the memory system supporting slow encoding of rigid associations, due to the repetitive nature of learning and the lack of flexibility in acquired responses. This system relies on the basal ganglia, cerebellum, parahippocampal gyrus and neocortex, which are sufficient to assure encoding, consolidation and retrieval of the memory in the absence of hippocampal function. However, it has been demonstrated that activation of the hippocampus during tasks that are used to measure this types of memories and that can be acquired by individuals with hippocampal lesions (both humans and animals) speeds up learning, probably by binding the memory to an episodic-like context.

- **Semantic memory.** This system belongs to the declarative group and process the form of memory that is generally known as “knowledge”. In fact, information hold in semantic memory is generic, factual, abstract and devoid of its context and any personal (i.e. of the subject memorizing) meaning. Lack of contextualization means that, in its ultimate form, semantic memory is composed of unitized items and rigid associations, thus it can be ascribed to the memory system supporting slow encoding of rigid associations. As for procedural memory, this system relies on the basal ganglia, cerebellum, parahippocampal gyrus and neocortex and is independent from the hippocampus, especially when semantic knowledge is acquired as the result of multiple learning sessions. However, semantic memory can also be the result of a process of progressive transformation of episodic memories (called “semanticization”), which succumb to loss of detail and gain in abstraction over time: in this case in particular (but, to a certain extent, also in the case of repetitive learning), the hippocampus is involved in memory encoding and in the first phase of consolidation and retrieval, facilitating learning (Moscovitch et al., 2005).
- **Episodic memory.** This system belongs to the declarative group and process personal experiences considered in their spatiotemporal and emotional context (so called “episodes”). Based on the procedural modes, Henke identifies episodic memory with the memory system supporting rapid encoding of flexible association, as a single exposure is sufficient to form complex and flexible composite memories. Eliminating the consciousness criterion, it allows to include also recognized forms of unconscious episodic memory (Hannula and Ranganath, 2009). This system relies on the hippocampus and neocortex for encoding, consolidation and retrieval.
- **Working memory.** Unlike the other four, working memory is not a form of long-term memory but of short-term memory (a term that is actually disappearing from the scientific jargon, oftentimes substituted by the term working memory itself because of its more specific and less confounding nature). This system can hold a large but limited amount of items (up to 5-7 at a time) useful for completing a cognitive task within a limited amount of time. Unlike long-term

forms of memories, the subdivision in the three phases of encoding, consolidation and retrieval are not of relevance related to working memory, as no persisting trace of it can be identified in the brain. However, working memory can be trained in order to expand its capacity in terms of number of items contemporaneously held and length of the retention time (Constantinidis and Klingberg, 2016). Items are either specific features present in the environment and collected through senses or cognitive rules and/or previous knowledge stored and retrieved from other forms of long-term memory, which are flexibly blend together during the reduced amount of time in which working memory is solicited (Tsutsui et al., 2016). The main theory regarding working memory is that its expression can be identified as a sustained activity of certain assemblies, representing each item, in the neocortex. The processing modes classification does not include working memory as it concentrates on long-term memory systems.

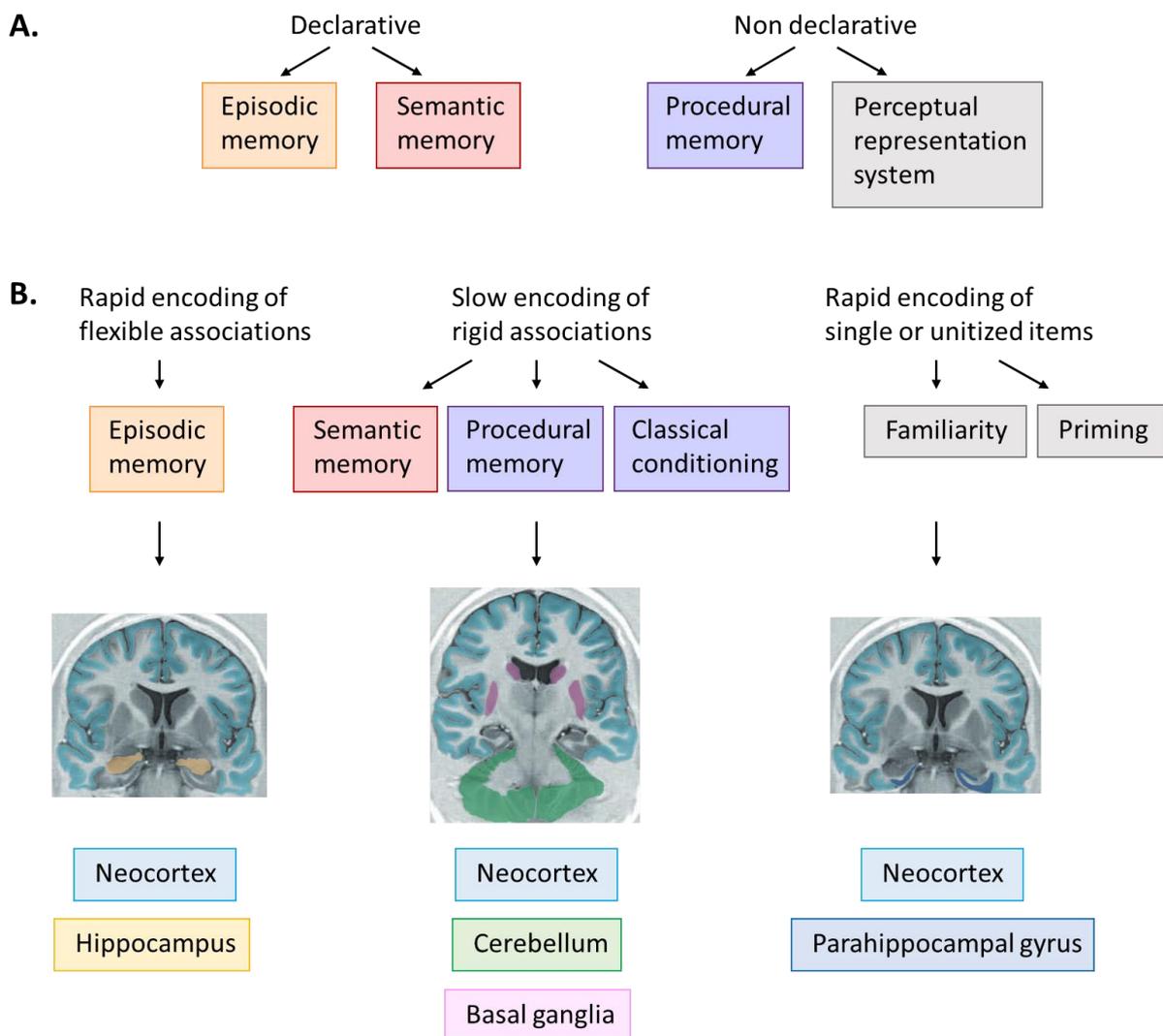


Figure 1. Long-term memory systems **A.** Classification of memory systems proposed by Schacter and Tulving, 1994. **B.** Classification of memory systems proposed by Henke, 2010. (adapted from Henke, 2010)

1.3 Memory Investigation in Rodents

For obvious reasons, human's research is limited in terms of invasiveness of adopted procedures and active manipulation is almost entirely forbidden, with the exception of drug administration in clinical trials, thus animal models are needed for more thorough research. Nowadays, rodents are the most common animal model used in research because of a multitude of characteristics (e.g. quick reproductive cycle, simple housing conditions, easy handling and manipulation and, especially for mice, proneness to genetic modification) and are therefore a commonly used model also for cognitive and memory research. As stated in the preceding paragraph, a major issue in translation of memory research in humans to animals and vice versa is represented by the pivotal role played by consciousness in traditional human's memory research: because of the difficulty of translating this concept into animal behaviour, rodent's research has traditionally been funded on a more brain regions-centred dichotomy, distinguishing hippocampal-dependent and hippocampal-independent forms of memories, sustained by distinct memory systems (Schacter, Daniel L. and Tulving, Endel, 1994). In this context, dependency on the hippocampus has to be intended as an integral hippocampus is required for encoding, consolidation and early retrieval of the memory. The distinction stems from the fundamental work conducted on patient H. M. throughout the years following his medial-temporal lobe resection, during which many tests were performed in order to discriminate, among different memory forms, those that had been spared from those that were irreversibly damaged (Corkin, 2002). The first group comprehends memories that are now considered hippocampus-independent, such as:

- emotional memories (e.g. cued fear conditioning)
- recognition/familiarity memories (e.g. novel object recognition)
- short-term memory (e.g. working memory)
- motor memory (e.g. visuomotor skills)

The second group, instead, comprehends a variety of memory forms that can be all traced to two major branches:

- declarative memory, in both forms of episodic and semantic memories
- spatial memory

Spatial memory deserves an insight on its own as it is not included as a separate form of memory in any description of memory systems, but it is a key concept in rodents' studies. Spatial memory is the ability to learn and remember spatial locations and to associate them with other stimuli (Bannerman et al., 2014). This is a fundamental ability for survival, allowing animals to recognize the environment surrounding them and not only correctly locate places of interests (such as their nest or food location), but also to recall the best path to follow to reach them. Spatial navigation through an environment exploits spatial cues, that can be defined as complex multimodal representations of the environment that comprise information from different sensory modalities. Based on the way the animal relates these cues between them and to itself, spatial navigation can be of two natures (Rinaldi et al., 2020):

- Egocentric. Based on self-references, such as turning right or left or moving toward or away from certain stimuli. Exploiting this type of navigation, the animal is always referring each encountered cue to itself, thus it is strictly dependent on the initial position of the animal for success.
- Allocentric. Based on external references: cues are linked together and exploited to build a cognitive map of the environment, inside which different places of interest (e.g. food well) are located. Unlike in egocentric perception, cues are put into relationship one with the other in a way that is independent from the current position of the animal, thus it is always able to create a path through the environment even from different starting points.

Proof of a heavy implication of the hippocampus in spatial navigation was given by the discovery of place cells during the '70s (O'Keefe and Nadel, 1978). Place cells are located inside the dorsal hippocampus and have the peculiarity to fire at specific location inside an environment. Clusters of space cells can encode whole environments, inside which each neuron present a "place field", meaning a specific location at which it is activated, characterized by high spatial selectivity (Strange et al., 2014). This place field remains stable across subsequent explorations and is totally independent from the orientation of the animal. For this reason, place cells activity is hypothesized to contribute to the building up of a cognitive map of the environment that refers to extended boundaries rather than local features (Bird and Burgess, 2008), thus putting the hippocampus in a crucial position for allocentric spatial navigation, but not egocentric navigation (Bannerman et al., 2014). This map is preserved inside the hippocampus for several weeks, determining that the place field of each neuron remains unvaried upon multiple visits of a familiar environment and even when orienting cues are removed. However, substantial changes within the familiar environment can lead to an abrupt change in place cells firing pattern, suggesting a re-mapping, adapting to the environment that is now considered as new (Bird and Burgess, 2008). Remapping can also take place following a change in the value of one of the features of the environment, for example changing the position of a food reward at different location changes the value of each location and induces remapping of place cells (Dupret et al., 2010). Formation of the cognitive map of the surrounding environment is a dominant feature of animal behaviour, which means that any task that presents a spatial connotation becomes hippocampal dependent, even if directly derived from hippocampal-independent tasks. A classic example is the dichotomy between cued fear conditioning, that relies entirely on the amygdala, and contextual fear conditioning, where the need to recognize and associate the environment to the unconditioned stimulus requires also the activation of the hippocampus (Kitamura et al., 2017).

Due to their dependency on the recollection of a spatial map to be completed, throughout the years most spatial tasks have been defined as episodic-like, because their dependency on an integral hippocampus led researchers to equate them to what was considered to be the most hippocampal-dependent memory system: the episodic one. However, the hippocampus has also been implicated in the early phases of encoding of non-spatial associations (Bradfield et al., 2020), corroborating Henke's statement that the

hippocampus is involved with the memory system supporting slow encoding of rigid associations to speed up learning in the early stage (Henke, 2010), which she identifies as the system supporting procedural and semantic-like memories.

1.3.1 The Delayed Spatial Alternation Task

When faced with the choice of an appropriate task to study the relationship between the hippocampus and cortical associative areas during memory consolidation, we identified a few criteria to guide the choice:

- The task needed to be hippocampal-dependent and solicit known associative areas for its completion
- It needed to rely on a semantic-like rule that could be encoded, consolidated and retrieved on testing days
- It needed to be simple enough to reach optimal performance within a day of training in order to allow clear temporal distinction among the three phases of memory
- It could not be a one-trial task because we needed to be able to observe a progressive learning behaviour, as a direct measure of ongoing encoding mechanisms

The Delayed Spatial Alternation (DSA) task is a spatial task that is commonly used to assess hippocampal but also neocortical integrity in rodents, particularly used to assess function of the prefrontal cortex (Zhang et al., 2017). The task is delivered in a Y-shaped 3 arms maze and demands the rodent to alternate between left arm and right arm choices in order to collect 10 food rewards. A 30 seconds delay separate each trial from the next (see Materials and Methods for more detailed explanation). The behavioural training phase is preceded by a habituation phase during which the rodent constructs a cognitive spatial map of the environment (presenting environmental visual cues surrounding the maze for vision-guided navigation), that it will later exploit for goal-directed maze exploration during behavioural training. This task presents 2 components:

- Working memory is solicited during the 30 seconds delay to hold the information about the previously chosen arm; integrity of the working memory system is therefore needed for correct behavioural expression at each moment of the training.
- The most efficient way to collect all ten rewards is to follow an “alternation rule” that the rodent learns by trial and error over multiple trial repetitions; acquisition of this rule falls in the description of “slow encoding of rigid association” and can be viewed as a semantic-like type of knowledge; in later phases of the training, the rodent actually starts to perform in a procedural-like manner (i.e. the behavioural response is executed quickly, as opposed to the initial phase of the training where hesitation is considered to be a landmark for cognitive engagement during navigation), which is however afferent to the same memory system.

Integrity of this system, relying, among others, on the neocortex, is therefore needed for encoding, consolidation and retrieval of the rule (combined with working memory in the latter phase) and hippocampal activity is needed to speed up the learning process, more so as the task relies on exploitation of a spatial map for completion.

The protocol we followed guaranteed a progressive learning of the rule governing efficient reward collection over the first day of training, while optimal behavioural performance was maintained on following days and could be tested. We were thus able to clearly temporally separate a progressive phase of encoding (training on day 1), consolidation (mainly taking place on the resting phase between day 1 and 2) and retrieval (training on following days).

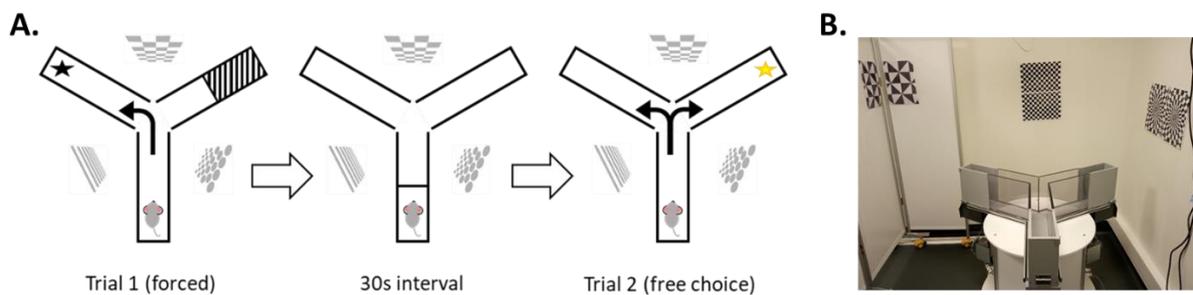


Figure 2. The Delayed Spatial Alternation (DSA) task. **A.** Scheme representing the protocol of the DSA task by mean of showing the principle of alternation in the first two trials. **B.** Picture of the Y-shaped maze and surrounding visual cues used for the DSA task.

Chapter 2: Anatomical Substrates for Memory

2.1 The Concept of Memory Engram

As can be observed, the definition of each phase supposes the existence of a “memory trace”. The concept that memories repose on a physical substrate within the brain has been first introduced by Richard Semon at the beginning of the XX century (Semon, Richard, 1904) and has been debated over multiple times since. Semon hypothesized that experiences imprint on neurons by inducing specific modifications on a given subset and postulated that, to produce an engram, these modifications had to comply to two requirements: being persistent and tagging cells for specific reactivation upon triggering during memory retrieval. Part of the reason why the existence of such a physical trace of memory as engrams has been questioned is due to the fact that the search for the engram has resulted to be such an elusive matter throughout the last century, for a few good reasons.

First of all, memory systems tend to be redundant and allow for compensatory mechanisms. Most tasks naturally encountered can be solved through multiple strategies, possibly involving different brain areas. For example, a single problem demanding spatial navigation could be resolved using an allocentric strategy (i.e. exploiting environmental cues) or an egocentric strategy (i.e. referring to the position of the subject and the actions needed to modify it in a goal directed way, independently from the surrounding environment): the first strategy mainly activates the hippocampus, the second one the lateral striatum and parietal cortex (Rinaldi et al., 2020). This issue explains why behavioural results in lesion or silencing experiments might be confusing and sheds a light on the importance of carefully crafting behavioural tasks in order to refine their memory type-specificity.

Second, redundancy and compensatory mechanisms are at work also within the brain areas being involved in the memory. Cell assemblies representing one specific memory aren't a fixed and never changing unit, but single neurons composing it dynamically reorganize even on a short period of time (i.e. days), undergoing loss and gain of activity and, less frequently, even changing behavioural significance (Driscoll et al., 2017; Schoonover et al., 2021). Computational models confirmed that activity's drift of single neurons composing the memory trace constitutes the most optimized model to achieve long-term storage of memories in a context that is still highly permissive for new learning (Fusi et al., 2005). These models predict that what is fundamental for the permanence of a memory is not a specific set of neuronal connections but the activity pattern elicited by those connections, that can be achieved by many different and ever-changing combinations (Ajemian et al., 2013).

It appears clearly that the second point seriously jeopardizes the persistence requirement postulated by Semon, leading to a third point: rather than a readily identifiable physical location within the brain, “engrams” are defined, today, as dynamic network connections encompassing multiple brain areas, continuously rearranging as memory are solicited and reconsolidated. Furthermore, some areas are involved in early phases of memory (encoding, consolidation and retrieval of temporally close

memories) but not in later phases (reconsolidation and retrieval of temporally far memories): a classical example of this phenomenon is the hippocampus (Bradfield et al., 2020; Kitamura et al., 2017).

Focus in engram research is therefore gradually switching from interrogating the properties of single neurons in single locations to an integrated framework where neuronal populations are considered in their relationships between each other's as part of a dynamic and ever-changing network (Eichenbaum, 2016). This leads to the need for simultaneous investigation of multiple brain areas as an inescapable feature for this type of research, as the individual properties of each area are not as important as the relationships governing their inter-areas interactions.

When approaching the study of memory, and of memory consolidation in particular, from a network prospective, two regions emerges as inescapable: the hippocampus and the medial prefrontal cortex. The hippocampus has emerged early on as a fundamental hub for memory encoding, consolidation and early retrieval, while the medial Prefrontal cortex is the most studied among neocortical associative regions because of its implication in most higher brain functions, including memory, a field in which a unique role for this region has emerged along the years (Euston et al., 2012). However, other associative cortices might reveal a similarly pivotal role if adequately studied. The posterior parietal cortex is a much less studied neocortical associative region which in humans' and primates' studies have been implicated in navigation and working memory (Lyamzin and Benucci, 2019). Hence, the delayed spatial alternation task is a perfect model task to study whether and how the posterior parietal cortex might play a major role in memory consolidation.

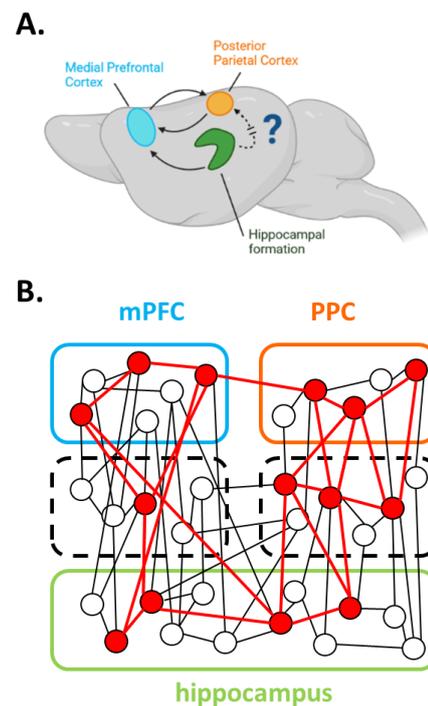


Figure 3. Memory engrams. **A.** Schematic representation of the hypothesised network encompassing hippocampus, the medial prefrontal cortex and the posterior parietal cortex. **B.** schematic representation of the engram (red dots); red lines signify stronger network communication; two unspecified regions that might rely the excitatory drive are also represented.

2.2 The Hippocampus

The hippocampus is a medial temporal lobe structure and part of the forebrain that is present in a conserved way in all mammalian orders. Some of its features and functions have made it one of the most studied areas of the brain, allowing to highlight its participation in many different processes, many of which I have listed above; to resume:

- it is part of the limbic system for expression of behavioural and emotional responses (Strange et al., 2014)
- it presents neurons specifically deputed to encoding of a precise location within an environment (i.e. place cells), determining the creation of a cognitive map exploited for spatial navigation (O'Keefe and Nadel, 1978)
- its integrity is necessary for encoding, consolidation and, to different degree, retrieval of certain forms of memory (Holtmaat and Caroni, 2016)

From a technical point of view, research in the hippocampus is facilitated by its simplified cortical structure (presenting a single layer of principal cells surrounded by strictly organized layers of processes and interneurons) and by the rigidly ordered connectivity between its different regions, granting easily recognizable and distinguishable cell types and a certain facility in experiments reproducibility.

2.2.1 Anatomy of the Hippocampus

In humans and non-human primates, the hippocampus is composed by small dispersed structures bilaterally buried under the neocortex in the medial temporal lobe. Based on their position and functionality, these structures are divided in posterior and anterior regions. In rodents, instead, it has a characteristic bilobate and curved structure that develops along a principal longitudinal axis,

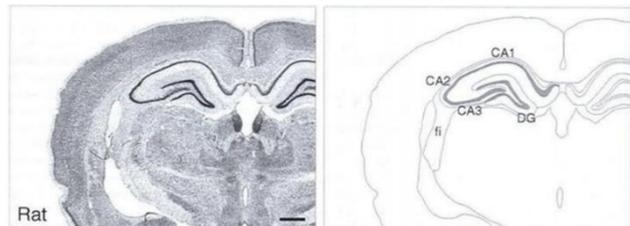


Figure 4. Hippocampus Brain slice (left) and schematic representation (right) of a rat's rostral part of the dorsal hippocampus. (adapted from O'Keefe and Nadel, 1978)

along which it is divided into dorsal and ventral regions (corresponding, respectively, to posterior and anterior hippocampus in humans and non-human primates), rotated of 90° with respect to the human's structures (Strange et al., 2014). Multiple evidences rose the hypothesis that this distinction is not only anatomical, but most importantly functional: the dorsal hippocampus (and the corresponding posterior region in humans) hosts the majority of place cells' populations and has thus been linked to spatial navigation; on the other hand, the ventral hippocampus (and the corresponding anterior region in humans) shows the higher degree of connection with neocortical areas and with the limbic system and therefore has been more strongly related to declarative and, in particularly, emotional memory (Fanselow and Dong, 2010). The hippocampus is part of the hippocampal formation, which

comprehends the subiculum, the entorhinal cortex, the dentate gyrus and the hippocampus proper – or *cornu ammonis* - (Topolnik and Tamboli, 2022). Because of their tight physiological connection through the tripartite synapse, the dentate gyrus and the *cornu ammonis* are often described together as two intertwining U-shaped regions: the *cornu ammonis* is located on the external – ventricular – surface, while the dentate gyrus on the internal one.

2.2.1.a General Organisation

The hippocampus is part of the archeocortex, which is mainly characterized by the presence of a single layer containing the soma of excitatory neurons, instead of the two normally present in the neocortex. The cellular architecture is different between the dentate gyrus and the *cornu ammonis* (O’Keefe and Nadel, 1978):

- Excitatory neurons in the dentate gyrus are called granule cells and are characterized by a small, round body and the presence of an apical but not a basal dendritic arborisation. These characteristics lead to the distinction of three cortical layers (from external-most to internal-most):
 - *molecular layer*, containing the apical dendrites of granule cells, intertwined with the projections of afferent neurons, which can be both local interneurons or long-range projections from other brain regions, such as the entorhinal cortex
 - *granular layer*, containing the cell bodies of granular cells, densely packed
 - *polymorph layer*, containing the first segment of the axon of granule cells, that will later bundle to form mossy fibres; in the *hilus*, this layer merges with the CA4 region of the hippocampus proper; it also hosts the cell bodies of inhibitory interneurons of the dentate gyrus
- Excitatory neurons within the *cornu ammonis* are pyramidal cells (as in the neocortex), characterized by a pyramid-shaped soma, which can be smaller or larger depending on the sub-region of the *cornu ammonis*, and by both apical and basal dendritic arborisations. These characteristics lead to the distinction of 5 layers, incremented to 6 in the CA3 region (from internal-most to external-most):
 - *stratum lacunosum/moleculare*, containing the distal apical dendrites of pyramidal cells
 - *stratum radiatum*, containing the proximal apical dendrites of pyramidal cells; the relative importance of each of these two layers of the dendritic tree depends on the species considered and their morphological distinction is mainly based on the orientation of the axons contacting the dendritic tree and coming from different regions
 - *stratum lucidum*, containing the initial segment of the apical dendrites of CA3 pyramidal cells, which is characterized by the presence of thorn-like spines serving as contact point for the mossy fibres arriving from granule cells of the dentate gyrus, which are

perpendicular to the dendritic tree of CA pyramidal neurons; this layer is not present in CA1 or CA2 regions because pyramidal cells of these two regions are not contacted by mossy fibres, nor in the CA4 region because of its unstructured nature (which actually prevents the identification of any of the other 5 layers)

- *stratum pyramidale*, containing the cell bodies of pyramidal cells and some inhibitory interneurons
- *stratum oriens*, containing the basal dendrites of pyramidal cells and the cell bodies of most inhibitory interneurons, together with projections from both local circuits interneurons and long-range projecting neurons from the septum
- *alveus*, containing the axons of pyramidal cells, directed toward the fimbria (rostral efferents) or the subiculum (caudal efferents) and a few afferent projections

The dentate gyrus has been divided into an exposed (external) blade and buried (internal) blade, but this division does not correspond to a substantial difference in the structure of the two blades, which are mostly uniform. On the other hand, the *cornu ammonis* has been divided into four sections based on different morphology and connectivity of pyramidal cells present with each sub-region (O'Keefe and Nadel, 1978):

- CA1: represents the dorsal-most region in the hippocampus of rodents (most lateral in that of primates) and is composed by pyramidal neurons whose soma is small and the apical dendritic tree is characterized by a main, undivided branch from which project many small side branches.
- CA2: presents pyramidal neurons whose morphological features are similar to CA3 pyramidal neurons, but lack inputs from mossy fibres.
- CA3: presents pyramidal neurons whose soma is large and whose apical dendritic tree is characterized by a singular, spiny proximal segment where mossy fibres make contact and that then divides into a highly branched structure in which no principal branch can be identified.
- CA4: unlike the rest of the *cornu ammonis*, this region is not strictly structured into layers and pyramidal neurons (CA3-like) results scattered and dispersed inside the *hilus* of the dentate gyrus; apical dendrites are reached by mossy fibres.

2.2.1.b Neuronal Population

I will focus now mainly on the cellular population of the hippocampus proper, as it is one of the regions I targeted for my study, in particular the dorsal portion due to its implication in spatial navigation.

Pyramidal neurons (PN) represent the vast majority of neurons present in the hippocampus and are glutamatergic excitatory projecting cells. They have historically been considered a fairly homogenous group, sharing recognizable morphological, molecular and physiological properties also with PNs belonging to neocortical regions, however this view has started to be challenged by more recent

experiments, highlighting a heterogeneity that should not be underestimated and that is hypothesized to explain the differential specialization of PNs belonging to different regions (such as place cells vs emotion-related cells) (Cembrowski and Spruston, 2019). The few morphological differences highlighted in the previous paragraph are the result (or the cause) of different spatial distribution of afferent synapses: in fact, synapses to PNs can be located on their dendrites, soma or axon and the relative abundance and distribution of these afferences determines the pattern of integration of the different information within the pyramidal neuron and, therefore, its firing output. While the almost entirety of excitatory input contact PNs on their dendrites, inhibitory inputs are much more varied in their targeting and are thought to be the fine regulator of information integration through spatial and temporal organization of inputs (Klausberger and Somogyi, 2008).

At least 21 different classes of inhibitory interneurons (INs) have been identified in the hippocampus (Klausberger and Somogyi, 2008), even if they represent only 10-15% of the total neuronal population of the hippocampus (Topolnik and Tamboli, 2022), and enumeration of their individual characteristics is well beyond the scope of this introduction, as nowhere during my research I was able to distinguish between different classes of interneurons from my electrophysiological data. However, even in general terms, interneurons are widely accepted as being the master regulators of brain oscillatory patterns and plasticity at their excitatory afferences is thought to be crucial for memory processing in the hippocampus (Topolnik and Tamboli, 2022). Interneurons receive excitatory inputs from projecting excitatory neurons from different brain regions or from PNs located in the same sub-region and send local inhibitory outputs to PNs, constituting circuits of feedforward inhibition (when the main input to IN is constituted by external afferents) or feedback inhibition (when the main excitatory input is constituted by synapses from local PNs themselves). Interneurons can also send inhibitory projections to other interneurons and this, together with the fact that different INs' classes project to different locations on pyramidal neurons, allows the creation of complex local circuit mechanisms that have implicated in fine population tuning and oscillatory patterns generation (Topolnik and Tamboli, 2022).

2.2.1.c Internal Connectivity

The main excitatory pathway within the hippocampus is one of the most studied excitatory circuits and it is known as the "trisynaptic circuit" (O'Keefe and Nadel, 1978). It actually starts outside of the hippocampus with afferent projections coming from the entorhinal cortex (EC, which is the closest neocortical region) through the perforant path and contacting granule cells of the dentate gyrus in the molecular layer. Granule cells projection, bundled into mossy fibres, then project to the CA3 region of the cornu ammonis in the *stratum lucidum* and CA3 pyramidal neurons relay the information to the CA1 region through their projecting axons, known as Shaffer collaterals, which reach the apical dendrites of CA1 pyramidal neurons mostly in the *stratum radiatum*. The CA1 is the main output region of the hippocampus, projecting through the *alveus* to multiple subcortical and cortical regions (see next section), including the entorhinal cortex, thus closing this excitatory loop. Another branch of the

perforant path directly connects the EC with the CA1 pyramidal neurons in the *stratum lacunosum/moleculare*, however no direct excitatory projection from the dentate gyrus to the CA1 region has never been observed, nor a “reverse” excitatory pathway connecting CA1 to CA3 and eventually to the dentate gyrus: hence, this circuit is thought to directionally polarize the transmission of the information within the hippocampus (O’Keefe and Nadel, 1978). Aside from this main excitatory pathway, a “recurrent” excitatory pathway has been observed in the CA3 region of the *cornu ammonis*, meaning that excitatory pyramidal neurons of the CA3 project to other excitatory pyramidal neurons of the CA3, in the *stratum radiatum*. This represents a unique case not replicated in other sections of the hippocampus and is hypothesized to be pivotal for the autonomous generation of oscillatory patterns that can be observed in this region (Le Duigou et al. 2014).

2.2.1.d Main Afferent and Efferent Pathways

Both dorsal and ventral parts of the hippocampus presents reciprocal, topographically arranged connections with the entorhinal cortex (O’Keefe and Nadel 1978; Strange et al. 2014). The entorhinal cortex represents the link between the hippocampus and other neocortical areas and, through reciprocal projections, works as a hub for both highly processed sensory information (received though the mediation of the perirhinal cortex) and information from associative areas (e.g. the prefrontal cortex). Furthermore, the EC is deeply involved in spatial navigation through firing of specific neurons named grid cells (Hafting et al., 2005). Thus, the hypothesis is that the hippocampus and the entorhinal cortex form a heavily connected system that is fundamental for the correct completion of hippocampal functions in memory and spatial navigation. The EC itself receives topographically arranged afferences from other neocortical regions: for example, in the rat, the medial section of the entorhinal cortex receives inputs from the infralimbic and prelimbic areas of the cingulate cortex, which are more involved in emotional processing, while the lateral section of the EC receives inputs from the retrosplenial region of the cingulate cortex, more involved in spatial processing (Jones and Witter, 2007). Accordingly, reciprocal projections between EC and hippocampus follow a continuous gradient where more dorsal regions of the hippocampus are connected to more lateral sections of the entorhinal cortex, while more ventral regions of the hippocampus to more medial sections of the EC (Cenquizca and Swanson, 2007), maintaining the distinction between a dorsal hippocampus more heavily implicated in navigation and a ventral one more heavily implicated in emotional processing (Strange et al., 2014). The same topographic gradient is observed for projections to the subiculum, which is the region receiving the denser proportion of projections from the CA1 field of the hippocampus, throughout its whole longitudinal axis (Cenquizca and Swanson, 2007) and which can serve as a relay between the hippocampus and the entorhinal cortex (Strange et al., 2014).

The subiculum can also have a relay function toward the lateral septum and hypothalamus, maintaining the same ordered topographic structure of projections (Cenquizca and Swanson, 2007), however the lateral septum also receives direct projections from the hippocampus through the fimbria (O’Keefe and

Nadel, 1978). The lateral septum later projects to the thalamus, creating a network that which is involved in memory and spatial navigation, but also in other behavioural outcomes more related to the associative and emotional part or the hippocampus (Risold and Swanson, 1996). The medial septum, which is considered, together with the supramammillary nuclei, the region controlling the initiation of theta rhythms that are later propagated to the hippocampus, projects instead toward CA1 and CA3 neurons located prevalently in the dorsal part or the hippocampus (Buzsáki, 2002).

This functional gradient of segregation is also maintained in the pattern of projections to other neocortical areas. The density of projections from the hippocampus gradually augments going from the dorsal to the ventral portion. The ventral portion of the hippocampus send direct projections to different sensory neocortices (auditory, visual, olfactory, somatosensory, gustatory and visceral), the amygdalar system (involved in emotional processing) and association cortices in the prefrontal and cingulate areas, with a complex system of projections that can belong either to the fornix or to the longitudinal association bundle (Cenquizca and Swanson, 2007). Projections from the dorsal portion are much less dense and are directed primarily toward the retrosplenial cortex: this is again in line with the functional segregation between a more spatially involved dorsal hippocampus, while the ventral hippocampus mainly participates in emotional processing (Cenquizca and Swanson, 2007).

2.2.2 Hippocampus and Memory

The hippocampus emerged as an important focus for memory-related studies during the 1950s, thanks to the case of patient H.M. (Scoville and Milner, 1957). Most of the lines of research which emerged from this medical case has been already discussed in the first chapter of this introduction, which on its own highlight the central role played by the hippocampus in any memory-related discussion. As stated before, the hippocampus has become first in line for scientific investigation of memory: it is thought to be the pivotal structure for encoding and early consolidation of episodic memory and to play an active helping role in speeding up learning of procedural rules and semantic knowledge (Henke, 2010). In rodents, its involvement has been demonstrated in all contextual and spatial navigation tasks, but also in some forms of associative learning.

An important aspect of patient H. M. amnesic condition that has not been discussed yet in this dissertation is the fact that patient H. M. became affected by both retrograde and anterograde amnesia following resection of the medial-temporal lobe (Scoville and Milner, 1957). Anterograde amnesia refers to the inability to form new memories, which in patient H. M. was mostly restricted to declarative memories, while he preserved the ability to acquire new skills, even though in the complete absence of recollection of practicing them. The implications of this distinction have already been discussed and led to the division of memory forms into hippocampal-dependent and hippocampal-independent, which is still standing, especially in rodent's research (Schacter, Daniel L. and Tulving, Endel, 1994). Retrograde amnesia, instead, refers to the loss of previously acquired memories and in the case of patient H. M. was progressive, meaning that his ability to recall past events failed the more recent were the events he was

asked to recall, while very old memories from his infancy were preserved. The fact that he did not undergo a complete retrograde amnesic process, but that his amnesia was actually temporally graded led to the hypothesis that the hippocampus is required for long-term storage of memories, but only for a limited time period and that after a certain amount of time memories are transferred from the hippocampus to a different brain district. This multi-step and multi-area process of memory consolidation was formalized into the theory of system consolidation (Squire, 1992). In its most recent form, system consolidation theory states that the hippocampus is required to provide context to a memory and, at the moment of encoding, it stores an index of the cortical activity registered during the experience (Tonegawa et al., 2018). On the other hand, the neocortex would hold from the beginning information about the abstract and semantic features of the memory. In this view, the relevant behavioural content would be stored from the beginning within neocortical networks, while the hippocampus would be required to provide the correct pattern of neocortical activation. Consolidation would then consist in reactivation of the neocortical network following indexed instructions contained within the hippocampus, eventually leading to strengthening of the neocortical network itself and progressively demeaning the need for the hippocampus to initiate the reactivation pattern (Euston et al., 2012; Tonegawa et al., 2018). Controversies around the classical vision of a progressive passage of memory storage from the hippocampus to the neocortex have always arose because of contradictory behavioural results, with examples of hippocampal-dependent long-term memory retrieval that can be found in literature (Bayley et al., 2005). Development of optogenetics tools helped with a more thorough insight into this issue. For example, in a classic experiment of contextual fear conditioning, an engram of DG cells was identified based on their activation during fear conditioning: the same cells were reactivated upon recent retrieval (after 1 day) but not upon remote retrieval (after 14 days), when instead a subset of prefrontal neurons was activated to support the behavioural response (Kitamura et al., 2017). However, two additional interesting results were found: 1) neurons forming the neocortical engram at remote retrieval were the same that had been activated at the moment of fear conditioning 2) optogenetics activation of DG engram cells at remote retrieval triggered a freezing response. These two results corroborate the hypothesis that relevant behavioural information is present within the neocortex from encoding, but suggest also that the memory trace within the hippocampus is not lost after a certain amount of time, it is just silent (Kitamura et al., 2017). The current hypothesis is that upon multiple reactivations of the neocortical network holding the abstract or semantic side of the information its activation can be triggered by external stimuli in the absence of the contextual pattern provided by the hippocampus (Tonegawa et al., 2018). However, this does not result in erasure of the mnemonic trace from the hippocampus, on the contrary during the consolidation process each reactivation would trigger the formation of a new contextual trace within the hippocampus. This hypothesis, called multiple trace theory, would explain the otherwise contradictory results obtained with patients and animal models affected by partial hippocampal disruption, forecasting that memories that have undergone a higher number of replay events have the higher probability to survive (Nadel and Moscovitch, 1997).

However, the subject is still debated and theories surrounding involvement of the hippocampus in remote retrieval are ever evolving.

2.3 The Medial Prefrontal cortex

The medial Prefrontal cortex (mPFC) is a neocortical associative region considered to be involved in most higher brain functions, including emotion, reward, motivation and cognition. It is anatomically subdivided in 4 regions, from dorsal-most to ventral-most: Medial precentral cortex (PrCm), Anterior Cingulate cortex (ACC), Prelimbic cortex (PL) and Infralimbic cortex (IL) (Van Eden and Uylings 1985). The two dorsal-most areas, PrCm and ACC, compose the dorsal division of the mPFC and have been more heavily implicated in motor behaviour, while PL and IL, forming the ventral division, have been related to emotional, mnemonic and cognitive processes (Heidbreder and Groenewegen, 2003).

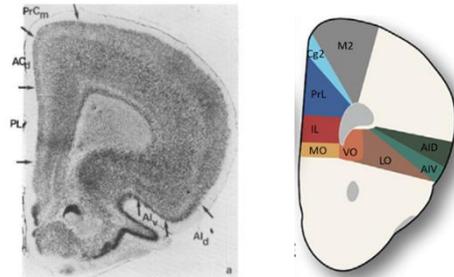


Figure 5. Medial prefrontal cortex Brain slice (left) and schematic representation (right) of a mouse's medial prefrontal cortex; the schema highlights the main anatomical subdivisions of this region. (left: adapted from Van Eden and Uylings, 1985; right: adapted from Le Merre et al., 2021)

2.3.1 Anatomy and Connectivity of the Medial Prefrontal Cortex

Neocortex is classically subdivided in 6 layers, from the one closer to pial surface to the deepest one (Anastasiades and Carter, 2021):

- Layer I (or molecular layer), containing the apical dendrites of pyramidal neurons and characterized by a very scarce population of cell bodies
- Layer II (or external granular layer) and layer III (or external pyramidal layer) are often impossible to distinguish anatomically and are therefore always considered together; they contain the cell bodies of local interneurons and pyramidal cells projecting to other cortical areas
- Layer IV (or internal granular layer), which is considered to be the main input site of the neocortex, where axons projecting from other structures makes synapses
- Layer V (or internal pyramidal layer), which is considered the main output site of the neocortex, characterized by the presence of large cell bodies of pyramidal neurons projecting to subcortical target regions
- Layer VI (or polymorph layer), characterized by a high neuronal diversity and presenting neurons which also project to extra-cortical regions, in particular to the thalamus

These layers can be easily observed and distinguished in sensory cortices but not as much in associative ones. mPFC, in particular, lacks layer IV (it is therefore classified as an agranular cortex), presenting instead a more homogeneous distribution of input fibres. This, together with the distribution of somas of projecting neurons from layer II to layer VI without a clear laminar organization, makes the appearance of this region of the neocortex fairly homogeneous when subjected to Nissl staining (Anastasiades and Carter, 2021; van Eden and Uylings, 1985).

The prefrontal cortex in its whole (comprehensive of the orbitofrontal cortex) projects to and from all major regions of the brain, cortical and subcortical, being the cortical region which receives inputs from and sends outputs to the highest number of different regions, therefore displaying the highest number of reciprocal connections (Le Merre et al., 2021). mPFC (especially IL and PL) is considered to be the final target of a medial cortical network conveying information from the subiculum, through the relay of Retrosplenial cortex (RSC) and Anterior Cingulate cortex (ACC) (Zingg et al., 2014). It is also considered to be the final target of a lateral cortical network involving the Insular cortex, a region with which mPFC shares dense reciprocal projections. Together, these two networks are hypothesized to serve as an interface for integration of external and internal sensory stimuli (Zingg et al., 2014).

mPFC also shares reciprocal connections with the Temporal Association area (TEa) and the lateral Entorhinal cortex, the latter possibly serving as relay for the information to the Hippocampus, as no direct afferences from mPFC to HPC have been identified (Zingg et al., 2014). On the other hand, IL and PL regions both receive inputs from the ventral hippocampus, while direct inputs from the dorsal HPC are sparser and potentially not sufficient to assure correct communication without a supporting polysynaptic alternative pathway (Anastasiades and Carter, 2021). mPFC and ventral Hippocampus, together with the Basolateral Amygdala, form a network involved in emotional processing and emotional learning (Ghashghaei et al., 2007). Dense reciprocal connections with the multiple thalamic nuclei are thought to be fundamental for support of many cognitive functions, among which working memory, attention and learning (Anastasiades and Carter, 2021). Finally, mPFC shares reciprocal connections with important centres of the neuro-modulatory system, such as the dorsal raphe nuclei, ventral tegmental area and locus coeruleus, which are hypothesized to be important for adaptative response to stress and reward (Euston et al., 2012)

By virtue of its large connectivity and because perturbation of PFC activity has been demonstrated to affect cortex-wide connectivity patterns (Allen et al., 2017), the prefrontal cortex has been hypothesized to be a source of top-down regulation of many if not all of its cortical targets, inducing appropriate behavioural adaptation through feedback projections to other cortical regions (Le Merre et al., 2021). A “competence”-division has also been hypothesized among prefrontal regions, corroborated by the pattern of reciprocal connections between the three prefrontal subdivisions (dorsal mPFC, ventral mPFC

and OFC) (Zingg et al., 2014), but convincing experimental results confirming or rejecting this hypothesis have yet to be produced (Le Merre et al., 2021).

2.3.2 The Medial Prefrontal Cortex and Memory

As much as the medial Prefrontal cortex has been attributed a crucial role in cognition, it also has been in memory, with many experiments testing its participation in a wide variety of learning and memory tasks (Euston et al., 2012) and being the main neocortical area involved in experiments based on the system consolidation theory (Tonegawa et al., 2018). Classically, the role of mPFC has been related to remote memory retrieval, stemming from the observation that activity of certain neocortical areas is stronger during memory retrieval after longer (25 days after learning) rather than shorter (5 days after learning) time-lapses (Bontempi et al., 1999), among which mPFC showed the strongest activity. Accumulation of evidences supporting a pivotal role for mPFC in remote retrieval led to the hypothesis that mPFC may play in this matter a role similar to that played by the hippocampus in recent retrieval, providing other cortical areas, which would store the information in an abstract form, with context and an indexed pattern for activation (Frankland and Bontempi, 2005). This view, which implies the unicity of the medial Prefrontal cortex and is generally widely accepted, might be challenged by accumulating evidence on the implication of mPFC also in recent retrieval of certain memory types, which would rather suggest that the region is implicated in engrams of specific memories from an early stage, like any other cortical area, instead of assuming a mastermind role over memory in general later on (Euston et al., 2012). However, several inactivation experiments have been successful in suppressing remote retrieval but not recent retrieval in a plethora of different tasks (Euston et al., 2012), while evidence of involvement of mPFC in recent retrieval is scarcer and the effect is generally weaker than in remote retrieval (Quinn et al., 2008).

What has been established, instead, is the activation of mPFC engram neurons during encoding (Kitamura et al., 2017), as described before. Because these engram neurons cannot be activated by natural triggers during early phases, they are considered to be silent and are thought to undergo a process of maturation, corresponding to the progressive acquisition of retrieval strength by neocortical neurons and disengagement of the hippocampus (whose engram neurons become progressively silent) (Tonegawa et al., 2018). The process of maturation is hypothesized to be strictly related to memory consolidation (Euston et al., 2012; Tonegawa et al., 2018). Multiple experiments have highlighted that mPFC inactivation right after learning leads to deficits in memory retrieval even after short time-lapses (24-48 hours), suggesting that this silent engram is required for consolidation of the memory (Euston et al., 2012). In both long-term storage and memory consolidation, this prominent role might be played by mPFC by virtue of its preferred targeting by monosynaptic afferent projections from the hippocampus (Cenquizca and Swanson, 2007).

Finally, the medial Prefrontal cortex, especially in the dorsal division, has been linked to working memory and inserted in a fronto-parietal network displaying sustained activity related to item identity

during delay periods in working memory-guided tasks (Constantinidis and Klingberg, 2016). Findings on this subject have mainly been collected in humans and primates, but lesion studies in rats have confirmed that mPFC impairment prevents correct task execution when working memory is required (Horst and Laubach, 2009).

2.4 The Posterior Parietal Cortex

The Posterior Parietal cortex (PPC) is one of the main associative cortices of the mammalian brain, along with the prefrontal and temporal cortices. Anatomically, it is located between the somatosensory and visual cortices on the antero-posterior axis, from which it can be distinguished because

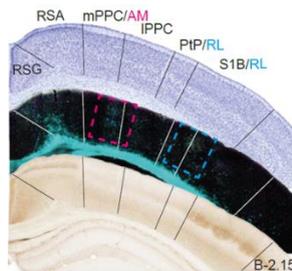


Figure 6. Posterior parietal cortex Brain slice of a rat's posterior parietal cortex stained with Nissl stain for anatomical definition. (adapted from Hovde et al., 2018)

of the strong number of callosal projections from cortical areas (Lyamzin and Benucci, 2019). It presents a wide network of reciprocal connections with different cortical and subcortical areas, which grants its participation in multiple cognitive processes, among which: sensory-motor integration, early motor planning, spatial attention, spatial navigation, representation of spatial relationships, working memory and decision making (Whitlock, 2017).

2.4.1 Anatomy and Connectivity of the Posterior Parietal Cortex

Like mPFC, PPC presents a homogeneous lamination when subjected to Nissl staining, even though in more lateral sections a denser layer II/III presenting smaller cells and a sparser layer V presenting larger cells emerge (Gilissen et al., 2021; Hovde et al., 2018).

PPC is often regarded to as a relay region between sensory cortical areas and “higher” associate areas involved in more cognitive tasks by virtue of its connectivity pattern (Lyamzin and Benucci, 2019). It shares reciprocal connections with both visual and auditory sensory areas and might be a site of integration of these two types of information before relay to the Orbitofrontal cortex (OFC), configuring as an alternative pathway to the direct input of information from sensory cortices to OFC (which share, however, reciprocal direct connections) (Zingg et al., 2014). In this context, it has been inserted within a cortical medial subnetwork which also comprise two others associative regions with whom PPC shares reciprocal connections: the Retrosplenial cortex (RSC) and the Anterior Cingulate cortex (ACC). Altogether, this subnetwork is hypothesized not only to deliver integrated sensory-motor information to the OFC, but also to be heavily implicated in spatial navigation, as all associative areas involved have been independently demonstrated to be required for this type of task (Clark et al., 2018; Zingg et al., 2014).

As far as the neocortex is concerned, other, albeit less dense, reciprocal connections exist between the PPC and the Temporal Association area (TEa) and with the lateral Entorhinal cortex (Zingg et al., 2014). PPC's projections also target subcortical regions, in particular the dorsal striatum, some thalamic nuclei (lateral posterior, laterodorsal and posterior complex) and the claustrum, from which it receives also dense afferent projections (Lyamzin and Benucci, 2019).

PPC is not directly connected to the Hippocampus, neither to the dorsal nor the ventral division of the latter. The relay might be represented by the lateral EC, in line with its general role as gateway for neocortical information to the hippocampus, but an alternative, interesting hypothesis is represented by the Retrosplenial cortex, which shares dense reciprocal connections with PPC and receives afferent projections from the dorsal portion of the Hippocampus (Cenquizca and Swanson, 2007). This relay would be, therefore, possibly heavily implicated in spatial navigation.

It is of interest to notice that PPC and mPFC have been ascribed to two different cortical medial subnetworks. RSC and ACC are associative relay areas that are shared between the two and therefore assure their interaction. However, the main target region of the PPC, the OFC, is segregated from the mPFC by a lack of reciprocal connections, suggesting PPC and mPFC might actually serve unique and independent contributions to information processing and behaviour (Zingg et al., 2014).

2.4.2 The Posterior Parietal cortex and Navigation

Ordered patterns of neuronal activity have been observed in the Posterior Parietal cortex during navigation in humans, primates and rodents. In rodents, subsets of PPC neurons express specificity for position of the animal within the environment and with respect to beginning and end of a spatial trajectory, specific navigational movement (e.g. a turn on the left or on the right) and head and body orientation in the environment (Krupin et al., 2018; Nitz, 2006; Whitlock, 2014). In one case (Nitz, 2006), navigational firing patterns were conserved even in dark conditions, therefore demonstrating to be independent from environmental visual cues. Altogether, evidences point toward the insertion of PPC within a network deputed to egocentric navigation, corroborated by its strong connection to the striatum (Rinaldi et al., 2020). However, PPC has also been involved in visual information integration and its integrity is required to solve visual decision tasks (Driscoll et al., 2017) and a subset of PPC cells display firing tuned to the direction of goal location with respect to head direction (Krupin et al., 2018), thus a participation, albeit minimal, to allocentric navigation processes cannot be ruled out, also by virtue of the dense reciprocal connections shared with the Retrosplenial cortex, which might represent a point of integration between the two navigation systems (Clark et al., 2018).

2.4.3 The Posterior Parietal cortex and decision making

PPC has been implicated in decision making in a plethora of different tasks involving a binary choice guided by visual, auditory or multimodal cues, in combination or not with navigation (Lyamzin and Benucci, 2019). In these tasks, PPC neurons displayed a choice-specific activity, with different subsets tuning in to either one of the behavioural responses (Harvey et al., 2012). Most convincing results come from experiments involving tasks demanding evidence accumulation (Hanks et al., 2015) and delay periods (Goard et al., 2016), during which a sustained neuronal activity was observed, supporting cue holding in working memory. Sustained activity in the region has also been linked to conservation of a lasting trace of previous trials choices and outcomes (Akrami et al., 2018), used as history bias to guide behaviour. These results are in line with studies in humans and primates which led to the insertion of the PPC in a fronto-parietal network sustaining working memory (Constantinidis and Klingberg, 2016). Participation of PPC to any phase of the processing of long-term memories of any type is much more debated and much less studied. In humans, PPC is strongly activated during retrieval of episodic memories, but patients with PPC lesions do not display amnesic symptoms (Sestieri et al., 2017). However, recent experiments in rodents revealed that behaviour-relevant firing patterns in the PPC are replayed during sleep following a learning experience, a phenomenon that is commonly associated with memory consolidation (Wilber et al., 2017).

Chapter 3: Mechanisms for Memory Encoding and Consolidation

3.1 The Synaptic Plasticity and Memory Hypothesis

When Richard Semon first formalized the concept of engram he stated that, to grant its formation, neuronal substrates had to undergo persistent changes that tag them for later reactivation upon triggering (Semon, Richard, 1904). It was already suggested by the scientific community that said changes had to imply a growth or strengthening of the connections between neurons and since the discovery of Long Term Potentiation (LTP) (Bliss and Lømo, 1973) synaptic plasticity events have been the privileged field of investigation for mechanisms leading to the creation of memory engrams (Takeuchi et al., 2014). The Synaptic Plasticity and Memory (SPM) hypothesis states, in its general formulation, that activity-dependent synaptic plasticity events are generated at a subset of synapses during an experience and that they are both necessary and sufficient to induce encoding and storage of the memory of said experience in the area of the brain where plasticity can be observed (Martin et al., 2000). This hypothesis has guided research in the field for the past 50 years, building a pool of evidences demonstrating that synaptic changes imputable to plasticity can be observed in the hippocampus after learning, such as AMPA receptors insertion at the post-synaptic density (Matsuo et al., 2008). Furthermore, administration of NMDA receptors blocker – again in the hippocampus, but also in other brain areas implicated in the learning circuit - at a concentration that matches LTP induction's blockade prevents learning (Morris et al., 1986) while LTP induction in the dentate gyrus after learning induced memory erasure (Brun et al., 2001), based on the principle that, in order to be efficacious for memory encoding, synaptic plasticity has to regard only a subset of neurons and a specific experience-related timeframe, while generalized and prolonged excitation “drowns” the relevant trace signal and results in encoding failure (Staubli and Chun, 1996).

An exciting challenge for the SPM hypothesis is determining when and where synaptic plasticity takes place and characterize the peculiarity of events distinguishing, for example, encoding from consolidation or memory formation within the hippocampus from neocortical areas. A widespread theory differentiating between synaptic plasticity events determining encoding and consolidation is the synaptic tagging and capture (STC) hypothesis (Redondo and Morris, 2011). Synaptic tagging happens during memory encoding at stimulated synapses. Although tagging is generally co-occurring with early LTP expression, the mechanisms underlying the two phenomena seem to be separated (Kopec et al., 2006; Sajikumar and Frey, 2004), and tagging is heavily dependent on Calcium/Calmodulin II kinase (CaMKII) activation (Redondo et al., 2010). The exact nature of the tagging is unknown: it has been long thought that it came down to one or a few molecules able to attract plasticity's stabilizers at a later time, but it is now considered more probably to be a transient structural state of the synapse, which for a lifespan of around 90 minutes makes the synapse “permissive” for long-term synaptic enhancement (Redondo and Morris, 2011). Concurrently to tagging, therefore still during encoding, LTP induces the

transcription and translation of Plasticity-Related Products (PRPs), a plethora of proteins and mRNA including kinases, receptor subunits and cytoskeleton-associated protein Arc that are necessary for stabilization of long-term potentiation at synapses. Due to the delay imposed by transcription and translation processes, PRPs become available to the synapse around 45 minutes after LTP induction and their presence is still detected 90 minutes after LTP induction, in line with the “permissive” window assured by the tag (Frey and Morris, 1998). Synaptic capture takes place during this time-window, which might also correspond to the time-window for consolidation, being severely delayed with respect to encoding, and consist in long-lasting structural and functional plastic changes induced by PRPs which are “captured” by tagged synapses, assuring the specificity of stable long-term potentiation only to synapses already solicited at the moment of encoding (Redondo and Morris, 2011). Synaptic capture would be dependent on consistent reactivation of neurons during consolidation. Even though STC theory is mostly mentioned with regard to synaptic potentiation, the existence of “negative” tags has been hypothesized and negative PRPs, promoting synaptic depotentiation, have been observed (Okuno et al., 2012). An interesting aspect of this formulation of the STC is the distinction between functional early-LTP, synaptic tagging and PRPs’ translation induction, which are described as interconnected but substantially independent events (Redondo and Morris, 2011). This independence has two consequences: on the one hand, when multiple events inducing LTP happens at a short time-delay, PRPs are equally shared by all tagged synapses at the moment of capture, which means that time-coupling of a low-meaning event (which would not normally be remembered upon testing 24 hours later) to a highly-meaning event would lead to a strong encoding of both events. This has been demonstrated by multiple experiments showing that novelty-exploration enhances the consolidation of an unrelated but time-coupled experience, both when exerted before or after the other experience (Park et al., 2021; Wang et al., 2010). On the other hand, even when an event fails to express a strong functional LTP during encoding, or LTP is experimentally blocked after induction bringing the synapse back to its functional baseline, if the experience was able to induce synaptic tagging PRPs’ translation-induction from unrelated events in nearby synapses could rescue the memory.

A main drawback of the STC theory is that most evidences have been cumulated in *in vitro* models, while *in vivo* experiments only served indirect proves of the concept (Redondo and Morris, 2011). Even though supported by much more *in vivo* evidence, the SPM hypothesis in general suffers from similar concerns, especially in regard of LTP duration, which, apart from very few exceptions, has been observed to decay back to baseline within a few hours from induction, also due to its high characterization using *in vitro* models, but which is in stark contrast with the persistence of long-term memories for days, months or even years. Another general concern of the SPM is that synaptic plasticity involvement in memory has been studied at the cellular level within the hippocampus, but the link with system consolidation involving neocortical areas has been difficult to harmonize, as the second has been primarily studied at the level of neuronal populations and network activity (Takeuchi et al., 2014).

Overall, the SPM is a blatant example of the challenges represented by the dramatic need to change the “level” of investigation, both from *in vitro* – where synaptic plasticity is well characterized within a readily measurable framework – to *in vivo* experiments – where direct markers of plasticity are more difficult to evaluate and often measurement are indirect or rely on loss of function experiments – and from single neurons’ level to network’s level. Experiments centred on synaptic plasticity often focus on single neurons – even single dendritic spines, thanks to novel high-resolution techniques -, implying a privileged connection that, once initiated, strengthen itself. On the other hand, from a network level engrams have been showed to be ever-drifting entities where new neurons are continuously recruited while others are dismissed (Driscoll et al., 2017; Schoonover et al., 2021). Furthermore, even though examples of isomorphism between experience-induced synaptic strengthening and behavioural response have been observed, such as fear conditioning in the amygdala (Johansen et al., 2010), memories are often much more complex spatio-temporal associations consolidated in a distributed network which relies on a complex pattern of multiple excitatory and inhibitory interconnections (Klausberger and Somogyi, 2008). Bridging the gap between synaptic plasticity, network activity and behaviourally-relevant system consolidation is one of the exciting challenges of modern neurosciences.

3.2 Synaptic Plasticity

Synaptic plasticity is defined as the activity-induced modification of strength or efficacy of synaptic transmission at pre-existing synapses (Citri and Malenka, 2008). Many different parameters determine the efficacy of a synaptic connection, some of which are pre-synaptic (such as action potential propagation, firing pattern, probability of release of neurotransmitter from vesicles), other are post-synaptic (such as global excitability of the cell, shape and size of the dendritic spine, number and position of neurotransmitter receptors), depicting a complex situation where different types of changes can take place. At the middle of the 20th century, Donald Hebb hypothesized that synchronism in the firing between the pre- and post-synaptic neurons was a mandatory requisite for synaptic plasticity, especially for changes inducing a strengthening of the connection, resuming his hypothesis in the famous statement “what fires together, wires together (Donald O. Hebb 1949)(Morris, 1999). Hebbian plasticity causally links synchronous discharge of action potentials from the two sides of a synapse to bilateral functional and structural changes at the level of the synaptic bouton. Therefore, hebbian synapses can be considered coincidence detectors, that are strengthened only when both neurons participating to the synapse are activated at the same time (Morris, 1999). Most forms of plasticity that were discovered in the following years fall within the canon described by Hebb, the most famous example being Long Term Potentiation (LTP), however examples of non-hebbian forms of plasticity have been found, where plastic changes at synapse can be observed even in the lack of either a pre-synaptic or a post-synaptic spike. One interesting form of non-hebbian synaptic plasticity is synaptic scaling, where weakening of a synapse is induced by strengthening of a nearby one and is realized through reduction of AMPA receptors at the post-synaptic site, hence it is an entirely post-synaptic mechanism (Whitt et al., 2014).

Different forms of synaptic plasticity have been classified based on their characteristics. Beside the division between hebbian and non-hebbian forms of plasticity, a main division can be drawn between mechanisms that strengthen a synaptic connection or that weaken it. LTP is again the most famous example of plastic synaptic strengthening and is countered by a mirror mechanism called Long Term Depression (LTD) that instead operates a weakening of the interested synapse (Alger and Teyler, 1976). The relationship between LTP and LTD is quite interesting, as they can be induced by similar protocols delivering the same stimulation pattern but with different timing. In fact, in line with their hebbian nature, both LTP and LTD are Spike Timing-Dependent forms of Plasticity (STDP), differing in the timing of coordinated activation of the pre- and post-synaptic site: when the pre-synaptic spike precedes the post-synaptic one, LTP can be observed, while when is the post-synaptic spike the one leading the pattern LTD verifies (Bi and Poo, 1998). This was a huge confirmation of Hebb's theory and showed how the same mechanism can be tuned to obtain very different and even opposite results. Both LTP and LTD are forms of long-term plasticity, whose effects lasts for hours or even days (even though given that most experiments were conducted *in vitro*, evidences for longer durations are scarce), as opposed to short-term plasticity, which is extinguished in a matter of milliseconds-to-minutes. Most common forms of short-term plasticity involve pre-synaptic mechanisms, such as facilitation and short-term depression, which are induced by changes in Calcium levels in the pre-synaptic bouton, determining a higher or lower rate of vesicular release (Citri and Malenka, 2008).

Lastly, synaptic plasticity can be divided into functional and structural forms. Functional plasticity regards all mechanisms that alter the strength of a synapse without permanently altering its structure, such as alteration of the rate of vesicular release at the pre-synaptic site or of receptors' composition at the post-synaptic site; structural synaptic plasticity, instead, is mainly studied at the post-synaptic site and regards all structural and cytoskeletal alterations that require protein synthesis and results, for example, in alteration of the size of a dendritic spine or even in the formation of a new one (Sala and Segal, 2014). Structural plasticity has long been considered to be a late stage of LTP, but research in the last decades highlighted that the interaction between functional and structural plasticity is more complex than a sequential step organization and that the two relies on independent, however interconnected, mechanisms (Redondo and Morris, 2011).

A thorough discussion of all different forms of synaptic plasticity, but even of the different forms of LTP alone, is beyond the scope of this introduction, as they are very vast subjects on themselves. I will limit myself to the description of AMPA receptors- and NMDA receptors-dependent LTP at excitatory synapses, as it is the one form of LTP that we addressed during this study.

3.2.1 Post-synaptic Long-Term Potentiation at Excitatory Synapses

Long Term Potentiation was first described by Bliss and Lomo in the 1970s, who observed a persistent (lasting several hours) increase in the amplitude of both Excitatory Post-Synaptic Potentials (EPSP) and

spikes and an increase in spike frequency in granule cells of the dentate gyrus following repetitive stimulation of the perforant path in the rabbit brain (Bliss and Lømo, 1973). Since then, different forms of LTP have been characterized, of which post-synaptic NMDA receptor-dependent LTP is the most studied.

NMDA (N-methyl-D-Aspartate) receptor is a transmembrane glutamate receptor that can be found at excitatory post-synaptic sites. It is a tetrameric ion channel which allows transmembrane diffusion of both Sodium (Na^+), Potassium (K^+) and Calcium (Ca^{2+}) and which is double-gated, demanding both ligand-binding and trespassing of a voltage threshold to be activated (Vyklícky et al., 2014). When Glutamate binds on NMDA receptors it triggers the opening of the ion channel, which is however obstructed by a Magnesium (Mg^{2+}) ion, preventing transmembrane diffusion: removal of Mg^{2+} is triggered by depolarization of the post-synaptic membrane. It is evident that NMDA receptors function as coincidence detectors of pre- and post-synaptic activation, respectively expressed as Glutamate release and membrane depolarization, and thus stand out as the perfect substrate for investigation of hebbian-LTP. Post-synaptic depolarization might be the result of other Glutamate receptors activation at the same synapse or the reflection of a wider excitatory activity that interest surrounding spines in the dendritic tree or the effect of back propagation of action potentials, thus being the expression of a more generalized post-synaptic neuron activation (Magee and Johnston, 1997). In either case, the main glutamate receptors assuring basal synaptic transmission are AMPA (2-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) receptors (Greger et al., 2017). AMPA receptor is a transmembrane tetrameric ligand-gated ion channel permeable to Na^+ and K^+ and activated by glutamate binding. It displays a rapid kinetic of activation and inactivation, designing a sharp curve, and AMPA receptors-blocker have been demonstrated to completely abolish neuronal response to excitatory stimuli (Greger et al., 2017). AMPA receptors most commonly found at synapses during basal conditions are either GluA1/2 or GluA2/3 heteromers (Huganir and Nicoll, 2013). The GluA2 subunit of AMPA receptors presents a bulk amino-acidic mutation which make GluA2-containing AMPA receptors Calcium-impermeable, thus preventing Ca^{2+} -mediated signalling cascade at the post-synaptic site during basal excitatory transmission conditions (Greger et al., 2017).

In order for LTP to take place, basal cationic conductance through AMPA receptors must trigger a strong-enough depolarization to induce Magnesium-removal from NMDA receptors ion channel and allow Ca^{2+} entrance at the post-synaptic site. This step of LTP is referred to as LTP induction and is NMDA receptor-dependent. Ca^{2+} transients at the post-synaptic site initiate a cascade of events that will result in LTP expression, which is a composite event independent from NMDA receptors but dependent on AMPA receptors, at least in its functional component (Nicoll, 2017). The first step in the cascade is Calcium binding to the Calcium/Calmodulin II kinase (CaMKII), which is considered the main effector of LTP-related synaptic changes (Herring and Nicoll, 2016). CaMKII phosphorylate multiple downstream targets, among which different subunits of AMPA receptors, modifying their ion conductance and mobility pattern, for example facilitating the insertion of new AMPA receptors at the

post-synaptic membrane (Huganir and Nicoll, 2013). Addition of new AMPA receptors strengthen the efficacy of synaptic transmission as it augments the pool of receptors that can be activated during basal transmission and is seen as the main marker of functional LTP expression. Conversely, low Calcium concentration at the post-synaptic site would induce dephosphorylation and reduction of the pool of AMPA receptors, determining long-lasting depression of the synapse (Herring and Nicoll, 2016). Other downstream targets of CaMKII are actin and the transcription factor cAMP Response Element Binding protein (CREB), which respectively account for early cytoskeletal remodelling and late protein synthesis, both components of structural LTP (Kandel, 2012).

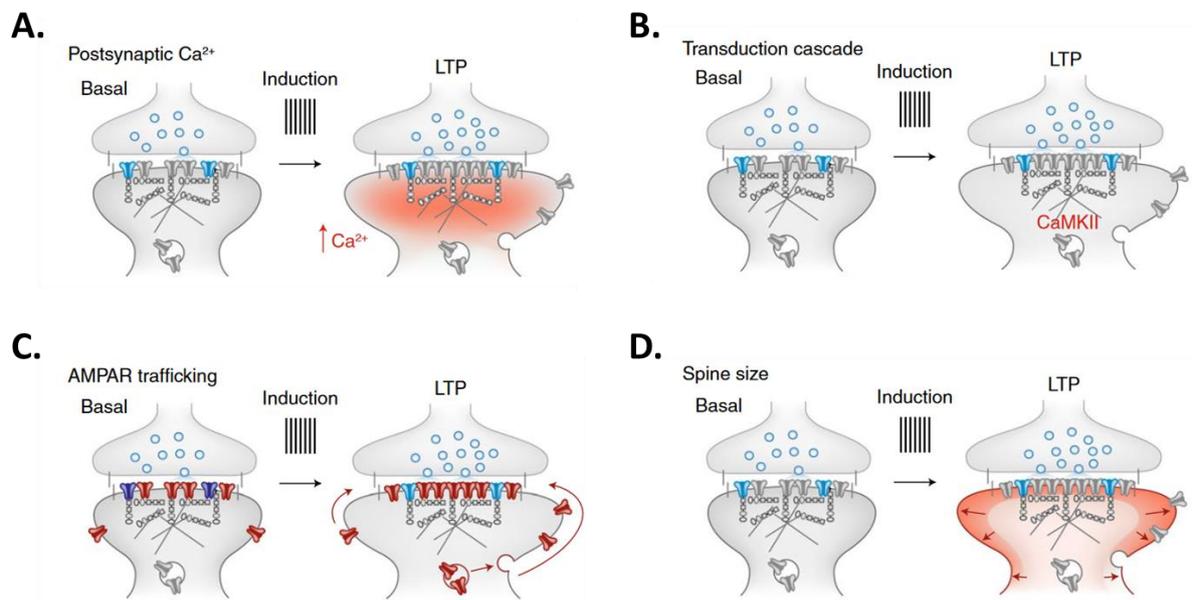


Figure 7. Post-synaptic long-term potentiation at excitatory synapses. **A.** NMDA receptors opening results in an increase in post-synaptic Calcium's concentration. **B.** CaMKII translocation to the post-synaptic site and its binding to Calcium initiate the transduction cascade leading to late phases of LTP expression. **C.** AMPA receptors' trafficking from the vesicular pool to the cell membrane and laterally from the peri-synaptic space to the post-synaptic density increases, resulting in an increase of the number of AMPA receptors available at the post-synaptic density. **D.** Rearranging of the local cytoskeleton results in spine-size's increase as a result of late LTP expression.

3.2.2 AMPA Receptors' Trafficking at Synapses and LTP Expression

Dendritic excitatory synapses are organized in spines and present a zone where concentration of scaffolding proteins, cell-adhesion proteins and glutamate receptors is higher, called the post-synaptic density (PSD) (Suratkal et al., 2021). This zone aligns with the portion of the pre-synaptic site showing the higher concentration of neurotransmitter vesicles and has therefore been identified as the site where synaptic transmission actually takes place (Jang et al., 2017). The surface of the spine surrounding the PSD is called peri-synaptic space, while spines' neck and the rest of the dendritic shaft make up the

extra-synaptic space (Suratkal et al., 2021). AMPA receptors localize at all three of these compartments and also to a cytosolic pool constituted by endosomal vesicles (Choquet, 2018). In basal conditions, around 50% of the total AMPA receptors population at the cellular membrane display a high mobility index, freely diffusing among the three transmembrane compartments following Brown's laws, while the other 50% is reversibly anchored to cytoskeletal elements that stabilize its position, with the most stable AMPA receptors localizing at the PSD (Tardin, 2003). Altogether, in basal conditions AMPA receptors dynamically pass from a stable anchored state to free diffusion and a constant ratio of PSD/peri-synaptic/extra-synaptic receptors is maintained. During Ca^{2+} transients due to LTP induction, phosphorylation of AMPA receptors at the PSD stabilizes their anchorage, preventing AMPA receptors lateral diffusion from the PSD; at the same time, new AMPA receptors diffusing to the PSD are more easily trapped and stabilized, overall inducing an increase in the number of AMPA receptors at the post-synaptic density and, therefore, augmenting the transmission strength of the synapse (Opazo et al., 2010). AMPA receptors trapping at the post synaptic density is considered to be the main effector for expression of functional LTP (Choquet, 2018). Multiple experiments have observed a subunit preference for LTP-related receptor trapping, with GluA1 homomeres being the preferential type of AMPA receptor moving to the PSD following LTP induction, while GluA2-containing heteromeres would follow at a later time-point (Plant et al., 2006). However, molecular replacement experiments, where individual GluA subunits have been knocked-out and replaced with other GluA subunits, have failed to identify a specific subunit that would be necessarily required for LTP expression (Granger et al., 2013). Furthermore, immobilization of GluA2-containing AMPA receptors at the cellular membrane, preventing lateral diffusion, have been shown to be sufficient to prevent LTP both *in vitro* and *in vivo* (Penn et al., 2017). Interestingly, the same paper showed that only continuous infusion of the AMPA receptor blocker completely prevented LTP, while washout of the blocker prior to LTP induction completely abolished short-term potentiation but allowed an attenuated expression of early LTP. This little potentiation is probably due to AMPA receptors newly inserted into the plasma membrane from the endosomal compartment, in line with the discovery that LTP expression is dependent on an intact exocytic machinery (Kopec et al., 2006). This dependency is actually staggering: in fact, peri-synaptic and extra-synaptic pools of AMPA receptors should be large enough to cover for the enhanced demand from the PSD. Furthermore, lateral diffusion of receptors is quicker than exocytosis, even more as exocytic events tend to take place at the extra-synaptic compartment, and to a lesser extent to the peri-synaptic one, but not at the PSD, therefore still demanding lateral diffusion of newly inserted receptors to the PSD.

In recent years, my team collaborated to the characterization and application *in vivo* of AMPA receptors' trafficking-blocking agents, more specifically a divalent antibody directed against the GluA2 subunit of endogenous AMPA receptors and a knock-in mouse strain expressing Acceptor Protein (AP)-tagged GluA2 subunits that can be targeted with tetravalent Neutravidin (Getz et al., 2022; Penn et al., 2017).

Both strategies share the same trafficking-blocking principle: the agent induces crosslinking of two or more neighbouring AMPA-receptors, constituting aggregates that have a lower facility of diffusion within the plasma membrane and a high probability of containing at least one receptor stably trapped to the cytoskeleton, thus inducing stabilization of AMPA receptors at their current location. These methods have the advantage to not affect basal transmission, as the proportion of AMPA receptors present at each compartment does not change, just their mobility index is affected. However, following LTP induction, cross-linking prevents lateral diffusion of AMPA receptors toward the PSD, thus preventing LTP expression (Penn et al., 2017). At the behavioural level, this resulted in memory impairment in both fear conditioning (Penn et al., 2017) and delayed spatial alternation task (El Oussini et al., 2023, **Annexes**). Interestingly, in this second work, not only AMPA receptors' cross-linking did not affect learning of the spatial task (even though retrieval was affected on the following day), suggesting that early LTP expression might not be required for learning, but injection of the blocking agent after training but before rest was sufficient for impairing memory performance after 24 hours, suggesting that AMPA receptors diffusion to the PSD is necessary for stabilization of long-term potentiation during memory consolidation (El Oussini et al., 2023, **Annexes**).

The interest of developing a knock-in strategy instead of using the antibody lies in the higher target precision that can be achieved with the former. In fact, binding of biotin to the Acceptor Protein is mediated by biotin ligase BirA, which is not endogenously expressed in the mouse brain and whose stereotaxical injection can be restrained to the region of interest. Furthermore, BirA expression can be made dependent on a cell-type specific promoter, restraining even more the investigation to a specific cell type (Getz et al., 2022).

3.2.3 Synaptic plasticity in the neocortex

In the neocortex, all most common forms of synaptic plasticity mechanisms have been observed and characterized in a way that is not different from corresponding synaptic plasticity forms in the hippocampus. However, most studies have been conducted focusing on synaptic plasticity events taking place during sensory development or sensory deprivation experiments, collecting evidence mainly in sensory cortices (Feldman, 2009) and only in recent years the interest in studying learning- and memory-related synaptic plasticity in associative cortices has been awakened. Increase in the functional strength of specific synapses following learning has been observed in many neocortical regions, including prefrontal and anterior cingulate cortices. Recently, structural LTP disruption in the anterior cingulate cortex 24 hours but not 8 hours after inhibitory avoidance learning paradigm resulted in memory impairment (Goto et al., 2021), consistent with the delayed role played by neocortical engrams in system memory consolidation. Engram neurons of the medial prefrontal cortex generated after fear conditioning resulted to be more strongly synaptically connected than non-engram neurons at 14 and 28 days but not 7 days after fear conditioning (Lee et al., 2023). The same experiment demonstrated that neocortical synaptic strengthening was abolished in the absence of hippocampal inputs.

3.3 Mechanisms for System Consolidation

Almost all theories proposed in this introduction are founded on the idea that the process of memory consolidation takes place a certain time after encoding of the experience. Uncoupling of encoding and consolidation phases is clever from a computational level: in fact, it prevents neurons from the complex handling of multiple simultaneous consolidation cascades all at a different step in their progression because of the sequential nature of experiences (Redondo and Morris, 2011). Instead, establishing tags that will later be captured during experience-free time-windows allow for the allocation of a precise spot dedicated solely to consolidation of all experiences faced since the end of the previous consolidation spot. Naturally, this experience-free time-window has been hypothesized to coincide with Sleep.

3.3.1 Sleep and Memory Consolidation

Brain States have been distinguished and classified based on prominent oscillatory frequencies in the Local Field Potential (LFP) trace, which are the image of coordinated network activity within the region. Most common oscillatory frequencies are delta waves (0.5-4Hz), theta waves (6-12Hz), beta waves (12-30Hz) and gamma waves (30-100Hz). Based on distinctive oscillatory pattern, sleep can be divided in two distinct phases (Rasch and Born, 2013):

- Rapid Eye Movement (REM) sleep, characterized by high theta power. Because of some of its characteristics similar to wake (including the predominance of oscillation in the theta range) it is called paradoxical sleep and, in humans, is considered to be the lightest phase of sleep, while in mice always precede a waking epoch.
- Non REM sleep, also called Slow Wave Sleep (SWS) because of the characteristic oscillations in the delta range that can be observed all throughout the brain, especially in the neocortex. Other oscillatory patterns typical of SWS are thalamic spindles (12-18Hz) and high-frequency oscillations (100-250Hz) called Sharp Wave-Ripples (SPW-Rs) in the hippocampus. In humans can be divided in three different phases based on depth but convincing evidence of a similar subdivision in mice has not been proposed yet.

SWS has been the privileged subject for memory consolidation investigation. Sharp-Wave-Ripples events happening during this phase have been hypothesized to be pivotal for synaptic capture by triggering widespread patterns of cell reactivation in both the hippocampus and the neocortex (Buzsáki, 2015). However, in more recent years a new field has opened investigating the role of REM sleep in consolidation, which has been long neglected (Boyce et al., 2016). The most interesting line of investigation in this sense is the newfound evidence that REM sleep is characterized by a higher rate of protein translation than SWS, suggesting that following synaptic capture, REM sleep is needed for effective translation of proteins that would be needed for stabilization of long-term structural potentiation (Li et al., 2017).

Sleep has been also implicated in global downscaling of the brain excitatory drive. In fact, multiple evidences point toward the idea that during wake the brain undergoes a global increase in neuronal excitability (Vyazovskiy et al., 2009). Increased excitability is a form of synaptic plasticity, but the exact mechanisms regulating increase of global excitability during wake or if it is a homogeneous process at all are not clear. A widely accepted hypothesis is that it reflects priming of neurons activated during wakeful experiences, whose excitability threshold would be lowered to facilitate replay during consolidation periods (Seibt and Frank, 2019). In this hypothesis, priming of whole neurons would be concurrent to but independent from synaptic tagging and would equally interest neurons whose synapses have been tagged for potentiation or depression (Seibt and Frank, 2019). Hence, during sleep process of synaptic potentiation and synaptic depression would be equally ongoing, leading to a general homeostatic effect important for conservation of physiological firing range for neurons, preventing excitotoxicity (Whitt et al., 2014). Homeostatic synaptic events during sleep would eventually result in removal of tags and priming effects from neurons and synapses, bringing the network back to a state permissive for future experience integration (Seibt and Frank, 2019). All of these events would take place on a global scale encompassing the whole brain.

3.3.2 The Role of Sharp Wave-Ripples in Memory Consolidation

Sharp Wave-Ripples events are the image of the rapid, synchronous activation of a vast population of hippocampal neurons and can be observed in both the CA3 and CA1 regions of the hippocampus during Slow Wave Sleep but also during periods of “consummatory” behaviour during wake, such as feeding, grooming or simple immobility (Buzsáki, 2015). Fast oscillations are autonomously generated within the CA3 field via the activation of recurrent excitatory connections among pyramidal neurons, which eventually result in bursts of synchronous population activity. Through fibres of the Shaffer collateral, this activity is propagated to the CA1 field, inducing a massive, non-rhythmic depolarization of apical dendrites in the *stratum radiatum* which is detectable as a “Sharp Wave” in the LFP trace (Girardeau and Zugaro, 2011). Excitatory signals from the Schaffer collateral also activate local interneurons of the CA1 which engage in a pattern of synchronous inhibitory firing that, together with the excitatory drive coming from CA3, generate the fast oscillations (150-250Hz) called “Ripples” (Buzsáki, 2015). The commonly used name Sharp-Wave-Ripples, therefore, refer to two distinct but concurrent and causally linked oscillatory events typical of the CA1 field of the hippocampus.

SPW-Rs have long been regarded as the privileged substrate for memory consolidation because of their hippocampal localization and because their intrinsic frequency is coherent with the induction of synaptic modifications such as LTP in downstream neurons. Furthermore, during SPW-Rs events sequences of hippocampal place cells corresponding to behavioural trajectories explored during the day are replayed in a compressed manner (Wilson and McNaughton, 1994), satisfying the requirement for cell reactivation to trigger synaptic capture. Replay of compressed neuronal sequences synchronised to SPW-Rs activity has also been observed in the neocortex (Peyrache et al., 2009; Wang and Ikemoto,

2016; Wilber et al., 2017), suggesting that SPW-Rs might play a pivotal role in system consolidation. In particular, a huge focus is put on the bursting activity displayed by SPW-Rs, that are usually detected in burst of two or three events separated by a short delay (70-150ms) (Buzsáki, 2015), which is hypothesized to be the perfect substrate for memory association within both the hippocampus and the extended memory network including the neocortex. SPW-Rs' bursts incidence and amplitude are augmented by high frequency stimulation of the Shaffer collateral in a NMDA receptor-dependent way, suggesting that synaptic plasticity events may modulate SPW-Rs physiology (Ishikawa et al., 2014). Previous experiments conducted within my laboratory reached similar conclusions by blocking AMPA receptors trafficking during sleep and observing a decrease in the occurrence rate of SPW-Rs *in vivo* (El Oussini et al., 2023, **Annexes**). Pairing of SPW-Rs and depolarization of a single pyramidal cell of the CA1 region resulted in enhanced participation of said neuron to subsequent SPW-Rs events (King et al., 1999), strengthening the hypothesis that SPW-Rs might act as a pivotal mechanism for synaptic capture by providing a coordinated frame of reactivation of primed neurons.

Coherent with the supposed role in consolidation of novel memories, SPW-Rs occurrence rate during SWS increases following training in a behavioural paradigm and after novelty exploration, while repeated exploration of the same environment leads to the decrease in the occurrence rate of SPW-Rs after a few days (Ramadan et al., 2009). Within the hippocampus, this increase is also associated with a stronger participation to SPW-Rs events of neurons belonging to trajectories explored during the daily training than neurons that have not been particularly solicited during the experience (Dupret et al., 2010). Furthermore, neurons participating to the training more strongly correlate with neuronal patterns participating to SPW-Rs during the rest period that follows training than that that precedes training (Wilson and McNaughton, 1994). However, this change in coupling interest only a small portion of neurons and, overall, the mean population activity is conserved throughout days, probably due to homeostatic mechanisms serving conservation of the physiological firing range of the population (Buzsáki, 2015). NMDA receptor-blockers administration in the hippocampus before training but not before rest following training abolished the preferential participation of neurons activated during training to SPW-Rs and also prevented memory retrieval on the following day (Dupret et al., 2010), confirming both the importance of correct SPW-Rs physiology for memory consolidation and the dependence of this mechanisms on an intact apparatus for synaptic plasticity. Interestingly, disruption of SPW-Rs occurring during wake periods through stimulation of the ventral hippocampal commissure prevented efficient learning of a working memory task but did not disrupt SPW-Rs replay pattern during subsequent SWS periods (Jadhav et al., 2012). The same method had been adopted to disrupt SPW-Rs during SWS following behavioural training, resulting in impairment of memory consolidation (Girardeau et al., 2009).

3.3.3 Sharp Wave-Ripples and System Consolidation

SPW-Rs show a complex relationship with neocortical oscillatory patterns typical of SWS, i.e. slow waves and spindles (Buzsáki, 2015).

Slow waves are typical of the neocortex and are the image of the bimodal distribution of neocortical neurons across two states of membrane potential: a depolarized state, called UP state, and a hyperpolarized state, called DOWN state, corresponding respectively to high synchronous activity and almost complete “silence” (Steriade et al., 1993). Hippocampal SPW-Rs typically occur at the transition from DOWN to UP state in the neocortex: this correlation is stronger for DOWN-to-UP state transitions recorded in the entorhinal cortex than in the neocortex, suggesting that slow waves may propagate from more frontal neocortical areas to the entorhinal cortex and finally the hippocampus, modulating the occurrence rate of SPW-Rs (Sirota et al., 2003).

Spindles, instead, are faster oscillations (12-18Hz) originated in the thalamus which propagate to the neocortex and coordinate the activation of thalamic nuclei thanks to neocortical feedback (Steriade et al., 1993). They eventually reach the hippocampus through the neocortical-entorhinal pathway and modulate the activity of the whole hippocampal formation. SPW-Rs are often phase-locked to spindles' cycles but the nature of their interactions is complex and vary based on localization: thalamic and parietal spindles tend to precede SPW-Rs, while prefrontal spindles, which are also slower (9-14Hz), tend to follow (Sirota et al., 2003). In both cases, coordination might be the result of modulation of both oscillatory frequencies by neocortical slow waves.

SPW-Rs events have been correlated to spindles and Ripple-like fast oscillatory activity (80-200Hz) in both the medial prefrontal cortex and the posterior parietal cortex (Khodagholy et al., 2017). The same research showed that coherence between hippocampal SPW-Rs and Ripple-like activity in the PPC was increased following learning of a spatial task. SPW-Rs have also been associated to compressed replay of neurons active during daily behavioural training in both regions (Peyrache et al., 2009; Wilber et al., 2017), suggesting that SPW-Rs might exert for neocortical neurons a similar role of coordination of primed neurons and promotion of synaptic capture as they do for neurons within the hippocampal circuit.

Aim of the study

We started the project with the idea to perform an integrative study for bridging the gap between synaptic plasticity and system consolidation.

At the time of the start of my PhD, the laboratory was completing the experiments described in El Oussini et al. (Annex to this dissertation) and my work was developed as a follow up to those results, to which I contributed in their final stages. The paper focuses on the manipulation of AMPA receptors' mobility within the hippocampus, exploiting a strategy that had already been showed to affect LTP expression, and describes its effects on the behavioural performance of mice training in the Delayed Spatial Alternation (DSA) task and on Sharp Wave-Ripples physiology. To resume, injection of an antibody (IgG) directed against the GluA2 subunit of AMPA receptors prevented memory consolidation both when injected 1 hour before the beginning of the first day of behavioural protocol and at the end of it, preceding a rest period that was electrophysiologically recorded for the first 3 hours, resulting in behavioural performance impairment during the second day of protocol; injection of the IgG did not affect the behavioural performance on the day of injection neither when injected 1 hour before the beginning of the first day of protocol nor 1 hour before the beginning of the second day of protocol. Electrophysiological recordings of mice injected with the IgG 1 hour before the beginning of the first day of protocol revealed a decrease in the occurrence rate of SPW-Rs recorded in the CA1 region of the hippocampus during Slow Wave Sleep (SWS) phases of the rest period of the same day. Similar results were obtained with a different strategy involving a knock-in (KI) mouse strain expressing an AP-tagged GluA2 subunit of AMPA receptors, by injecting tetravalent neutravidin in the CA3 region of the hippocampus at the end of the first day of behavioural protocol.

From these solid base, we wanted to explore the modulatory role of hippocampal SPW-Rs on neocortical associative regions and eventually manipulate synaptic plasticity in neocortical engrams.

We kept the DSA task for consistency with previous experiments and individuate medial Prefrontal cortex (mPFC, areas InfraLimbic and PreLimbic) as our primary area of investigation among associative cortices, because of its implication in memory consolidation in general and in this task specifically (Zhang et al., 2017). Interest in another associative area, the Poster Parietal Cortex, arose for its implication in visuo-spatial navigation (Krumin et al., 2018), even though there were no direct evidence of its implication in the DSA task. Comparative study of these two areas is particularly interesting because they have been linked in a fronto-parietal network for working memory execution in humans and non-human primates, only partially confirmed in rodents (Hanks et al., 2015), and have a very different pattern of connection with the hippocampus, with the mPFC receiving afferences, even monosynaptic, mainly from the ventral hippocampus (Cenquizca and Swanson, 2007) and the PPC being a main projection output for the Retrosplenial cortex, which is targeted mainly by projections from the

dorsal hippocampus (Zingg et al., 2014). We found intriguing the hypothesis of possibly unravelling a full network for system consolidation of the semantic-like rule governing efficient reward collection in the DSA task, but we were also open to the possibility of observing and characterizing two distinct networks, possibly working in parallel for consolidation of different features.

We expected hippocampal SPW-Rs to exert a modulatory activity on the firing of single neurons within the two neocortical areas, and we expected this modulation to be increased after acquisition of the behavioural rule and an enrichment of behaviourally relevant neurons among the modulated proportion. To tackle this last question, we implemented electrophysiological recording also during behavioural sessions, which had not been done in the previous set of experiments.

Finally, once the pattern of modulation established, we aimed to manipulate synaptic plasticity within the neocortex through the AP-GluA2 KI strategy, by injecting tetravalent neutravidin in the neocortex following the behavioural protocol and observing effects both the animal behaviour and on modulation of neocortical neurons by hippocampal SPW-Rs.

RESULTS

Hippocampal Sharp Wave-Ripples Differentially Modulate Medial Prefrontal Cortex and Posterior Parietal Cortex After Acquisition of a Semantic-Like Rule

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Abstract

Consolidation of memory is a complex process that encompasses various brain regions and brain states. A widely accepted theory postulates that the hippocampus is the main actor in consolidation of episodic and spatial memories, coordinating different networks of neocortical regions for long-term storage of memories through synaptic plasticity mechanisms. The present study approaches the investigation of system consolidation mechanisms in a network encompassing the hippocampus and two associative neocortical areas in the context of consolidation of the rule governing efficient reward collection in a spatial working memory task (DSA task). We used mice implanted with single electrodes in the dorsal Hippocampus (dHPC), medial Prefrontal Cortex (mPFC) and Posterior Parietal Cortex (PPC) to record Local Field Potential (LFP) and single neurons' activity both during the task and a three-hour long rest period at the end of each behavioural training day. We observed that mPFC neurons, but not PPC neurons exhibited positive modulation during hippocampal Sharp Wave-Ripples (SPW-Rs) following learning. Investigating neuronal activity during the task, we observed that PPC neurons were mostly engaged in navigation, while mPFC neurons engaged with more cognitive features of the task. Crucially, a high proportion of mPFC neurons active during the behavioural protocol were positively modulated around SPW-Rs' peaks during sleep, while PPC neurons activated during the task followed the activation distribution of the general population around SPW-Rs' peaks. We concluded that the two associative regions play very different roles in the DSA task and that PPC might be excluded from the consolidation network.

Introduction

Consolidation of memories is a complex and multistep process which involves a distributed network within the brain. The system consolidation theory postulates at least two distinctive steps in memory

consolidation, separated in time by the order of days: consolidation of recent memories, taking place mainly within the hippocampus, and consolidation of remote memories, supported by the gradual stream of information and transfer of the engram from the hippocampus to neocortical areas (Frankland and Bontempi, 2005). A pivotal role in both steps is played by sleep, in particular by Slow Wave Sleep, which is characterized by bursts of massive neuronal firing within the hippocampus, which design both a sharp, ample wave of depolarization and a high frequency oscillatory pattern (150 - 250 Hz), two phenomena collectively called Sharp Wave-Ripples (SPW-Rs) (Girardeau and Zugaro, 2011). Perturbation of SPW-Rs during Sleep efficaciously prevents memory consolidation and memory trace stabilization following learning (Girardeau et al., 2009).

SPW-Rs entrain the replay of neurons participating to trajectories explored during a behavioural protocol in both the hippocampus (Wilson and McNaughton, 1994), subcortical regions (Girardeau et al., 2017) and the neocortex (Peyrache et al., 2009; Wang and Ikemoto, 2016; Wilber et al., 2017). This process is thought to induce synaptic plasticity at reactivated neurons, tying-in to the synaptic tagging and capture hypothesis (Redondo and Morris, 2011). This hypothesis postulates that, during encoding, neuron's activation would create tags at appropriate synapses; the nature of these tags is not completely understood, but they are associated to synaptic plasticity changes that would last a few hours without stabilization. Stabilization of tagged synapses would happen during consolidation and would consist in mostly structural synaptic plasticity that would result in the stable long-term potentiation or depression. Synaptic tagging during encoding would also be accompanied by priming, a series of modification that lower the spiking threshold of neurons, putting them in a "permissive" state for reactivation at the moment of synaptic capture (Seibt and Frank, 2019).

Indeed, NMDA receptors-blocker administration before a spatial task efficiently prevents SPW-Rs enrichment with neuronal patterns expressing trajectory explored during the behavioural paradigm during the following SWS period (Dupret et al., 2010), suggesting that tagging is NMDA receptors-dependent and that it is needed to assure the reactivation during subsequent SPW-Rs of neurons participating to behaviourally relevant trajectories during the day. AMPA receptors' crosslinking, which is efficient in preventing LTP (Penn et al., 2017), lowers SPW-Rs frequency and prevents memory consolidation (El Oussini et al., 2023, **Annexes**). On the other hand, neocortical engrams are formed at the same time as the hippocampal ones, but need a maturation time before being naturally activated by memory triggers (Kitamura et al., 2017). Formation and maturation of these engrams need hippocampal input (Lee et al., 2023), however, whether and how this input induces synaptic plasticity at neocortical synapses has not been established.

In this study, we investigated the modulation of neocortical neurons by SPW-Rs detected in the dorsal hippocampus in the context of consolidation of a spatial working memory rule for efficient reward collection in a Y maze. We chose to address two associative neocortical regions: the medial

Prefrontal Cortex (mPFC) and the Posterior Parietal Cortex (PPC). These two regions are not connected in any functional network in the mouse (Zingg et al., 2014), but have been connected in a fronto-parietal network for working memory in humans and non-human primates (Whitlock, 2017). The mPFC is heavily implicated in all higher cognitive functions (Euston et al., 2012) and is thought to play a pivotal role in consolidation of remote memories (Tonegawa et al., 2018), even comparable to that played by the hippocampus in recent memories. The PPC is heavily implicated in sensory integration (Driscoll et al., 2017) and navigation (Harvey et al., 2012; Rinaldi et al., 2020). SPW-Rs induces neuronal replay in both areas (Peyrache et al., 2009; Wilber et al., 2017) and are coherent with specific oscillatory patterns in each area, specifically with Slow oscillations and Spindles in the mPFC (Binder et al., 2019) and with high-frequency Ripples-like oscillations in the PPC (Khodagholy et al., 2017). With our approach we unravel the original contribution of each one of these two areas to acquisition and consolidation of a Delayed Spatial Alternation (DSA) task demanding working memory and spatial navigation for execution and leading to the consolidation of a semantic-like rule for efficient reward collection.

Materials and Methods

1. Biological models

The experiments described in the Results section of this manuscript were conducted on a total of 26 male mice belonging to two strains: C57BL6/J wild type strain and C57BL6/J transgenic AP-GluA2 knock-In (KI) strain, detailed below. Mice were kept on a 12-hour light/dark cycle and provided with *ad libitum* food and water, except when food restriction was demanded by the protocol (detailed within the description of each procedure). Mice were housed with littermates respecting the original number of mice per cage (3-5), except when demanded otherwise by the protocol (detailed within the description of each procedure). Manipulations (surgery, behaviour, drugs administration, perfusion, histology) were conducted on young adult/adult mice aged between 6 weeks and 4 months (further detailed within the description of each procedure). The experimental design and all procedures were in accordance with the European Guide for the care and use of laboratory animals. All procedures were validated by the ethical committee of animal experimental of Bordeaux Universities (CE50; PIV-EXPE APAFIS 18507-201901118522837; A1 APAFIS 4552 2016031019009163A; Magendie and PIV-EXPE 13515-2018021314415739)

C57BL6/J wild type strain

C57BL6 is the most commonly used wild type mouse strain, assuring a higher degree of comparison and reproducibility of data shared by the scientific community. The transgenic strain AP-GluA2 KI (Getz et al., 2022) that was employed for part of the experiments was derived from C57BL6 ancestor, thus employment of C57BL6/J mice as wild type control subjects limits to the minimum the differences between the two strains involved in my experiments, enhancing as much as possible the opportunity of comparing the 2 datasets.

C57BL6/J AP-GluA2 KI transgenic strain

AP-GluA2 KI strain was developed and validated as a mouse model to study AMPA receptors surface trafficking (Getz et al., 2022). AP-GluA2 KI mice are similar to wild type C57BL6/J mice in terms of weight, size, growth or fertility, but also for their behavioural phenotype, assessed through SHIRPA protocol (Getz et al., 2022). At the genetic level, this strain presents a substitution of the endogenous GluA2 subunit of the AMPA receptor by a genetically modified one bearing an AP- (Acceptor Protein) tag on the extracellular domain of the subunit. The AP can bind Biotin, which is endogenously present inside neurons of the murine brain, however only in the presence of the BirA ligase enzyme, which is not endogenously expressed by the same cells. Thus, expression of AMPA receptors bearing biotinylated GluA2 subunits is restricted to neurons in which BirA ligase has been introduced by viral transfection, making biotinylation region-specific. Intracranial administration of tetrameric Neutravidin (further detailed in the “Chemicals” and “Surgery” sections of this Materials and Methods) eventually leads to cross-linking of multiple AMPA receptors and their immobilization at the synaptic and peri-synaptic space.

2. Surgery

Different surgery protocols were performed on different mice depending on the aim of the procedure, dividing into two major subgroups: stereotaxic injections and stereotaxic implantations. All types of surgeries, however, shared some common steps, hereby listed. The same surgery protocol was observed for both mouse strains.

Mice were anaesthetised through exposure to the anaesthetic gas agent Isoflurane (concentration: 4% mixed with air) for 4 minutes and anaesthesia was maintained all throughout the surgery through administration of the same anaesthetic agent (concentration: 2% mixed with air) via a vaporising mask. Mice were positioned in the stereotaxic apparatus (David Kopf Instruments) on a 37° heating pad and received a subcutaneous injection of Buprenorphine (100µl, 0.1mg/Kg) and a local injection of Lidocaine (100µl, 0.4mg/kg) for analgesia. The scalp was rinsed with Betadine to prevent infections.

After incision and opening of the scalp, Bregma and Lambda point were identified in order to be able to identify the region of interest using Paxinos atlas coordinates. To conclude the surgery, sutures were applied to close the incision point, except where noted otherwise. After the end of the surgery, mice were subcutaneously injected with another analgesic agent, Carprofen (100µl, 4mg/kg) or Meloxicam (200µl, 20mg/kg), fed with powdered-nutrient enriched food and left recovering inside a recovery cage positioned on a heating pad for 30 to 120 minutes, depending on the length and severity of the surgery. A post-surgery care routine was observed for 2-6 days following the surgery, depending on its length and severity, during which the weight and general presentation of mice were monitored and analgesic drugs (Carprofen or Meloxicam) were administered if needed.

Stereotaxic injection

Stereotaxic injection was performed on mice aged 6-8 weeks except when coupled to electrodes implantation, because this latter procedure demands slightly older mice (8-10 weeks). For the purpose of this dissertation, stereotaxic injection was never performed alone, but always coupled to an implantation.

Viruses were charged inside 1ml graduated glass pipets (960 01 05 5µl, Hirshman ringcaps) and pressurized via a 5ml syringe (Terumo). The pipette was manually descended into the target region at a speed of approximately 20µm/s (mPFC: AP: +1.94, ML: ±0.4, DV: -2.5/-2/-1.5). Injection of 250nl of the product was performed manually by applying light and constant pressure on the syringe. The pipette was maintained in position within the target region for 5 minutes after the end of the injection to allow local diffusion of the injected product, then retracted at a slow speed.

When combined with other surgical procedures, stereotaxic injection always preceded stereotaxic implantation.

Stereotaxic implantation

Stereotaxic implantation was performed on mice aged 6-8 weeks (cannulas) or 8-10 weeks (electrodes, alone or coupled with cannulas). Age difference was due to the considerable length of electrodes' implantation surgery (averagely: 2.5 hours) compared to guide cannulas' implantation surgery (averagely: 1.5 hours). Various types of implants were used, all of which are detailed in the following section ("Implanted materials"), but surgical procedures common to all or multiple types of implants are hereby listed.

Prior to proper implantation, the skull was prepared by briefly applying Peroxidase RED ACTIVATOR (Super-Bond, Sun Medical Co) (3-5 seconds, then the chemical was promptly washed to avoid long-lasting bone damage) to remove the periosteum and by generously scratching the skull to augment

the surface for cement bonding. Single guide cannulas were manually descended into the target region at a speed of approximately 20 μ m/s (mPFC: AP: +1.94, ML: \pm 1.65/2.05, DV: -1.42, 30° angle). Electrodes were descended into the target region with the aid of a micromanipulator (Narishige) which consented to regulate descent speed to a steady pace of 10 μ m/s (first 2/3 of the descent) and 1 μ m/s (last third), to reduce to the minimum tissue damage caused by the implantation (mPFC: AP: +1.94, ML: -0.4, DV: -2.0; PPC: AP: -2.0, ML: -1.5, DV: -0.3; dHPC: AP: -2.0, ML: -1.4, DV: -1.7); ground references were implanted in the Cerebellum without any precision instrument (AP: -5.6, ML: \pm 0.3, DV: \sim -2.0; grounds from different connectors were implanted in opposed hemispheres). All implants were held in place with the aid of the descending support during dental cement application for chronic fixation of the implant. Guide cannulas were fixed with dental cement (Super-Bond, Sun Medical Co), while for electrodes' fixation a layer of Paladur resin (Kulzer) was put on top of the layer of Super-Bond cement, in order to augment the volume of material protecting the implant. Once the cement solidified, implants were gently released from their supports. To end the surgery, the scalp of mice implanted only with cannulas was sutured, resulting in partial coverage of the cemented region with skin. The skin of mice implanted with electrodes, instead, was glued to the skull in the region surrounding the cemented implant, to prevent contact with the cement in its still unsolidified state. Post-surgery care for implanted mice always included 1-4 days of Carprofen (100 μ l, 4mg/Kg) or Meloxicam (200 μ l, 20mg/kg) subcutaneous injection, depending on the severity of the surgery and the mouse's rate of recovery. After implantation mice were housed alone to prevent implants' damaging.

3. Implanted materials

Guide cannulas

Stainless steel guide cannulas (Mouse Guide cut 2.5MM below pedestal 26 gauge, 1.5mm of length, Bilaney) were used for implantation. Prior to the surgery, guide cannulas were kept in alcohol to minimize the risk for bacterial contamination and plugs were maintained on them at all time to avoid penetration of external material.

Electrodes

Electrodes were crafted starting from an 18 male connector (nano 18 positions 2 guides ISC-DISTREL SA, Omnetics) compatible with the Intan recording system (further detailed in the "in vivo electrophysiological recording" section of this Materials and Methods). Each pin of the connector was wrapped with the naked end of a nichrome wire (diameter: 13 μ m, Sandvik Kantal) and silver paint (RS Components Ltd) was added to strengthen conductivity. Wires targeted to the same region were organized in bundles and passed through a stainless steel tube (diameter 0.25mm external /0.12mm

internal x 1.2mm length, Unimed) serving as a guide to protect them from damage and enhance the rigidity of the structure. Guides bearing bundles were either glued on the side of the connector or left unattached, allowing a certain freedom in positioning the connector on the mouse skull during surgery without threatening correct targeting of the bundle (deported-bundles). All exposed section of nichrome wires, with the exception of the portion of the bundle aimed to be implanted in the mouse's brain, were either protected with nail paint (when glued to the side of the connector) or with silicone (floating portion of the wires in deported bundles). Silicone was also applied on the top portion of the connector englobing all of the connected or unconnected pins to assure electrical insulation. A silver wire (diameter: 70 μ m, A-M Systems) of about 2cm length was naked (i.e. the insulating plastic envelope was removed) on both ends and welded on a dedicated pin to serve as ground reference for whole brain electrical activity generated by movement and physiological activities of the mouse (e.g. breathing). Silicone was also applied to the naked portion of the ground reference closer to the connector to assure electrical insulation.

Between 48h and 2h before implantation, bundles were plated with a solution of gold particles (Sifco) and carbon nanotubes (Cheap Tubes) via an electrolytic process, in order to reduce the impedance of each nichrome wire to an optimal value of 60k Ω , to assure a better signal to noise ratio and enhance spike collection.

For this study, three types of electrodes were used: a connector with a single glued bundle of 15 wires targeted to the mPFC; a connector with a single deported bundle of 16 wires targeted to the mPFC; a connector with a 12 wires-bundle and a 4 wires-bundle, both glued and targeted to the PPC and dHPC respectively. dHPC/PPC connectors were always used in combination with either a glued-bundle or a deported-bundle mPFC electrode. Deported-bundle electrodes were used in combination with guide cannulas implantation in the mPFC. A total of two electrodes were implanted in each mouse.

4. Intracranial injection

Intracranial injection was performed on awake mice freely moving inside their home-cage. Plugs closing the implanted guide cannulas were removed and injection cannulas (Internal Cannula FIS 2.5mm guide, Bilaney; 0.5mm of projection) were introduced inside guides. Injections were performed via an automatic pump (Legato 101, Kd Scientific Inc.) that applied a constant pressure on two 1 μ l Hamilton syringes (7101 KH), allowing the regulation of injection speed to 50nl/min. Pre-rest injections were performed immediately after the last session of the first day of behavioural protocol.

5. Chemicals

Viral vectors

All viral vectors used for the experiments described in the Results section are Adenoviruses and their engineering is detailed in Getz et al. 2022. Ongoing production was assured either by the viral core facility of the Bordeaux Neurocampus IMN or by Charité Universitätsmedizin Berlin or viral vectors were ordered on Addgene. All viruses were stocked at -80°C for long-term storage, conserved at 4°C during surgery preparation and injected at room temperature. 250nl per injection site of viral vector solution were administered through stereotaxic injection during surgery.

pAAV9 α -pSyn-BirA-ER-IRES-eGFP (5.6 x 10¹³ gcp/ml, IMN). The pSyn promoter allows the expression of the BirA enzyme in all neuronal types without distinction. BirA ligase expression promotes biotinylation of the extracellular portion of the GluA2 subunit of AMPA receptors, thus inducing AMPA receptors cross-linking in the presence of Neutraavidin. eGFP is used as a tag to identify neurons expressing the enzyme.

Others

Tetravalent Neutraavidin. Texas Red-conjugated tetravalent Neutraavidin (8.33 μ M; Invitrogen, A2665) was used to operate cross-linking of AMPA receptors in the AP-GluA2 KI mouse model, due to its predisposition to theoretically bind up to 4 GluA2 subunits (potentially belonging to 4 different receptors) at a time. 500nl per injection site of tetravalent Neutraavidin solution were administered via intracranial injection in the awake, freely moving mouse.

Saline Physiological solution. Saline physiological solution was used as control for cross-linking in the AP-GluA2 KI mouse model. 500nl per injection site of Saline solution were administered via intracranial injection in the awake, freely moving mouse.

6. Food restriction

Food restriction was required to assure mice's motivation during the Delayed Spatial Alternation task (described in the next section). Mice were weighted right before food withdrawal and this weight was used to calculate the 80% of weight-loss limit that was fixed for protocol termination. On the first day of restriction, mice were fed with ~30 pellets (either Dustless precision pellets, purified, Chocolate, PLEXX; or dehydrated pasta pellet, Panzani) of the same type as those that were used to bait the maze during the behavioural task, in order to habituate them to the new food. On subsequent days, mice were fed at the end of all behavioural manipulation with 2-3g of powdered nutrients-enriched food, in order to maintain their weight around 85% of their initial weight.

7. Behavioural protocols

Delayed Spatial Alternation (DSA) task

The DSA task is a delayed non-matching-to-place task used to assess spatial navigation and cognitive functions in rodents (Zhang et al., 2017).

Materials. A semi-transparent white PVC Y maze was used for the task (custom made). All three arms are identical (40cm length, 8cm width, 15cm high walls), except for an additional rectangular chamber (15cmx25cm) which is accessible from the bottom of the “Starting arm” via a pivot door. Arms are spaced by a 120° angle. An opaque blue Falcon top was positioned at the end of each “Goal arm” to serve as food well for reward delivery. Environmental cues are positioned on the three walls surrounding the maze (one positioned in the middle between the two goal arms, one on the left of the maze and one on the right). Video recordings are realized through an infrared camera (1 Basler USB camera -ac1920-155um - Noldus) positioned on the ceiling upon the centre of the maze. The experience was realised in conditions of dim light and an infra-red lamp was lighted and indirectly reflecting light on the behavioural apparatus to allow infrared-camera recordings via the Media Recorder (Noldus) system.

For behaviour coupled to electrophysiological recordings, a partially automated Y-shaped maze was used for the task (Imetronic Systems). All three arms are identical (40cm length, 8cm width, 20cm high walls on the two sides) and equally spaced by a 120° angle. The proximal 2/3s of each arm present walls built with transparent PVC (hence, the centre of the maze present completely transparent walls), while the distal third presents opaque-grey PVC walls on all three sides; this same third can be closed by an automatically raising and descending opaque sliding door, hence mice could be restrained in this restricted area providing a darker, completely opaque “box” at the end of each arm. An opaque-blue falcon top was positioned within the distal third of each arm to serve as food-well for reward delivery. Environmental visual cues were positioned on the three walls surrounding the maze (one positioned in the middle between the two goal arms, the other two behind each of the goal arms).

Doors were operated throughout a software interface provided by the same enterprise which built the maze (Imetronic Systems). Video recordings were realized through an infrared camera (Dalsa Genie Nano-M800 CS-Mount, Teledyne Dalsa) positioned on the ceiling upon the centre of the maze (slightly on the right side, as the centre is occupied by a turning collector for compensation of the electrophysiology recording system). The same software operating the maze also operates the camera used for visual recording of each behavioural session.

All behavioural experiences were realised in conditions of dim light and an infra-red lamp was lighted and indirectly reflecting light on the behavioural apparatus to allow infrared-camera recordings.

Habituation. Habituation lasted for 5-8 days, depending on the training reactivity of each individual mouse, and was divided into 3 phases. A first phase, starting before food restriction, consisted in 2-3

days of handling by the researcher, in order to habituate the mouse to be manipulated and hold onto the head (for injection cannulas insertion and/or electrodes plug-in). Proper habituation for the task started on the second day of food restriction and consisted in multiple sessions of free exploration of the maze, aimed at familiarizing mice with the environment and the environmental visual cues. 1 or 2 sessions per day were administered, of progressively reducing length as the session was stopped as soon as the mouse had eaten a reward food-pellet in all three baited arms (the three arms were treated as identical during this phase; during early session a time-limit of 15 minutes was given). This phase ended when the mouse was able to collect the corresponding reward upon its first entry inside a yet unexplored arm (generally after 3-6 sessions). The last phase of habituation consisted in a single trial where the mouse was positioned inside the “start arm” (defined by position with respect to environmental visual cues) and had to collect a reward food-pellet in each of the two “end arms”, with a time-limit of 1 minute. If the time-condition was not met, the trial was repeated after an interval of at least 1 hour and, possibly, on following days. For animals submitted to electrophysiological recordings, habituation was stopped two days prior to the beginning of the DSA protocol to allow unbiased electrophysiological recordings of baseline brain activity during sleep.

Task. The DSA task consists in 10 trials in which the left and right end arms are alternatively baited with a rewarding food-pellet. During the first trial, the choice is forced toward the baited arm, setting the pattern of alternation (i.e. the reward-zone of the un-baited arm is made inaccessible through positioning a PVC slide at the entrance of the proximal portion of the arm or by closing the sliding door; each consecutive session alternatively starts with a forced right or left choice). The 9 following trials rely on the mouse free choice of one of the two arms. A single trial can be repeated up to 6 times (each individually called “run”) if the mouse makes consecutive mistakes, of which the sixth consists in a forced run in the baited arm direction. Once the mouse has reached the reward-zone of the chosen arm, access to the reward-zone of the unchosen one is restricted and the mouse is let spontaneously come back to the distal portion of the starting arm. Here, the mouse is collected and put back in his home-cage and a delay of 30 seconds is respected before the mouse is allowed to explore the maze again (to either repeat the trial in case of a wrong choice or to pass to the next trial). For mice submitted to electrophysiological recordings during the task, once the distal portion of the starting arm has been reached the sliding door is closed, confining the mouse inside the distal portion (serving as “start box”) where it will be left during the 30 seconds delay. During this delay period, the maze is cleaned with ethanol (4% of concentration) for odours saturation, to prevent navigation following the trace of body odour of previous choices. On the first day of training, 4 sessions are realized, spaced by 30 minutes (S1-S2 and S3-S4) or 1 hour (S2-S3); on the 2 following days, 2 sessions per day are realized, spaced by 30 minutes (see Fig.).

Behavioural training was administered between 8a.m. and 1p.m.; for each individual mouse, priority was given to a consistent timing for the end of behavioural testing on each day (and not to its starting), to maximise homogeneity of electrophysiological recordings of rest periods.

Analysis. Mice were scored during behaviour for direction (right-turn vs left-turn), reward collection (success vs error) and display of Vicarious Trial and Error (VTE) behaviour. All sessions were recorded and videos were later watched to confirm the behavioural scoring and for manual annotation of frames at which automated doors were closed or opened. This annotation was used to more precisely align videos with the electrophysiological trace recorded, as automatic door closing and opening generated a TTL signal that was captured by OpenEphys recording system and stored as a timestamp in seconds. VTE behaviours were assessed manually and recognized within a range of possible behaviours at the choosing point (i.e. the centre of the maze), from full stops to rapid head turning in both directions. In literature, VTE is defined as a perceivable hesitant behaviour that entrain the rapid replay of neurons involved in all possible future trajectories. Head turning is particular important to this behaviour, as heading toward a particular direction is associated to replay of the neuronal pattern associated to that future trajectory (Redish, 2016).

In vivo electrophysiological recording

Electrophysiological recordings were realized by plugging a headstage (INTAN) containing 16 unity-gain operational amplifiers to each connector. Recordings were realised through the recording system OpenEphys. On the behavioural apparatus for the DSA task, cables are suspended through a turning collector (Imetronic Systems) which also provides a weight-compensatory system based on loose elastics and a pulley. This apparatus allows the mouse to autonomously explore the whole length of the maze without getting entangled and minimizing the weight of the electrophysiology recording system.

Recording of rest periods is realized in the same room as the behavioural task, inside a closed box (50x35cm) presenting only a very small circular hole on the top to allow cables suspension and a larger squared window on the side to allow positioning of the infrared lamp required by the camera (Basler USB camera - ac1920-155um - Noldus). Even though dim light was still lighted in the room, the mouse was almost completely in the dark during rest periods, due to the conformation of the box. Mice were put in this apparatus inside their unaltered home-cage and were given access to the food ration for the day and a sufficient amount of water. Given the restricted size of the home cage, two elastics were sufficient to serve as compensatory system for the weight of the electrophysiology recording system.

Mice were plugged and recorded all along the habituation period to get used to navigate the maze with the weight of the recording system. On the two days preceding the start of the proper behavioural task, recording of one rest period of three hours per day was realised inside the rest apparatus, to establish baseline activity. 18 wild type Mice were recorded all throughout the behavioural task, being plugged at the beginning of each session and unplugged at its end. Recording of rest periods started averagely 10 minutes after the end of the last training session of the day. All rest recording started between 11a.m. and 1p.m., trying to have a high consistency for each individual mouse. 8 AP-GluA2 KI mice were not recorded during the task and were submitted to rest recording only on the day prior to the behavioural protocol (to establish the baseline) and at the end of the first day of behavioural protocol.

Electrophysiological output consists in a continuous data file for each recorded channel, sampled at a rate of 30kHz to allow collection of both Local Field Potential and single spikes. During task sessions, the recording was started by an input TTL signal sent from the software operating the maze at the start of camera recording, to assure synchronization between the video and the electrophysiological trace. No such precise synchronisation was possible for recording of rest periods.

8. Perfusion and Histology

Mice were anaesthetized with a mix of Ketamine (0.1mg/g) and Xylazine (0.01mg/g) diluted in NaCl; 10 μ l of solution per gram weighted by the animal were administered via intra-peritoneal injection. Perfusion with Paraformaldehyde (PFA, concentration: 4%) was realised on the anaesthetized mouse and brain were initially collected without being evicted from the scalp. After 48h of storage in PFA at 4°C temperature, implants and scalp were removed and the brain was washed three times in PBS solution (concentration: 1%) and then stored in PBS for 24-72 hours at 4°C. Slicing was performed with a vibratome (Leica VT1200s). Coronal slices of 60 μ m thickness were collected at a speed of 30-50 μ m/s from the regions of interest and stored in PBS for 24h before being mounted on slides and covered with Fluoromont-G (complemented with DAPI for cellular nuclei staining, Thermofisher Scientific). Image acquisition of slides was performed with an epifluorescence microscope (Nikon Eclipse NI-U) coupled to an illumination system (Intensilight C-HGFI, Nikon) and a camera (Zyla sCMOS, Andor Technology, Oxford Instruments).

9. Analysis of electrophysiological data

LFP processing and Sharp Wave-Ripples detection

Electrophysiological data in the continuous data format were imported on Matlab and down-sampled to 1kHz for storage and analysis speed convenience.

Ripples detection was performed on Matlab using scripts originally wrote by Cyril Dejean and modified to adjust to the specificities of the data and the type of information collected. The scripts first subtract the mean of all hippocampal channels oscillations to each individual channel, in order to eliminate noise oscillations common to all channels; a notch filter at 50Hz is also realized to eliminate signal in the band of environmental electricity. A band-pass filter in the Ripple's frequency (100-250Hz) is then realized and on this filtered trace the peak of a single Ripple event is detected as the point of maximal amplitude of each event, defined by the following criteria:

- an amplitude higher than 5 standard deviations from the mean amplitude of oscillations on the band-passed trace
- the event must be at least 30ms long
- two individual peaks must be separated by an interval of at least 45ms

Ripple's characteristics are then computed, creating an excel output containing the following information for each Ripple event: timestamp of the peak, intrinsic frequency, number of oscillations, mean amplitude (both on the filtered and the integrated trace), area under the integrated curve, duration (total and of each part preceding and following the peak), half prominence.

Single unit sorting and processing

Electrophysiological data in the continuous data format were converted to binary files (one file containing all of the recorded channels for a single session) via a Python script provided by Stephane Valerio (Aquineuro). Binary files were later concatenated through another Python scripts to pool multiple sessions together and strengthen the power of single neurons follow up across days and sessions. Because of computing capacity limits, not all sessions from a single mouse could be concatenated together, thus files were pooled as follow: 1) rest of D-1 (second baseline), behavioural sessions of D1 (S1-4) and rest of D1; 2) behavioural sessions of D2 (S5-6) and rest on D2; 3) behavioural sessions of D3 (S7-8) and rest of D3. Binary files were uploaded on Kilosort software (<https://github.com/cortex-lab/KiloSort>) for unit sorting, then results were visualized with the Phy gui (<https://github.com/cortex-lab/phy>) for quality check and refining. Extracellular voltage traces were preprocessed with common average referencing. Each set of events was detected by a template and then manually curated on Phy. If the events corresponding to a unit were judged to correspond to noise (near-zero amplitude, non-physiological waveform and event detected on every channels), the unit was discarded. Both single-units (considered to represent the activity of one single neuron) and multi-units (considered to represent the activity of multiple neurons whose wave cannot be disentangled) were downloaded from the software and uploaded on Matlab for further processing, but multi-unit activity was not included for further analysis. From this analysis pipeline, we could easily

perform longitudinal analysis on pooled sessions but not between pools, therefore for longitudinal analysis we focused on baseline rest on D-1, rest on D1 and the first 4 sessions of the behavioural task.

Analysis of Sleep phases

Video recordings of rest phases were analysed with a Matlab script (Lanore et al., 2021). The script computes an index of motion by comparing the pixel composition of each individual frame with the one immediately following it.

Motion index data and LFP data in the form of Matlab matrices were processed with “The State Editor” Matlab toolbox (created by Andres Grosmark at Gyuri Buzsaki’s laboratory, 2012) to compute sleep phases. The toolbox’s interface displays three heatmap representations of the power spectrogram of the LFP trace of three channels belonging to the hippocampal region and a scatterplot representation of the motion index value in function of time. Slow Wave Sleep (SWS) phases were visually identified as having relatively high power in the delta and spindle bands, while relatively lower theta; Rapid Eye Movement (REM) sleep phases were visually identified as having very high power in the theta band and low power in the delta and low-gamma bands. SWS and REM phases were only identified in prolonged periods of immobility, meaning periods during which the motion index value was low (<0.04) for at least 60s (this doesn’t equate to either SWS or REM periods being at least 60s long, as from the power spectrum punctual waking episodes can be identified which do not necessarily result in significant mouse movement).

Analysis of single neurons within the neocortex

For analysis of reactivation around Sharp Wave-Ripples (SPW-Rs) peak, the firing rate of single-units on a time window of 1000ms around the peak of SPW-Rs (500ms preceding and following the peak of SPW-Rs) was binned into bins of 10ms each and z-scored to a reference average computed for each single-unit individually by calculating the average bin count of the first 20 bins (200ms; as SPW-Rs events have a duration of 50-150ms, this part of the considered window was reasonably far from the peak and could be considered a good sample for the activity of the single-unit outside of SPW-Rs’ event-window). Different populations of single-units were computed by calculating the mean of the z-scored firing rate on the bins surrounding the peak of SPW-Rs (50ms, 25ms before and 25ms after the SPW-Rs’ peak) and distinguishing the three populations based on the value of the obtained mean z-scored firing rate: single-units displaying a value >1.96 were classified as “significantly activated”, <-1.96 as “significantly inhibited” and all the other single-units as “non-modulated”.

For DSA sessions, a cut-off of single units with a firing rate <0.2 Hz during the analysed session was performed in order to avoid confounding results deriving from very sporadic firing (less than once per

run). The firing rate of single-units on a time window of 40s (30s preceding and 10s following the timestamp for the beginning of each run; intervals were chosen as runs' length was around 10s on average and prior to it mice were retained for 30s within the starting box, representing an inter-trial interval during which exploration was very limited) was binned into bins of 1s and z-scored to a reference average computed for each single-unit individually by calculating the average bin count from bins representing the middle of the inter-trial interval (bins 10-20, 11s). Single-units were classified as "activated in the maze" if the z-score of at least one of the bins representing the run (bins 31-40) was $>2SD$ from the reference average and "inhibited in the maze" if at least one value was $<-2SD$ from the reference average.

For analysis of "behaviourally relevant" single-units during DSA sessions, the maze was subdivided in spatial bins of 2cm and firing rate at each spatial bin was computed taking into consideration occupancy time with the help of functions from the FMA toolbox in Matlab (Michaël Zugaro, General Public Licence). Preference for a specific firing location within the maze ("maze preference criterion") was computed by calculating the average firing rate across all bins and comparing the firing rate value of each individual bin to this reference: if the value was $>2SD$ from the reference for at least one bin belonging to a spatial sub-location of the maze, the single-unit was classified as having a firing preference for the whole sub-location and being significantly activated within it (sub-locations: start box, bins 1-7; start arm, bins 8-19; centre, bins 20-25; end arm, bins 26-37; end box, bins 38-45); similarly, a single unit was considered significantly inhibited at a location if its firing rate was $<-2SD$ from the reference average in at least one of the bins composing it. The same firing frequency maps were divided into clusters based on paired behavioural attributes for the computation of the "behavioural attribute criterion" (pairs of features: success vs error runs; right-turns vs left turns runs; VTE vs non-VTE runs). Clusters were uploaded to GraphPad Prism and a Two-Way ANOVA analysis was conducted for each single-unit in order to produce multiple comparisons among the clusters. When a single unit showed a significant difference in the firing rate map of two paired clusters, the unit was classified as being significantly modulated by that behavioural attribute.

Statistics

All statistical analysis was performed in the Prism environment (GraphPad). Detailed statistics are described for each figure. Significance levels were defined as $p < 0.05$.

Results

Not all mice trained for the Delayed Spatial Alternation reached learning criterion within the first day of protocol

18 C57BL6/J wild type mice were unilaterally implanted with electrodes bundles in three regions – dorsal Hippocampus (dHPC), medial Prefrontal Cortex (mPFC) and Posterior Parietal Cortex (PPC) – for electrophysiological recordings both during the task and during a total of five 3 hours-long rest periods, one at the end of each day of behavioural protocol and two baselines on the two preceding days (see Materials and Methods). During the three days of behavioural protocol, mice underwent training in the Delayed Spatial Alternation (DSA) task as previously described (El Oussini et al., 2023, **Annexes**), during which they were asked to collect a food reward at the end of one arm of a Y maze following a pattern of alternation between left and right arm choices (**Fig. 1B-C**). Mice performed 4 sessions (S1-4) on the first day and two sessions (S5-6 and S7-8) on each of the two following days (**Fig. 1A**). Acquisition of the rule for efficient reward collection (i.e. spatial alternation between the two end arms) takes place during the first day of behavioural protocol, while the two following days are used as tests for the acquisition and consolidation of said rule.

We observed that not all mice were able to reach the required learning criterion (mean errors' number per trial < 0.5) at the end of the fourth sessions. We therefore decided to divide the mice in three groups: Learners (n = 10), No Learners (n = 3) and Slow Learners (n = 5), the latter including mice that did not reach the learning criterion on S4 but did reach it by S8 (mean errors' number per trial: S4: Learners 0.3 ± 0.05 , Slow Learners 1.2 ± 0.2 , No Learners 1.3 ± 0.3 , t-test: $p = 0.00012$ Learners vs No Learners, $p = 0.00002$ Learners vs Slow Learners, $p = 0.69854$ Slow Learners vs No Learners; S8: Learners 0.4 ± 0.09 , Slow Learners 0.5 ± 0.1 , No Learners 1.5 ± 0.4 , t-test: $p = 0.00005$ Learners vs No Learners, $p = 0.522$ Learners vs Slow Learners, $p = 0.00673$ Slow Learners vs No Learners; **Fig. 1D-E**). Poor behavioural performance is explained by a higher number of repetitive errors. The protocol allows mice to do up to 5 consecutive errors for each trial, before being forced into a run in the right direction. Zhang et al. classify series of 3 or more consecutive errors as high-rank errors and observed that they are indicative of a compulsive behaviour which characterized mPFC-impaired mice, while is rarely showed by control animals (Zhang et al. 2016). Mice from the Learners group show a marked reduction of high-rank errors from S2 (percentage of high-rank error runs per session: $22 \pm 6\%$ S1; $3 \pm 0.7\%$ S2-8), while the reduction in Slow Learners is progressive along training days (percentage of high-rank error runs per session: $22 \pm 9\%$ S1; $11 \pm 2\%$ S2-4; $8 \pm 2\%$ S5-6; $4 \pm 1\%$ S7-8) and never takes

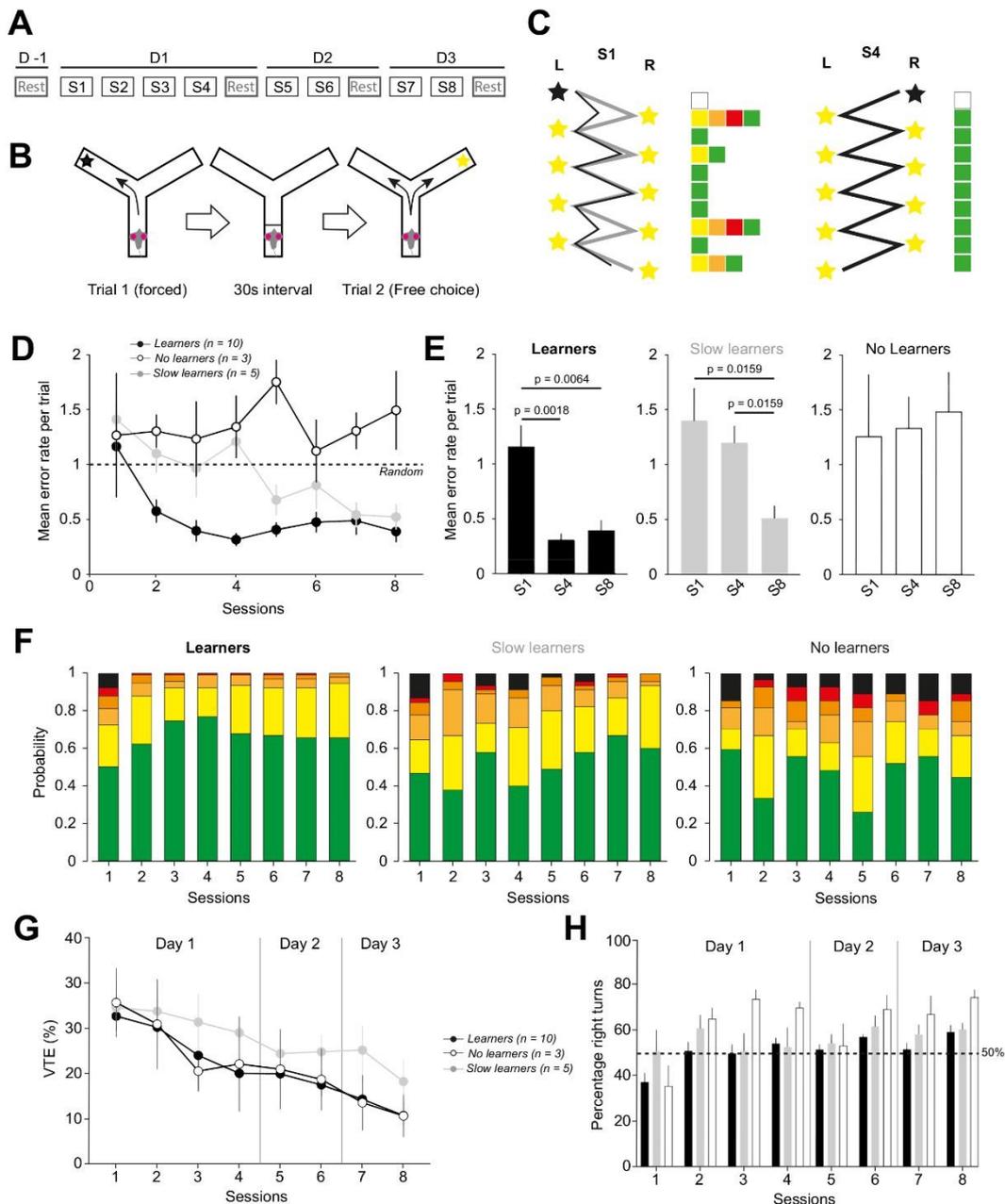


Figure 1. Mice trained in the DSA task segregates into 3 groups based on behavioural performance **A.** Timeline of the behavioural sessions over days. **B.** Schema of the DSA task principle: at the beginning of each session the mouse faces a forced trial in a given direction, on subsequent trials the mouse is faced to free choices and would have to alternate between left and right choices in order to collect the reward; all runs are separated by a 30s interval, characterized by retention within the starting arm; error runs are repeated a maximum of 5 times. **C.** Example of score charts for S1 (left) and S4 (right) for a mouse in the learners group. **D.** Behavioural performance plotted as mean number of errors per trial within each session. **E.** Column plots representing the mean number of errors per trial on S1, S4 and S8 for Learners group. **F.** Percentage distribution of first correct choices and different ranks of errors for Learners (left), Slow learners (center) and No learners (right) groups. **G.** Average percentage of VTE per run within each session for the three groups. **H.** Percentage of right arm choices within each session for the three groups.

place for mice belonging to the No Learners group (percentage of high-rank error runs per session: $21 \pm 2\%$ S1-8; **Fig. 1F**).

We noticed that some mice exhibited a tendency in turning preference for one of the two arms of the maze, the most displaying a bias toward right arm-choices, and we hypothesized that a turning bias might be an explanation for the high number of high-rank errors. Indeed, mice from the No Learners group exhibit a preference for right-arm choices in all sessions with the exception of S1 (**Fig. 1H**, mean right-arm preference across all sessions: $63 \pm 13\%$) and in 5 sessions this preference is significantly higher than that of the Learners group (multiple t-test: $p = 0.0391$ S2; $p = 0.00039$ S3; $p = 0.01716$ S4; $p = 0.02464$ S7; $p = 0.02169$ S8). However, the other two groups never display a clear turning bias (mean right-arm preference across all sessions: $51 \pm 6\%$ Learners; $56 \pm 4\%$ Slow Learners) and for none of the groups the percentage of right arm choices during a given session significantly correlates with the success rate of the same session (data not shown), thus excluding the possibility that a turning bias might be the sole explanation for a poor learning performance. We also hypothesized that the difference in behavioural outcome might be the image of different levels of engagement in completing the task. Average speed in completing each run progressively increased from S1 to S8 for all three groups and without significant differences among them (**Supp Fig. 1**), suggesting similar levels of familiarization with the task and general motivation. However, speed is not a convincing enough proxy for engagement and we tested if other behavioural parameters might highlight differences among groups. Vicarious Trial and Error (VTE) is a typical mouse behaviour consisting in a stop at the choice-point accompanied by rapid head movement toward the different possible future directions. VTE has been associated to compressed replay of neuronal firing patterns representing all the possible alternative routes in the hippocampus and is considered a common mark of cognitive engagement in a spatial task (Redish, 2016). During the first session of the task all three groups exhibited a high proportion of VTE-runs (percentage of VTE runs over total runs: $65 \pm 6.7\%$ Learners, $69 \pm 6.7\%$ Slow Learners, $71 \pm 15.1\%$ No Learners; **Fig. 1G**), which progressively declined to $\sim 20\%$ of total runs being VTE-runs in S8 for Learners and No Learners groups ($21 \pm 6.2\%$ Learners, $21 \pm 9.3\%$ No Learners), suggesting that mice engage in a procedural behavioural response, which do not demand constant cognitive engagement anymore. Interestingly, the percentage of VTE-runs of the Slow-Learners group did not decline as sharply, even though it is significantly different from Learners group only on S7 (percentage of VTE runs over total runs in S7: $28 \pm 6.6\%$ Learners, $50 \pm 10.6\%$ Slow Learners, $27 \pm 12.1\%$ No Learners; t-test: $p = 0.04089$ Learners vs Slow Learners, $p = 0.15214$ Slow Learners vs No Learners). A possible explanation is that, contrary to mice from the No Learners group, mice from the Slow Learners group do not lose cognitive engagement early on but keep trying to optimize their strategy for reward.

We took advantages of this classification based on behavioural performance to have an internal negative control for learning, helping us to determine if there were any changes in hippocampo-neocortical coupling during the learning process.

Hippocampal Sharp Wave-Ripples are stable across days.

First, we focused on the characterization of the functional properties of Hippocampal Sharp Wave-Ripples (SPW-Rs) events detected during Slow Wave Sleep (SWS) which are known to be associated to memory consolidation. We detected SPW-Rs events by filtering the Local Field Potential (LFP) trace of dHPC's channels in the 100-250 Hz oscillatory band and identified peaks of activity based on the amplitude of the oscillation (see Materials and Methods). We then restrained our analysis to SPW-Rs falling within periods of SWS, identified from the combination of mice's immobility (measured through a motion index) and a low theta/delta ratio in the power spectrogram of dHPC's LFP channels (See Materials and Methods and **Fig. 2A**).

We first tested the stability of sleep quality across days. Total time spent in SWS was mostly comparable across days and across groups, with two noticeable exceptions: 1) total time spent in SWS on the first baseline (D-2) by the No Learners group resulted statistically lower than the time spent by mice from the Slow Learners group, but not from the Learners group; 2) total time spent in SWS on the second day of behavioural training (D2) by the No Learners group was statistically higher than the time spent in SWS by the same group on both baselines (D-2 and D-1; **Fig. 2B**). Overall, a slight tendency to increase the total time spent in SWS following behavioural training compared to baseline was observed in all groups, but it was not statistically significant in any other case. Mean length of SWS periods was stable across all days and groups, as well as total time spent in REM sleep (**Fig. 2B**). Overall, our data showed that the structure of the rest periods, at least during the 3h we recorded immediately following the DSA task, was not affected.

We then checked the intrinsic functional properties of SPW-Rs. Both Learners and Slow Learners groups exhibited comparable measures of intrinsic frequency, mean oscillatory amplitude and event duration stable across days (**Fig. 2C**). No Learners group SPW-Rs' characteristics were stable across days, but both mean oscillatory amplitude and event duration were significantly lower than respective measures for Learners and Slow Learners groups (t-test: No Learners vs Learners: $p = 0.0084$ D-2, $p = 0.01005$ D-1, $p = 0.01881$ D1, $p = 0.01216$ D2, $p = 0.02165$ D3; No Learners vs Slow Learners: $p = 0.00142$ D-2, $p = 0.00212$ D-1; $p = 0.00086$ D2; $p = 0.00078$ D3), while intrinsic frequency was comparable to that of the other two groups. The difference was already in place during the baseline, thus it cannot be a consequence of failed learning. Last, we examined the occurrence rate of SPW-Rs during SWS periods. All groups exhibited comparable measures of occurrence rate which were stable

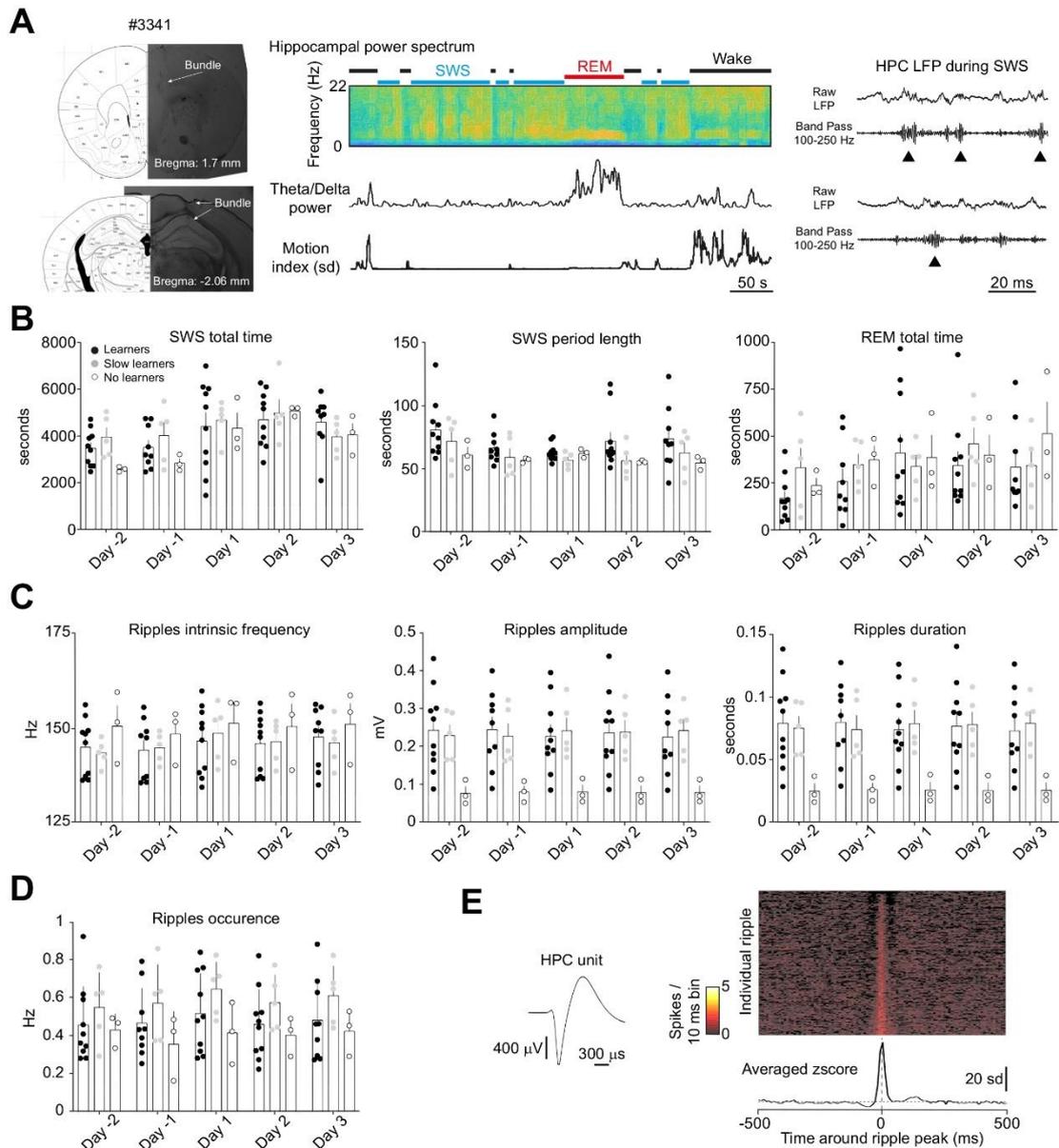


Figure 2. Sleep and SPW-Rs properties are stable across days and groups. **A.** Pictures of fixed tissue showing the wire bundle implantation in the mPFC and PPC. Example of the data analysis for the identification of SWS periods and SW-Rs detection. The upper band of the first panel shows periods of wake (black), SWS (light blue) and REM (red) identified from the combination of the three other bands, showing respectively from top to bottom: heatmap of the power spectrogram of the LFP trace for one dHPC's channel, plotted between 0 and 22 Hz oscillatory frequency, ration of theta/delta frequency bands of the power spectrogram and motion index computed from the video recording of the resting session; the second panel shows the two examples of the unreferenced LFP trace of the same hippocampal channel (upper trace, black) during a portion of the time window captured in the power spectrogram, accompanied by the filtered trace of the same channel in the SW-Rs band (100-250 Hz, centre trace, blue) and highlighting SPW-Rs with black arrow heads. **B.** Sleep quality evaluation across days and groups: total time spent in SWS (left), mean length of SWS periods (centre) and total time spent in REM sleep (right). **C.** SPW-Rs characterization across days and groups: mean intrinsic frequency (left), mean amplitude (centre) and mean event duration of SPW-Rs. **D.** SPW-Rs occurrence rate across days and groups. **E.** z-scored mean firing rate of dHPC's neurons around SPW-Rs' peak.

across days (SPW-Rs occurrence rate \pm standard deviation for Learners group: 0.46 ± 0.20 Hz D-2, 0.47 ± 0.18 Hz D-1, 0.52 ± 0.21 Hz D1, 0.46 ± 0.19 Hz D2, 0.48 ± 0.21 Hz D3, multiple comparisons of one-way ANOVA: $p > 0.99$ for all pairs; Slow Learners group: 0.58 ± 0.18 Hz D-2, 0.57 ± 0.20 Hz D-1; 0.65 ± 0.14 Hz D1, 0.57 ± 0.15 D2, 0.61 ± 0.16 D3, multiple comparisons of one-way ANOVA: $p > 0.99$ for all pairs; No Learners group: 0.43 ± 0.09 Hz D-2, 0.36 ± 0.17 Hz D-1, 0.41 ± 0.16 Hz D1, 0.40 ± 0.10 Hz D2, 0.42 ± 0.12 Hz D3, multiple comparisons of one-way ANOVA: $p > 0.99$ for all pairs; paired t-tests between groups all handed $p > 0.05$; **Fig. 2D**). These data are coherent with results obtained in previous experiments (El Oussini et al., 2023, **Annexes**). However, they contrast with previous literature highlighting an increase in SPW-Rs occurrence rate following a spatial learning paradigm (Eschenko et al., 2008). To confirm the accuracy of our detection, we plotted the z-scored firing frequency of single hippocampal neurons identified within the dHPC and observed that their peak of activity perfectly coincides with the peak of SPW-Rs events (Data not shown; **Fig. 2E**). Despite differences in the No-Learners group, our data show that SPW-Rs properties are stable across days of learning and recordings.

Hippocampo-neocortical coupling is increased after learning in the mPFC but not in the PPC.

Hippocampal SPW-Rs events have already been associated to replay of neocortical neurons during SWS following behavioural training, suggesting the relevance for hippocampal-neocortical connections for system consolidation (Peyrache et al., 2009). The impact of SPW-Rs on neocortical spiking might also be seen as a proxy for synaptic plasticity mechanisms at work during the consolidation process. Therefore, we tested if we could detect enhanced neuronal firing within mPFC and PPC concurrent to SPW-Rs events detected in the dHPC, restraining our investigation to events detected during periods of SWS.

Mean z-scored firing frequency of mPFC's neuronal population of the Learners group showed an increase of firing discharge starting approximately 200ms before the peak of SPW-Rs and peaking concurrently to SPW-Rs' peak, to then sharply come back to baseline. This pattern of progressive increase in the level of excitation of the population was observed with similar features on all recorded days, from the baseline on D-1 to D3 of the behavioural protocol (**Fig. 3A**) and was similarly observed in mPFC neuronal populations of Slow Learners and No Learners groups (**Supp. Fig. 2**). The increase in mean z-scored firing rate did not reflect the firing pattern of the totality of neurons composing the population. In fact, we could distinguish 2 sub-populations: one significantly activated around the peak of SPW-Rs (mean z-score in a window of 25ms before and after SPW-Rs' peak >1.96) and one which showed no modulation around SPW-Rs' peaks. Both populations represented roughly half of the total population in all groups across all days (significantly activated neurons percentages: Learners: 49 % D-1, 46 % D1, 60 % D2, 60 % D3; Slow Learners: 47 % D-1, 54 % D1, 42 % D2, 54 % D3; No Learners: 57 %

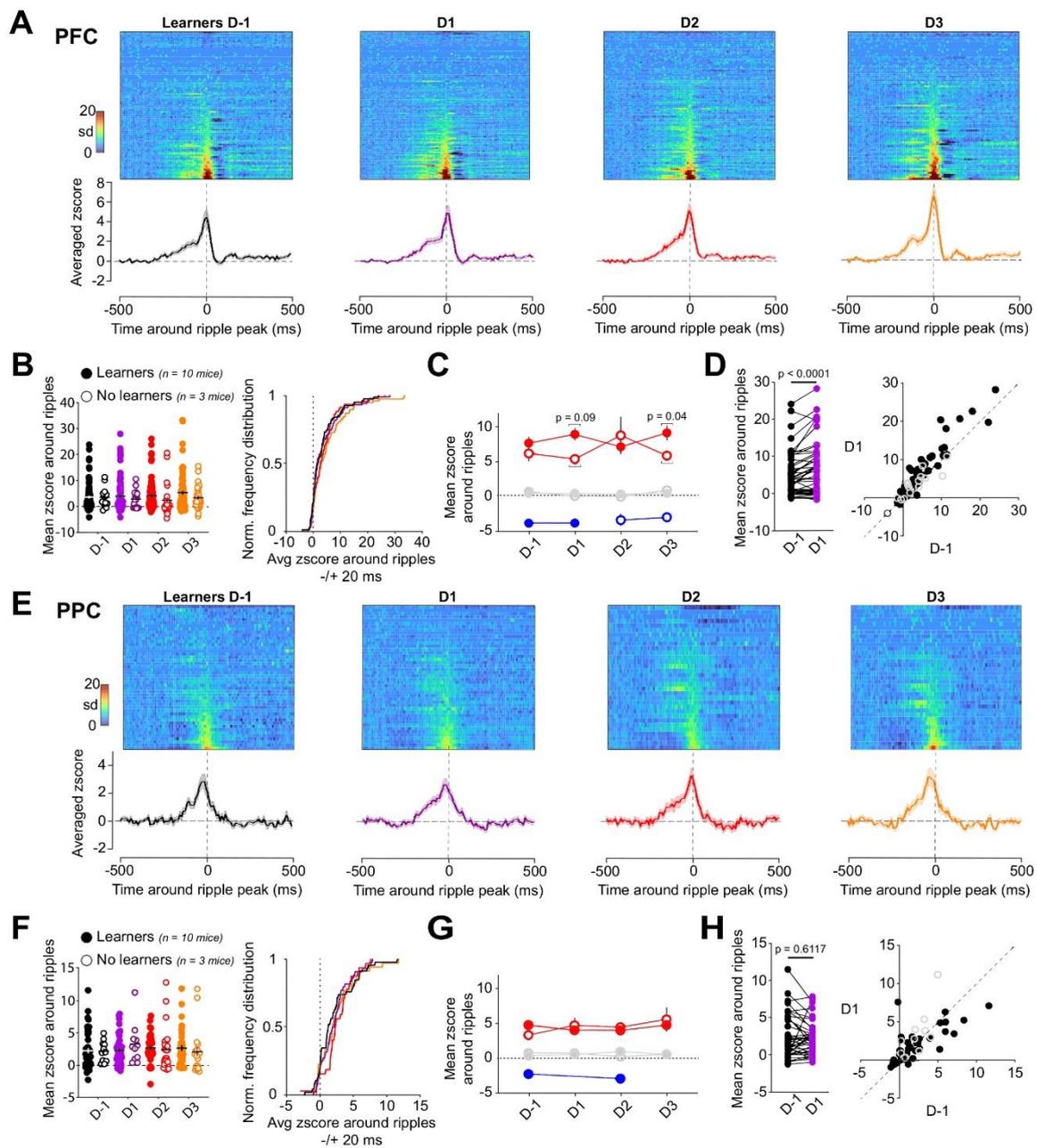


Figure 3. Neocortical single units' activity coordinates with hippocampal SPW-Rs' peaks in mice from the Learners group **A**. From left to right, heatmap (upper panel) and mean z-scored firing frequency (lower panel) of single mPFC's neurons around SPW-Rs' peaks during SWS recorded during rest periods from D-1 to D3 for the Learners group; event window: 500 ms before and after the peak of SPW-R events (D-1: 82, D1: 102, D2: 93 and D3: 77 single-units). **B**. Individual plot points of z-scored firing frequency of mPFC's neurons around the peak of SPW-Rs (25 ms before and after the peak, left panel) and cumulative distribution of the same values (right panel). **C**. Mean z-scored firing frequency of mPFC's neurons around the peak of SPW-Rs (25 ms before and after the peak) divided by the three identified population of significantly activated neurons (red), significantly inhibited (blue) and not significantly different from baseline (grey) across days and groups. **D**. Individual plot points of z-scored firing frequency around the peak of SPW-Rs (25 ms before and after the peak) of mPFC's neurons detected on both D-1 and D1 and scatterplot of the same values. **E-H**. Same analysis performed for PPC neurons (D-1: 46, D1: 44, D2: 32 and D3: 35 single-units).

D-1, 59 % D1, 34 % D2, 60 % D3); neurons significantly inhibited around SPW-Rs' peaks were too sporadic to allow any type of analysis (less than 1 neuron per group per session). Increased firing around SPW-Rs' peak suggest positive modulation from the hippocampal excitatory drive provided by SPW-Rs.

The mean z-scored firing rate around the peak of SPW-Rs events of the whole population of mPFC neurons of the Learners group was not different across days, but their cumulative distribution revealed a statistically significant shift toward the right for rest periods recorded after behavioural training compared to the baseline, with the exception of D2 (Kolmogorov-Smirnov test: $p = 0.0022$ D-1 vs D1, $p = 0.1370$ D-1 vs D3, $p < 0.0001$; **Fig. 3B**). Furthermore, the z-scored firing frequency of mPFC neurons around the peak of SPW-Rs events significantly increased on D1 compared to D-1 (Wilcoxon test: $p = 0.0001$; **Fig. 3D**). mPFC's neuronal populations recorded in mice from the Slow Learners and No Learners groups did not display any statistical significance in their z-scored firing rate around SPW-Rs' peaks between D-1 and D1 (**Supp. Fig. 2**). On D3, z-scored firing frequency of mPFC neurons of the Learners group around SPW-Rs' peaks was significantly higher than the same measure for the No Learners group (Mann-Whitney test: $p = 0.0451$; for the other days: $p = 0.9892$ D-1, $p = 0.0957$ D1, $p = 0.9921$ D2; **Fig. 3C**). All of these results suggests that the positive modulation provided by the hippocampus is enhanced after learning of the rule governing efficient reward collection in the DSA task, but not after the same behavioural task has failed in inducing learning.

Mean z-scored firing frequency of PPC's neuronal population of the Learners group showed a similar pattern of augmentation, starting slightly later (around 150ms before the peak of SPW-Rs), peaking at the SPW-Rs' peak and coming back to baseline levels less sharply. As in the mPFC, patterns similar to this were common across days (**Fig. 3E**) and across groups (**Supp. Fig. 2**). Roughly half of the PPC's neuronal population was significantly activated around the peak of SPW-Rs across days and groups (significantly activated neurons percentage: Learners: 43 % D-1, 50 % D1, 59 % D2, 49 % D3; Slow Learners: 42 % D-1, 41 % D1, 51 % D2, 14 % D3; No Learners: 50 % D-1, 64 % D1, 51 % D2, 30 % D3) with the noticeable exception of D3 for Slow Learners and, to a lesser extent, No Learners, which displayed only 14 % and 30 % of significantly activated neurons, respectively.

The mean z-scored firing rate around the peak of SPW-Rs events of the whole population of PPC neurons of the Learners group was not different across days, but their cumulative distribution revealed a statistically significant shift toward the right of values recorded on D3 compared to the baseline recorded on D-1 (Kolmogorov-Smirnov test: $p = 0.0030$ D-1 vs D1, $p = 0.0010$ D-1 vs D3, $p < 0.0060$; **Fig. 3G**). However, comparing the firing frequency of the same neuron identified on D-1 and D1, the z-scored firing frequency of PPC neurons around the peak of SPW-Rs events was not significantly different between D-1 and D1 (Wilcoxon test: $p = 0.6117$; **Fig. 3I**). The same analysis for Slow Learners and No Learners groups showed comparable results (**Supp. Fig. 2**). Finally, z-scored firing

rate around the peak of SPW-Rs was never significantly different between Learners and No Learners groups (Mann-Whitney test: $p > 0.99$ for all days; **Fig. 3G**). Overall, these data suggest that the hippocampus indeed exerts a positive modulation on the PPC, but that this modulation is not increased after learning of the rule governing efficient reward collection in the DSA task and that, on the contrary, it might weaken after a few days of repetition of the same behavioural protocol.

Neocortical neurons that display a behaviourally relevant firing pattern during the task are preferentially reactivated in the mPFC but not in the PPC.

Even though the proportion of neocortical neurons significantly activated at the peak of SPW-Rs did not change across days, we wanted to test if behavioural training induced a change in the composition of this population.

First, we identified neurons displaying a behaviourally relevant firing pattern during the 4 sessions of the first day of behavioural training. We defined the behavioural relevance of the firing pattern of a given neuron based on two alternative criteria (see Materials and Methods): 1) *maze preference criterion*, based on the identification of a significant peak of activity at a specific sub-location within the maze (start box, start arm, centre, end arm or end box) 2) *behavioural attribute criterion*, based on the identification of a significant divergence in the firing map of a given neuron when runs were pooled based on pairs of behavioural attributes (success vs error runs; right-turn vs left-turn runs; VTE vs no-VTE runs). Neurons that corresponded to either one of the two criteria were considered to display a behaviourally relevant firing pattern. Due to the relatively low number of neurons and apparently comparable results across groups, we pooled all neurons from all groups together for this analysis.

56 % of investigated neurons in the mPFC corresponded to either or both criteria for behavioural significance. In particular, 24 % of mPFC neurons resulted modulated by the behavioural attribute criterion, with neurons divided quite evenly among the three comparative pairs (9 % responded differently to success vs error runs, 6 % to VTE vs no VTE runs and 9 % to right-turn vs left-turn runs), while 46 % resulted modulated by the maze criterion, but with proportion clearly skewed toward neurons firing in either the start box (23 %) or the end box (7 % significantly activated and 13 % significantly inhibited), while populations for other locations were almost inexistent. Interestingly, among neurons that were significantly more active within the start or the end boxes, 38 % were overall significantly inhibited during runs compared to the inter-trial interval, suggesting their role might not be strictly related to navigation.

52 % of investigated neurons in the PPC corresponded to either or both criteria for behavioural significance. A low proportion, 14 % of PPC neurons, resulted modulated by the

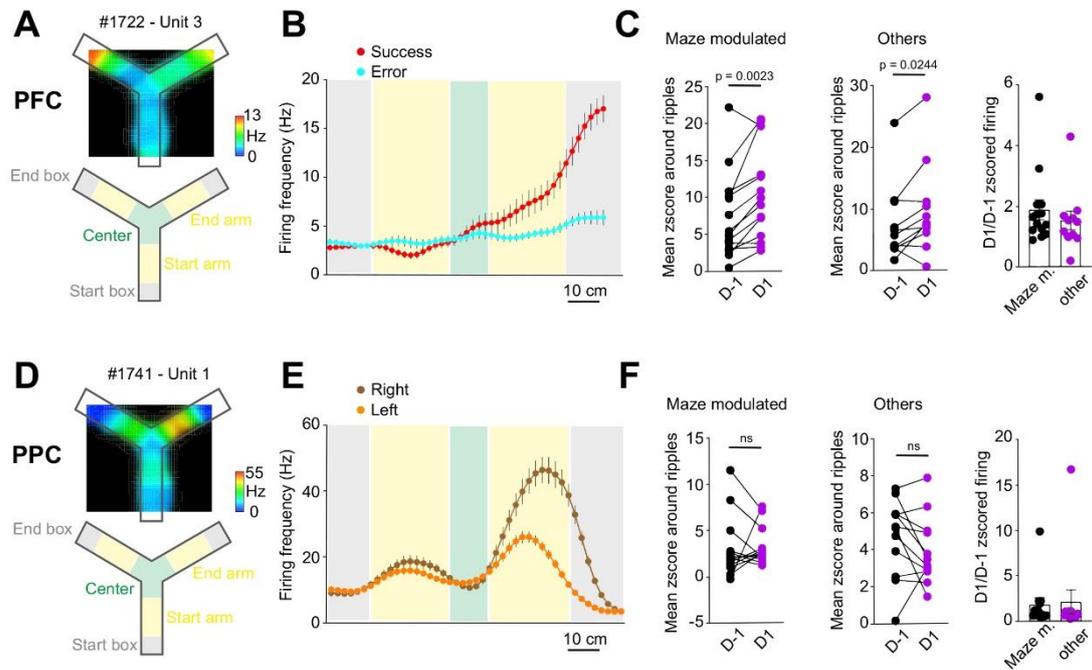


Figure 4. Neocortical neurons are modulated by task's features during DSA training A-B. example of mPFC neuron modulated by the behavioural attribute “success vs error”. A. 2D firing-map of the neuron’s spiking within the maze during run-time. B. Plot of the linearized firing-map for the same neurons highlighting the different firing patterns on success vs error runs. C. Individual plot points of z-scored firing frequency around SPW-Rs’ peak (25 ms before and after the peak of SPW-Rs) of behaviourally relevant mPFC’s neurons detected on both D-1 and D1. D-E. example of PPC neuron modulated by the behavioural attribute “right-turn vs left-turn” and displaying a firing preference for the end arm. D. 2D firing-map of the neuron’s spiking within the maze during run-time. E. Plot of the linearized firing-map for the same neurons highlighting the different firing patterns on right-turn vs left-turn runs. C. Individual plot points of z-scored firing frequency around SPW-Rs’ peak (25 ms before and after the peak of SPW-Rs) of behaviourally relevant PPC’s neurons detected on both D-1 and D1.

behavioural attribute criterion, with a clear preference for the right-turn vs left-turn pair comparison (8 %, while only 2 % responded differently to success vs error runs and 3 % to VTE vs no-VTE runs). 50% of PPC’s neurons, instead, resulted modulated by the maze criterion, showing a distribution of preferred firing-location more even than the neuronal population of the mPFC but still quite biased toward a preference for the start box (start box: 22 %; start arm: 9 %, centre: 2 %; end arm: 4 %; end box: 10 % significantly activated and 1 % significantly inhibited). 57 % of neurons displaying their firing peak in either the start or end box were overall significantly inhibited during runs compared to the inter-trial interval.

We then investigated if behaviourally relevant neurons were more likely to undergo activation around the peak of SPW-Rs during the rest period following learning. Proportions of behaviourally relevant neurons from either of the two neocortical regions significantly active around the peak of SPW-Rs were not significantly different across the two days (mPFC: 76 % on D-1, 84 % on D1; PPC: 39 % on D-1, 41

% on D1). However, while the proportion of positively modulated behaviourally relevant PPC neurons reflected that of the general population, a significantly greater proportion of mPFC's behaviourally relevant neurons than that of the general population was activated around the peak of SPW-Rs. We then questioned if activation around the peak of SPW-Rs of behaviourally relevant neurons was qualitatively different than reactivation of neurons that were not specifically engaged during the behavioural task. Within mPFC, among neurons positively modulated around SPW-Rs' peaks, both behaviourally relevant and not behaviourally relevant neurons were significantly more activated around SPW-Rs' peaks on D1 compared to D-1 (Mann-Whitney test: $p = 0.0023$ for behaviourally relevant neurons, $p = 0.0244$ for other neurons); the magnitude of the increase was not statistically different between the two groups (**Fig 4C**). The same results were not observed in PPC, where neurons positively modulated around SPW-Rs' peaks in D-1 show no significant increase nor decrease of z-scored firing rate on D1 (**Fig. 4F**).

Injection of AMPA receptors' mobility blockers in the mPFC at the end of the first day of behavioural training had no effect on memory consolidation.

The system consolidation theory hypothesizes that the excitatory drive provided by the hippocampus to neocortical areas during SPW-Rs induces synaptic plasticity within the neocortical engram. To test this hypothesis, we used an AMPA receptors-blocking strategy based on intracranial injection of tetravalent neutravidin in a knock-in mouse strain expressing AP-tagged GluA2 AMPA receptor subunit and the enzyme BirA ligase (see materials and Methods). We injected mice through guide cannulas chronically implanted in the mPFC at the end of the first day of behavioural training with either neutravidin (NA) or Saline, as control group to test whether the increase in firing frequency observed in D1 around SW-Rs during SWS (**Fig. 3D**) could be explained by the induction of long term synaptic plasticity.

Unexpectedly, mice from the NA-injected group did not display any sign of memory impairment during the second day of behavioural protocol, suggesting that AMPA receptors immobilization within the mPFC during the resting period did not impact memory consolidation (**Fig. 5A**).

Two mice from this batch were also implanted with electrodes in both dHPC and mPFC, so we could make a preliminary investigation of the impact of AMPA receptors-blocking on single neurons within the mPFC, especially around the peak of hippocampal SPW-Rs. Having just one mouse per batch I will limit myself to describe preliminary results without any attempt at quantification. We checked that basic parameters such as time spent in SWS and SPW-Rs' occurrence rate were constant and comparable to wild type measures (Data not shown). We then investigated the relationship between SPW-Rs' peak and neuronal firing within the two neocortical areas. Z-scored firing frequency described

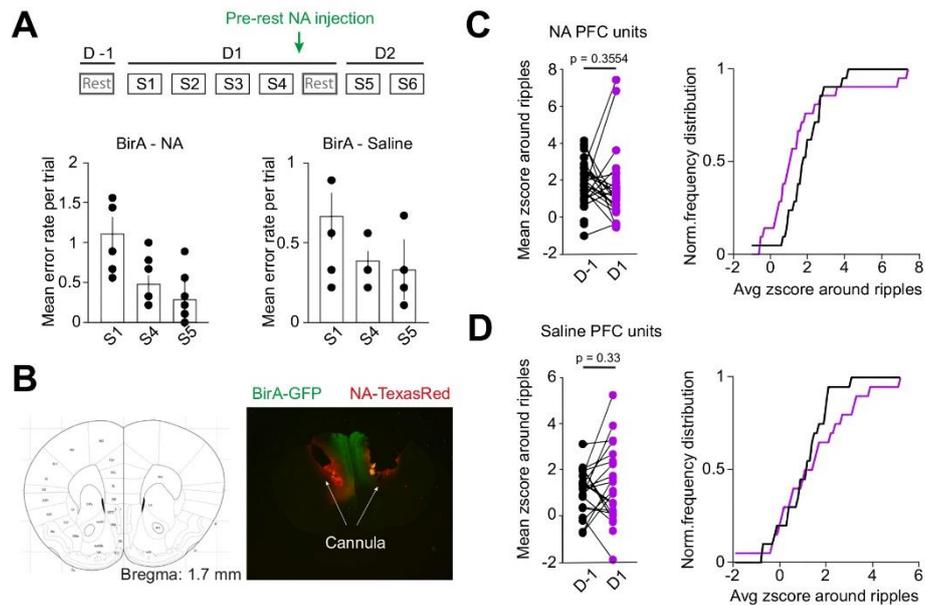


Figure 5. (preliminary) Blockage of LTP in the mPFC during the resting period does not affect mice's behavioural performance on the next day **A.** Column plots representing the mean number of errors per trial on S1, S4 and S5 for neutravidin-treated mice (left) and saline-treated mice (right). **B.** Left panel: coordinates of cannulas implantation on the Paxinos Mouse Brain Atlas; right panel: histology of BirA-GFP infection (green) and neutravidin injection (red). **C.** Individual plot points (left) and cumulative distribution (right) of z-scored firing frequency around SPW-Rs' peak (25 ms before and after the peak of SPW-Rs) of mPFC's neurons from the neutravidin-treated group detected on both D-1 and D1. **D.** Individual plot points (left) and cumulative distribution (right) of z-scored firing frequency around SPW-Rs' peak (25 ms before and after the peak of SPW-Rs) of mPFC's neurons from the saline-treated group detected on both D-1 and D1.

a curve similar to that of wild type mice in both regions, even though increased firing at the peak of SPW-Rs events was greatly attenuated in the mPFC compared to wild type animals (Data not shown). No apparent increase or decrease in z-scored firing rate around the peak of SPW-Rs between D-1 and D1 was observed in any of the two regions (**Fig 5C-D**). However, the proportion of neurons significantly activated around the SPW-Rs' peak on D1 dropped only for mPFC neurons and only in the NA-treated mouse (46 % on D-1, 24 % on D1).

(Disclaimer: this study has been first produced in the form of a paper during the redaction of this PhD dissertation; given that it presents limitations related to the advancement of analysis and experiments that are entirely due to PhD's time-constraints, all of the technical aspects will be discussed in the general discussion of this thesis and not mentioned in the discussion of the paper, even when they are of relevance to the paper itself.)

Discussion

We investigated system consolidation of a semantic-like and spatial working memory-based rule governing efficient reward collection in a Y-maze Delayed Spatial Alternation task.

Behavioural training solicited both the neocortical areas of our interest: in fact, around 50% of the recorded neuronal population in both mPFC and PPC showed a behaviourally relevant firing pattern during navigation for behavioural training, tuning-in either to a specific location within the maze or to an attribute of the run (success vs error, right-turn vs left-turn, VTE vs no-VTE) or to both. However, the information encoded in each of the two areas was qualitatively different. PPC neurons tuned-in to spatial and navigation-related features: neurons preferentially firing at all sub-locations of the maze could be found in this region, while the percentage of neurons changing their firing pattern as a function of behavioural attributes of the run was low (14%) and dominated by neurons tuning-in to the right-turn vs left-turn attribute (58%). mPFC neurons, instead, mostly had a peak of activity in either the start or end boxes, which by task-design are charged also with other inherent meaning beside the spatial information, which cannot be disentangled, the end box as the site of reward collection and the start box as the site where the mouse was retained during the inter-trial interval. Furthermore, a higher proportion of neurons (24%) were tuned-in to behavioural attributes and weights were more evenly distributed across the three pairs of attributes. For these reasons, we hypothesized for the mPFC a more cognitive role. These results are not surprising, as fall perfectly in the range of roles that are attributed to these two regions in literature (Le Merre et al., 2021; Lyamzin and Benucci, 2019) and were expected when mPFC and PPC were chosen for this investigation, even though mPFC activity has also been associated to spatial navigation in other type of tasks (Fujisawa et al., 2008).

Coordination between hippocampal SPW-Rs and spiking activity in the neocortex was taken as a proxy of system consolidation, based on the evidence in literature that projections from the hippocampus propagate the excitatory drive of SPW-Rs to neocortical areas, inducing coherence in the pattern of oscillations and neuronal replay (Binder et al., 2019; Khodagholy et al., 2017; Peyrache et al., 2009; Wilber et al., 2017). Indeed, in both neocortical areas we observed that the firing rate of 40 - 60 % of neurons increased around the peak of SPW-Rs, resulting in a net increase in the mean firing rate of the whole population. We restrained our investigation to neurons that we could reliably detect on two consecutive days and observed that mPFC's neurons from mice of the Learners group increased their firing rate around SPW-Rs' peak after completion of the behavioural protocol on D1 compared to the baseline recorded the day before. No such increase was observed for mPFC's neurons from mice of the Slow Learners or No Learners group. This learning-dependent positive modulation confirms that acquisition of the rule governing efficient reward collection in the DSA task induces hippocampal-

driven memory consolidation in the mPFC. This conclusion is strengthened by the fact that the majority (70 – 80 %) of behaviourally relevant neurons were positively modulated at the peak of SPW-Rs. Interestingly, these neurons were already part of the pool of positively modulated neurons prior to any learning protocol during baseline recording, they were not specifically recruited by SPW-Rs following their activation during the task. Evidence collected in the hippocampus showed that hippocampal neurons which participate to SPW-Rs' sequence activations during a baseline recorded right before the learning protocol are more likely to participate to trajectory sequences during the behavioural experience, suggesting the idea that new learned trajectories would emerge from a set of prefabricated neuronal sequences rather than be *de novo* created during exploration (Dragoi and Tonegawa, 2011). This perspective is efficient from a computational point of view and implies that encoding strengthens the connections and activation drive of neurons that are already highly permissive for sequential activation. Stretching this concept, a similar situation might verify also within the mPFC, where neurons that receive stronger hippocampal input would somehow be primed to participate into neuronal assemblies and new neocortical engrams.

Surprisingly, PPC's neurons from mice of any group did not show any sign of firing modulation following completion of the behavioural protocol compared to their baseline. As we showed, PPC's neurons are activated during the task in ways that are qualitatively different but quantitatively comparable to mPFC's neurons, however we could not find any evidence of increased communication between the hippocampus and this neocortical area. It is possible but extremely unlikely that memory consolidation within the PPC would be regulated by hippocampus-independent mechanisms, also because experiments conducted exploiting other model tasks showed an increased positive modulatory effect from the hippocampus on this neocortical area (Wilber et al., 2017), therefore the conclusion most likely to be correct is that the network involved in consolidation of the rule governing efficient reward collection in the DSA task does not include PPC. PPC's activity during the task might be due to the participation of this region to processes such as attention, egocentric navigation or working memory but none of these components would be part of the semantic-like rule consolidated during the following rest period. Certainly, a loss of function study would be needed to determine the dependence of the DSA task on an intact PPC, to rule out the possibility that the patterns of activation we found during the task were purely correlative or that the PPC only serve a supportive and redundant role in navigation during this task.

We hypothesized that consolidation of the rule governing efficient reward collection in the DSA task would be dependent on synaptic plasticity within the hippocampo-medial prefrontal network. A recent work from the laboratory demonstrated that preventing AMPA receptors mobility within the hippocampus led to disruption of the physiology of SPW-Rs and to memory impairment upon retrieval, suggesting a lack of consolidation (El Oussini et al., 2023, **Annexes**). Prior to that,

blockage of AMPA receptors' mobility was showed to prevent LTP at excitatory synapses in the CA1 region (Penn et al., 2017). We decided to use a recently developed AMPA receptors' mobility blockage strategy (Getz et al., 2022) to prevent LTP within the neocortex. The strategy is based on a strain of KI mice expressing an AP-tagged GluA2 subunit for biotinylation of AMPA receptors in the presence of the enzyme BirA ligase, whose expression was induced in the mPFC via injection of a viral vector and under the promoter pSyn, to assure expression in all neuronal types. AMPA receptors crosslinking to prevent mobility is induced via acute injection of tetravalent neutravidin. Our main hypothesis was that preventing AMPA receptors' mobility we would prevent LTP at hippocampus-to-mPFC projections, disrupting consolidation. However, our experiment design does not allow any control over the affected synapses, as the enzyme BirA ligase is expressed in mPFC neurons irrespective of their input pattern, thus an equally valid hypothesis would be that LTP suppression would affect local excitatory synapses between neurons of the mPFC engram. Disentangle the two possibilities is very difficult with our approach.

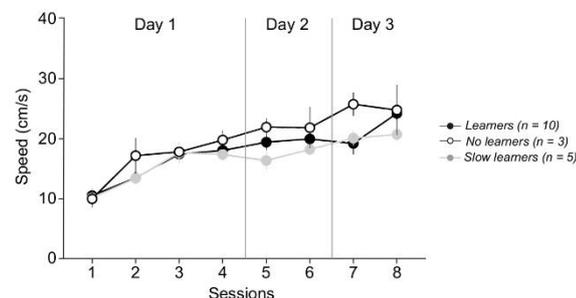
Surprisingly, injection of neutravidin at the end of the first day of protocol did not affect memory retrieval upon testing on the second day of protocol. Indeed, a recent research showed that preventing structural LTP within the anterior cingulate cortex 24 hours but not 8 hours after the learning paradigm disrupted behavioural performance in an inhibitory avoidance task by affecting memory consolidation (Goto, 2022). Likewise, AMPA receptors-dependent functional LTP might sustain memory consolidation within the prefrontal cortex 24 hours but not immediately after the acquisition of the rule governing efficient reward collection in the DSA task, therefore testing the effect of neutravidin injection later in the protocol would be definitely interesting. Alternatively, AMPA receptors-dependent functional synaptic plasticity might not have a role after that of LTP expression during synaptic tagging at the moment of encoding. This is unlikely, because AMPA receptors addition at the potentiated synapse is hypothesised to take place also during synaptic capture, as new spots are allocated to AMPA receptors within the reorganized cytoskeleton at the post-synaptic density, and because of the extensive effects of AMPA receptors' crosslinking in the hippocampus on memory consolidation found by El Oussini et al. However, it is possible that memory consolidation would be regulated by different mechanisms in the hippocampus and in the mPFC or that AMPA receptors' crosslinking would have an effect on SPW-Rs' physiology in general but not specifically on tagged synapses of engram neurons. This question might be partly answered by analysing the effects of AMPA receptors' crosslinking on the modulation of mPFC's neurons around SPW-Rs' peaks. Indeed, physiological effects induced by crosslinking might not translate to a behavioural phenotype, as it is often the case with silent neocortical engrams during consolidation of recent memories (Kitamura et al., 2017). Unfortunately, we could not collect enough electrophysiological data to draw any conclusion upon, even if we remarked a promising tendency of decreasing of the firing rate on D1 compared to

the baseline in D-1 in mPFC's neurons in the neutravidin group. An increase in the number of animals is certainly needed.

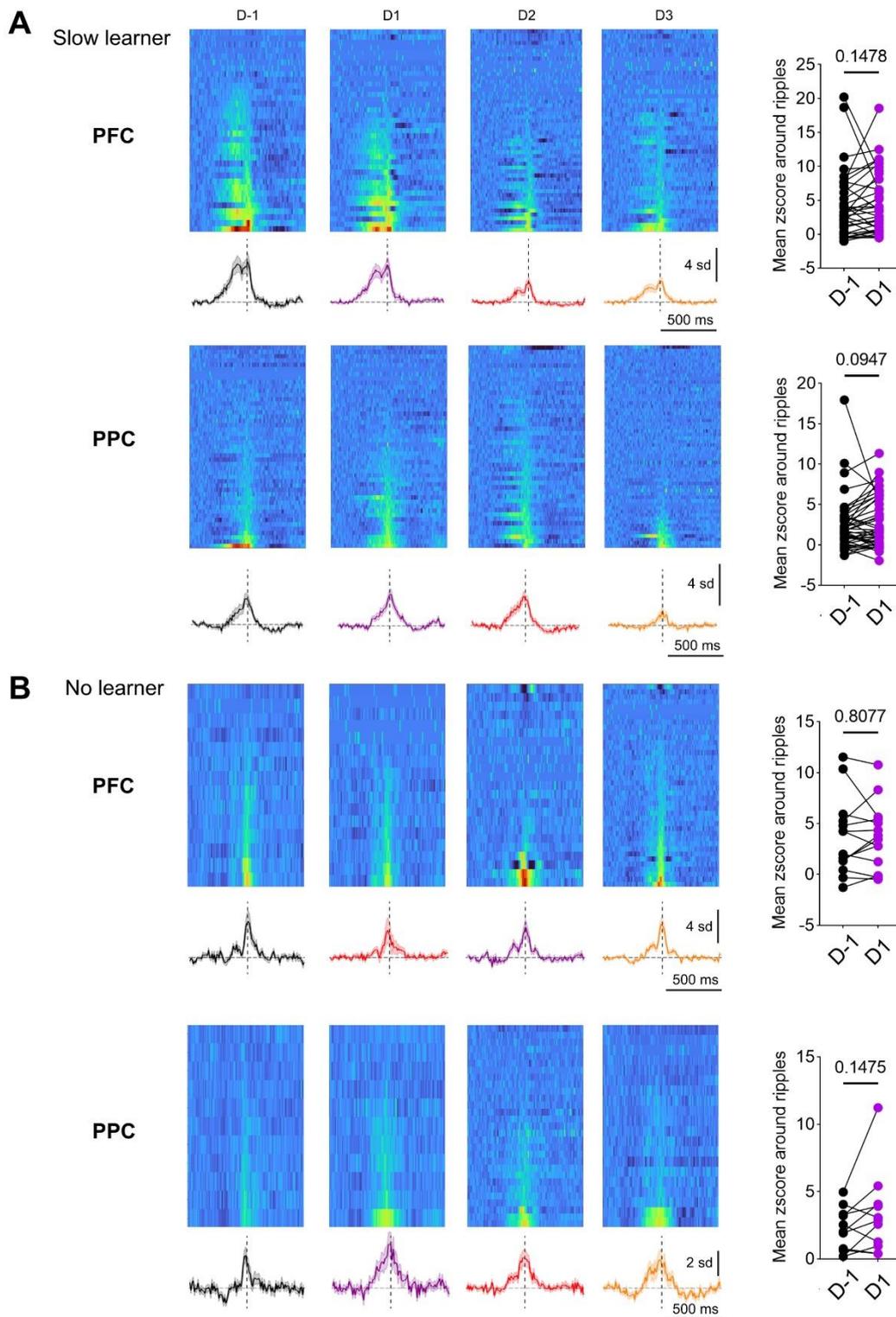
Conclusions

Spatial working memory-guided navigation solicits both mPFC and PPC, but only the prior is positively modulated by hippocampal SPW-Rs during the rest periods following rule acquisition, excluding PPC from a functional network of consolidation of this type of memory. Memory consolidation is not dependent on AMPA receptors' mobility within the mPFC at least during the rest period immediately following rule acquisition, while further experiments will be needed to determine mPFC's involvement at later phases of consolidation and the effects of AMPA receptors' crosslinking on the modulation of mPFC's neurons by hippocampal SPW-Rs, possibly also with a yet silent neocortical engram.

Supplementary Figures



Supplementary Figure 1. Average speed per run within each session for the three groups.



Supplementary figure 2. Heatmaps and z-scored firing frequency of mPFC and PPC neurons during rest periods from D-1 to D3 for the Slow Learners (**A.**) and No Learners (**B.**) groups; individual plot points of z-scored firing frequency around the peak of SPW-Rs (25 ms before and after the peak) of mPFC's neurons detected on both D-1 and D1 (right panels). Slow learners: PFC: D-1: 34, D1: 39, D2: 52 and D3: 44 single-units; PPC: D-1: 47, D1: 46, D2: 45 and D3: 58 single-units; No learners: PFC: D-1: 14, D1: 17, D2: 23 and D3: 40 single-units; PPC: D-1: 11, D1: 11, D2: 29 and D3: 20 single-units.

DISCUSSION

Discussion

We wanted to investigate the role of hippocampal sharp wave-Ripples in modulating neocortical areas within a network involved in memory consolidation. We chose a behavioural task (the Delayed Spatial Alternation task) that easily allowed us to discriminate between a progressive but rapid phase of learning which could be included in a single day of behavioural training, to which we attributed the encoding phase of memory formation, and an extended resting period allowing memory consolidation, before testing for memory acquisition on the following day. We recorded Local Field Potential with single wire electrodes from the dorsal hippocampus to collect information on SPW-Rs activity in the area CA1, and from medial prefrontal cortex and posterior parietal cortex to collect spiking activity both during behavioural training and resting periods prior to and following behaviour.

Unexpectedly, even though we used 18 wild type mice without any particular condition nor treatment, only 55% were able to readily learn the task within the allocated 1-day time-frame, while 45% displayed either delayed learning (28%) or no learning at all throughout the three days of protocol (17%). This is in contrast with previous results obtained within the laboratory with the same protocol, where control mice were pooled without distinctions (Zhang et al., 2017)(El Oussini et al., 2023, **Annexes**). The learning impairment seems to be caused by different mechanisms in the two groups: mice from the Non-Learners group showed a natural bias toward right-turn choices which impaired their behavioural performance in both mean error-per-trial rate and number of high-rank consecutive errors; furthermore, they seemed to disengage quite quickly from the cognitive part of the task by reducing the number of runs during which they displayed VTE (vicarious trial and error) behaviour, which is a sign of choice-making under cognitive control (Redish, 2016). Their rate of decrease of VTE-runs was comparable to that of mice from the Learners group and is generally interpreted as a loss of cognitive control in favour of procedural choices guided by a stereotypical behaviour once the most efficient strategy for collecting reward has been implemented. In the absence of acquisition of this optimized strategy by mice from the Non-Learners group, we interpreted their loss of VTE as cognitive disengagement from the task and, possibly, proceduralization of a random pattern of exploration or a low-rewarding strategy. On the other hand, mice from the Slow-Learners group did not display any turning bias that could explain their poor behavioural performance, but retained a higher proportion of VTE-runs all throughout the behavioural protocol. This result suggests that, in the absence of an efficient strategy to collect reward and of confounding factors (i.e. internal or external biases), mice retain cognitive control over choices during the DSA task longer, possibly as long as needed to figure out the best strategy.

Overall, the poor behavioural performance of a significant proportion of animals might be explained by the logistic of combining maze exploration and electrophysiological recordings. The compensatory system used was not well calibrated to relieve the mouse from the weight of cables used for recordings

and pull effortlessly up to the distal edge of each arm, making navigation physically more engaging than for mice that were not recorded for electrophysiology during the task.

Sharp wave-Ripples are at the core of investigation for memory consolidation both within the hippocampal circuit and from a systemic point of view (Buzsáki, 2015), therefore we naturally addressed their characterization and modulation during our behavioural protocol. Multiple papers pointed out that the occurrence rate of SPW-Rs events augments during slow wave sleep following novelty exploration or execution of a behavioural task, especially after acquisition of the learning criterion (Eschenko et al., 2008). We did not observe this augmentation in SPW-Rs, as we didn't either in our previous research employing the same protocol (El Oussini et al., 2023, **Annexes**). This result is rather puzzling, as the DSA task is a hippocampal-dependent spatial navigation task which rely on an intact hippocampus to be executed, as we verified personally by injecting muscimol within the hippocampus, resulting in learning disruption (El Oussini et al. supplementary figures). One would therefore hypothesises that hippocampal SPW-Rs are needed during consolidation at least for coordinating the local circuit. Indeed, El Oussini et al. showed that by altering SPW-Rs physiology through injection of an AMPA receptor-blocking agent memory consolidation was disrupted, thus even though SPW-Rs' occurrence rate was not enhanced following learning they still played a pivotal role in consolidation of the cognitive rule governing the task. A simple explanation to the discrepancy with bibliography might be that most, if not all, research in this field has been conducted in rats and mice's SPW-Rs' physiology might be slightly different or the difference in occurrence rate smaller and therefore more difficult to detect. Other SPW-Rs' features used to characterize their physiology were stable across days and across groups, with the exception of SPW-Rs' amplitude and duration for mice of the No Learners group. This difference is striking and does not rely on a difference in site of recording, as showed by histological slices: it is not an effect of lack of learning, as it is already present in recorded baselines, and might highlight an underlying issue shaping mice's poor behavioural performance from a physiological point of view. Indeed, longer SW-Rs during wake consummatory behaviours in the inter-trial interval of a spatial alternation task have been associated to a higher content of neurons participating in behavioural trajectories and prolongation of SW-Rs was effective in increasing the accuracy of the behavioural performance (Fernández-Ruiz et al., 2019). However, the two measures, even though comparatively low, are not outside the physiological bounds and SPW-Rs activity seems normal in any other aspect, from occurrence rate to correlation with neocortical neurons, hence it might be due to a random coincidence related to the low number of mice composing the No Learners group.

The sleep pattern of mice, in terms of time spent in SWS and REM phases or length of SWS intervals, was consistent across days and groups, even though a non-significant tendency to an increase in time spent in SWS was detectable between rest following the behavioural protocol and baselines, most likely due to the more demanding and experience-filled nature of days interested by the behavioural training.

A possible mechanism for system consolidation relies on the modulation of the firing pattern of neocortical neurons by hippocampal SPW-Rs. In particular, much as it is within the local circuit of the hippocampus, SPW-Rs are hypothesized to entrain burst of compressed neocortical neuronal replay of primed and tagged neurons to promote synaptic capture, and a few researches have indeed found correlations between SPW-Rs and neocortical firing (Peyrache et al., 2009; Wang and Ikemoto, 2016; Wilber et al., 2017).

Our results point toward the conclusion that the hippocampus coordinates with the medial prefrontal cortex for consolidation of the cognitive rule determining the most efficient strategy for reward collection in the DSA task, but this functional network does not include the posterior parietal cortex nor the hippocampus shows any particular positive modulatory activity toward this region during consolidation of this rule. In fact, even though roughly 50% of PPC's neurons showed significantly higher z-scored firing rate around the peak of hippocampal SPW-Rs, the mean of the population activity was still quite low and single neurons did not show any significant change, neither positive nor negative, in their firing pattern around SPW-Rs' peaks throughout the days of the protocol. On the contrary, on D3 we observed an abrupt drop in the proportion of PPC neurons significantly more active around the peak of SPW-Rs events than outside of SPW-Rs' event-window, at least for the Slow-Learners and Non-Learners groups. Strengthening of coherence between dHPC and PPC in the high-frequency range following spatial learning has been shown (Khodagholy et al., 2017), however, while the DSA task certainly relay on spatial navigation during the behavioural performance, the nature of the rule to be encoded, i.e. alternate between left and right arm choices, seems to be exquisitely semantic and its consolidation would not require PPC's engagement, which has rather been implicated in navigation and sensory association (Lyamzin and Benucci, 2019). Progressive semanticization of the strategy to efficiently solve the DSA task, marked by stereotyped and procedural choices, would further reduce the spatial and navigational components of the task, possibly explaining the decrease in the proportion of neurons significantly activated around the peak of hippocampal SPW-Rs on the last day of protocol. The mPFC, on the other hand, shows a higher mean population activity around the peak of SPW-Rs, even though also in this region roughly 50% of total detected neurons displayed a z-scored firing rate significantly higher around the peak of SPW-Rs events than outside of SW-Rs' event-window, a proportion that remains constant across days and groups. Single mPFC's neurons displayed a significantly higher z-scored firing rate around SPW-Rs' peak on D1 compared to the baseline on D-1 and almost all neurons that were already significantly positively modulated around SPW-Rs' peak on D-1 significantly enhanced their firing rate around SPW-Rs on D1. The mPFC is the main neocortical investigation subject for memory consolidation, because of its role in cognitive control (Euston et al., 2012) and cumulating evidences potentially suggesting a pivotal role in long-term memory storage, similar to that played by the hippocampus on a shorter time-frame (Tonegawa et al., 2018). It is not surprising that there seems to be a stronger and functionally relevant connection between mPFC and dHPC that was not detected for PPC, also because, unlike this latter region, mPFC receives dense

monosynaptic projections at least from the ventral hippocampus, while projections from the dorsal hippocampus are scarcer. Furthermore, mPFC is part of the memory system regulating encoding and consolidation of semantic memories (Henke, 2010), therefore its stronger correlation with hippocampal activity during SWS suggests that the rule governing efficient execution of the DSA task that has to be consolidated is indeed semantic.

Characterization of the interplay between dHPC and neocortical regions would greatly benefit by an analysis of the coherence between these areas in different oscillatory frequencies, both during the task and subsequent rest periods. Indeed, it would be interesting to reproduce observations on the coherence in the theta range between dHPC and mPFC during navigation (Benchenane et al., 2010) as a marker of coordinated participation to the same network for task execution and inspect whether a similar coherence pattern is shared also by dHPC and PPC. Furthermore, analysis of LFP's oscillations might give a deeper insight into the participation of PPC to the network for execution of the DSA task, even in the absence of loss of function studies that might redeem the question in a more direct way. We would look specifically at the coherence between the two neocortical regions, as a sign of coordinated interaction that would be expected during execution of a working memory task (Yamamoto et al., 2014).

Evidences, collected primarily within the hippocampus, point out that neurons implicated in SPW-Rs-induced replay of activity are those most activated during the experience encountered during the preceding waking period (Dupret et al., 2010). We tackled this question by considering the behavioural relevance of neurons active during the DSA task, as we specifically focused on neurons that displayed a peculiar and significantly different firing pattern either at a specific location within the maze or in runs characterized by one out of a pair of behavioural attributes (left-turn vs right-turn, success vs error, VTE vs no-VTE). We included PPC neurons in the analysis because, even though we excluded this region from the functional network for memory consolidation, it did undergo activation during the task and we considered that a deeper insight on the activity of this neocortical region during the behavioural task might further explain the lack of correlation with hippocampal SPW-Rs activity during SWS. Indeed, PPC's neurons displayed activity patterns during the DSA task that could be attributed to navigation. Only 14% of neurons resulted to be positively modulated by the behavioural attribute criterion, among which neurons modulated by left-turn vs right turn choices, which could either be view as a cognitive feature from the choice-making prospective or yet another spatial feature, were predominant. Furthermore, most of these neurons actually showed a double modulation, being significantly more active also at a specific location within the maze. Location-specific neurons represent 50% of total PPC neurons and all location are represented, even though not equally. The most represented location is the start box. Start box and end box cannot be strictly considered completely spatial and navigational location, as the first one represents the space were mice are retained during the inter-trial interval, while the second is where reward is collected, therefore they have a strong cognitive connotation. However, this high proportion of PPC's neurons firing at location not entirely related to space and navigation

might be explained by an analysis bias: to compute temporal edges of single runs, we took the two timestamps at which, respectively, mice crossed the border of the start box and the border of the end box and added 2 seconds to each extreme. Therefore, a certain amount of time not strictly spent in exploration was included in each run. The hypothesis of a contamination of data from the inter-trial or foraging intervals seems to be confirmed by the fact that 57 % of neurons positively modulated in either the start box or the end box were overall inhibited during runs compared to the inter-trial interval.

mPFC neurons, instead, were more strongly modulated by the behavioural attribute criterion (23 %) and neurons differentially modulated in success vs error runs were as numerous as neurons differentially modulated in right-turn vs left-turn runs. 46 % of mPFC's neurons were modulated at a specific location within the maze, but only one of them outside of either the start or end boxes. Contamination from neurons activated during the inter-trial interval was lower than for the PPC, interesting 38 % of total mPFC neurons modulated in either one of the two boxes. Interestingly, neurons modulated within the end box were equally distributed among significantly activated and significantly inhibited, suggesting that reward, or absence of reward, processing might require activation of certain neurons and inhibition of others. Indeed, half of the neurons differentially modulated in success vs error runs were also significantly modulated (either activated or inhibited) in the end box. Furthermore, most neurons inhibited in the end box were detected in mice from the Learners group, therefore inhibition at reward location might be heavily implied in correct reward evaluation. Overall, these results confirm our hypothesis that, in the DSA task, the PPC plays a role which is primarily related to navigation, while the mPFC is more cognitively engaged.

We restricted the analysis only to the first day of behavioural protocol because of the choices made at the moment of unit sorting, when we were able to concatenate data from the baseline on D-1 and the totality of the first day of behaviour (4 behavioural sessions and the rest period) in a single file that was sorted as a unique input, assuring consistency of the detection of a particular neuron across different electrophysiological sessions. Data from the two following days of behavioural protocol were excluded from this joint sorting, therefore individuation of single neurons that could be diachronically followed on all days of the protocol requires further attentive refinement steps that have not been performed yet. It would be certainly interesting to assess whether and in which proportion neurons activated during the task undergo a drift in activity saliency, as suggested by other researches (Driscoll et al., 2017; Schoonover et al., 2021), or if the behavioural association are fixed and stable across the whole protocol. What is lacking from the current analysis is also an assessment of the evolution of the firing pattern of single neurons within the first day of protocol, whether, for example, behaviourally relevant neurons reveal their preferential behavioural association from the beginning of if it is refined with runs' progression and, in this latter case, whether it correlates with the progression in acquisition of the behavioural rule. Finally, it would be interesting to assess whether repetitive activation of the same behaviourally relevant neurons on consecutive days would augment their enrichment among SPW-Rs-modulated neurons, which might be interpreted as sign of reconsolidation, or decrease it, possibly

indicating that, once the main phase of consolidation of the behavioural rule has taken place, neurons that represent consolidated behavioural trajectories are no-longer preferred for SPW-Rs-modulated activity.

All of these are intriguing results, however the low number of neurons accessible for this analysis makes very difficult to draw any clear cut conclusion, or even to properly differentiate among the different patterns of activity of the three behavioural groups. Detection of a low number of single neurons is indeed a weakness of the project design, as 16 single wire electrodes do not have the throughput power of silicone probes bearing hundreds of recording sites, but, on the other hand, methods for chronic implantation for recording of freely moving animals of this type of probes are not easy to implement and a boost in this direction have been made only in very recent years (Steinmetz et al., 2021), obliging to a compromise. Furthermore, we can still take more information out from the dataset we have already recorded. In fact, for time-constraint reasons, in the present manuscript only the analysis of single-units was included, while multi-units detected simultaneously were put aside. Kilosort software sorts as multi-units all spiking activity that is considered to be the result of two or more neurons firing in close proximity, in a way that do not allow disentanglement of the individual firing patterns, resulting in patterns of activity that do not display any physiological inter-trial interval. Multi-units can be considered clusters of neurons functionally connected together, thus their analysis might handle some interesting results. We detected almost as much multi-units as single-units during our sorting, thus including them in our investigation might significantly increase the size of the dataset. Furthermore, a good proportion of multi-units was found in the hippocampus, where, instead, single-units' detection rate was low due to project design (only four electrodes were implanted in this region) and to the densely packed nature of this region, which makes disentanglement of the activity of individual neurons more difficult. Analysis of multi-units activity might even allow us to detect multi-units firing tuned to behavioural trajectories in the hippocampus and analyse the participation of behaviourally-relevant hippocampal neurons to SPW-Rs.

Based on our results, we hypothesized that consolidation of the DSA task relies on a network which includes the hippocampus and the medial prefrontal cortex. Previous experiments conducted within the laboratory had already established that preventing AMPA receptors mobility induces SPW-Rs disruption and abolishes consolidation of the rule governing efficient reward collection in the DSA task (El Oussini et al., 2023, **Annexes**). This time, we wanted to block AMPA receptors mobility in the mPFC to test if preventing AMPA receptors-dependent synaptic plasticity in the neocortex we could still disrupt memory consolidation. We hypothesized that either neocortical neurons reactivation leads to new NMDA receptor-dependent LTP induction and expression, or that following synaptic capture in tagged synapses of primed neurons, AMPA receptors mobility would be needed to occupy the new receptor-slots created at the post-synaptic density as a consequence of late structural LTP. As a model

for the experiment, we used knock-in mice expressing both AP-tagged GluA2 subunit and BirA ligase enzyme for biotin fixation chronically bilaterally implanted with guide injection cannulas in the mPFC. Unexpectedly, injection of neutravidin at the end of the first day of protocol did not induce an impairment in behavioural performance on the following day, suggesting that the AMPA receptors mobility-blocking agent had no effect on memory consolidation. A possible explanation is that, even if the mPFC is engaged early on during encoding and engram neurons are already detectable, it does not yet play an active role in memory consolidation, as suggested by the two stage system consolidation theory. Indeed, prevention of structural synaptic plasticity within the mPFC was effective in disrupting memory consolidation in an inhibitory avoidance task 24h but not 8h after conditioning (Goto et al., 2021). We could collect only a very limited amount of electrophysiological data, but, even though we could not find any significant difference in single mPFC neurons' z-scored firing rate around the peak of SPW-Rs between baseline recording and the rest period following the first day of behavioural protocol and neutravidin injection, we observed that the proportion of mPFC neurons significantly activated around the peak of SPW-Rs dropped by half following neutravidin injection. A similar drop was not detected in PPC neurons, that we kept recording as a control neocortical region. This result is certainly preliminary and partial, but it is also very intriguing as, if confirmed, would mean that AMPA receptors-crosslinking does affect the physiology of hippocampo-neocortical communication, possibly preventing synaptic plasticity at hippocampus-to-neocortical synapses. However, this disruption has no effect on recent memory retrieval. It would be interesting to explore if this single manipulation would be effective at preventing memory retrieval at a later point, when hippocampal engrams are generally dismissed in favour of neocortical engrams for remote memory, suggesting that early neocortical plasticity is indeed needed for long-term memory consolidation, or if physiological activation during rest periods at the end of the following days of protocol would be sufficient to induce consolidation of long-term memory, suggesting a model where memories are reconsolidated after each reactivation and different consolidation phase are not regulated by any particular hierarchy.

We are aware that gain or loss of modulation is an indirect measure of synaptic plasticity, especially when the phenomena observed are mostly correlatives and our project design did not allow us to do any manipulation of specific synaptic connections. For this reason, for the future progression of the project we started testing optogenetics methods to exert manipulation or to directly test properties of individual engram neurons and we took a special interest in the CaMPARI and Cal-Light approaches.

CaMPARI is a photo-convertible molecule which switches from emitting green light to emitting red light after concurrent illumination with UV light and raise in Calcium concentration, resulting in a measurable red/green ratio of infected neurons which is proportional to the firing rate of a given neuron during the illumination period (Fosque et al., 2015; Trojanowski et al., 2021). We performed preliminary experiments where we simply illuminated the mPFC during each run (from the moment the mouse exited from the start box to the moment it entered the end box) and we obtained the photo-conversion of a

reasonable number of neurons after illuminating during all four sessions performed on the day 1 of protocol, even though we did not perform any quantification yet. We plan to test the membrane properties of photo-converted and non-photo-converted neurons at rest and in response to stimulation from hippocampal or local projections to collect evidence of strengthened coupling between the hippocampus and the mPFC and within the neocortical engram. This approach is still limited by the survival time of the photo-conversion (24h) and by its proportionality to the firing rate of the neuron, which would mask the contribution from slow-spiking neurons.

Cal-Light is a photo- and Calcium-sensitive cocktail of proteins for induction of transcription in the presence of blue light and Calcium transients (Lee et al., 2017). Again, we only performed preliminary experiments to check if our illumination pattern was effective in inducing reporter gene expression, but we plan to use this strategy to induce the expression of opsins in neocortical engram neurons and manipulate their firing pattern to either strengthen or weaken connections within the local engram network.

CONCLUSIONS

Conclusions

The results here presented are mostly correlative but indirectly sustain a model network for memory consolidation indicating in the hippocampus the master-modulator of other brain areas through the excitatory drive provided by sharp wave-Ripples. This view is widely shared by the scientific community. We could not demonstrate the implication of synaptic plasticity mechanisms for modulation of activity of neocortical neurons, but we will modify the conditions of the experiment in order to test other hypothesis, notably if consolidation takes place at a delayed time-scale in the neocortex. We were expecting the posterior parietal cortex to play a more prominent role in the task, instead our results relegate its role to navigation, with little to no hint of an engagement in cognitive processing. Probably other types of tasks, more associative, will handle more interesting results.

Future experiments will be focused on assessing and manipulating synaptic plasticity in neocortical engram neurons both *in vivo*, with the AP-GluA2 KI strategy, and *in vitro*, through CaMPARI and Cal-Light optogenetics strategies.

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ANNEXES

CA3 hippocampal synaptic plasticity supports ripple physiology during memory consolidation.

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Abstract

Consolidation of recent memory depends on hippocampal activities during resting periods that immediately follows the memory encoding. There, Slow Wave Sleep phases appear as privileged periods for memory consolidation as hosting the ripple activities, some fast oscillations generated within the hippocampus and that inactivation leads to memory impairment. If a strong correlation exists between these replays of recent experience and the persistence of behavioural adaptations, the mobilisation, the localization and the importance of synaptic plasticity events in this process is largely unknown. To question this issue, we used cell-surface AMPAR immobilisation to block post-synaptic LTP within the hippocampal region at various steps of the memory process. 1- Our results show that hippocampal synaptic plasticity is engaged during the consolidation but is dispensable during the encoding or recall of a working memory based spatial memory task. 2- Blockade of plasticity during sleep leads to apparent forgetting of the encoded rule. 3- *In vivo* recordings of ripples activities during resting periods show a strong impact of AMPAR immobilization solely, prominent when a rule has been recently encoded. 4- *In situ* examination of the interplay between AMPAR mobility, hippocampal plasticity and spontaneous ripple activities pointed that post-synaptic plasticity at CA3-CA3 recurrent synapses support ripple generation. As crucial results were reproduced using another AMPAR blockade strategy, we propose that after rule encoding, post-synaptic AMPAR mobility at CA3 recurrent synapses support the generation of ripples necessary for rule consolidation.

Introduction

The importance of activity-dependent synaptic plasticity (SP) in the memorization process is generally admitted. This statement is supported by multiple studies reporting the behavioural impact of pharmacological treatments targeting SP-related molecular mechanisms, SP-related genetic inactivation, or more recently molecular approaches specifically affecting long term potentiation¹⁻³. The examination of the physiological consequences of these inactivation strategies in living animals generally point to alterations of behavioural performance at testing time^{2,3}, an impact that however depend on the type of memory and the extent of molecular manipulations onto one or more implicated brain regions². Recent reports also challenged the link existing between neurobiological consequences of synaptic plasticity blockade and animal performances. For example, Kaganovsky and colleagues recently proposed that blocking synaptic plasticity in the CA1 region of the hippocampus did not impacted animal performance in a variety of behavioural tests, even though some cellular and network proxies for learning were affected in the hippocampus². This may result from functional redundancy between brain regions to achieve cognitive robustness, but it also certainly complexify the interpretation of the impact of these SP interference methods on memory.

One intriguing possibility would be that the process of memory that includes multiple steps – encoding, consolidation and retrieval – would correspond to sequential steps of synaptic plasticity formation and maintenance^{4,5}. There, Hebbian synaptic tagging would be the immediate response to coincident neuronal activities supporting rapid adaptation of animal behaviour in response to new situations. Then synaptic capture, necessary for the maintenance of the plasticity, would occur during quiescent - awake or sleeping - states, allowing consolidation of a memory that can be retrieved after hands. In this line, the importance of hippocampal ripples appears as central. Ripples intrinsically contain both the prerequisite for synaptic capture, namely a capacity in replaying behaviourally relevant spatial sequences encoded during the awake state⁶, but also to allow broadcasting of these information enabling expression of plasticity related molecules important for synaptic capture⁴. Recent findings confirmed that ripple content depends on recently acquired memories⁷, reactivating neuronal ensembles in cortices, such as those implicated in the running of specific rules⁸.

Physiologically, hippocampal ripples are short network oscillations at 180-250 Hz corresponding to synchronized neuronal activation generating synaptic waves that can be evidenced if recording in the dendritic fields – *i.e. stratum radiatum*- of hippocampal CA3 and CA1 regions⁶. Interestingly, ripples can reach cortical regions - through direct or indirect projections - where they synchronize with other sleep related oscillations, such as spindle and delta waves⁶, a process that is reinforced by newly encoded learning⁹. In the hippocampus, ripples are generated in structures – such as in hippocampal

CA3 region – rich in recurrent connectivity and depends both on excitatory and inhibitory local inputs that constitute the feedback loops necessary for fast oscillations building¹⁰. As such, impairing¹¹ or prolonging⁷ ripples can be achieved by manipulating specific interneuron populations .

Even if *in situ* preparations do allow spontaneous oscillations that share numerous characteristics with ripples recorded *in vivo*^{10,12,13}, the relation between synaptic plasticity and ripple physiology has not yet been explored specifically *in situ* by using methods avoiding confounding factors such as effects on basal glutamatergic transmission¹. We here used two alternative strategies to abolish synaptic plasticity depending on AMPAR mobility (AMPARM): the first, based on multivalent IGGs directed against the GluA2 subunit of AMPAR has been proved to be efficient in blocking LTP at Schaeffer collaterals to CA1 synapses¹⁴. Similarly, blocking AMPARM using biotinylation of GluA2 subunit and presence of tetravalent neutravidin in the external medium did not affect basal transmission but led to a complete absence of LTP¹⁵. We thus used these strategies in the dorsal hippocampus to explore the link between synaptic plasticity, ripple physiology and learning and memory processing.

Results

AMPAR immobilization in the dorsal hippocampus impairs memory consolidation

Based on our previous reports showing that AMPAR immobilization at neuronal surface was efficiently blocking post-synaptic expression of hippocampal long-term potentiation, we used intra-hippocampal infusions of AMPAR cross-linkers to test for the implication of synaptic plasticity in the memorization process of a spatial task. For this, mice were cannulated bilaterally above the dorsal hippocampus, and trained for working memory-based delayed spatial alternation task (DSA¹⁶). In this task, food deprived mice are learned to find food rewards in a Y-maze according to a simple rule: reward location is alternating between right/left ending arms (**Figure 1a**). A delay of 30 seconds between consecutive runs is imposed, forcing the animals to remember the previous location before engaging in the following run. In control conditions, a training day of 4 sessions - about 40 trials or reward positions - is sufficient for the animals to decrease its number of errors and reach its maximal performance which is maintained the following days (**Figure 1d**). To mediate AMPAR immobilization in the dorsal hippocampus, we performed bilateral, intra-cerebral injections of AMPAR cross-linkers (anti-GluA2 IgGs) or their controls (anti GluA2 monovalent Fabs) at key timings of the learning process (**Figure 1b-c**): Immediately before the first learning session of day 1 (Pre-learning), immediately after the end of the first training day (Pre-rest), and immediately before the first session of day 2 (Pre-test). Our aim was to test the importance of hippocampal AMPARM-dependent plasticity in the encoding, the consolidation and the recall of DSA rule respectively. Collectively, results pointed to an impact of

AMPA cross-linking onto memory consolidation: Indeed, pre-learning injections of AMPAR cross-linkers did not impact animal performance on day 1 (**Figure 1d and 1e_{left}**), but rather on the following day, choices returning to random level (**Figure 1d_{left} and 1e_{right}**). A similar effect was observed when injections were performed immediately after session#4 (**Figure 1d_{middle}, and 1f**), but not if done before the test in day2 (before session session#5, **Figure 1d_{right} and 1g**). This last experiment indicates that memory retrieval was not impacted by AMPAR cross-linking, but more importantly that crucial AMPARM-dependent process occurs during resting period that support memory consolidation.

An important question to address is the origin of the lack of performance observed in day 2 in pre-learning and pre-rest injected animals. Indeed, an increase in the number of errors can have various origins such as disorientation, disengagement, bad animal state, up to the complete forgetting of the alternation rule. To further dissociate between these options, we thoroughly analysed animal behaviour along the DSA rule acquisition (**Figure 2**). As other groups using T and/or Y mazes to test for mice cognitive abilities^{16,17}, we noticed that runs can be separated in two groups: those in which the animals were running in the maze with almost constant speed and those in which hesitation can be observed at the crossing point, with significant changes in head orientation and speed, called Vicarious Trial and Error runs or VTE runs (**Figure 2a**) that are predictive for good choice accuracy, as testified by the difference in the error rates in VTE and non VTE runs (**Figure 2a_{right}**). Interestingly, probably because no rule was clear initially, animals exhibited more no-VTE runs at the beginning of the learning day (**Figure 2b**).

Next, to get insights in the origin of the performance loss of pre-learning treated animals, we examined the occurrence and choice quality of VTE and no-VTE runs in session#5 (**Figure 2b-e**): Surprisingly, the lack of performance of pre-learning IgG-injected animals was associated with reinstatement of initial values, suggesting an apparent DSA rule amnesia (**Figure 2c-e**): **i**) in IgG-treated mice, the occurrence of no-VTE runs in session#5 was similar to its initial session#1 value (**Figure 2b,c**), as if the animal had to re-establish a rule, rather than being unable to apply the beforehand encoded one; **ii**) the error rate of VTE runs in session#5 also increased, returning to the level observed in session#1 (**Figure 2d**), pointing that when wanting to apply the rule, animals performance was as when encountering it initially (**Figure 2e_{bottom}**) and **iii**) Whereas with training the no-VTE runs performance improved and progressively diverge from random choices, they also returned back to initial values in session#5 of IgG-injected mice (**Figure 2e_{top}**). Importantly, in day1, all these parameters evolved similarly between IgG- and control-injected animals, suggesting that DSA rule encoding process is ongoing normally in absence of AMPARM (**Figure 2b-e and Supplementary Figure 1**). Thus, we propose that a hippocampal AMPARM-dependent mechanism is at play during post-training resting periods that support memory consolidation, and that dHPC AMPAR immobilization leads to a total forgetting of the acquired DSA rule rather than to an incorrectly encoded or played DSA rule.

AMPA immobilization in the dorsal hippocampus impairs ripple physiology during slow wave sleep.

Hippocampal ripples are fast oscillations that are developing during slow wave sleep (SWS) phase and that are considered as offline replays of immediately preceding experiences^{6,8,18}. They are generated in CA2/CA3 regions of the hippocampus, and propagate in CA1 before broadcasting to cortical regions^{9,19}. Interestingly, their interplay with immediately preceding synaptic tagging is unknown, even if specific downscaling and NMDAR-dependent synapse refinement have been reported in *in situ* preparations¹¹.

Thus, we wanted to examine the impact of IgG treatment and DSA learning on dHPC ripples (**Figure 3**). To achieve that, animals were implanted bilaterally with wire bundles medially to injection cannula (**Supplemental Figure 2a**). dHPC Local Field Potentials (LFPs) were recorded for 3 hours immediately following Y maze habituation (“habituation” in Day-1 or D-1) or testing sessions (“DSA” in Day1 or D1, **Figure 3**). At first, we separated awake and resting/sleeping state in the home cage using animal tracking (mobility, **Figure 3_{top}** and **Supplemental Figure 2c**). Then, Slow Wave Sleep (SWS) and Rapid Eye Movement (REM) sleeping phases were separated using a Theta/Delta ratio defined on hippocampal LFP spectra (**Figure 3_{middle}** and **Supplemental Figure 2c**), REM periods hosting robust theta oscillations absent in SWS periods, that are characterized by pronounced Delta waves (for a typical example see **Supplemental Figure 2c**). Importantly, as expected, SWS periods correlate nicely with the occurrence of hippocampal ripples (see methods, **Figure 3_{bottom}** and **Supplemental Figure 2c**).

Then we tested if the DSA protocol and AMPAR cross-linking were leading to alterations of ripple frequency and amplitudes (**Figure 3c-f**). At first, we tested if our recordings were stable over time: indeed, and not surprisingly, neither the amplitude nor the frequency of detected ripples differ between two basal consecutive days (Controls D-2 and D-1 recordings, **Figure 3c**). Some reports described that spatial learning or retrieval was leading to increases in dHPC ripple frequency²⁰. However, no noticeable changes in recorded ripples were observed in animals submitted to DSA learning, and injected with control constructs or non-injected (see specific mentions in **Figure 3d**). We next tested if the blockade of AMPAR in the dHPC was perturbing ripple physiology in DSA trained animals (**Figure 3e**) or non-trained mice (**Figure 3f**). Importantly, in both cases, as for control injections, IgG injections were done several hours before the recorded resting periods, the only difference being that non-trained animals are solely positioned in the maze for a similar time duration, but with no specific rule to learn (see methods). Surprisingly, we detected a significant impact of IgG injections on ripple amplitude and frequency that were significantly decreased, but only when learning was present (compare **Figure 3e** and **3f**). Because ripple inactivation during SWS has been proved to impair spatial memory consolidation²¹, this decrease in ripple content may explain the lack of consolidation observed in pre-learning IgG injected animals (**Figure 1d**). Importantly, we did not detect any change in the total

time of SWS between groups in D1 within the first three hours of resting period (data not shown). Importantly, this early SWS phase has been shown to elicit ripples that are of particular importance for recent memory consolidation¹⁸. Thus, our data support that in the dHPC, DSA-consolidation mobilize AMPARM-dependent plasticity events that support the genesis and strength of hippocampal ripples.

AMPARM-dependent plasticity at CA3 recurrent support ripple activity *in situ*.

Most of what we know about cellular and synaptic contribution to ripple physiology comes from acute *in situ* preparations in which ripple like oscillations are spontaneously generated^{10,12,13}. If some aspects of *in vivo* ripples are obviously lacking, such as their cognitive content⁶, they recapitulated most of the *in vivo* ripple properties and keep some link with *in vivo* experience¹¹⁶.

We setup and used *in situ* hippocampal preparations exhibiting spontaneously ripples and combined them with synaptic “tagging” by inducing LTP at CA3→CA1, CA3→CA3 and DG→CA3 synaptic contacts with or without presence of AMPAR cross-linkers (**Figures 4 and 5**). Among the addressed questions is the interplay between AMPARM-dependent plasticity and ripple physiology to get insights on our *in vivo* results. In optimized *in situ* preparations¹³, ripple-like activities – here called SPW-Rs – can be stably and robustly recorded using field recording pipettes positioned in the CA3 and CA1 regions (**Figure 4a-e**). To be included, SPW-Rs recordings has to have stable occurrence frequency, showing co-detected CA3 and CA1 events, present a constant delay between CA3 (first) and CA1 (delayed) responses (**Figure 4c_{middle}**), and have a good amplitude matching between both signals (**Figure 4b and 4c_{right}**). Some other criteria were eventually respected when present: **i**) the signal polarity in the CA1 region was depending on the recording location: positive in the *stratum pyramidale*, and negative in the *stratum radiatum*, confirming that incoming CA3 activities were generating a significant synaptic field response in CA1 (**Supplementary Figure 3a**), **ii**) both evoked and spontaneous SPW-Rs eventually engaged CA3 unitary activities (**Supplementary Figure 3b**), **iii**) when tested, stimulations in the CA1 *stratum radiatum* that generated SPW-Rs were interfering with spontaneous SPW-Rs, generating a refractory period (**Supplementary Figure 3c**). Importantly, after a 20 minutes’ period in the recording chamber, all parameters were stable for more than an hour, allowing the combination of SPW-Rs recordings with HFS application and/or pharmacological manipulations (**Figure 4 and 5**).

We previously showed that AMPAR immobilisation at neuronal surface in the CA1 region impaired LTP expression at CA3→CA1 synapses¹⁴. We first wanted to reproduce and extend this finding to other synapses eliciting post-synaptic LTP expression. Interestingly, in CA3 region, pyramidal neurons receive two major excitatory afferent that are intrinsically different: Mossy fibres originating in the dentate gyrus generate “detonating” synapses expressing a huge rate-dependent facilitation that can be prolonged by a sustained potentiation of presynaptic origin (presynaptic release probability increase²²). In contrast, recurrent synapses emitted by distant or neighbouring CA3 pyramidal cells are

classical Hebbian synapses, expressing post-synaptic LTP²². We thus tested the impact of AMPAR cross-linking on LTP in our *in-situ* “SPW-Rs” preparation (**Supplementary Figure 4**). Not surprisingly, we observed that AMPAR X-linking led to an absence of LTP at synapses postsynaptic to CA3 axons (CA3→CA1 and CA3→CA3) but not at DG→CA3 projections, that were solely affected by PKA blockade using Rp-cAMP preincubations (**Supplementary Figure 4**).

In the absence of learning-associated synaptic tagging, AMPAR immobilization did not lead to changes in ripple frequency or amplitude (**Figure 3f**). Acute slice preparations from naive mice are often used as models to study molecular and cellular mechanisms of LTP induction and expression at hippocampal synapses²³. It is then often considered that in naive mice, no significant synaptic tagging – endogenously triggered LTP - is present. We thus tested the effect of AMPAR cross-linking in SPW-Rs containing naive preparations by locally infusing anti-GluA2 IgGs in CA1 or CA3 *stratum radiata* (**Figure 4f-i**). Importantly, the efficacy of this injection procedure on LTP expression was previously validated in CA1¹⁴, and reiterated here in CA1 and CA3 regions (**Supplementary Figure 4**). As compared to basal conditions, these injections had no effect on SPW-Rs frequency or amplitude (**Figure 4f-i**), the local effect of IgG injection on amplitude being attributable to the one/two pipette(s) procedure (**Supplementary Figure 5**). Thus, in great coherence with the absence of AMPAR immobilization on basal synaptic transmission¹⁴, and *in vivo* results obtained in naive mice, our *in-situ* result suggest that basal SPW-Rs did not depend on AMPARM in absence of specific synaptic tagging.

Next, we wanted to test for the effects of synaptic tagging – here generated by HFS applications enabling LTP induction (**Supplementary Figure 4**) – on SPW-Rs frequency (**Figure 5**). Interestingly, CA1 HFS stimulations did not impacted SPW-Rs frequency or amplitude (**Figure 5a, c, d**), indicating that synaptic strength at CA3→CA1 synapses may not be a prominent SPW-Rs frequency determinant. In contrast, the same procedure applied at CA3→CA3 recurrent synapses led to a strong increase in SPW-Rs frequency (**Figure 5b, c**), prominent in case of low basal SPW-Rs frequency (**Figure 5d**). Furthermore, the effect of HFS on synaptic strength and SPW-Rs frequency seems to be temporally disconnected, the increase in evoked EPSP amplitude being detectable as early as in the 0-5 post-tetanic period, whereas the effect on SPW-Rs frequency was not yet detectable (**Figure 5d_{bottom}**). This suggest that the reinforcement of CA3→CA3 recurrent synapses progressively increase CA3 region excitability, more prone to generate ripples.

Finally, we tested the impact of AMPAR immobilization on SPW-Rs modulations by CA3-HFS to test if they would be independent. We applied HFS-CA3 stimulations in SPW-Rs expressing slices in which local CA3 infusions of anti GluA2 IgGs were performed (**Figure 5e-g**). Under AMPAR immobilization, as found for LTP at CA3→CA3 synapses, HFS-associated effect onto SPW-Rs frequency was absent (**Figure 5e-g**), suggesting that their physiology depend on AMPARM-dependent CA3 recurrent synaptic strength. Importantly, a contribution of synaptic potentiation at DG→CA3 synaptic inputs is unlikely,

as we systematically tested for 1Hz frequency facilitation in our evoked synaptic responses (Data not shown), and also because they harbour a form of plasticity that is insensitive to AMPAR cross-linking (**Supplementary Figure 4**). Thus, we would like to propose that AMPARM-dependent LTP at CA3 recurrent synapses positively controls ripple activity *in situ*. Together with *in vivo* data, it suggests that CA3→CA3 synaptic tagging is triggered during DSA learning, allowing ripple-mediated consolidation to occur during consecutive sleep phases.

AMPA mobility in CA3 area is necessary for memory consolidation.

Based on our *in situ* data, we ambitioned to restrict AMPAR cross-linking to the CA3 area to evaluate if impairing plasticity only in this region would be sufficient in impairing memory consolidation and ripple physiology. However, specific antibody-based AMPAR cross-linker strategy lacks of spatial and temporal resolution: Indeed, to maximize efficiency, multiple *in vivo* injections were performed on a dorso-ventral axis to cover most of the dHPC¹⁴. In addition, secondary, unwanted effects of anti-GluA2 antibodies on AMPAR composition²⁴ may be present that can lead to misinterpretation of the data (**see discussion**). Thus, we used a recently developed approach to cross-link endogenous GluA2-containing AMPAR using biotin/streptavidin complexes¹⁵ (**Figure 6a**). In Knock-in mice expressing AP-tagged GluA2 subunits, the presence of an exogenous enzyme – BiRA^{ER}, brought by viral infections – allow the biotinylation of GluA2-containing AMPAR, that can be cross-linked in the presence of tetravalent streptavidin added in the extracellular space (**Figure 6a**). This cross-linking approach that has been validated *in vitro* and *in vivo*¹⁵ and among other advantages, will help in getting improved spatial resolution, as combining viral expression and drug delivery through intracerebral cannula (**Figure 6b**). We tested our capacity in targeting CA3 area by infusing NA-texasRed (red-tagged tetravalent neutravidin through cannula implanted above the CA3 regions of BIRA-expressing mice (**Figure 6c**). Indeed, red labelling was almost completely restricted to the CA3 region, in a subpart of the green expressing region (**Figure 6c**). Then, we tested the capacity of mice in retaining DSA rule in various control and CA3 cross-linking conditions. Because the time course of NA action is not yet known, we privileged pre-rest injections (**Figure 6b**), and GluA2 KI animals being slow in establishing alternating behaviour, we mixed sessions #1-2 and #5-6 to get more robust behavioural outcomes. When compared to session#1-2 – a time point at which no learning is achieved – the error rate of control animals was significantly lower in sessions #5-6 (**Figure 6d**) indicating that encoding and consolidation have been successfully achieved. In contrast, error rates at these two time points remain close to random values in X-linking conditions (**Figure 6d**) a phenotype that is again accompany by an apparent forgetting of the DSA rule, as the accuracy of VTE runs, that improved in control mice, did not show any evolution in the X-linking conditions (**Figure 6e**).

To confirm that the lack of consolidation is associated with impairment of ripples physiology, we combined this novel cross-linking method with dHPC recordings using the same recording methodology as for IgG experiments (**Figure 7**). Ripples occurring during SWS were extracted and counted in habituation (D-1) and after DSA (D1) sessions (**Figure 7**). This last recording starting immediately after drug delivery, it was important to control for unspecific effects of drug actions: importantly, NA application on GFP-only, and saline or mSA delivery on BiRA expressing dHPCs were not leading to changes in ripple frequency, mirroring the lack of effect on DSA consolidation (**Figure 6**). However, NA delivery in BiRA expressing CA3 areas were associated with a pronounced decrease in ripple frequency, remembering the effect observed upon pre-learning and pre-rest IgG injections (**Figure 3**). Thus, by two different approaches, we demonstrated that AMPARM in the CA3 region is necessary for memory consolidation and support ripple physiology during slow wave sleep.

Discussion

In order to understand the link between synaptic plasticity and learning and memory it is essential to analyse separately the various phases of memory encoding, consolidation and retrieval, and to use specific tools that perturb plasticity without affecting basal synaptic transmission. With this in mind, we develop molecular strategies that can be used *in vivo* to address these issues. We tested two different methods to impair postsynaptic long-term potentiation in the dorsal hippocampus, and uncover that during the process of acquiring a spatially guided rule to get food rewards, AMPAR mobility was necessary during the consolidation phase and was an important physiological mechanism that support ripple activity consecutive to new rule encoding. From our *in-situ* experiments, an interplay between AMPARM, LTP at CA3→CA3 recurrent synapses and ripple physiology emerged, suggesting a model (**Figure 8**) in which rule encoding, possibly through synaptic tagging, condition/organize subsequent ripple activity that will develop during rest to consolidate memory. This would require the occurrence of AMPARM-dependent LTP at CA3 recurrent synapses, as the immobilization of AMPAR in CA3 *in vivo* during consolidation leads to a learning-dependent loss of ripple activity and complete forgetting of the acquired rule. Thus, we bring a new mechanism by which synaptic plasticity contribute to learning and memory.

Controls for AMPAR X-linking strategies. Some antibodies against GluA2 subunits have been reported to modify AMPAR composition within several hours²⁴, a time windows that may correspond to the consolidation phase of the DSA rule in case of pre-learning injections. Thus, one can attribute the observed effects onto consolidation to important changes of hippocampal connectivity/activity due to the replacement of GluA2-containing heteromers by GluA1 homomers. However, we observed a very

similar effect on animal performance when intracerebral IgG injections were performed before or after DSA rule encoding (**Figure 1d-f**), a time at which the IgG-dependent changes in AMPAR composition might not have yet occurred. The same reasoning applies to the ripple recordings in **Figure 3**: The application of pre-learning IgG in absence of DSA learning did not impact the network capacity in generating ripples. In fact, the impact of both strategies blocking AMPARM appears to be specific on the actual cross-link capacity (see the various control conditions for both antibody- and neutravidin-based strategies) at the time of offline rule consolidation.

A methodological issue regarding *in vivo* strategies and ripple detection is that local delivery of drugs affects the electrophysiological recordings due to volume injection, an issue that was identified in our *in-situ* experiments (**supplementary Figure 5**). If occurring *in vivo*, a global decrease in LFP amplitude caused by tissue movements would have potentially affect our capacity to detect ripples after injection. At first, we tried to minimize this effect by decreasing the injection speed (see methods) and temporally separate the injection from the recordings (pre-learning injections). We also designed a number of control conditions that would account for this effect: we injected animals with either saline, various monovalent or divalent antibodies, but also with IgG without submitting the animals to DSA learning (**Figure 3**). In all of these conditions, no changes in ripples properties was observed.

Comparison between *in vivo* and *in situ* recordings. Another issue concerns the comparison of SWRs recorded *in vivo* and *in vitro*⁶. Indeed, in our *in vivo* recordings, we essentially characterized changes in frequency and amplitude of oscillations recorded in the CA1 region after a 150-250 Hz band pass filtering (**Figures 3 and 7**), whereas focusing on the occurrence frequency of CA3/CA1 sharp waves *in situ* (SPW-Rs, **Figures 4 and 5**). Sharp-wave are proposed to reflect the dendritic depolarization evoked by the synchronous activity of subgroups of excitatory afferents from the CA3 region, whereas the ripple oscillations are thought to be generated in the CA1 region, in response to the sharp-wave-associated excitatory inputs¹². In the retained *in-situ* recordings, the vast majority of SPW-Rs are co-detected in CA3 and CA1 regions with a significant and stable delay (**Figure 4**). In CA1, a 150-250 Hz band pass filtering of SPW-Rs eventually give rise to a ripple-like oscillation (**supplementary Figure 3a**), but that appears to be highly unstable. Another issue is that in some CA3 *stratum pyramidale* recordings, 150-250 Hz ripples were contaminated by recorded spikes (**supplementary Figure 3c**). Thus, the use of 150-250 Hz ripples *in situ* appears to be less reliable than the associated co-detected waves. *In vivo*, the presence of dendritic responses – waves – is present in some but not all recordings, as the wires implanted wires are separated by 200 μm in the dorso-ventral axis, thus radially to the CA1 region (**supplementary Figure 2a**). However, because waves and ripples are reflecting the same intrinsic network events, we believe that effects observed on *in vivo* ripples and *in situ* sharp waves frequencies can be compared, especially the fact that they share the same lack of sensitivity to AMPAR

X-linking in basal condition, whereas being impaired by the same treatment after DSA learning (*in vivo*, **Figures 3** and **7**) or LTP induction (*in situ*, **Figure 5**).

Synaptic plasticity in memory phases. Surprisingly to us, the effect of AMPAR X-linking on ripple activity is present only if the DSA rule is encoded (**Figure 3**). This suggests that ripple activity is different if salient cognitive events have to be consolidated. One intriguing possibility would be that the learning process directly or indirectly “condition” or “imprint” the hippocampal network, especially the CA3→CA3 recurrent synapses, to drive DSA-related ripples to be efficiently generated offline (**Figure 8**). A likely mechanism by which this could happen is the occurrence of synaptic “tagging” –activity-dependent synaptic plasticity events – during online rule acquisition, that will have to be “captured” during the offline consolidation phase of memory^{4,5}. It has yet been suggested that *in vivo* synaptic modifications would protect these synaptic contacts from ripple-mediated synaptic downscaling during sleep, allowing cognitive map refinement¹¹. Several aspects of our findings need to be discussed regarding this conceptual line, especially the fact that memory encoding seems to occur even if synaptic plasticity is blocked during rule encoding.

Synaptic tagging is thought to rely on cellular and molecular mechanisms associated with synaptic plasticity induction and expression, that includes AMPAR trafficking at the plasma membrane¹. However, we yet reported that AMPAR cross-linking was not impacting LTP induction¹⁴ as NMDA receptors are kept functional. Thus, coincident neuronal activations may have activated transduction cascades necessary for synaptic tagging even if no LTP was expressed.

It remains quite surprising that we did not observe an impact of hippocampal AMPAR cross-linking on DSA rule encoding (**Figure 1d_{left}**). It would suggest that synaptic tagging associated with rule acquisition does not depend on activity-dependent changes in synaptic strength in the hippocampus, a structure that is assumed to bind all components of experience into one unique episodic memory²⁵.

A first hypothesis would be that rule encoding is independent on AMPAR-dependent Hebbian plasticity. Some report yet described that spatial map reorganisation upon rule acquisition can be independent from NMDA-dependent plasticity: Dupret and colleagues²⁶ injected rats with an NMDAR antagonist in order to interfere with their spatial memory. Learning performance was unaffected, but the animals failed to remember the newly-learnt locations, suggesting that the newly-acquired representations of goal locations, that when replayed during sleep predicted memory performance¹⁸, did not stabilize.

A second explanation would be that our cross-linking strategy did not perturb some hippocampal synaptic contacts crucial for rule encoding. Our two strategies target GluA2-containing AMPAR. Therefore, a number of excitatory synapses may have escape from the effect of cross-linking, such as excitatory inputs onto interneurons that can be GluA2 independent²⁷. It is of note because some

pyramidal cell-interneuron coupling changes have been reported during spatial rule encoding²⁸. Thus, to have a complete and comprehensive view of AMPARM impact onto learning-dependent ripple physiology, further experiments using cross-linking strategies targeting GluA1-containing AMPAR, or specific cell types for example using conditional expression of the BiRA under IN or PN promoters will be necessary. In the same line, we observed that DG→CA3 LTP was preserved in presence of anti GluA2 IgG, that would possibly support rule encoding and relay abroad synaptic tagging within the CA3 region. Further *in vivo* experiments using specific blockers of DG→CA3 plasticity, such as Rp-cAMP (**supplementary Figure 4**) would allow deciphering the role of these particular connections in the encoding of the DSA rule in the hippocampus.

Another possible mechanism for this unexpected disconnection between learning ability and dHPC LTP blockade could reside in the resiliency of the system. A recent study using another strategy to block post synaptic LTP showed that even if CA1 plasticity was largely absent, and some of the learning-induced cellular rearrangements were lost, animals were still able to perform correctly².

Alternatively, the rule can first be encoded in another brain region than the dHPC, such as being hosted in the mPFC. For example, Peyrache and colleagues showed that in very similar conditions of a new rule learning in a Y maze, ripple activity was directed towards the reactivation of rule-related neuronal ensembles in the mPFC⁸, opening the possibility that synaptic tagging have been generated there. Indeed, replay of firing patterns in hippocampal neuron ensembles during sleep is thought to cause the gradual formation of stable representations in extra-hippocampal networks by enhancing connectivity between their elements²⁵. Thus, we can anticipate that pre-learning AMPAR X-linking experiments in the mPFC, by interfering with LTP-dependent synaptic tagging during encoding, would impair animal performance. Similar results would be obtained at pre-rest injections, by blocking ripple-mediated generation of DSA rule representations⁸. In our hands, complete pre-learning inactivation of dHPC or mPFC through bilateral pre-learning injections of muscimol was leading to a prominent – above chance level - number of errors linked to stereotyped choices (data not shown). This suggests that both structures are necessary to build the cognitive representation of the DSA rule. Importantly, this phenotype was not observed upon IgG-based pre-learning AMPAR cross-linking in dHPC, supporting that no such major effect on dHPC physiology was associated to the procedure.

Synaptic plasticity and memory maintenance. One interesting observation emerging from our data is the fact that in absence of correct consolidation, the DSA rule is apparently completely forgotten, as if the animal would be completely naive to the task (**Figure 2**). So far, experiment impacting ripples activity during sleep impaired the performance of the animal on the following days, but were not reported to lead to complete resetting, but more slowing down the behavioural performance²⁸. An intriguing possibility would be that in absence of synaptic capture, synaptic tagging would fade away,

bringing the neuronal network in the exact same “naive” state as the previous day, as yet suggested by Norimoto and colleagues¹¹. Interestingly enough, this would open a time window during which newly encoded memory would be accessible to erasure if one can specifically act onto ripple physiology.

Conclusion

This study is bringing a first piece of evidence that consolidation of recently acquired memory depends on AMPARM in the hippocampus. Our result points to the importance of CA3 region in this process. Our results are embedded in the more global framework of the tagging and capture synaptic hypothesis that is now more and more discussed in terms of encoding / consolidation of memory and awake / sleeping state of the animals. The importance of the cortico / hippocampal dialog in this process is of fundamental interest, and the deciphering of its intimate mechanisms will certainly profit from the development and use of *in vivo* applicable molecular strategies interfering with plasticity *in vivo* with good temporal and spatial control.

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Acknowledgment

Figure legends

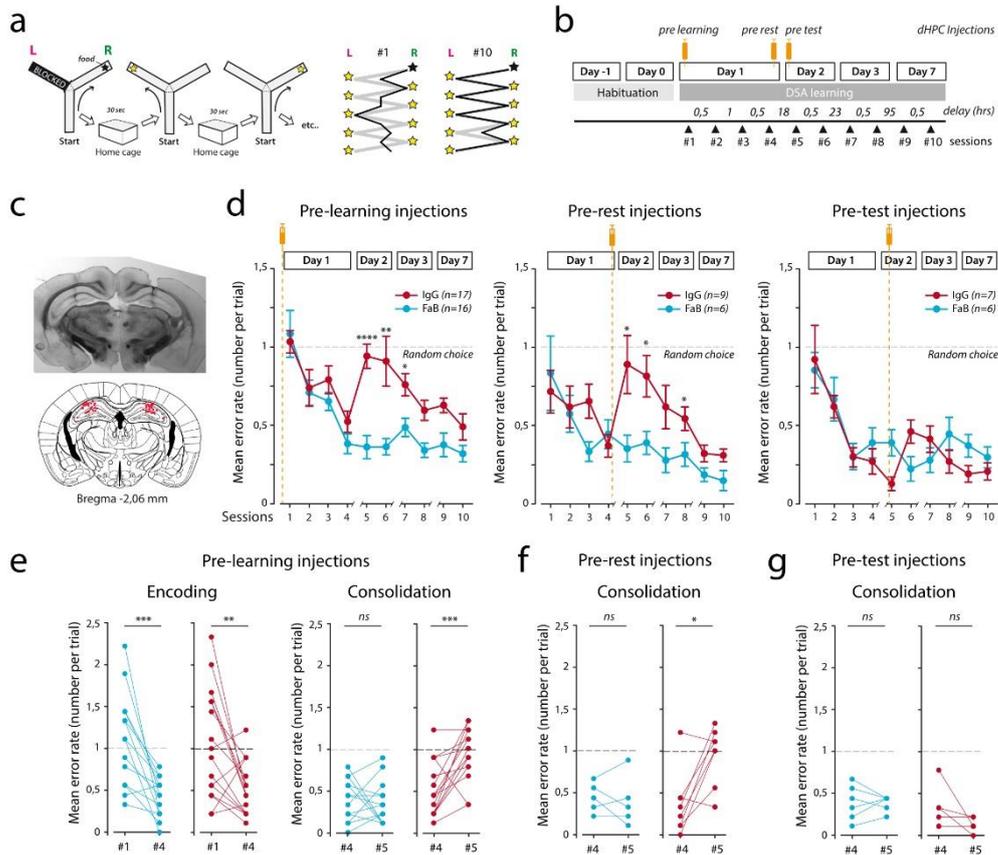


Figure 1

Figure 1: AMPAR surface mobility in the dorsal hippocampus is necessary for consolidation of a delayed spatial alternation rule.

a: schematic of the DSA (Delayed Spatial Alternation) rule that animals have to learn. In each session, after a first forced choice, nine food-rewarded positions are set, alternatively in the right or left ending arm. Up to five error runs are permitted per reward position before the animal is forced to enter in the rewarded arm. In between runs, the animal is positioned in its home cage for 30 seconds. After 10 training sessions (session#1 to session#10) allocated within a week, animals are alternating almost perfectly (right panels).

b: Intracerebral bilateral injections in the dorsal hippocampus were performed at three different time points of the DSA training: pre learning (before session session#1), pre-rest (after session session#4) and pre-test (before session session#5) (syringes).

c: histological description of the injections. Two cannulas were implanted above the dHPC, and anti GluA2-IgGs or control compounds injected (top). In order to cover a large portion of the dHPC, multiple injection points were used in the ventral-dorsal axis (middle). Entry points of the injection cannulas were mostly above the CA1 area (bottom).

d: Behavioural results obtained in the various cohorts expressed as mean error rates. Injections of anti GluA2 bivalent IgGs (red) or monovalent Fabs (blue) were performed before session session#1 (pre-learning, Left), after session session#4 (pre-rest, middle) or before session session#5 (pre-test, Right).

*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. N numbers represent the number of injected animals.

e: Single animal data are shown for crucial behavioural steps. Memory encoding (left) is supposed to be achieved within the first day (after 4 sessions) as the error rate is minimal at session session#4 (Figure 1d). Error rates between sessions session#4 and session#5 are similar in FaB-injected control animals, suggesting optimal memory consolidation. In contrast, Error rate returned to chance level in IgG-injected mice, denoting lack of rule consolidation. *ns*: not significant, **: $p < 0.01$, ***: $p < 0.001$.

f-g: Same presentation as in **e**. Results for consolidation - sessionsession#4 VS session session#5 – in pre-rest (**f**) and pre-test (**g**) injected cohorts are shown. *ns*: not significant, *: $p < 0.05$.

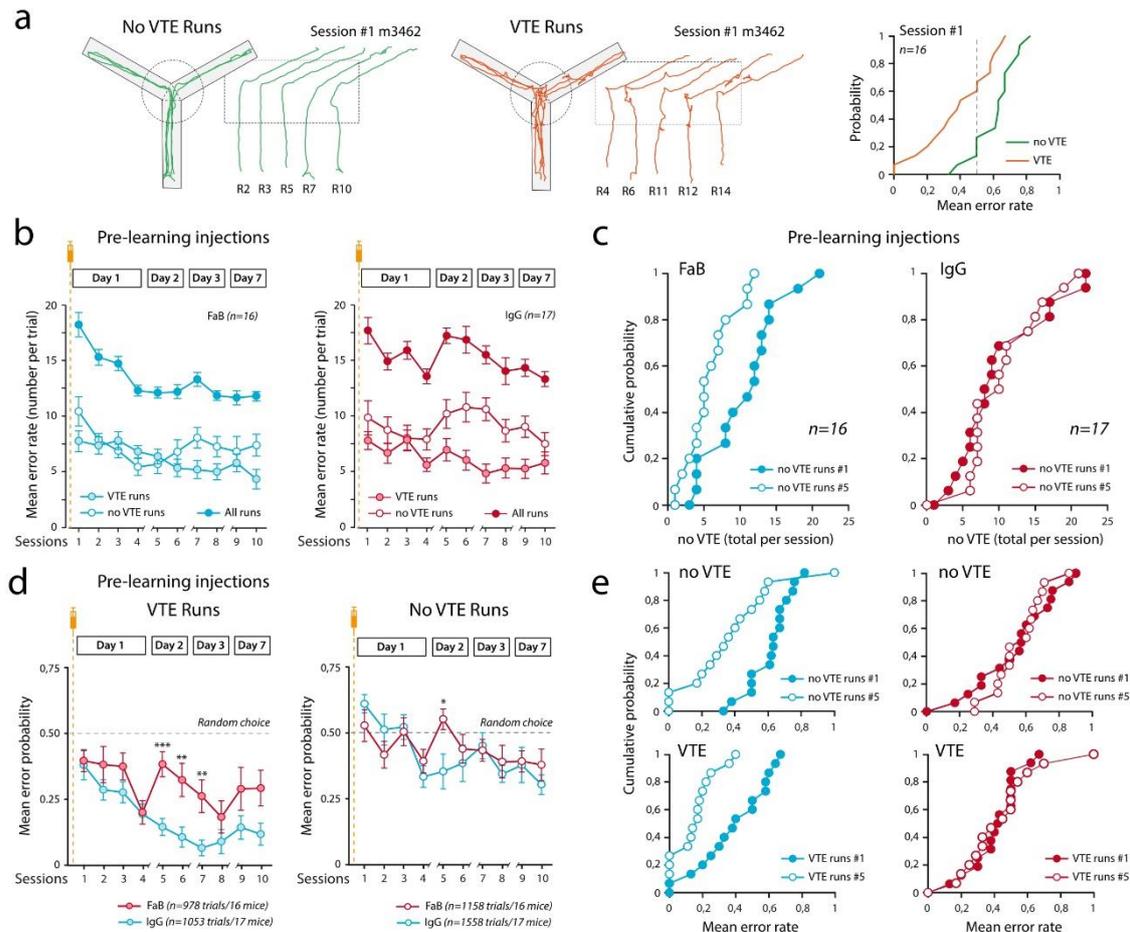


Figure 2

Figure 2: Immobilization of AMPAR in the dorsal hippocampus led to complete forgetting of the acquired DSA rule.

a: DSA runs can be separated in two groups according to animal hesitation in the middle of the maze. As defined by¹⁷, Vicarious Trial and Errors behaviour indicate the cognitive engagement of the mouse in the task.

b: For pre-learning injections cohorts, number of VTE and no VTE runs was analysed along the DSA sessions. Note that upon IgG injections, the number of no VTE runs at session session#5 was similar as in session session#1.

c: Cumulative single animal data for no VTE run numbers in session session#1 and session session#5. Note that curves are superimposable following pre-learning IgG injections.

d: Choice acuteness during VTE and no VTE runs was analysed along the DSA sessions in the pre-learning injection cohorts. The lack of animal performance at session session#5 (see Figure 1d) is associated with a strong decrease in VTE runs accuracy that returned to its initial value in session

session#1. A similar effect is observed for no VTE, with a lesser extent. *: $p < 0.05$, **: $p < 0.01$. ***: $p < 0.001$.

e: Same presentation as in c for choice accuracy at VTE and no VTE run numbers in session session#1 and session session#5. Note that curves are superimposable following pre-learning IgG injections.

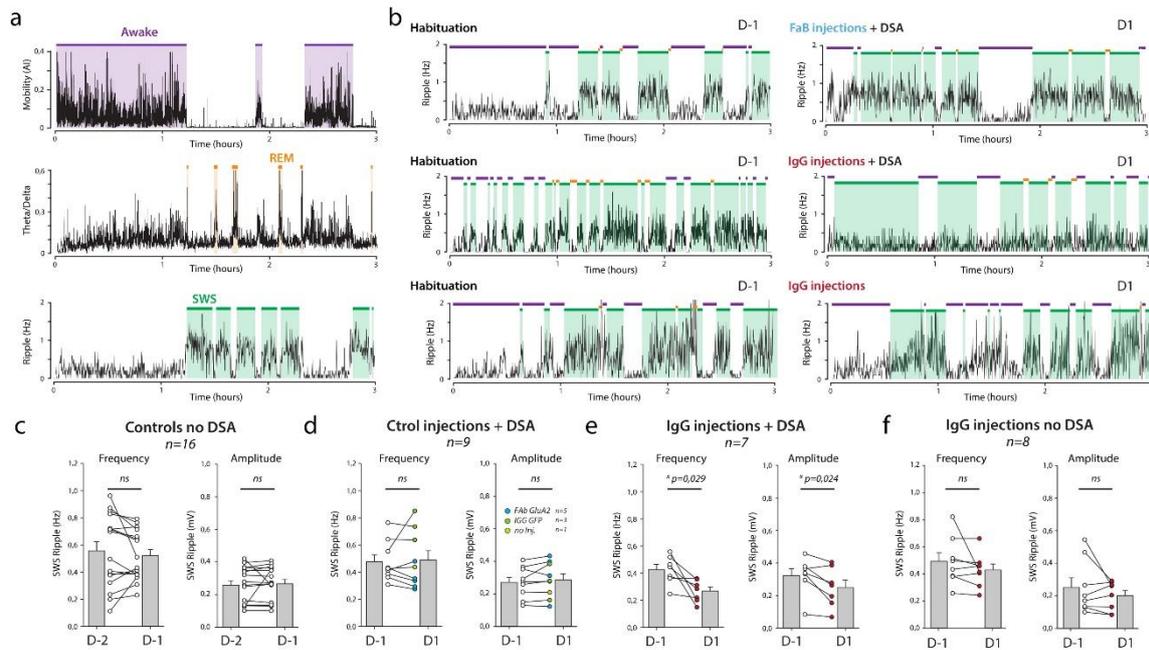


Figure 3

Figure 3: Immobilization of AMPAR in the dorsal hippocampus led to learning-dependent impairment of ripple activities.

a: During resting periods in the home cage, animal tracking and dHPC electrophysiological LFP recordings were performed, to temporally define three animal states: awake state is defined by animal mobility, whereas resting states were separated in REM (Rapid Eye Movement) and SWS (Slow Wave Sleep) according to the Delta/Theta ratio of dHPC LFPs. LFP signals were also filtered at 150-250 Hz to extract ripples, the frequency of which is closely correlated with SWS periods⁶.

b: Bilateral dHPC LFPs were recorded for three hours resting periods before (habituation, D-1) or after DSA encoding (after sessions session#4, D1). Typical examples of ripple frequency in pre-learning FaB (Top) and IgG injected (middle) animals, or in IgG injected animals that were not subjected to DSA learning (bottom). Note the decrease in SWS-ripple frequency in D1 of the IgG injected DSA-trained animal.

c-f: Amplitude and frequency of ripples during SWS periods were extracted in D-1 and D1 resting periods in four different groups: **c:** day to day recordings with no injection nor DSA learning. **d:** before and after control drug injections and DSA learning, **e:** before and after IgG injections and DSA learning, **f:** before and after control IgG injections but no DSA learning. *ns*: not significant, ***: $p < 0.05$. Number of recorded animals is indicated.

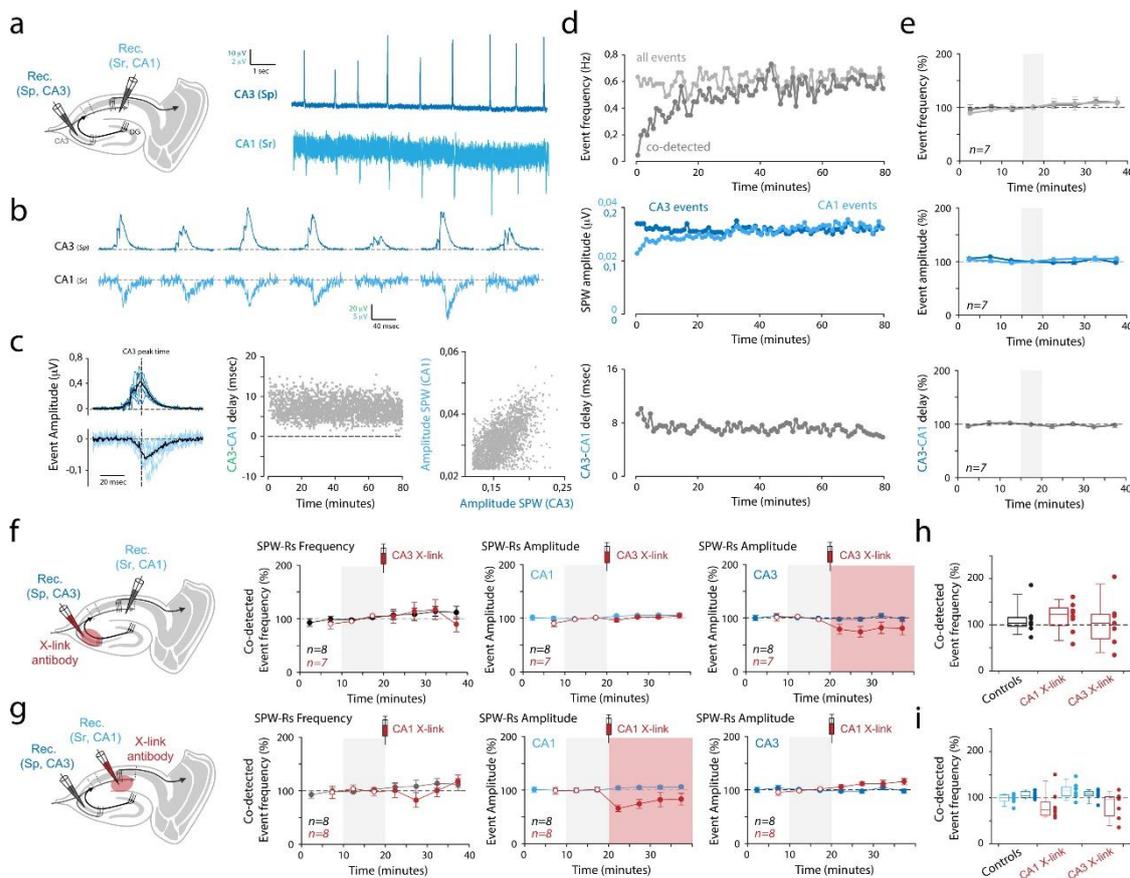


Figure 4

Figure 4: Immobilization of AMPAR in the dorsal hippocampus did not affect spontaneous ripple activities in naive *in situ* preparations.

a: Spontaneous Sharp waves events (SPW-Rs) are recorded in fresh *in situ* hippocampal preparations (see methods) using extracellular field electrodes. **b:** Examples of recorded events. **c:** Simultaneously recorded events showed a significant delay, and correlated amplitudes denoting their propagation from CA3 to the CA1 area.

d-e: Single example (d) and averaged (e, n=7 similar experiments) measures showing the stability of SPW-Rs frequency (Top), amplitudes (middle) and delay (bottom) with time. All pharmacological experiments are starting after respecting a 20 minutes period required for SPW-Rs stabilisation.

f-g: Effect of AMPAR X-linking on spontaneous SPWRs was tested in in situ preparations by pressure injection of anti-GluA2 IgGs. Left: schematic of the experiment. Right: Time course of SPW-Rs frequency (CA3-CA1 co-detected events) and amplitude in CA1 and CA3 regions. The “light red” area indicate that events are recorded in the IgG-injected area. All except red colour indicate time course of the same parameter in control conditions. Open red circles are the IgG preparations before the injections.

h-i: All single experiments and average values 20 minutes in control or after IgG injections. Note that a significant decrease in SPW-R amplitude is observed at the locus of IgG injection (see **supplementary Figure 5**).

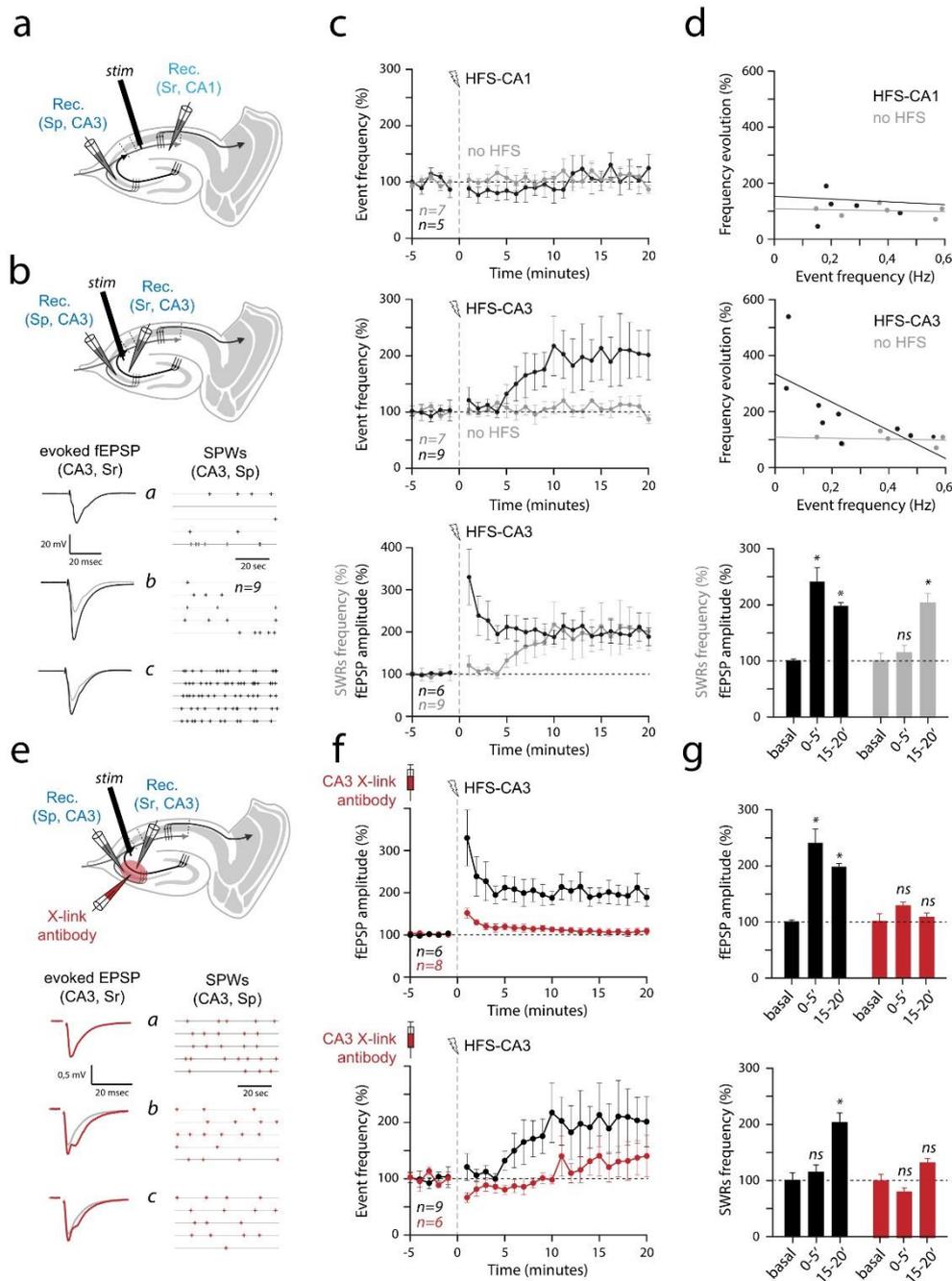


Figure 5

Figure 5: Interplay between plasticity induction, spontaneous SPW-Rs and AMPAR mobility in hippocampus *in situ*.

a: Spontaneous Sharp Waves Ripples were recorded in hippocampal acute slices. After stabilisation, application of HFS in the *stratum radiatum* of CA1 (CA1-sr) was applied to induce LTP at CA3→CA1 synapses (see **Supplementary Figure 4**). No major impact of CA1-HFS onto SPW-Rs frequency or amplitude was observed. Grey zone: time period for baseline calculation; Yellow zone: time after HFS application.

b: Top: same presentation as in **a**, for HFS application in CA3-sr. Note the increase in SPW-Rs frequency and amplitude after HFS application. Bottom: Left: Typical example of the effect of CA3 HFS on evoked CA3 responses, and frequency of spontaneous SPW-Rs. A significant delay exists between synaptic potentiation (evoked f EPSCs, middle; SPW-Rs amplitude, right) and SPW-Rs frequency increase that can be better appreciate in the time courses expressed by minutes.

c: Results presented in **a** and **b** are summarized.

d: Top: the effect of CA3 HFS (black dots) depends on the initial SPW-Rs frequency. SPW-Rs frequency evolution with time did not depend on initial frequency (grey dots). Bottom: A tendency for a co-evolution exist between CA3-HFS effects on SPW-Rs frequency and amplitude.

e: Same presentation as in **b**. Before electrophysiological recordings and CA3-HFS applications, IgG injections were performed in the recorded CA3 region (CA3 X-link antibody). Note that all effect triggered by CA3-HFS in control conditions are absent in presence of the IgG. Number of recordings is indicated.

f-g: Same presentation as in **c** and **d**. The experiments run in presence of AMPAR X-linkers were added.

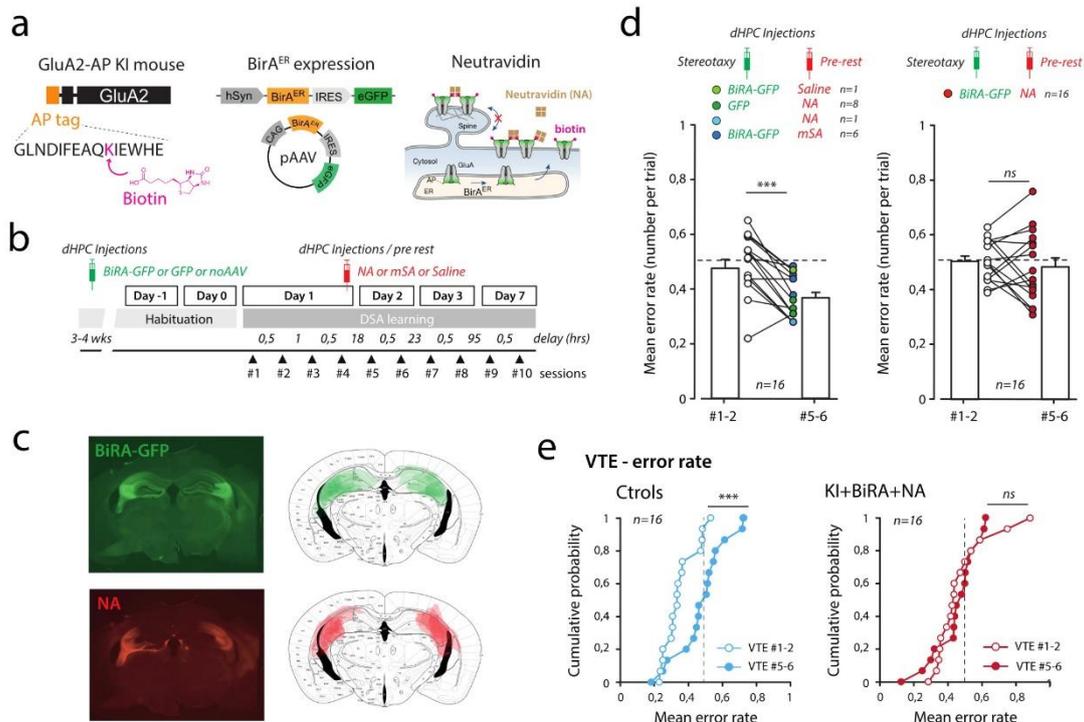


Figure 6

Figure 6: An alternative AMPAR X-linking strategy allowing a better targeting to the CA3 area also induced complete forgetting of DSA rule.

- a:** We recently developed a new strategy for AMPAR X-linking. Knock-in mice expressing endogenous AP-tagged GluA2 AMPAR subunits can be biotinylated in presence of BiRA^{ER}, and once exported to the cell surface can be immobilized in presence of external neutravidin (NA, cross-linking condition).
- b:** similar *in vivo* pharmacological experiments as in **Figure 1d** were performed, combining early stereotaxic dHPC injections of AAV-BiRA-GFP or AAV-GFP, and pre-rest injections of saline, mSA or NA.
- c:** histological controls for the mSA and NA staining on top of the AAV-GFP expression. The combination of both injections better restrict AMPAR immobilization to the CA3 area.
- d:** Mean error rates were compared between session session#1 and session session#5 to evaluate the retention of the DSA rule upon various pharmacological treatments (as indicated by colour coding).
- e:** As in **Figure 2**, we reported the number of no VTE runs that were observed in sessions session#1 and session#5. The amount of no VTE runs was similar between sessions session#1 and session#5 in the KI/BiRA/NA condition (right) pointing for memory forgetting, whereas different in the control group (Left).
- f:** The error rate in VTE runs was analysed and reported in sessions session#1 (Left) and 5 (Right) for control and cross-link groups. Note that they were identical in Session session#1, but diverge significantly in session session#5, suggesting memory impairment in absence of AMPARM.

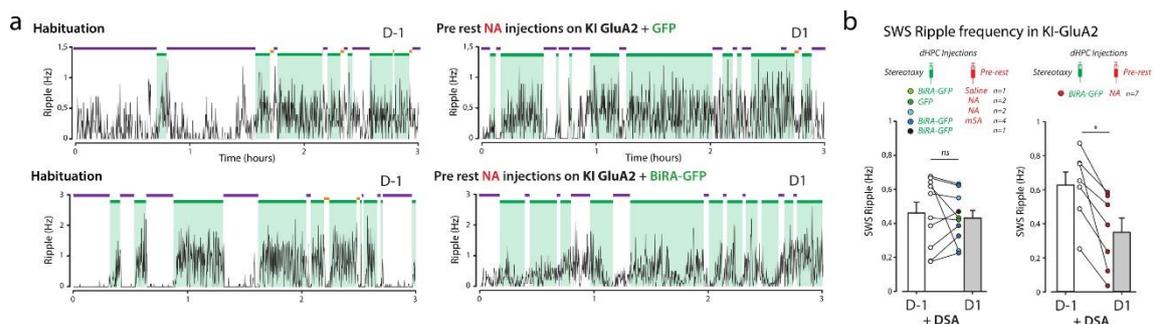


Figure 7

Figure 7: An alternative AMPAR X-linking strategy allowing a better targeting to the CA3 area affected SWS ripple activity.

- a:** Same presentation as in Figure 3b. Bilateral dHPC LFPs were recorded for three hours resting periods before (habituation, D-1) or after DSA encoding (after sessions session#4, D1). Typical examples of ripple frequency in control (Top and bottom) and X-linking (middle) conditions. Note the decrease in SWS-ripple frequency in D1 of the KI-BiRA-NA DSA-trained animal.

b: Same presentation as in Figure 3c. SWS ripple frequency was analyzed and reported for single experiments (dots) and averaged (bars). Conditions and animal numbers are indicated. *ns*: not significant, *: $p < 0.05$.

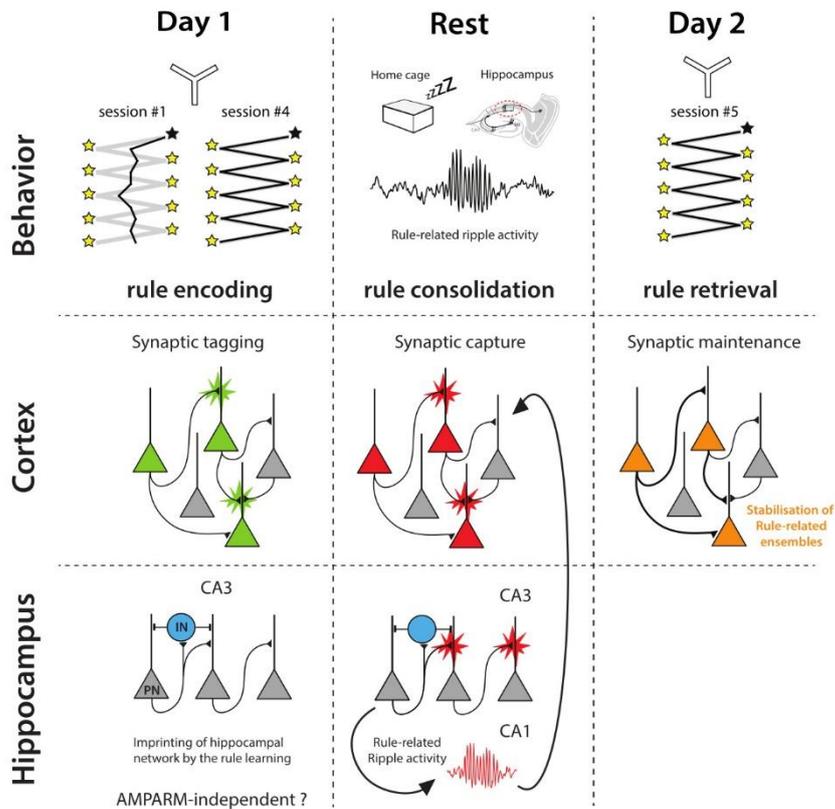
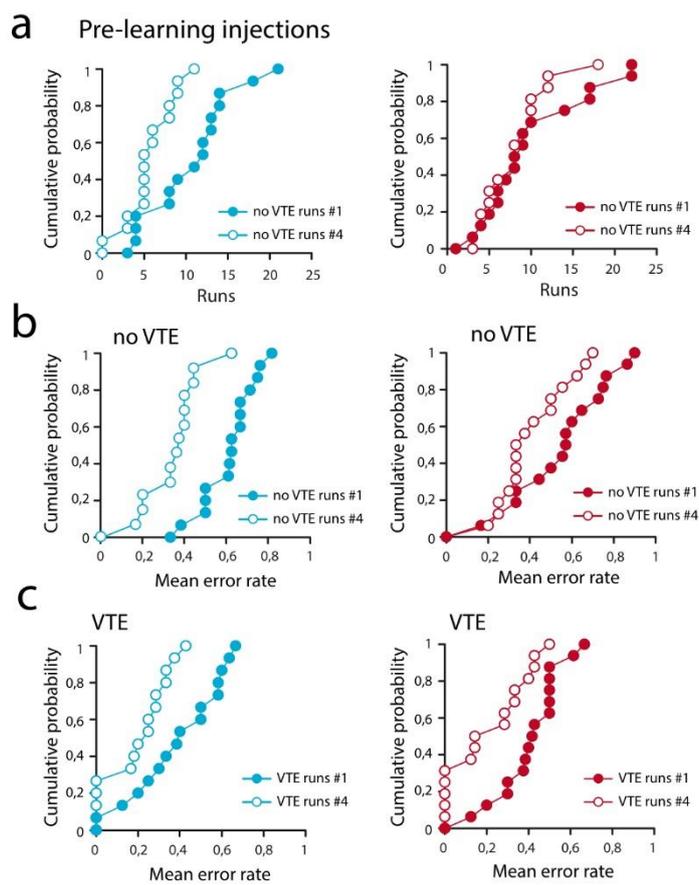


Figure 8

Figure 8: Working model. Model proposed for the action of AMPAR X-linking onto DSA memory consolidation. DSA rule encoding lead to synaptic tagging in the cortical areas, including the mPFC. Hippocampal remapping that is possibly occurring would remain insensitive to GluA2-dependent AMPAR immobilization (EX-IN CA1→CA1 synapses or DG→CA3 synapses). During SWS, ripples necessary for synaptic capture in the cortical areas (through replays-dependent reactivation of neuronal ensembles) would be impaired as plasticity at CA3→CA3 recurrent is impaired.

Supplementary figures



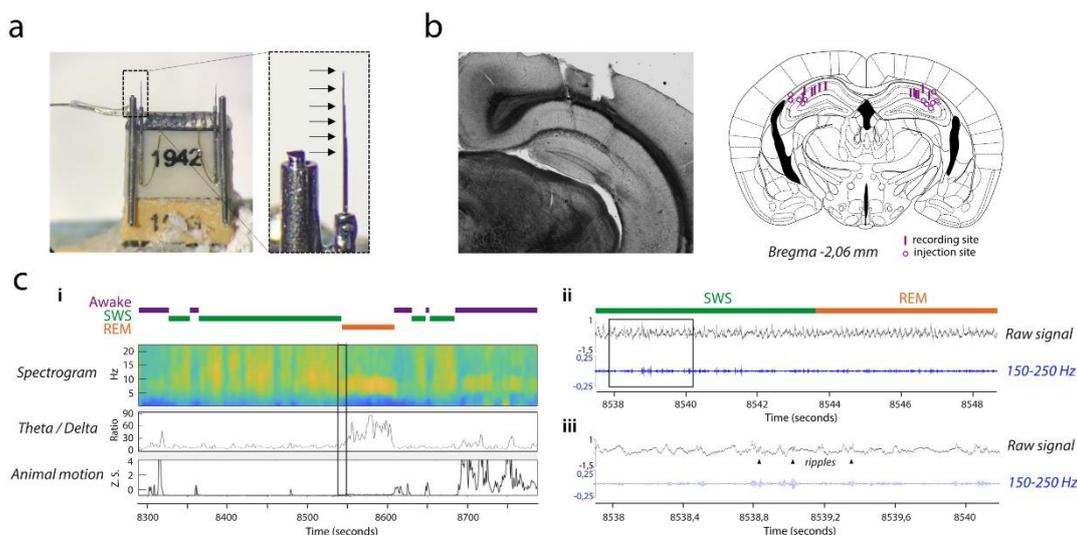
Supplementary Figure 1

Sup. Figure 1: dHPC AMPAR X-linking preserve DSA rule encoding.

Same presentation as Figure 2.

a: Cumulative single animal data for no VTE run numbers in session session#1 and session session#4.

b-c: Choice accuracy at VTE and no VTE run numbers in session session#1 and session session#4.



Supplementary Figure 2

Sup. Figure 2: Combined dHPC electrophysiological and pharmacological experiments.

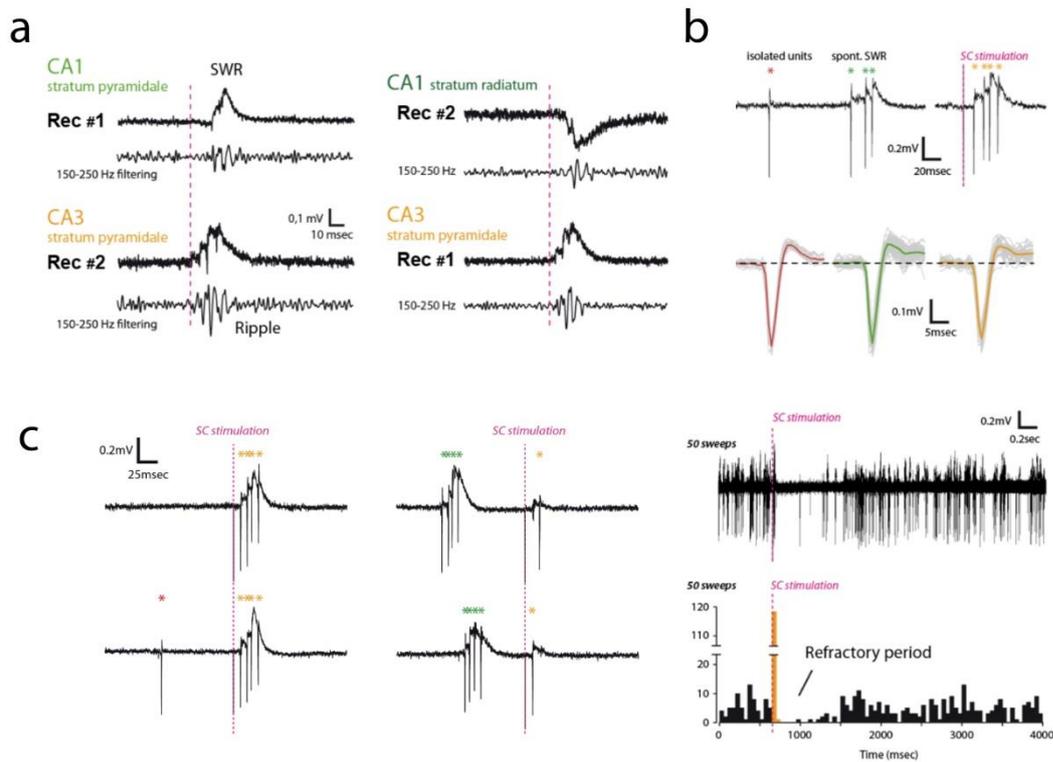
a: photograph of the design implant. Ripple recordings are performed *via* 2x6 wires cut at 200 micrometres intervals in order to target the stratum pyramidale of the CA3 region. Lateral to each bundle, are positioned cannulas that will be used for drug injection.

b: micrograph of coronal slice of implanted mouse brain. Arrows identified the bundle of recording wires, and the tip of the implanted cannula. To perform injection, an injection cannula projects out of 1 millimetre within the hippocampus and is retracted back during the injection in order to cover a large portion of the dHPC.

c: Example of injection of anti-GluA2 antibody that is detected by immunofluorescence (see methods for more details).

d: Analytical pipeline for sleep and ripple detection. Animal tracking (motion) allows separating awake and sleeping states. Within sleep periods, LFP analysis of the Theta/Delta range separate REM (high theta/low delta) and SWS (low theta/high delta) phases.

e: In parallel, LFP are band pass filtered between 150-250 Hz in order to extract ripples oscillations (see methods). A zoom on detected ripples is shown in **f** (triangles).



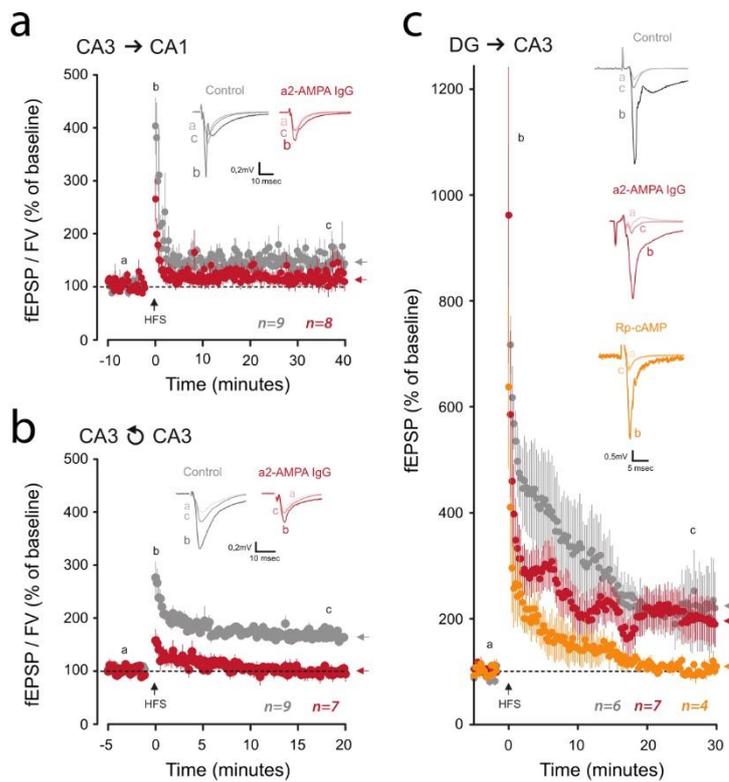
Supplementary Figure 3

Sup. Figure 3: Characteristics of SWRs recorded in hippocampal acute slices.

a: SPW-Rs events recorded in acute hippocampal slice are composed of dendritic synaptic wave that polarity is changed depending on electrode position (upward in CA1-sp (left), downward in CA1-sr (right)). Filtering at 150-250 Hz shows high frequency oscillations (ripples) associated to the wave. Note that a significant delay exists between CA3 SWRs and those recorded in CA1 area, suggesting that they are occurring first in the CA3 region.

b: Typical example of a CA3-sp single unit recording showing that spontaneous or evoked (through CA3 axons stimulations) SWRs are indeed leading to focal neuronal activation. Bottom: waveforms of the single unit in the three conditions (alone, spontaneous SWRs and evoked SWRs).

c: interaction between spontaneous and evoked SWRs. Typical example of refractory period observed for consecutive SWRs that probably limit their activity in acute slices. Left: examples of collision between spontaneous and evoked SWRs. Right: No spontaneous SWR is observed after the generation of an evoked SWR, suggesting that these oscillations may require time-dependent regenerative mechanisms.



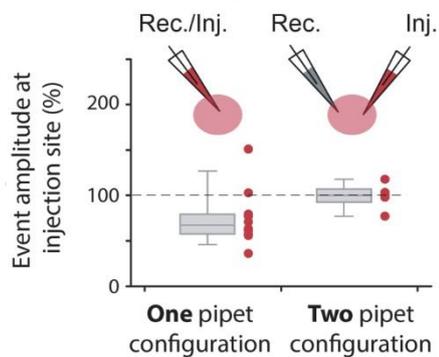
Supplementary Figure 4

Sup. Figure 4: AMPAR X-linking impairs LTP at CA3→CA1, CA3→CA3 but not DG→CA3 synapses.

a: Time course of fEPSP/FV slopes ratio before and after high frequency stimulations (HFS) in control (grey) and AMPAR X-linking conditions (red). Note the absence of potentiation in presence of anti GluA2 IgGs (arrows). In insert, representative traces obtained during baseline (a), immediately after HFS (b) and 35 minutes after HFS (c) in both conditions.

b: Same presentation as in **a**. HFS stimulation was applied within the CA3 *stratum radiatum* to isolate CA3→CA3 recurrent synapses. Note the absence of potentiation in presence of anti GluA2 IgGs (arrows). In insert, representative traces obtained during baseline (a), immediately after HFS (b) and 20 minutes after HFS (c) in both conditions.

c: Same presentation as in **a**. HFS stimulation was applied within the CA3 *stratum lucidum* to isolate DG→CA3 synapses. Note the potentiation that is still present in the anti GluA2 IgGs condition, but not when slice was preincubated with Rp-cAMP to block PKA activity (arrows). In insert, representative traces obtained during baseline (a), immediately after HFS (b) and 20 minutes after HFS (c) in all conditions.



Supplementary Figure 5

Sup. Figure 5: AMPAR X-linking effect on SWR amplitude is due to injection procedure. We compared the local effects of anti-GluA2 antibodies on SWR amplitude in experiments in which it was introduced via the recording pipet (One pipet configuration) or using another pipet than the recording one (two pipet configuration). As can be seen, the decrease in amplitude of SWRs was due to the positive pressure applied in the pipet that probably moved away the tissue locally. Thus we conclude that, as previously observed¹⁴ that AMPAR X-linking was not affecting basal transmission, and thus leave spontaneous SWRs unaffected.

Materials and Methods

10. Biological models

Experiments in this manuscript were conducted on 1,5 to 4 months old male mice belonging to two strains: C57BL6/J wild type and C57BL6/J transgenic AP-GluA2 knock-In (KI, maintained on a C57BL6/J background) strains. Mice were kept on a 12-hour light/dark cycle and provided with *ad libitum* food and water, except for food restriction associated with behavioural testing (see below). Mice were housed with 3-5 littermates except when demanded by the protocol. The experimental design and all procedures were in accordance with the European Guide for the care and use of laboratory animals. All procedures were validated by the ethical committee of animal experimental of Bordeaux

Universities (CE50; PIV-EXPAPAFIS 18507-201901118522837; Animalerie A1 APAFIS 4552 2016031019009163A; Animalerie Magendie puis transfert au PIV 13515- 2018021314415739).

AP-GluA2 KI strain was developed and validated as a mouse model for AMPA receptors mobility in¹⁵. AP-GluA2 KI mice are similar to wild type C57BL6/J mice in terms of weight, size, growth or fertility, but also for tested cognitive abilities¹⁵. At the genetic level, this strain presents a substitution of the endogenous GluA2 subunit of the AMPA receptor by a genetically modified one bearing an AP- (Acceptor Peptide) tag on the extracellular domain of the subunit. In the presence of the BirA ligase enzyme, which is not endogenously expressed, AP can bind Biotin, yet present in the murine brain. Thus, expression of AMPA receptors bearing biotinylated GluA2 subunits is restricted to neurons in which BirA ligase has been introduced by viral transfection, allowing targeting of AMPAR cross-linking. Indeed, presence of extracellular tetrameric Neutravidin consecutive of intracranial administration leads to immobilization of AMPA receptors at the synaptic and peri-synaptic space (see¹⁵).

11. Surgery

Various surgery protocols were performed depending on the aim of the procedure, dividing into two major subgroups: stereotaxic injections and stereotaxic implantations. They eventually shared some common steps, hereby listed. Surgery protocol were similarly applied for both mouse strains.

i-Common surgery procedures

Mice were anaesthetised through exposure to the anaesthetic gas agent Isoflurane (4% mixed with air) for 4 minutes and anaesthesia was maintained all throughout the surgery by isoflurane 2% mixed with air. Mice were positioned in the stereotaxic apparatus (David Kopf Instruments) on a heating pad and received a subcutaneous injection of Buprenorphine (100µl, 0.1mg/Kg) and a local injection of Lidocaine (100µl, 0.4mg/kg) for analgesia. The scalp was rinsed with Betadine to prevent infections. After incision and opening of the scalp, Bregma and Lambda point were identified in order to identify the region of interest using atlas coordinates (Paxinos). Finally, sutures were applied to close the incision point, and mice were subcutaneously injected with analgesic agent (Carprofen, 100µl, 4mg/kg), fed with powdered-nutrient enriched food and left recovering inside a recovery cage positioned on a heating pad for 30 to 120 minutes. A post-surgery care routine was observed for 2-6 days following the surgery, during which the weight and general presentation of mice were monitored and analgesic drugs were administered if needed.

ii-Stereotaxic injection

Stereotaxic injection was performed on mice aged 6-10 weeks. Viruses to be injected were charged inside 1ml graduated glass Hirschman pipets (ref. 960 01 05, Germany) and pressurized via a pressurized via a 5ml syringe (Terumo). The pipette was automatically descended into the target region at a speed of approximately 20µm/s (3 injections sites in the dorsal CA3; coordinates: AP -2.35; ML

± 2.65 ; DV $-2.5/-2.0/-1.5$). Injection of 250nl of the product was performed manually by applying low but constant pressure on the syringe. The pipette was maintained in position within the target region for 5 minutes after the end of the injection to allow local diffusion of the injected product, then retracted at a slow speed. When combined with other surgical procedures, stereotaxic injection always preceded stereotaxic implantation.

iii-Stereotaxic implantation

Stereotaxic implantation was performed on mice aged 6 to 10 weeks. Various types of implants were used, all of which are detailed in the dedicated “Implanted materials” section, but surgical procedures were common to all. Prior to proper implantation, the skull was prepared by briefly – 3 to 5 seconds - applying Peroxidase RED ACTIVATOR (Super-Bond, Sun Medical Co) to remove the periosteum. Single guide cannulas were manually descended into the target region at a speed of approximately $20\mu\text{m/s}$ (coordinates CA1: AP -1.95 ; ML ± 2.25 ; DV -0.55 ; angle 30° ; coordinates CA3: AP -2.35 ; ML ± 2.65 ; DV -1.2). Electrodes were descended into the target region using a micromanipulator at $1\mu\text{m/s}$ for the last third of the descent, to reduce tissue damage caused by the implantation (coordinates: AP -2.35 ; ML ± 2.3 ; DV -1.7 ; glued guide cannulas coordinates: AP -2.35 ; ML ± 2.65 ; DV -1.2). Guide cannulas and electrodes were fixed with dental cement (Super-Bond, Sun Medical Co). After implantation mice were housed alone to prevent implants’ damaging.

12. Implanted materials

i- Guide cannulas

We used stainless steel guide cannulas (Bilaney 26 gauge, 1.5mm of length; PlasticOnes). Prior to the surgery, guide cannulas were kept in alcohol to minimize the risk for bacterial contamination and plugs were maintained on them at all time to avoid penetration of external material. When intracranial injections had to be combined with extracellular field recording, Guides were glued directly on the electrode connector and were obstructed with a metallic dummy cannula to avoid penetration of external material. Intracranial injection was performed on awake mice, either loosely held in the manipulator hands (for short injections) or free to move inside their home-cage. Injections were performed through injection cannulas (Internal Cannula FIS 2.5mm guide, Bilaney; 0.5mm projection) and via an automatic pump (Legato 101, Kd Scientific Inc.) that applied a constant pressure on two $1\mu\text{l}$ Hamilton syringes (7101 KH), allowing the regulation of injection speed (antibodies: 100nl/min ; Neutravidin: 50nl/min). Pre-Learning and pre-test Injections were performed 1 hour before the beginning of the behavioural protocol; pre-rest injections were performed immediately after the last session of the behavioural protocol.

ii-Recording Electrodes

For hippocampal ripples recordings, bundles of Nichrome wires (diameter: 13 μ m, Sandvik Kantal) were connected to a 18 males connector (nano 18 positions 2 guides ISC-DISTREL SA Omnetics) were passed through a guide cannula (see **supplementary figure 2**) to protect them from damage and spreading while entering into the brain.

13. Chemicals

i-Viral vectors

All viral vectors used for the experiments described in the Results section are Adenoviruses and their engineering is detailed in Getz et al. 2021. Ongoing production was assured either by the viral core facility of the Bordeaux Neurocampus IMN or by Charité Universitätsmedizin Berlin or viral vectors were ordered on Addgene. All virus were stocked at -80°C for long-term storage, conserved at 4°C during surgery preparation and injected at room temperature. 250nl per injection site of viral vector solution were administered through stereotaxic injection during surgery.

- **pAAV9a-pSyn-BirA-ER-IRES-eGFP** (5.6 x 10¹³ gcp/ml, IMN). The pSyn promoter allows the expression of the BirA enzyme in all neuronal types without distinction. BirA ligase expression promotes biotinylation of the extracellular portion of the GluA2 subunit of AMPA receptors, thus inducing AMPA receptors cross-linking in the presence of Neutravidin. eGFP is used as a tag to identify neurons expression the enzyme.
- **pAAV9a-pSyn-IRES-eGFP** (1.8 x 10¹³ gcp/ml, IMN). Lack of BirA ligase coding sequence makes this viral vector a control for un-catalysed Biotin binding to GluA2 subunits bearing the AP.

ii-Antibodies

Production and conservation of antibodies used for the experiments detailed in the Results section of this manuscript is detailed in¹⁴. All antibodies were stored at -80°C for long-term storage, conserved at 4°C for maximum 1week preceding injection and injected at room temperature. 500nl per injection site of antibody solution were administered via intracranial injection in the awake, freely moving mouse.

- **Antibody against GluA2 subunit of AMPA receptors (clone: 15F1)** (2.9mg/ml). This antibody is a monoclonal divalent IgG- κ directed against the extracellular domain of GluA2 subunit of AMPA receptors. The divalent nature of this antibody allows for binding of 2 target GluA2 subunits at the same time, therefore promoting AMPA receptors cross-linking. In vitro, a washout time of 8 hours due to internalization of clustered receptors has been observed.
- **Fragment Antigen-Binding (Fab)** (2.9mg/ml). The antigen-binding portion of the antibody directed against GluA2 subunits was isolated and used as monovalent control for cross-linking.

- **Antibody against GFP** (2.9mg/ml). This antibody is a divalent IgG-κ from murine clones 7.1 and 13.1 (11814450001, Roche). As murine neurons do not physiologically synthesize GFP, this antibody was used as control for unspecific antibody binding.

iii-Others

- **Neutravidin or NA.** Texas Red-conjugated Neutravidin (8.33μM; Invitrogen, A2665) was used to operate cross-linking of AMPA receptors in the AP-GluA2 KI mouse model. 500nl per injection site of Neutravidin solution were administered via intracranial injection in the awake, freely moving mouse.
- **monomeric Streptavidin or mSA** was produced and conjugated to STAR 635P (Abberior,ST635P) using N-hydroxysuccinimide ester-activated fluorophore coupling as previously described. 500nl per injection site of mSA solution (concentration: 8.33μM) were administered via intracranial injection in the awake, freely moving mouse.
- **Saline Physiological solution.** Saline physiological solution was used as control for cross-linking in the AP-GluA2 KI mouse model. 500nl per injection site of Saline solution were administered via intracranial injection in the awake, freely moving mouse.

14. Behavioural protocol.

Delayed Spatial Alternation (DSA) task. The DSA task is a delayed non-matching-to-place task used to assess spatial navigation and cognitive functions in rodents¹⁶.

i-Food restriction

Food restriction was required to assure mice's motivation. Mice were weighted right before food withdrawal and this weight was used to calculate the 80% of weight-loss limit that was fixed for protocol termination. On the first day of restriction, mice were fed with Perles pasta of the same type as those that were used to bait the maze during the behavioural task, in order to habituate them to the new food. On subsequent days, mice were fed at the end of all behavioural manipulation with 2-3g of powdered nutrients-enriched food, in order to maintain them to about 85% of their initial weight.

ii-Materials.

A custom-made, semi-transparent white PVC Y maze was used to the task. All three arms are identical (40cm length, 8cm width, 15cm high walls), except for an additional closable rectangular chamber (15cmx25cm) bridged to the "Starting arm". Arms are spaced by a 120° angle. An opaque small container was positioned at the end of each "Goal arm" to serve as food well for reward delivery. Environmental cues are positioned on the room walls surrounding the maze. Video recordings are

realized through an infrared camera (Basler USB camera - ac1920-155um - Noldus) positioned on the ceiling upon the centre of the maze. The DSA tests were realised in conditions of dim light.

iii-behavioural paradigm

- **Habituation.** Habituation lasted for 5-8 days, depending on each individual mouse, and was divided into 3 phases. A first phase, starting before food restriction, consisted in 2-3 days of handling in order to habituate the mouse to be manipulated, especially for injection cannulas insertion and/or electrodes plug-in. Proper habituation for the task started on the second day of food restriction and consisted in multiple sessions of free exploration of the maze. The sessions were stopped when the mouse had eaten food-pellet in all three baited arms. The last phase of habituation consisted in a single trial in which the mouse was positioned inside the “starting arm” (defined by position with respect to environmental visual cues) and had to collect a reward food-pellet in each of the two “goal arms”, with a time-limit of 1 minute.
- **Task.** The DSA task consists in 10 trials in which the left and right goal arms are alternatively baited with a rewarding chocolate pellet. During the first trial, the choice is forced toward the baited arm, setting the pattern of alternation (i.e. the reward-zone of the un-baited arm is made inaccessible through positioning a PVC slide at the entrance of the proximal portion of the arm; each consecutive session alternatively starts with a forced right or left choice). The 9 following trials rely on the mouse free choice of one of the two arms. A single trial can be repeated up to 6 times (“runs”) if the mouse makes consecutive mistakes, of which the sixth consists in a forced run in the baited arm direction. Once the mouse has reached the reward-zone of the chosen arm, access to the reward-zone of the unchosen one is restricted and the mouse is let spontaneously come back to the distal portion of the starting arm. Here, the mouse is collected and put back in his home-cage and a delay of 30 seconds is respected before the mouse is allowed to explore the maze again. During this delay period, the maze is cleaned with ethanol to prevent odour-based navigation. On the first day of training, 4 sessions are conducted, spaced by 30-60 minutes; on the 2 following days, 2 sessions per day are realized, spaced by 30 minutes (see **Figure 1**).

Behavioural training was conducted between 8a.m. and 1p.m.

15. *In vivo* electrophysiological recording

i-Recording sessions Electrophysiological recordings are realized by plugging a headstage (INTAN) containing 16 unity-gain operational amplifiers to each connector. Recordings were realised through the recording system OpenEphys. Recording are performed during resting periods in the mouse home cage positioned in an isolated closed box allowing cables suspension and infrared recordings by the camera (Basler USB camera - ac1920-155um - Noldus). 3 hours long rest sessions were recorded on the day preceding the start of the behavioural protocol (baseline) and at the end of

the first day of behavioural protocol. They started averagely 10 minutes after the end of the last habituation/training session of the day.

ii-LFP processing and Sharp Wave-Ripples detection

Electrophysiological data were imported on Matlab and down-sampled to 1kHz for storage and analysis speed convenience. Ripples detection was performed on Matlab scripts originally developed by Cyril Dejean. At first, referencing and band-pass filtering at 50 Hz eliminates noise oscillations common to all channels. Then, 100-250Hz band-pass filtering is used to detect ripple events that are selected if respecting following criteria: 1) its amplitude is higher than 5 standard deviations of the mean band-passed trace, 2) the event must be at least 30ms long and 3) two ripples must be separated by an interval of at least 45ms. Ripple's characteristics are then computed, including: timestamp of the peak, intrinsic frequency, number of oscillations, mean amplitude (both on the filtered and the integrated trace), area under the integrated curve, duration (total and of each part preceding and following the peak), half prominence.

16. In situ slice recordings

i- Preparation of hippocampal slices

Mice are males WT C57bl6/J mice aged from 4 to 9 weeks. The extracellular ACSF solution (Artificial Cerebro-Spinal Fluid) is composed of: 119 mM NaCl; 2.5mM KCl; 1.3mM MgCl₂; 2.5mM CaCl₂; 10mM glucose; 1mM NaH₂PO₄; 26mM NaHCO₃. The cutting solution is an ice-cold sucrose solution (1-4°C) composed of 2 mM KCl; 2.6mM NaHCO₃; 1.15mM NaH₂PO₄; 10mM glucose; 120mM sucrose; 0.2 mM CaCl₂ and 6 mM MgCl₂. Both solutions are oxygenated with carbogen (95% O₂, 5% CO₂, pH 7.4 at 37°C, 290-310 mOsm/L). For brain removal, mice are anesthetized with 5% isoflurane for 2 min before decapitation. The head is immersed in the iced sucrose solution. The removed brain is immersed for 4 minutes in iced oxygenated sucrose solution and then placed on a cellulose nitrate membrane to separate and position the hemispheres in the vibratome (Leica VT1200s) to obtain 400 µm horizontal slices (speed of 0.1 mm/s). Once produced, slices are semi-immersed in a dedicated incubation chamber, oxygenated and maintained at 35°C in a water bath for at least 2 hours before starting the recordings.

ii- In vitro electrophysiological recordings

Recordings are made in an S-shaped recording chamber, maximizing oxygenation while preventing slice movement caused by the 3.5 mL/min perfusion flow. Field recordings are obtained using glass micro pipettes stretched with a PC-10 (Narishige, Japan) and broken at their tip to decrease the resistance (<0.5 MΩ). Depending on the experimental configuration, the pipette is filled either with ACSF or supplemented with IgG α-GluA2, 15F1 (IgG) or IgG Fab (Fab). Electrophysiological recordings are obtained by a MultiClamp 700B (Molecular Devices, Foster City, CA) using Clampfit software (Molecular Devices, Foster City, CA). Electrical stimulation is provided by a CBCSE75 concentric bipolar

electrode (FHC, Phymep, France) and an external A.M.P.I Iso-flex stimulator (Scop Pro, France). Synaptic field are recorded in the stratum radiata (sr) of CA1 and CA3 to measure evoked fEPSPs, and to test the presence of propagated SPW-Rs. Depending on the configuration, stimulation electrodes are placed in the stratum radiatum of CA1 or CA3 to stimulate respectively CA3→CA1 and CA3→CA3 axons and elicit basal (0,1 Hz) or high frequency stimulations (HFS, 100 Hz, 1 sec train repeated 3 times each 30 sec) to induce long term potentiation (LTP). When stimulating CA3 sr, a train of 10 stimulations at 1 Hz is first applied to test for eventual contamination by DG mossy fibers that display frequency facilitation.

17. Perfusion and Histology

Mice were anaesthetized with a mix of Kétamine and Xylazine (100mg/20mg/kg) diluted in NaCl; 10µl of solution per gram weighted by the animal were administered via peritoneal injection. Perfusion with Paraformaldehyde (PFA, concentration: 4%) was realised on the anaesthetized mouse and brain were initially collected without being evicted from the scalp. After 48h of storage in PFA at 4°C temperature, implants and scalp were removed and the brain was washed three times in PBS solution (concentration: 1%) and then stored in PBS for 24-72 hours at 4°C. Slicing was performed with a vibratome (Leica VT1200s). Coronal slices of 60µm thickness were collected at a speed of 30-50mm/s from the regions of interest and stored in PBS for 24h before being mounted on slides and covered with Fluoromont-G (complemented with DAPI for cellular nuclei staining, Thermofisher Scientific). Image acquisition of slides was performed with an epifluorescence microscope (Nikon Eclipse NI-U) coupled to an illumination system (Intensilight C-HGFI, Nikon) and a camera (Zyla sCMOS, Andor Technology, Oxford Instruments).

Title: Study of the Link between Synaptic Plasticity and Behavioural Adaptation

Abstract: Memory can be defined as the ability to store information within the brain in a way that allows retrieval of such information and promotes behavioural adaptation. At the biological level, memorization is a complex mechanism which demands coordinated brain-wide interactions and is subject to progressive decline upon natural aging. It is a shared idea that understanding the mechanisms regulating formation and storage of memories could help in developing targeted approaches for mnemonic deficiency.

Different theories have been formulated to describe memory consolidation in an integrated system: 1) the engram theory postulates the existence of neuronal assemblies persistently modified by experience and memory consolidation; 2) the synaptic plasticity in memory theory postulates that these persistent modifications rely on synaptic plasticity mechanisms 3) the system consolidation theory postulates that engrams are, in fact, distributed across an interconnected network encompassing multiple brain areas. In each of these theories, memory consolidation is a multi-step process and the precise identification of whether, where, when and how a specific modification is produced inside this complex system is a hot topic in research. For each of these questions collected evidences have directed the focus toward precise lines of research. The hippocampus is considered to be main site of recent memory consolidation and the prefrontal cortex has progressively emerged as an important site for remote consolidation, making the communication between these two areas a main centre of interest. During sleep, the lack of additional experiences favours memory consolidation and the coordinated reactivation of neuronal assemblies of both the hippocampus and neocortical areas during fast-oscillatory hippocampal events called Sharp Wave-Ripples (SPW-Rs) is now taken as the principal hallmark of memory consolidation. At the molecular level, long-term potentiation (LTP) is the most studied mechanisms for the formation of long-lasting memories.

I studied the interplay between the dorsal hippocampus (dHPC) and two neocortical areas in the context of consolidation of the rule for successful completion of a Delayed Spatial Alternation (DSA) task. We aimed to answer the following questions:

- 1) Does the medial Prefrontal cortex (mPFC) and the Posterior Parietal cortex (PPC) participate in the engram sustaining acquisition of this rule? Is their participation equal or are they quantitatively or qualitatively differentially solicited?
- 2) Both areas display signs of hippocampal modulation, in the form of coherent oscillations and coordinated neuronal firing patterns during hippocampal SPW-Rs; how is this modulation modified by consolidation of this rule?
- 3) Does preventing LTP within the neocortex affect consolidation of this rule?

To answer these questions, we used mice implanted with single electrodes in the dHPC, mPFC and PPC to record Local Field Potential (LFP) and single neurons' activity both during the task and a three-hour long rest period at the end of each behavioural training day. We observed that, during behavioural training, PPC neurons were mostly engaged in navigation, while mPFC neurons engaged with more cognitive features of the task. Surprisingly, mPFC neurons, but not PPC neurons, exhibited an increase in positive modulation during hippocampal SPW-Rs following learning, with a high proportion of mPFC's neurons active during the behavioural protocol that were positively modulated around SPW-Rs' peaks during sleep. Last, preliminary data showed that prevention of LTP within the mPFC during the sleep period allocated to memory consolidation does not affect the behavioural performance on the following day, but might affect the modulation of mPFC's neurons around SPW-Rs' peak.

Keywords: Memory, hippocampus, associative cortices, behaviour, electrophysiology

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