Schema Driven Memory Consolidation: A Neurobiological Investigation Using the HexMaze Paradigm

Alejandra Alonso

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Alejandra Rocio Alonso Garrido
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DISSERTATION

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from Radboud University Nijmegen
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according to the decision of the Doctorate Board
to be defended in public on
Tuesday, March 18, 2025
at 10:30 am

by

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born on December 12, 1991
in Puente Alto (Chile)

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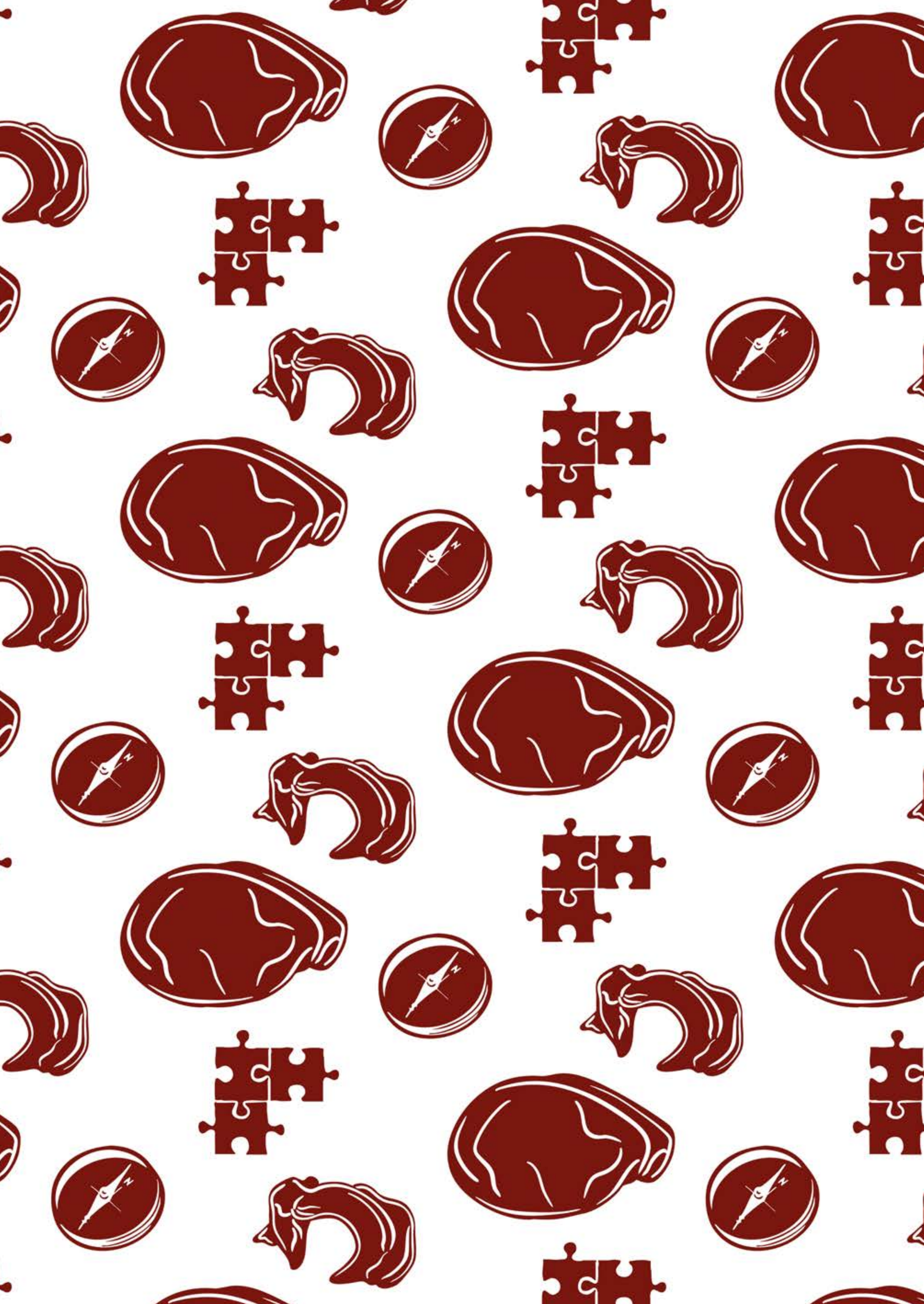
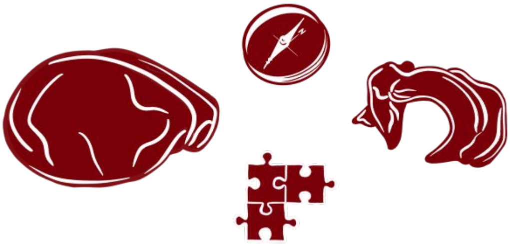
* **Appendices**





Chapter 1

Introduction

The process of memory formation has been long studied, but often a key element was being left out on memory theories: what is already known. Learning new things which are within our knowledge topics, is easier and faster than learning something totally unbeknown to us. **Studies in humans have shown that schemas facilitate the processing of new information, but not exactly how** (Harlow 1949, Bartlett and Bartlett 1995). While this concept has a historical presence in psychology, its exploration in animal models is relatively recent, and prevailing theories on memory consolidation often neglect the role of previous knowledge. In mice particularly, there is a lack of research involving previous knowledge, and the distinct contributions for brain areas different than hippocampus have not yet been investigated in this context.

In this thesis I will investigate **which brain areas are necessary to build and update a previous knowledge network in the context of spatial memory, as well as what task solving strategies are used**. Navigation and memory recall will be tested against inhibition of hippocampal and cortical regions known to be relevant for schemas. The memory substrate, the cells which activate together forming **the memory representation, will be visualized and analysed in different time points of the learning experience**, in an effort to understand better the neurobiology of schemas in memory consolidation.

This introduction will begin with the anatomical properties of the hippocampal formation and the neocortex. Next, the cognitive functions of these key brain regions, learning and memory, as well as navigation. Subsequently, an overview of memory consolidation theories will be presented, culminating in an outline of the overall thesis.

Anatomy

In mammals, the brain comprises five principal structures: the brainstem, cerebellum, cerebral nuclei, hippocampal formation, and neocortex (Fig 1A). This thesis places emphasis on the hippocampal formation and neocortex due to their significance in memory and navigation. The subsequent review will delve into the anatomical properties of these specific brain regions, elucidating their functional implications in the context of memory and navigation, with a particular emphasis in rodent anatomy.

Hippocampal formation

The hippocampal formation, positioned atop the brainstem and beneath the isocortex, faces the cerebral nuclei. As an archicortex, it represents the **phylogenetically oldest cortical area**. Comprising the hippocampal region and entorhinal area (Fig 2), the former encompasses cornu ammonis (CA) and dentate gyrus (DG), while the latter includes the entorhinal cortex and subiculum. Functionally, the entorhinal cortex serves as an interface between the neocortex and the hippocampal formation, facilitating the transfer of highly processed information from prefrontal and association cortices into the hippocampal region (Buzsáki and Moser 2013). Additionally, the hippocampal formation receives inputs from the amygdala, thalamus, septum, raphe, and locus coeruleus (O'Keefe and Nadel 1979).

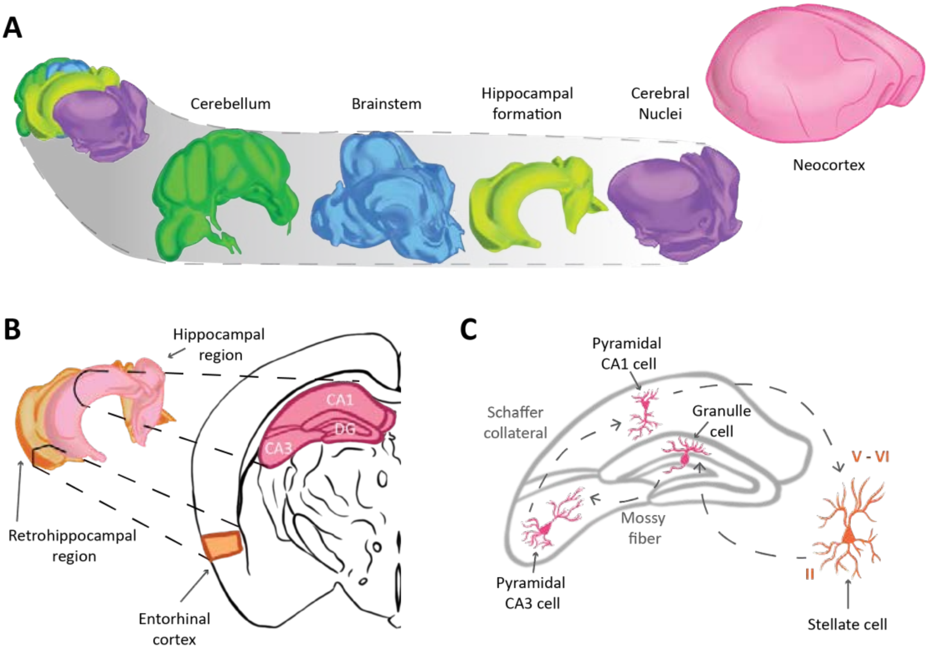


Figure 1 **A** Schematic overview of the mouse brain's key structures. The top-left section illustrates the position of the cerebellum, brainstem, cerebral nuclei, and the hippocampal formation. Individual brain areas are visually isolated below, as indicated by the grayscale strip The top-right corner illustrates the neocortex which envelops these structures. **B** The top left illustration shows the three dimensional shape of the hippocampal formation. Black lines indicate the location of the the coronal section shown on the right. The hippocampal region is marked in pink, and the entorhinal cortex is highlighted in orange. Dentate gyrus (DG) and cornus ammonis (CA) are labeled. **C** Diagram of the hippocampal region, outlining cell types and illustrating the circuit from layer II of the entorhinal cortex to dentate gyrus, CA3, CA1, and back to layers V and VI of the entorhinal cortex.

The **trisynaptic pathway of the hippocampus** (Fig 1C) constitutes one of the most extensively studied neural circuits (Basu and Siegelbaum 2015). This unidirectional circuit originates from the entorhinal cortex, progresses through the dentate gyrus (DG), CA3, and CA1, and concludes by returning to the entorhinal cortex. Specifically, the circuit commences with stellate cells in layer II of the entorhinal cortex. These stellate cells project to the granule cells of the dentate gyrus, characterized by their mossy fibers, which in turn synapse on pyramidal cells of the CA3 area. The Schaffer collaterals emanating from the CA3 pyramidal cells excite CA1, and information is then conveyed back into the entorhinal cortex. Notably, each structure within this pathway maintains direct connections back into the entorhinal cortex.

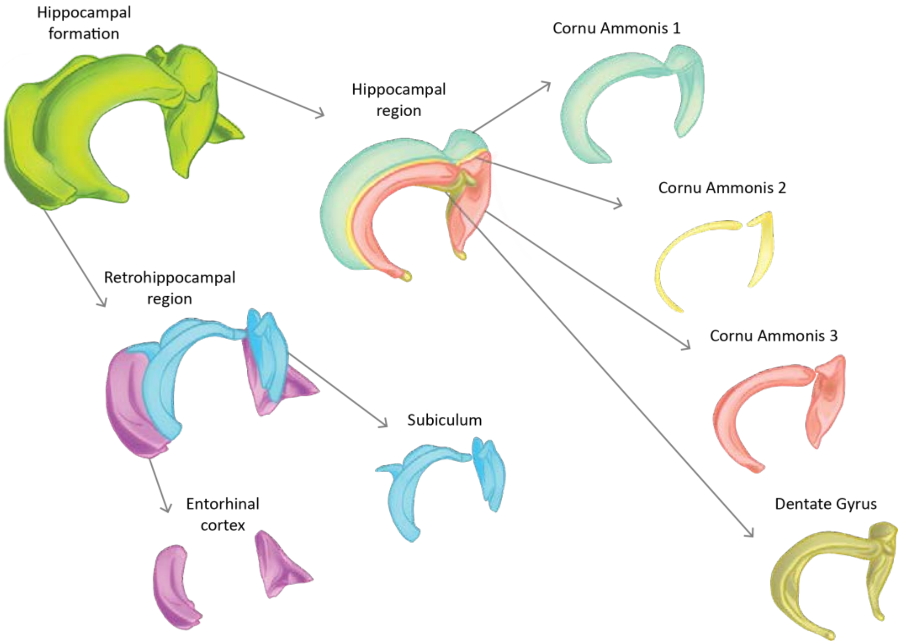


Figure 2 Illustration of the structural components within the hippocampal formation. The two primary regions of the hippocampal formation are the hippocampal and retrohippocampal regions. The hippocampal region comprises the dentate gyrus, cornu ammonis 1, 2, and 3. The retrohippocampal region is composed of the subiculum and entorhinal cortex.

Beyond the previously mentioned connections, additional intricacies exist within the hippocampus. Layer III of the entorhinal cortex establishes a direct pathway to both CA1 and the subiculum (Basu and Siegelbaum 2015). **In CA3, multiple recurrent connections form a robust auto-associative local network**. This implies that activation of one cell within a CA3 ensemble will propagate to activate all other neurons within that ensemble. CA1 receives input not only from CA3 but also from the less explored CA2 region, recognized for its relevance in social memory (Oliva, Fernandez-Ruiz et al. 2020). Inputs to CA2 originate from layer II of the entorhinal cortex, CA3, and directly from the dentate gyrus. The principal input to the hippocampus from the entorhinal cortex is facilitated through the **perforant path**. The entorhinal cortex, functioning as a sensory association area, receives a broad array of spatial and non-spatial inputs from various cortical regions, including the prefrontal and cingulate cortices, temporal cortex, parietal areas, and pyriform cortex. Additionally, visual and olfactory inputs converge in the entorhinal cortex (O'Keefe and Nadel 1979).

Inputs from the brainstem, originating in the median raphe nucleus and locus coeruleus, reach the hippocampus, releasing serotonin and noradrenaline, respectively. Septal inputs, releasing acetylcholine, and inputs from the fastigial nucleus of the cerebellum also project to the hippocampus (O'Keefe and Nadel 1979). Outputs from CA1 and CA3 extend not only to the entorhinal cortex but also to the lateral preoptic and hypothalamic areas, thalamus, mammillary bodies, amygdala, and rostral midbrain (O'Keefe and Nadel 1979) (Basu and Siegelbaum 2015). Additionally, through the fornix, connections to the septum and hypothalamus are established (Kandel, Schwartz et al. 2000). **Given this extensive connectivity, the hippocampus emerges as an ideal center for integrating information from both visceral and cognitive inputs, underscoring its pivotal role in the integration of diverse sensory and cognitive processes**.

The significance of the hippocampal formation gained considerable attention following the case of **patient HM** (Scoville and Milner 1957). HM, an adult man with epilepsy, underwent surgery to remove the hippocampus as it was identified as the source of his seizures. While the surgery successfully alleviated his epileptic symptoms, HM experienced profound amnesia. He could not recall events from the past ten years and was unable to form new declarative memories. Notably, he exhibited the ability to improve motor skills over time, even without conscious memory of practicing those motor tasks. This case spurred extensive research on the hippocampus, not only in the context of memory function but also in navigation.

In another light, the hippocampal formation is implicated in coding spatial representations, including place cells (O'Keefe 1976), grid cells cells (Buzsáki and Moser 2013), and head direction cells (Knierim, Kudrimoti et al. 1995). These elements contribute to the creation of an internal representation of the environment, supporting navigation. This line of inquiry will be further explored in the Navigation section, emphasizing the multifaceted role of the hippocampus in both memory and spatial cognition.

Despite the well-established association of the hippocampus with spatial processing and navigation, it serves as a versatile structure processing inputs from various modalities. The precise role of the hippocampus remains a subject of ongoing debate. This thesis adopts a perspective that contextualizes the hippocampus within the framework of previous knowledge to enhance comprehension of its role in memory. The focus will specifically be on the CA1 area for pharmacological interventions, given its role as the primary output from the hippocampal formation to the isocortex. Additionally, activity expression studies of immediate early genes will be conducted in the hippocampal region. This approach aims to contribute valuable insights into the nuanced functions of the hippocampus in memory processes.

Neocortex

Next, I will focus on the cerebral neocortex, exclusive to mammals and pivotal for **higher-order brain functions** encompassing cognition, perception, reasoning, language, and motor control. This structurally complex region can be categorized into distinct functional regions, including sensory areas, motor areas, and association areas (Fig 3). The association areas serve as intermediaries, facilitating communication between sensory and motor functions. This division underscores the intricate organization of the neocortex and its integral role in orchestrating diverse cognitive processes.

The neocortex exhibits a semi-hierarchical organization, where primary sensory cortical areas process sensory information before transmitting it to association areas. This organization occurs both in series, with each area serving as the source of information for the next, and in parallel, by receiving sensory inputs directly from the thalamus. Supplementary sensory areas contribute additional processing of raw sensory data and project to other association areas. Notably, receptive fields increase in size and complexity as one progresses from primary to supplementary sensory areas. Ultimately**, all these pathways converge on the hippocampus, emphasizing the integration of processed sensory information** in memory-related structures (Teyler and Rudy 2007).

In the 1800s, a prevailing scientific perspective posited that discrete structures within the brain, particularly the neocortex, held the key to understanding brain functions. Researchers like David Ferrier, Eduard Hitzig, and Hermann Munk were central to this line of thought. To gain insights into the role of the neocortex, decortication experiments were conducted on dogs and cats. The first documented cases of dogs surviving without a cortex date back to 1892, as reported by

Goltz (Goltz 1892). Subsequent decades saw further decortication experiments in cats, focusing on **assessing the role and significance of the neocortex in fundamental functions such as sleep, feeding, and navigation**. These experiments contributed to early attempts to decipher the specific functions of the neocortex in living organisms.

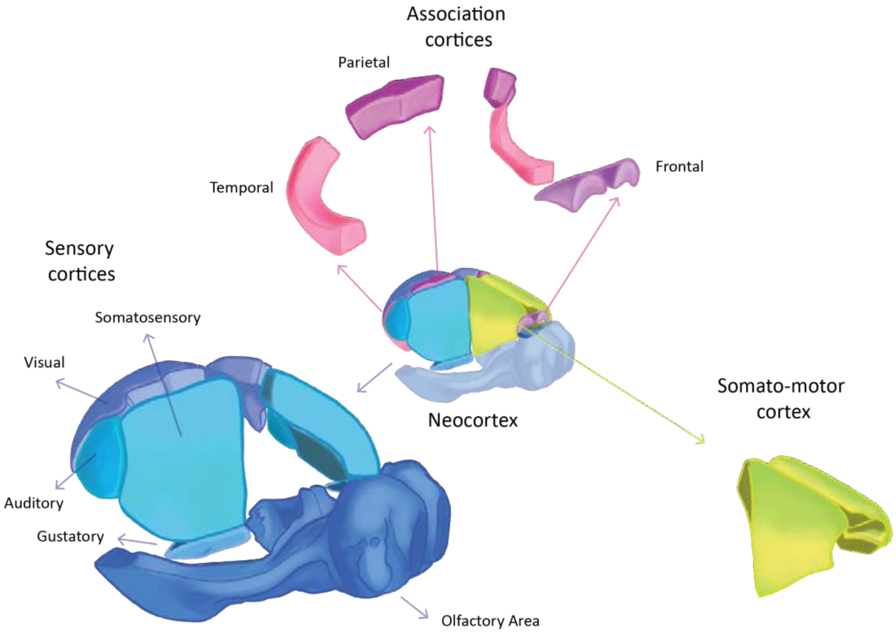


Figure 3 The neocortex encompasses the somato-motor, sensory, and association cortices. To note, the olfactory area does not technically fall under the classification of neocortex.

These studies reported that, following an initial stabilization period, animals could survive without a cortex for several months. Results within the same studies varied, with some animals initially displaying restlessness that decreased over days, while others exhibited an initial paralysis followed by an increase in activity over time. Undisturbed animals would stand or sit for extended periods, initiating persistent walking upon stimulation. During this phase, they showed a lack of obstacle avoidance, often hitting their heads against walls and changing direction randomly. After a few weeks of recovery, decorticalized animals learned to avoid obstacles. Only strong stimuli, such as feeding or defecation, would interrupt their walking (David McK 1938) (Villablanca 1972). These animals displayed excessive feeding and grooming behaviors and showed heightened reactivity to sounds and handling. When multiple stimuli were presented simultaneously, only the strongest stimulus would elicit a response, suggesting **a lack of integration and evaluation of presented inputs**.

The observed movements in decorticalized cats could be explained by reflexes dependent on the spinal cord. However, over time, the behaviors observed surpassed the simple sum of reflexes, indicating the **emergence of a new organizational pattern** (David McK 1938). Regarding sleep patterns in decorticalized cats, conflicting findings have been reported. Some studies, such as those by (David McK 1938), indicate that decorticalized cats tend to sleep more profoundly than intact cats. Conversely, other studies, like (Villablanca 1972), report reduced overall sleep time, particularly in rapid eye movement (REM) sleep.

In experiments where only one hemisphere of the cortex is intact, it took animals around four months to recover normal sleep patterns. During the first month, there is a notable decrease in sleep duration and shortening of sleep stages, with REM sleep being particularly affected, compensated by an increase in light sleep (Bogoslovskii 1985). These observations underscore the significance of **the cortex in regulating sleep patterns, which are tightly linked to memory consolidation**.

These experiments suggest that the role of the cortex is intricately connected to interpreting inputs and modulating responses. While decorticalized animals retained essential functions for survival, they **lacked directed goals without a cortex to guide them.** Of specific interest to this thesis is the frontal association area, specifically the prefrontal cortex, which plays a crucial role in executive functions and goal-directed behaviors (Dalley, Cardinal et al. 2004), as well as the retrosplenial cortex, which has been proposed to be the gateway between the neocortex and the medial temporal lobe, mediating perception and memory (Milczarek and Vann 2020), as well as essential for landmark-based navigation (Balcerek, Wlodkowska et al. 2021).

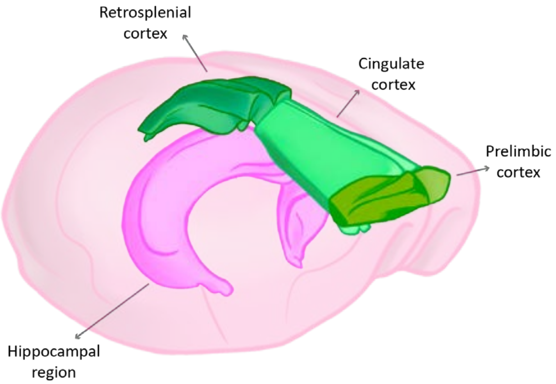


Figure 4 Illustration of prefrontal and posterior cortices. As a reference, the neocortex is shown in light pink, and the hippocampal region in bright pink.

The prefrontal cortex, situated in the frontal lobe, exhibits conserved functionality across rodents and primates. Its executive function governs cognition and associative processes. In simpler terms, **the prefrontal cortex processes inputs, compares them to past experiences, and selects the optimal output based on internal goals**. It considers both the physical demands required for an outcome and its value (Patai and Spiers 2021).

This region receives inputs from various brain areas, including cholinergic projections from the basal forebrain, adrenergic inputs from the locus coeruleus, serotonin from the raphe nuclei, dopamine from the ventral tegmental area, emotional values from the amygdala, sensory information from the thalamus, memories stored in the hippocampus, intrinsic homeostatic drives from the hypothalamus, interoception from insular areas, and inputs from other parts of the cortex (Euston, Gruber et al. 2012). **Its extensive connectivity underscores its essential role in executive function, reasoning, problem-solving, planning, and imagination.**

The retrosplenial cortex, situated above the hippocampal formation (Fig 4), comprises a granular region and an agranular region. The granular region of the retrosplenial cortex receives direct connections from the subiculum and CA1, reciprocally projecting back to these areas. In contrast, the agranular region receives direct inputs from the visual cortex (Balcerek, Wlodkowska et al. 2021). It has been proposed to serve as a gateway between the neocortex and the medial temporal lobe, **playing a crucial role in mediating perception and memory processes** (Milczarek and Vann 2020). Additionally, the retrosplenial cortex, particularly the agranular region, serves as one of the principal afferents to the entorhinal cortex (Jones and Witter 2007). This intricate connectivity underscores the retrosplenial cortex's role in integrating information between cortical and medial temporal structures.

The retrosplenial cortex exhibits robust connectivity with both the hippocampal region and the cortical default mode network. The default mode network, which is anti-correlated with attention networks, becomes active during rest states. In rodents, this network includes the parietal cortex, orbital cortex, and anterior cingulate cortex (Stafford, Jarrett et al. 2014). Studies suggest that the retrosplenial cortex’s function is associated with landmark-based navigation (Balcerek, Wlodkowska et al. 2021) and long-term storage (Milczarek and Vann 2020) (Mitchell, Czajkowski et al. 2018). **The retrosplenial cortex's connectivity with these networks underscores its role in facilitating processes related to navigation and long-term memory.**

In this thesis I contextualize the prefrontal and retrosplenial cortices within the structure of previous knowledge in order to deepen the understanding of its role in memory. The focus will specifically be on prelimbic and retrosplenial area for pharmacological interventions (Fig 4). Additionally, activity expression studies of immediate early genes will be conducted in anterior cingulate cortex, prelimbic cortex, retrosplenial cortex and posterior parietal cortex. This approach aims to contribute valuable insights into the nuanced functions of the neocortex in memory processes.

In summary, the hippocampal formation and neocortex exhibit distinct properties that influence their capacity to acquire and store memories over varying time frames and strengths. The hippocampus, characterized by high plasticity, rapidly encodes all experiences but experiences a quick turnover of spines, leading to relatively fast decay of memories (Holtmaat and Svoboda 2009). On the other hand, the neocortex, with a larger volume, demands stronger stimuli for encoding changes due to decreased plasticity (Tonegawa, Liu et al. 2015). However, once new information is incorporated, it tends to remain stable for extended periods of time. This contrast in properties highlights the **complementary roles of these structures in the overall process of memory formation and retention**. Next, I will introduce the cognitive functions which are supported by these structures.

Cognitive functions

Learning and Memory

Memory can be conceptualized as a cognitive function encompassing three main processes: encoding, consolidation, and retrieval. Encoding involves the recording of events, while consolidation selects specific events for long-term storage. Retrieval is the subsequent process of recalling stored information. In the context of survival, organisms rely on memory to remember crucial information about their environment, such as the locations of food resources or potential threats. **The ability to recognize and recall past experiences, predict outcomes, and adjust behavior accordingly is vital for adaptation and survival.** Given the dynamic nature of the environment, the capacity to adapt to changing circumstances becomes a critical aspect of effective memory function.

Memory can be categorized into different types, with an initial distinction between **short-term and long-term memories**, each serving distinct purposes in response to varying circumstances. Short-term memories are rapidly forgotten but play a crucial role in preventing redundant actions, such as checking the same spot for food multiple times in a short timeframe. In contrast, long-term memories endure for extended periods, shaping behavior based on experience, such as understanding optimal times of the year to find a preferred food source in a specific location.

Within long-term memories, two main categories emerge: **declarative and non-declarative memories**. Declarative memories are consciously recallable, enabling individuals to remember specific information, like identifying edible and poisonous berries. Non-declarative memory, on the other hand, cannot be explained verbally but is demonstrated through actions, such as the motor skills required to reach a berry surrounded by thorns without getting pricked (Kandel, Schwartz et al. 2000). This multifaceted classification highlights the diverse nature of memory and its adaptive functions in different contexts.

Declarative memory is further subdivided into **episodic and semantic memory**. Episodic memory involves detailed autobiographical events, such as the first experience of taking a bus (Squire 2004). **Episodic memories are rich in detail and typically associated with salient personal events**. Within episodic memory, there is a specific type known as flashbulb memories. These memories preserve vivid details, especially during highly salient events like witnessing a traffic accident. Flashbulb memories capture not only the event itself but also intricate details such as clothing, surrounding individuals, and even the music playing at the time. This phenomenon aligns with the tag and capture hypothesis, wherein memories that would typically fade quickly become reinforced due to the by-products of intense stimulation (Redondo and Morris 2011). Molecular changes that occur in a subset of cells encoding a strong memory are also experienced by neighboring ensembles coding for weaker memories.

Semantic memories, on the other hand, encompass factual and general world knowledge accumulated over time. They are believed to form through the extraction of commonalities across multiple experiences. For instance, knowing the routes of different buses without formal study, acquired over several years of riding around the city, exemplifies semantic memory. These memories are structured systems of general knowledge distributed throughout the cortex (Squire 2004, Sweegers, Takashima et al. 2014). The distinction between episodic and semantic memory highlights the diverse nature of declarative memory, encompassing both personal experiences and accumulated factual knowledge.

In this specific thesis, the focus will be on schema memories or previous knowledge networks, falling within the scope of semantic memory at a higher organizational level**. Schemas are associative network structures grounded in multiple episodes, lacking unit detail but adaptable with the acquisition of new related events.** They are dynamic frameworks constantly evolving and adjusting. Learning events associated with a particular schema is more rapid than learning entirely new information. Schemas guide behavior contextually and facilitate retrieval by enabling memory search and reconstructing missing information (Ghosh and Gilboa 2014). Throughout our lives, humans construct an intricate framework of knowledge, and it is rare for adults to learn something entirely disconnected from their previous experiences.

In rodents, research on previous knowledge is limited. **This thesis employs a behavioral task to assess previous knowledge, aiming to elucidate the roles of different brain areas and networks across various learning stages**. Given that coordinated activity in structures like the hippocampus and prefrontal cortex is essential for constructing this framework, the neural activity of the hippocampus, prelimbic, and retrosplenial cortex will be inhibited during early and late learning phases. This approach aims to test the memory's capacity to manifest in the absence of one of these structures. Further details on this type of memory will be extensively reviewed in Chapter 3. Throughout this thesis the terms previous and prior knowledge will be used equivalently.

Navigation

To investigate memory in humans, complex and nuanced tasks can be performed, and questions can be posed, such as which items can be remembered from a previously shown list. However, rodents lack the ability to communicate specific items they remember. Instead, researchers leverage natural tendencies of animals that express memory, such as an animal returning home after foraging. Various mazes and tests have been developed to understand the neural representations of physical space, commonly referred to as a cognitive map. The following section will introduce **the concept of navigation and outline some of the tasks that have been employed to study it**.

Navigation entails the traversal of an environment to achieve a specific goal, whether it be locating a food source or returning home. One navigational strategy involves using oneself as a reference point, termed egocentric navigation. In this approach, individuals learn a sequence of body turns, which can later be stored as an episodic memory. **Egocentric navigation is supported by path integration**, a mechanism that aids navigation by calculating distances between landmarks. It relies on internal cues of movement related to the environment, enabling the computation of the shortest path toward the goal (McNaughton, Battaglia et al. 2006). **Another strategy is based on distal cues in the environment, known as allocentric navigation** (Wilber, Clark et al. 2014, Grieves and Jeffery 2017). However, navigation must also be flexible, as the environment can unexpectedly change, or the priority of goals can shift, such as transitioning from finding food to seeking a mate. This adaptability is crucial for effective navigation in dynamic and evolving environments.

Both the hippocampal and parahippocampal areas play crucial roles in navigation, and recent attention has been directed toward understanding the role of the prefrontal cortex (Patai and Spiers 2021). The hippocampus is capable of representing place and time, interacting with the prefrontal cortex to adapt routes based on current environmental changes and plan ahead for guiding navigation (Patai and Spiers 2021). **Additional structures relevant to navigation** include the retrosplenial cortex, essential for landmark determination and their relation to cues (Czajkowski, Jayaprakash et al. 2014); the parietal cortex, responsible for processing sensory information from the environment (Barry, Coogan et al. 2016); frontal cortices such as the prelimbic cortex for route (Hok, Save et al. 2005), and the anterior cingulate cortex for making object-place associations (Weible, Rowland et al. 2012).

Deeper structures, such as the thalamic nuclei, regulate the level of awareness or attention (Aggleton and Nelson 2015). The septum, which connects the hippocampus with the ventral tegmental area, reinforces memories when a reward is found (Leutgeb and Mizumori 2002). The striatum is involved in flexible navigation (Gahnstrom and Spiers 2020), among many others (Grieves and Jeffery 2017). The **integration and collaboration of these various brain regions highlight the complexity of the neural network involved in navigation**.

One of the first mazes for rats (Fig 5A) resembled an actual hedge maze garden at Hampton Court in London (Small 1901). The maze featured multiple dead ends, with an open space in the middle. Initially, animals were allowed to freely explore the environment, and later, a reward was introduced at the center of the maze. Through repeated exposure, the animals became more adept at reaching the reward quickly and efficiently. Notably, these experiments were conducted in darkness as rats are nocturnal, indicating that they did not rely on visual cues to reach their goal.

The reward's location was subsequently moved closer, and the path was shortened. In an interesting twist, rats, accustomed to their usual path, ran into the newly placed wall after this modification, resulting in a distinctive "kerplunk" noise. These experiments, known as the kerplunk experiments (Watson 1907), led scientists to propose a **stimulus-response theory**. The animals were not relying on visual cues but instead on a set of predetermined turns, establishing direct associations between stimuli and rewarded responses.

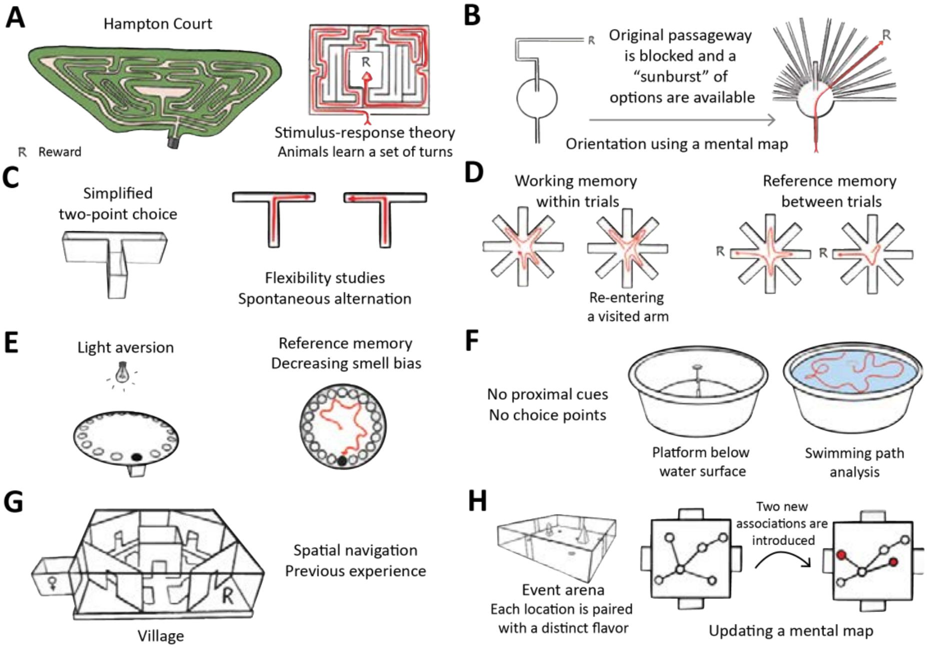


Figure 5A On the left, an illustration of the Hampton court maze in London seen from an aerial view. On the right, the maze designed inspired on the Hampton court. In red marked the route to the reward (R). Scientists proposed the stimulus-response theory, observing that animals were simply learning a predetermined set of turn to reach the goal. **B** Tolman sunburst maze. Left, animals learn a reward location through the only available route. Right, the original passage is blocked, and a radiating arrangement of new gangways is available. Most animals would take the most direct route to the reward, marked in red. Tolman believed that animals used a mental map for orientation, disputing the stimulus-response theory **C** Left, T-maze, a simplified two-point choice experiment. Right, animals are placed at the end of one arm and reach a bifurcation towards left and right. Rats naturally alternate visiting left and right arms on consecutive trials, as shown in red. However many variations can be used in this task to understand different nuances of learning and memory. **D** Radial arm maze. Left, eight (or more) gangways connected in a central octagon, the animal is placed in the middle to find a reward in one of the gangways. To test working memory within a trial, which gangways have been visited are tracked, as well as the time elapsed until reentering an already visited gangway, as shown in red. Right, to test reference memory, the same gangway is baited across several trials. **E** Barnes maze, devised to study reference memory while decreasing the smell bias observed in radial arm. Left, open round arena exposed to strong light with several holes at the edge. Only one hole leads to a tunnel away from the light stimulus, represented as a black hole. Right, a trajectory of an animal searching for the tunnel, shown in red. **F** Morris water maze. Left, circular pool with a submerged platform. Right, the platform is below the surface, and rodents need to swim around to locate the submerged platform. The path of the animals is shown as a red line, while the location of the platform as a red dashed circle. **G** Winocur Village. A complex behavioral setup with four different chambers offering food, water, females and toys. If animals are food-deprived, they will try to move quickly towards the food chamber. **H** Event arena. Left, arena equipped with intra and outer maze cues, and six available sandwells where a reward can be found. Each trial, the animal is given a flavor cue, and it should dig in the sandwell corresponding to that flavor. There are 6 flavor-place associations to learn initially. Right, after a learning period, two associations are replaced with new flavor-place associations, updating their mental map, which takes place after a single session. Not seen on figures B to H, all mazes present spatial cues available for orientation.

In 1948, Tolman challenged the stimulus-response theory, proposing that rats constructed a mental map, or a **cognitive map**, using cues and landmarks in the behavioral setups to facilitate navigation (Tolman 1948). He emphasized the significance of incentives, such as hunger and rewards, for animals to express their knowledge about the environment. In the absence of a clear goal, animals would explore rather than traverse the environment efficiently. Blodgett termed this phenomenon latent learning, asserting that animals, despite appearing to make mistakes, demonstrated knowledge of the environment when provided with an incentive, indicating learning without immediate rewards (Blodgett 1929). To reinforce his claim, Tolman observed that at decision points during critical learning stages, animals would pause, examine different choices, and engage in vicarious trial and error (Muenzinger 1938). This behavior suggested that animals would contemplate the correct choice before making a turn.

To further investigate the theory of mental maps and orientation abilities, innovative mazes were developed. In the sunburst maze (Fig 5B), rats were initially trained to follow a predetermined path to a reward. The original route was then blocked, and several radiating paths were offered. Most animals chose the path with the shortest length to the reward, **demonstrating their ability to orient themselves in relation to the reward even when the original route was blocked** (Tolman, Ritchie et al. 1946).

Ritchie expanded on this by testing spatial orientation in a maze with specific environmental cues and a fixed reward point. The maze was later turned around, presenting rats with a radial arrangement of passageways to reach their reward. In this scenario, animals did not simply take the shortest path but adapted their original direction based on the cues in the room. For example, if they initially had to turn left to reach their goal, they adjusted their turn to the right, leading them to the correct side of the room where their reward was located (Tolman, Ritchie et al. 1946).

In the 1950s, there was insufficient physiological evidence to fully support the theory of cognitive maps. However, with the **discovery of place cells** by O'Keefe in 1976 (O'Keefe 1976), this concept was reconsidered, leading to a rapid increase in experiments dedicated to spatial navigation and understanding cognitive maps. While the first mazes around 1900 were large and naturalistic, they were simplified over time, often utilizing the basic structure of a two-point choice, as seen in the T-maze originally developed in the 1920s by Hunter (Hunter and Randolph 1924) (Fig 5C). Rats tend to naturally alternate visits between the left and right arm, and with different variations, this task allows to study different memory and learning features (Dudchenko 2004).

Two extensively studied types of memory are **working memory and reference memory**. Working memory involves the retention and manipulation of information simultaneously, while reference memory maintains spatial information consistently over time. For instance, in the **radial arm maze**, animals search through several arms for a reward. Working memory is tested by evaluating how many arms the animal visits before re-entering the same one within the same trial, while reference memory is assessed by determining if the animal remembers over multiple trials which arm was baited (Fig 5D). Although the radial arm maze lacks intra-maze cues, odor cues left by the animals may assist in recalling visited arms (Olton and Samuelson 1976). To mitigate the influence of smell bias, the **Barnes maze** was developed to specifically study reference memory. The Barnes maze features a well-lit circular platform surrounded by 18 holes, with only one hole leading to an escape tunnel below the platform (Barnes 1979) (Fig 5E). In each trial, the animal begins within a specific tunnel, to later be placed in the middle of the maze. Given the aversive nature of light to rats, they are motivated to locate the tunnel hole. The starting tunnel changes position in each trial, and the maze can be rotated to prevent incidental cues that might aid the animal in orientation.

Water mazes were designed to minimize certain biases in navigation, such as excessive hungriness or reliance on odour cues. These mazes utilize the natural motivation of animals to find a place to rest from swimming. Water mazes can come in various shapes, with some repeating structures observed in dry mazes. One of the most popular water mazes is the **Morris water maze** (Morris 1981) (Fig 5F). This circular maze features a hidden platform beneath the water level. Animals are initially habituated to find the platform by placing a cue above it. In subsequent trials, the platform is relocated, and no cues indicate its location, compelling the rats to swim randomly around the maze to discover a platform. This design removes biases associated with proximal cues and choice points. Different variations of the task can be employed to assess various types of learning.

To assess the impact of previous experience in rats, more intricate environments were devised. For instance, a "village" featuring diverse compartments with rewards like food, water, toys, or females was employed to **evaluate the effect of prior exposure to an environment on spatial navigation** (Fig 5G). The arena could be relocated to different rooms to examine the use of allocentric cues, or cues within the same room were altered (Winocur, Moscovitch et al. 2005).

To further understand the role of previous experience, **the paired-associates task** was devised (Tse, Langston et al. 2007) (Fig 5H). Animals were required to learn six flavour-place associations, where each trial involved testing a specific association. The spatial relationships between different flavors had to be learned, and each trial commenced from a different side of the maze, necessitating the use of allocentric strategies to locate the correct reward. After several weeks of training, two of the flavor-place associations were altered, and animals successfully adapted to this change after only one session, whereas the original map had taken them several weeks. Hippocampal lesions were introduced 48 hours after learning the new associations, yet this did not prevent the rats from selecting the correct locations when tested, **challenging established notions about consolidation**. This task represents the initial exploration of previous knowledge networks and will be comprehensively examined in the subsequent chapter.

In the pursuit of a profound understanding of cognitive functions, particularly memory, the examination of natural behaviors such as navigation proves invaluable. The precise mechanisms underlying memory function remain elusive, prompting the proposal of various theories over the decades. The subsequent section will introduce some of these theories on memory consolidation, elucidating the evolution of these conceptual frameworks over time.

Theories on memory consolidation

Consolidation denotes the conversion of transient and labile events into enduring and stable memories (Squire, Genzel et al. 2015). At the cellular level, this is facilitated by synaptic consolidation, a process spanning minutes to hours. Concurrently, **systems consolidation** operates at a broader scale, intertwining connectivity across diverse brain regions, and unfolds over weeks, months, or even years. The ensuing discussion will introduce pivotal concepts in memory research, followed by an exploration of various theories on systems consolidation.

Memory processes encompass three fundamental stages: **encoding, consolidation, and retrieval**. Encoding involves representing all facets of an event within a subset of neurons; consolidation stabilizes this pattern of activation, ensuring enduring memory; and retrieval reactivates the previously stored pattern. Notably, as a trace is retrieved, a re-encoding of the retrieval occurs, engendering a recurrent and intricate loop known as reconsolidation, where stored memory traces are updated in response to novelty (Wang and Morris 2010). At the local and cellular level, the foundation of memory is thought to rely on long-term potentiation (LTP), strengthening communication between neurons (Bliss and Lomo 1973). While extensively studied in the hippocampus, LTP is also prevalent throughout the brain. At a global and systems level, connectivity among distinct neocortical areas representing diverse modalities of a memory must be strengthened, a phenomenon termed systems consolidation.

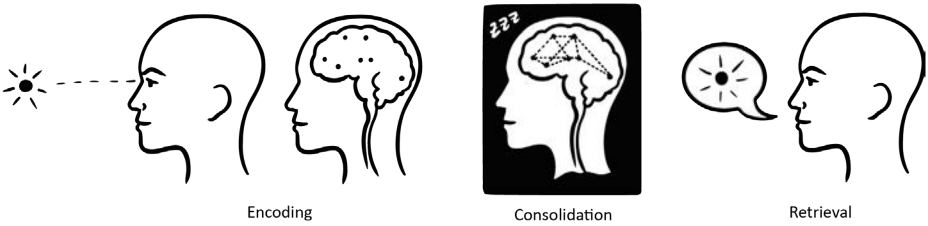


Figure 6 Encoding, consolidation and retrieval processes

To optimize behavior, not every experience warrants long-term storage; hence, a temporary associative memory briefly holds information, transferring it to long-term storage selectively. Early memory theories proposed dual memory systems: one for initial recording and another for categorization and storage (Marr, Willshaw et al. 1991). Generally, theories converge on simultaneous encoding in both hippocampal and cortical areas for new memory traces. However, **distinctions arise regarding the hippocampus' role post-consolidation and the similarity between hippocampal and cortical traces**.

An artificial neural network, based on the Rumelhart network, demonstrated the formation of memory structures. Comprising input, output, and hidden units, the network mirrors the levels in the nervous system. Sensory neurons correspond to input units, motor neurons to output units, and the rest of the neurons to hidden units. The network is trained to categorize inputs into desired outputs, capturing patterns of learning and generalization when presented with new information. Critical to retention is the rate of incorporation when faced with conflicting information. Rapid learning risks catastrophic interference, disrupting established knowledge. Instead**, a gradual interleaved presentation, reinforced with known information, ensures steady incorporation without disruption**.

This simulation aligns with the **Complementary Learning Systems theory** (McClelland 2013), asserting the necessity of two systems for efficient learning. The fast learner, represented by the hippocampus, records specific items and experiences, while the neocortex serves as the gradual learner, acquiring structured knowledge of the environment. Reactivations from the hippocampus of learned events are crucial for gradual learning, subtly strengthening neural connections and adapting cortical structures over time to avoid memory disruption. Both the hippocampus and neocortex experience memory decay, suggesting that the hippocampus, with a greater decay rate, plays a time-limited role in the formation of neocortical representations.

The aforementioned theory aligns with the **Standard Systems Consolidation Theory,** proposing that the hippocampus acts as a pointer toward cortical ensembles. In this framework, the hippocampus provides contextual information to the cortically encoded memory. Initially, episodic memories would depend on the hippocampus, and over time, these memories would be consolidated in their original form in the cortex, becoming hippocampal independent. This consolidation process involves replay, occurring during offline or quiet periods, where the hippocampus reactivates previously encoded events to the neocortex, reinforcing both hippocampal and cortical traces (Genzel and Robertson 2015). Replay takes place in the same temporal order of the event but in a compressed manner, during sharp-wave ripples in the hippocampus, correlating with cortical spindles (Frankland and Bontempi 2005, Cowan, Liu et al. 2020) (Squire 2004). However, evolving research has indicated that hippocampal and cortical memories are not identical, leading to the development of new theories.

The hippocampus as a cognitive map (O'Keefe and Nadel 1979), a term initially introduced by Tolman (Tolman 1948), is proposed to play a crucial role in long-term storage by constructing and maintaining allocentric maps of the environment. This model introduces the idea of an egocentric space, centered on the self-moving within an absolute space, representing a fixed framework. Within this framework, spatial memory is permanently stored in the hippocampus, supporting both a place system, facilitating navigation in a familiar environment, and a misplace system, detecting novel or absent cues or objects.

According to **memory indexing theory**, a new memory trace is established by a specific hippocampal ensemble projecting to a set of neocortical neurons, forming an index. Retrieval is initiated by activating a fraction of this index, which, in turn, activates the hippocampal ensemble. This ensemble then projects back to the remaining cortical modalities of the memory, facilitating retrieval of the episode. This process, known as pattern completion, also contributes to strengthening the memory trace. In this framework, episodic memories are represented by the synapses between the hippocampus and isocortex rather than among neocortical cells (Teyler and DiScenna 1985). The hippocampus, in this model, doesn't encode the context or experience but points to the neocortical cells coding for all details. The ability to distinguish similar yet distinct episodes is termed pattern separation, where similar experiences may share many cortical units but possess distinct hippocampal representations. The presence of an index pointing to these distinct but similar episodes allows for their differentiation as separate experiences, a distinction not achievable solely through associative networks (Teyler and DiScenna 1985). Over time, repeated activation of the neural ensemble strengthens neocortical activity patterns, leading to eventual disengagement of the hippocampus in retrieving the episodic memory**.**

Multiple trace theory suggests that the cortical memory is not an exact replication of the hippocampal trace but rather captures the essence or gist of a memory. The hippocampal trace preserves the details and context, forming a new trace each time the memory is reactivated. This new hippocampal trace indexes toward a very similar set of neocortical neurons as the original episode, enabling the extraction of commonalities and integration into semantic knowledge. Consequently, the hippocampus consistently plays a role in retrieving the spatial context of a memory, while the neocortex is crucial for the temporal frame frame (Nadel and Moscovitch 1997).

Following Tse's 2011 findings demonstrating that consolidation can occur within 48 hours when the memory aligns with prior knowledge (Tse, Langston et al. 2007), emerging theories began considering the impact of previous knowledge on the consolidation process.

The **trace transformation theory**, building on the Multiple Trace Theory, proposes that memories undergo dynamic changes over time. Both cortical and hippocampal traces coexist and interact, with highly detailed episodic memories relying on the hippocampus as long as its trace is present. The retrieval of either the detailed hippocampal trace or neocortical semantic knowledge depends on the specific task demands and conditions during retrieval (Moscovitch, Cabeza et al. 2016).

The **scene construction theory** suggests that the hippocampus is consistently involved in constructing and anticipating scenes, generating representations that extend beyond immediate sensory inputs. These constructed scenes can be atemporal, as long as they maintain a coherent spatial context, encompassing imagination of scenes from the past, future, or fictitious scenarios (Mullally and Maguire 2013).

Kumaran, Hassabis, and McClelland (Kumaran, Hassabis et al. 2016) expanded the complementary learning systems theory by incorporating a generalization role for the hippocampus, additional functions of replay, and an adaptation for situations involving prior knowledge, leading to an accelerated consolidation process.

Scene reconstruction theory (Barry and Maguire 2019), suggests that neuronal turnover leads to the gradual disappearance of hippocampal memory traces over time. Consequently, during memory retrieval, if the original trace is no longer present, a new hippocampal index is formed, incorporating various modalities of the memory as a reconstruction of the past experience.

Contextual binding theory (Yonelinas, Ranganath et al. 2019), suggests that episodic memory is not consolidated solely to the neocortex. Instead, gradual changes in context contribute to forgetting and extend encoding activity. The hippocampus plays a crucial role in binding context and events, considering various aspects of an episode that may change abruptly or gradually, such as transitioning to a different room or variations in lighting. Interference may occur when multiple episodes share a particular aspect or context. The hippocampus binds context and item information, while the cortex assesses familiarity, recognizes novelty (strengthening episodic memory), and supports semantic memory. This theory builds upon the Multiple Trace Theory but emphasizes the critical role of context in episodic memory and forgetting.

Theories regarding the process of consolidation continually evolve with new evidence, yet a consensus exists that the hippocampus promptly records every experience, concurrently with parallel encoding in the cortex. Cortical ensembles mature and stabilize over time through regular retrieval of specific memories. Given the extensive nature of the cortex with diverse modalities, the hippocampus serves as the binding point for all cortical modalities. Novel theories suggest replacing systems consolidation with "**memory system reorganization**" (Moscovitch and Gilboa 2022) due to demonstrated adaptations in memory over time, influenced by new experiences and interactions between different memory systems.

Studies on humans with brain lesions in the hippocampal or cortical areas are able to describe detailed experiences over an extended period, yet often lack complete lesions, and further interventions are limited. Rodent studies, offering more invasiveness, allow for thorough removal or silencing of brain areas but reduce the level of description to behavioral interpretation. Rodent studies are essential in neuroscience research in order to understand the nervous system and its processes in its natural context (Genzel, Froudarakis et al. 2024). While most rodent studies span a few weeks, limiting the evaluation of memory consolidation over extended periods**. This thesis constructs a memory network grounded in spatial navigation, demonstrating various learning levels, over an extended learning period.** By silencing different brain areas at key time points, their contributions to memory consolidation may be disentangled. To understand the extent of the memory network, immunohistochemical analysis labelling active cells during the task performance will allow me to visualize the engram over different learning points.

Thesis outline

The objective of this thesis is to characterize the involvement of cortical and subcortical structures in mice performing a previous knowledge task. The task is the **mouse HexMaze** (Alonso, Bokeria et al. 2021), which will be described in detail in Chapter 2 of this thesis. The task's objective is for mice to navigate a large environment efficiently to reach a food reward. As the animals form a mental map of the environment, adaptations such as goal relocation are introduced, prompting the modification of search strategies, engaging prefrontal cortices and the hippocampus. The changing position of the food reward every few weeks allows testing how quickly animals can learn a new goal location within a familiar environment. After 12 weeks, animals exhibit one-session learning leading to long-term memory. The task's nature, involving learning new goal locations, provides an opportunity to study encoding, retrieval, and the process of consolidation

Chapter 3 will comprehensively review the literature on previous knowledge networks or schemas (Alonso, van der Meij et al. 2020). **Schemas**, associative network structures, facilitate learning by expediting consolidation, conceding that the novel events fit into pre-existent knowledge. The review will encompass findings from human and rodent studies, **proposing a novel theory** on the continuous gradient interaction of cortical and hippocampal structures during memory consolidation. Various behavioral tasks for studying previous knowledge in rodents will be examined, including the paired-associates task from the Morris lab and the HexMaze for mice, the primary focus of this thesis.

In Chapter 4, the navigational strategy employed by the animals performing the mouse HexMaze will be extensively explained, detailing how their approach evolves over different learning stages. **The most efficient strategy is revealed to be a combination of a memory component, foresight, and a random walk component with occasional long diagonal runs**. Modeling this strategy demonstrated that foresight strengthens over time, while the random walk component persists.

The decision for conducting experiments exclusively with male animals is thoroughly discussed in the Interlude, providing a comprehensive review of sex and menstrual phase influences on sleep and memory (Alonso, Genzel et al. 2021). Brain areas with estrogen receptors are numerous, and as females experience fluctuating levels of estrogen and progesterone during estrous cycles, these hormonal variations significantly impact the behavioral output of female rodents. In navigation tasks, strategies employed to solve spatial challenges shift depending on estrogen and progesterone levels, with high hormone levels favoring allocentric strategies and low hormone levels favoring egocentric ones. Likewise, working memory is enhanced with higher hormone levels and deteriorates when hormone levels are lower. Given the distinct navigational strategies associated with hormone levels, it is imperative to consider this variable when analyzing behavioral results. The research initially focused on characterizing male behavior, with the intention to incorporate a more comprehensive sample, including both males and females, in future studies.

Following the development of the memory task, the investigation will delve into concepts like adaptability and prior knowledge. The specific roles of structures such as the area CA1 of the hippocampus, the prelimbic cortex, and the retrosplenial cortex will be explored in memory consolidation processes. This will be achieved through **pharmacological interventions** aimed at silencing these areas during the initial phases of the experiments or after 12 weeks of training. The outcomes of these inhibitory interventions will be detailed in Chapter 5, with a focus on assessing their impact on the processes of encoding and retrieval.

In Chapter 5, a comprehensive **histological analysis of neural markers related to activity** will be conducted to gain a deeper understanding of the neural substrates of memory. Mice underwent a final experience, tasked with recalling their latest goal location or learning a new one. Immunohistochemistry targeting activity-related proteins was employed to label active cells. Multiple time points were assessed throughout the task, and the quantification of active cells in hippocampal and cortical areas was performed, correlating the findings with different memory stages. This analysis was carried out in two distinct strains of animals, C57Bl6j, commonly used as a wild-type strain, and *FosTRAP2xRosa*, a transgenic line facilitating the labelling of active cells both in vivo during a specific time point of interest and through regular immunohistochemistry. This approach results in a brain sample with dual labels, capturing the in vivo state and the state of the brain cells during the animal's final experience.

In addition to slice immunohistochemistry providing representative quantification of cell activity in specific regions of interest, the iDisco whole brain clearing technique was employed. A brain hemisphere from the TRAP2 mice was immunolabeled against cFos, cleared, and subsequently imaged using a light sheet microscope. Although the analysis of these samples is intricate and time-consuming, it is essential to note that a dataset was generated and is currently being utilized in a computational project within our lab. The remaining hemisphere underwent slice immunohistochemistry and constitutes part of the data set presented in Chapter 5.

Chapter 6 delves into the implications and interpretations of this research, exploring concepts such as distributed networks and cortical-wide connectivity, as well as the implications this research may have for current theories on systems memory consolidation.

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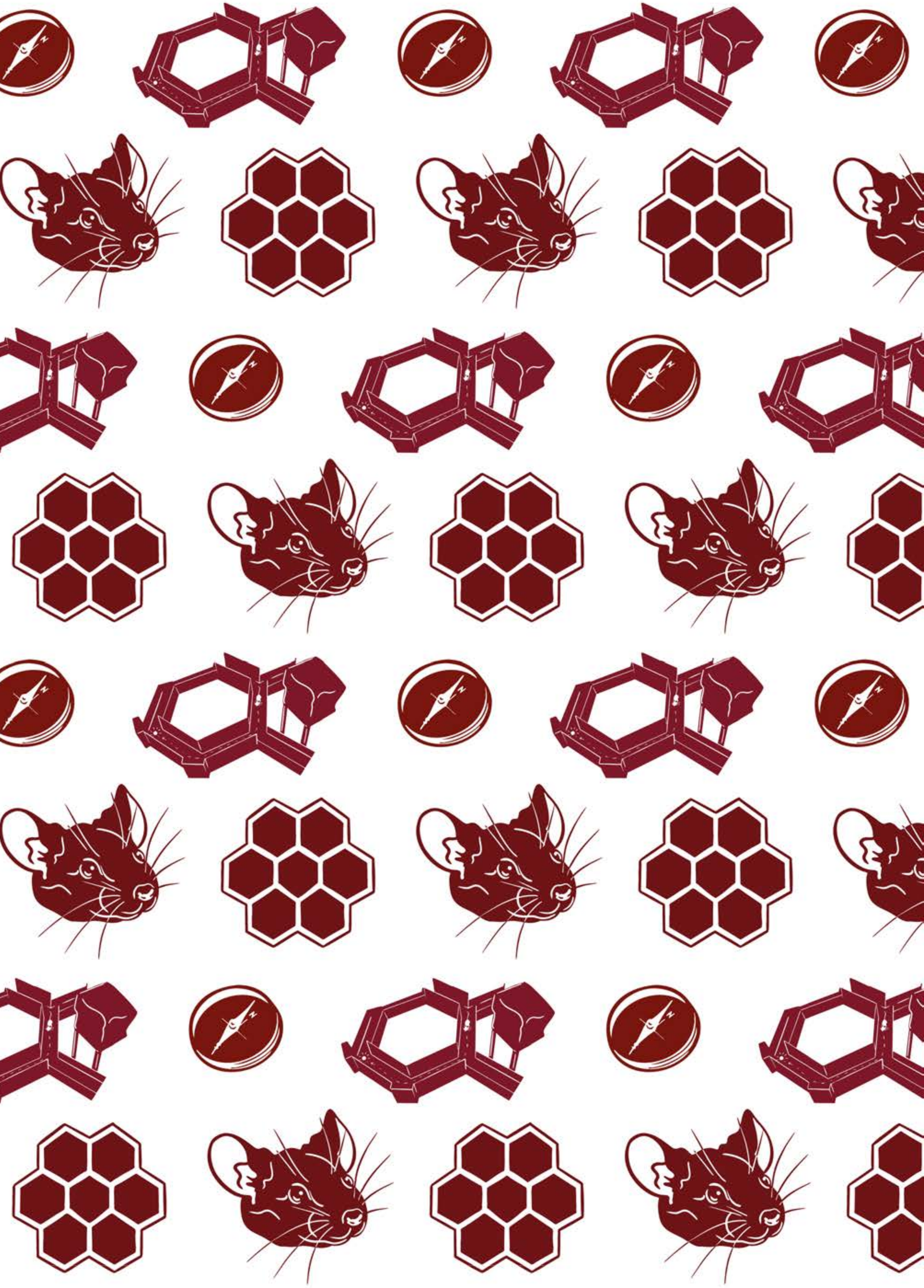

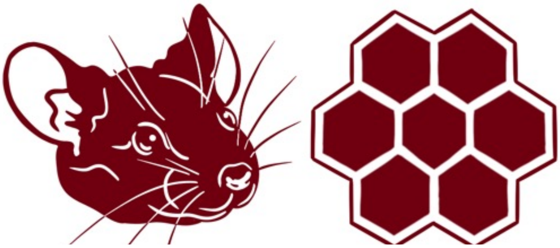

Chapter 2

The HexMaze: A Previous Knowledge Task on Map Learning for Mice

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Abstract

New information is rarely learned in isolation; instead, most of what we experience can be incorporated into or uses previous knowledge networks in some form. Previous knowledge in form of a cognitive map can facilitate knowledge acquisition and will influence how we learn new spatial information. Here, we developed a **new spatial navigation task where food locations are learned in a large, gangway maze to test how mice learn a large spatial map over a longer time period**—the HexMaze. Analyzing performance across sessions as well as on specific trials, we can show simple memory effects as well as multiple effects of previous knowledge of the map accelerating both online learning and performance increases over offline periods when incorporating new information. We could identify the following three main phases: (1) learning the initial goal location; (2) faster learning after 2 weeks when learning a new goal location; and then (3) the ability to express one-session learning, leading to long-term memory effect after 12 weeks. Importantly, we are the first to show that buildup of a spatial map is dependent on how much time passes, not how often the animal is trained.

Introduction

How does one learn new spatial environment? And once a spatial layout of an environment is learned, how is it used when incorporating new information? After infancy, we rarely acquire new information in isolation; instead, most of what we learn throughout our lives can be associated with previous knowledge. For example, Harlow (Harlow 1949) described learning sets as the "learning to efficiently learn" process of generalizing previous experience in a class of problems to new problems of the same class. Further, schemas, as proposed by Bartlett (Bartlett and Bartlett 1995) and expanded on by Ghosh and Gilboa (Ghosh and Gilboa 2014), are associated network structures based on previous experience that expedite long-term memory. Previous knowledge can also affect spatial and map learning: the more experience you have with an environment, the easier it will be to navigate through it and learn new goal locations (GLs) within it. In the past decade, more research into how previous knowledge affects learning in rodents has been provided, but how mice learn a very large, complex environment over a longer time period has not been investigated so far. The present project aims to tackle the question **in which steps map–knowledge affects learning** and provides a large, comprehensive dataset on mice spatial navigation for others to use with 16 mice over ~10 months with a total of +30 000 trials.

Outside laboratory settings, rodents will learn the spatial layout of their home environment with likely food and water resources as well as danger zones. Further, they will also learn the complex layout of their home burrow system. Surprisingly, laboratory tasks rarely tap into this spatial ability of learning large spatial environments. Further, most experiments using more complex spatial abilities have been done in rats and not mice. Rat burrows have been used to test for path integration and general navigation patterns (Zanforlin and Poli 1970, Zuri and Terkel 1996, Alyan and McNaughton 1999), and mazes composed of four or more connected square environments have been used to test whether rats take novel shortcuts (Roberts, Cruz et al. 2007, Grieves and Dudchenko 2013). Less has been done with mice. The most prominent spatial task with mice is the star maze by Rondi-Reig et al. (Rondi-Reig, Petit et al. 2006) and Fouquet et al. (Fouquet, Babayan et al. 2013). The star maze is a circular gangway maze that has five arms going off the main circular path. However, the maze is generally used to test how animals learn one single goal location with either an allocentric strategy based on cue-related navigation or a motor sequence strategy based on body turns. This goal location remains stable during training, and no changes are introduced to the environment. **How previous knowledge of the environment is used to incorporate new information, such as a goal location switch, has not been investigated so far**.

The distinction between early spatial learning and the incorporation of new information once the original spatial map has been established is critical. How much previous knowledge exists when learning something new will influence the rate of learning and consolidation as well as neural underpinnings. Relevant brain areas can show a shift in the presence of previous knowledge (Wang and Morris 2010, Van Kesteren, Ruiter et al. 2012, Squire, Genzel et al. 2015, Genzel and Wixted 2017, Alonso, van der Meij et al. 2020). In human research, the previous knowledge effect has been long established (Bartlett and Bartlett 1995), but it was not introduced to rodent research until the seminal study of the paired-associates task introducing the schema effect on system consolidation in rats (Tse, Langston et al. 2007). During the task, rats initially learn a small map of six flavor–location associations: they receive a flavored pellet in the start box and learn that more of the same flavored pellets can be found in one specific sand well in an open field environment. After learning six flavor–location pairs over 9 weeks creating a mental map of paired-associate locations or “schema,” this map can be updated with new flavor–location pairs. In a sequence of articles, it was shown that previous knowledge accelerates learning to a one-trial event as well as the rate of systems consolidation (i.e., the process of memories that are initially hippocampal dependent becoming hippocampal independent) from weeks to days (Bethus, Tse et al. 2010, Tse, Takeuchi et al. 2011). Further, in addition to the hippocampus, the medial prefrontal cortex needs to be active during encoding for memories to last (Tse, Takeuchi et al. 2011, Wang, Tse et al. 2012). In these experiments, the schema is based on the map of flavor locations and not simply on the rule that flavors will be associated with locations, as they could show in a critical control experiment with an unstable map. The involvement of the medial prefrontal cortex as a structure for the schema effect—the expedition of long-term memory—was then later confirmed in humans (van Kesteren, Fernandez et al. 2010, Ghosh and Gilboa 2014, van Buuren, Kroes et al. 2014). How experience of a complex spatial map will influence navigation and new learning has been investigated in humans (Patai, Javadi et al. 2019), but so far not in rodents. This is surprising, since the concept of a cognitive map representation in the brain is of long standing (O'Keefe and Nadel 1979). With place cells in the hippocampus and grid cells in the entorhinal cortex, we have learned about the basic building blocks of how the cognitive map is coded in the brain (McNaughton, Battaglia et al. 2006, Moser, Kropff et al. 2008). These same fundamental building blocks have been shown to also be harnessed for nonspatial memory representation and associations between these (Behrens, Muller et al. 2018). Therefore, **map learning can be the ideal model for us to understand how we build up as well as update (UP) our knowledge systems**.

In the present study, we aimed at **developing a new behavioral task in which we focus on map learning of a larger environment and how mice can use this type of previous knowledge to navigate to and flexibly update information about goals**. Further, in this task we can investigate the role of previous knowledge on new memory acquisition and consolidation across different time-points in training. To achieve this, it is important that during both initial buildup of the knowledge network as well as later updates, the difficulty of the task and thereby the cognitive load remain the same. Thus, we chose to train mice in a large environment to navigate to a single goal location. We expect to see different types of previous knowledge effects on the performance of the mice, reflected in the length of their navigational paths: **learning the general task principles** (static food location and allocentric navigation from different starting positions), **enhancing memory encoding** (increased performance on the second up to the last trial of a session), and **enhancing memory consolidation** (increased long-term memory and performance on the first trial of each session). To test how quickly new information can be incorporated into this map, we changed the goal locations every few sessions.

We could show that mice learn this complex spatial map in the following three main phases: (1) Learning the initial goal location; (2) faster learning after 2 weeks when learning a new goal location; and then finally (3) a third phase after 12 weeks to express one-session learning, leading to long-term memory. Importantly, the map buildup is dependent on how much time passes (weeks), not how often the animal is trained (training days). In addition to the enhancement of long-term memory after map acquisition, we can distinguish a simple memory effect, reflected by better performance across the first couple sessions of the first goal location. Furthermore, an initial learning set effect after 2 weeks of training is seen in the first goal location switch as well as a late learning set effect after 12 weeks of training. This initial learning set effect is not expressed in the first trial of a session (long term memory and thus different from the previous described effect) but does facilitate the increase of overall session performance. Finally, focusing on later learning after 12 weeks, we could show that the degree of overlap with previous knowledge influences navigational performance on the first session of a change (i.e., how quickly new information could be incorporated online). Thus, **the HexMaze task allows the distinguishing of four effects of previous knowledge on memory expressed across three phases in time, ranging from learning set to rapid consolidation and within-session updating**. With this task, we can provide a very rich dataset (130,000 individual trials) that allows the investigation of spatial navigation patterns of mice and how they develop within a session as well as across weeks of experience with the spatial map of the maze.

Materials and Methods

Subjects

Five cohorts with four male C57BL/6J mice in each (Charles River Laboratories), which were 2 months of age at arrival, were group housed in the Translational Neuroscience Unit of the Centraal Dierenlaboratorium at Radboud University (Nijmegen, Netherlands). They were kept on a 12 h light/dark cycle, and before training were food deprived overnight during the behavioral testing period. Weight was targeted to be at 90 to 85% of the estimated free-feeding weight of the animals. All animal protocols were approved by the Centrale Commissie Dierproeven (protocol #2016–014-018). The first cohort (coh 1) was used to establish general maze and task parameters, and was not included in the current analysis.

HexMaze

The HexMaze was assembled from 30 10-cm-wide opaque white acrylic gangways connected by 24 equilateral triangular intersection segments, resulting in a distance of 36.3 cm center-to-center between intersections (Fig. 1A). Gangways were enclosed by either 7.5- or 15-cm-tall white acrylic walls. Both local and global cues were applied to provide visual landmarks for navigation. Barriers consisted of transparent acrylic inserts tightly closing the space between walls and maze floor as well as clamped plates to prevent subjects bypassing barriers by climbing over the walls. The maze was held 70 cm above the floor to allow easy access by the experimenters.

Video acquisition and tracking

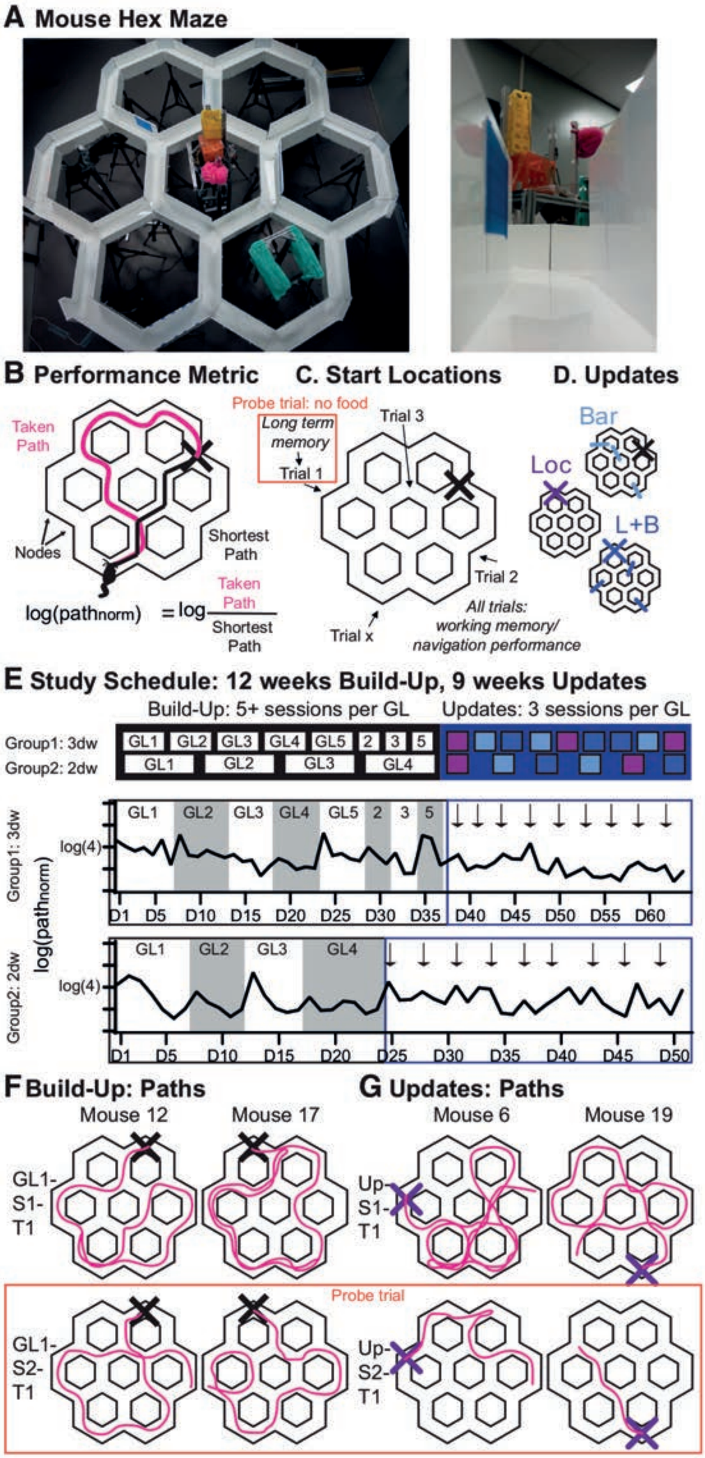
Two USB cameras (model C270, Logitech) were installed 2.1 m above the gangway plane with an overlapping field of vision (FOV) to provide full coverage of the arena and reduce obstruction of vision by maze walls. Image data (15 frames/s, 800 x 600 square pixels per camera) was acquired on a low-end consumer PC (Ubuntu version 19.04, AMD Ryzen 2200G processor, 8 GB RAM) with custom Python scripts (Anaconda Python version 3.7, OpenCV version 4.1.0) at controlled brightness and exposure levels. Images were immediately compressed and written to disk for offline analysis. In parallel, online tracking was applied for feedback to the experimenter and adjustments of the paradigm. Briefly, for each camera view a mask was generated at the beginning of the experiment based on the contrasting brightness of the maze and experimental room floor. This arena outline mask was applied to new frames, and a foreground mask was generated using the OpenCV MOG2 background estimation implementation (Zivkovic and van der Heijden, 2006). The resulting foreground mask was cleaned, and the centroid for the largest detected foreground object in a tracking search window was calculated as the putative location of the mouse in the maze. The location was smoothed over time using a Kalman filter, interpolating occasional occlusions by the maze walls and similar detection failure modes. The detected location was mapped to the closest node, and visually presented to the experimenter as well as logged for offline path analysis. Synchronization between cameras for offline analysis was enabled by presenting a blinking LED (1 Hz, 50% duty cycle) in the overlapping FOV of both cameras. Experimenters could indicate start and offset of trials using a remote presenter (model R400, Logitech).

Behavioral training

After arrival and before training initiation, mice were handled in the housing room daily for 1 week (until animals freely climbed on the experimenter, see videos on https:// www.genzellab.com/#/animal-handling/) and then habituated to the maze in two 1 h sessions (all four cage mates together) with intermittent handling for maze pickups (tubing; Gouveia and Hurst, 2017). Mice were trained either on Mondays, Wednesdays, and Fridays (coh 1–3, group 1) or Tuesday and Thursday (coh 4 and 5, group 2). Per training day (session), each mouse underwent 30 min of training in the maze, resulting in up to 30 trials per session. The maze was cleaned with 70% ethanol between animals (later clean wipes without alcohol to avoid damaging the acrylic and to encourage returning in the next trial), and a heap of food crumbles (Coco Pops, Kellogg's) was placed at a previously determined GL, which varied for each animal. GLs were counterbalanced across animals, as well as within animals across GL switches (e.g., one of four animals), and one of four GLs per animal would be located on the inner ring of the maze while the others were on the outer ring (to shape animal behavior against circling behavior). Start locations for each day were generated based on their relation to the GL and previous start locations (locations did not repeat in subsequent trials, and at least 60% of the trials had only one shortest path possible, the first trial was different from the last and first trial of the previous session, and locations had at least two choice point distances to each other as well as the GL). On average, 30 start locations, which were generated the day before training, were needed per day per mouse. After the mouse reached the food and ate a reward, the animal would be manually picked up with a tube, carried around the maze to disorient the mouse, and placed at the new start location. All pickups in the maze were performed by tubing (Gouveia and Hurst 2017). After placing the animal at the start location, the experimenter quickly but calmly moved behind a black curtain next to the maze to not be visible to the animal during training trials. Each cohort had multiple experimenters (bachelor and master interns, both female and male experimenters), and different cohorts were run by different sets of students. Each mouse was habituated to each experimenter before training in the maze. Each training day, the animals were brought to the training room at least 20 min before training start.

Training consisted of two blocks: Build-Up and Updates. During probe sessions [each second session of a GL switch and additionally in Build-Up; GL1, session 6 (S6); GL2, S5; GL3–5, S4], there was no food in the maze for the first trial of the day and each time for the first 60 s of the trial to ensure that olfactory cues did not facilitate navigation to the GL. After 60 s, food was placed in the GL while the animal was in a different part of the maze (to avoid the animal seeing the placement). All other trials of the day were run with food at the GL. Probe trial and GL switches were initially minimized to help shape the animal behavior. In the first trial of the day, animals would not find food at the last presented location for both the first session of a new GL as well as probe trial days (e.g., always the second session of a new GL); thus, these sessions were interleaved with normal training sessions with food present at the last known location in the first trial of the day to avoid the animals learning the rule that food is initially not provided.

> **Figure 1** The Hex Maze. **A**, Shows the maze with intramaze and extramaze cues (left) and the maze from the view of the mouse (right; also see Movie 1). **B**, The main performance metric is the log-normalized path (pathnorm), with the lengths of the paths taken by the animal divided by the shortest possible path to the GL (indicated by the X). Thus, for all subsequent figures the number in brackets of the log is the relative length of the path taken by the animal, with 2 indicating that the path was twice as long as the shortest possible path. **C**, During training, animals started each trial from a different location and had to navigate to a fixed GL. A first trial measures long-term memory performance and was used as a probe trial on critical sessions (no food present). Performance on all trials of the session measure general working memory/navigational performance in the known environment. **D**, After the animals had acquired the general maze knowledge during the Build-Up, Updates were performed with inclusion of new Bars, new goal Locs, or the inclusion of L1B. **E**, The general training schedule for all animals during the whole experiment. Animals were trained to one GL in a given session. For group 1, the GL was kept constant for seven sessions of GL1, then five or six sessions for GL2, and five or seven sessions each for GL3–5. Additionally, three of the initial five locations were repeated with each of three sessions. For group 2, the GL was kept constant for seven sessions of GL1, then five sessions for GL2–3, and seven sessions each for GL4. Finally, for all cohorts, each Update contained three sessions. The sequence of the Update types was counterbalanced across animals (session 1 of each update indicated with an arrow). Each Update type was repeated two to three times. Throughout all phases, the first trial of the second session and during Build-Up first trial of the fourth, fifth, or sixth session were used as probe trials. Group 1 was trained 3 d/week (3dw), group 2 was trained 2 d/week (2dw). **F, G**, Example paths of the Build-Up (**F**) and Updates (**G**) are shown (Movies 2, 3, 4, 5, 6, 7, 8, 9). T1, Trial 1. Data are in Extended Data Figure 1-1, available online.


A Mouse Hex Maze
B Performance Metric
Taken Path
Nodes
Shortest Path
log(pathnorm) = log Taken Path / Shortest Path
C. Start Locations
Probe trial: no food
Long term memory
Trial 1
Trial 3
Trial 2
Trial x
All trials: working memory/ navigation performance
D. Updates
Bar
Loc
L+B
E Study Schedule: 12 weeks Build-Up, 9 weeks Updates
Build-Up: 5+ sessions per GL
Updates: 3 sessions per GL
Group1: 3dw
Group2: 2dw
GL1 GL2 GL3 GL4 GL5 2 3 5
GL1 GL2 GL3 GL4
Group1: 3dw
Group2: 2dw
log(pathnorm)
log(4)
D1 D5 D10 D15 D20 D25 D30 D35 D40 D45 D50 D55 D60
D1 D5 D10 D15 D20 D25 D30 D35 D40 D45 D50
F Build-Up: Paths
Mouse 12
Mouse 17
GL1-S1-T1
GL1-S2-T1
G Updates: Paths
Mouse 6
Mouse 19
Up-S1-T1
Up-S2-T1
Probe trial

To measure the performance of the animals, the actual path a mouse took was divided by the shortest possible path between a given start location and the GL, resulting in the log of normalized path length (Fig. 1B) and functioning as a score value. Given a sufficient food motivation and an established knowledge network of the maze, a mouse should navigate the maze efficiently. A score of 0 indicated that the mouse chose the shortest path and navigated directly to the goal. On average, animals would improve from a 3 times to a 1.5–2 times longer path length than the shortest path, corresponding to 0.4 and 0.2–3 log values. Random walks (random choice at each node) through the maze are estimated with a model to result in a 4 times longer path (0.6 in log). A more refined random walk with random choices at each node and once in a while a long diagonal run are included in the companion article (Vallianatou et al., 2020, Chapter 4 of this thesis). The normalized path length of any first trial of a session was used to measure long-term memory since training sessions were 2–3 d apart.

The first trials of the second sessions (probe trials) of each goal location in Build-Up and Update phase were watched to score the number of times that animals crossed their current and previous goal location as well as the amount of time they dwelled there. As a control, the same method was applied to two other nodes, one on the inner ring of the maze and the other on the outer ring. These nodes were selected in such a way that they were not close to each other and to the goal locations, with at least three gangways between them. Further, to control a false-positive result, nodes that were in the way between goal locations were not chosen as a control.

Food motivation was ensured by restricting access to food for 8–24 h before training and confirmed by both the number of trials run each day as well as the count of trials during which the animal ate food at the first encounter with the food in each trial. If animals were not sufficiently motivated, the count of both would decrease. Additionally, animals were weighted three times a week and the average weekly weight was ensured to not fall below an estimated 85% free-feeding weight, which was adapted for the normal growth of each animal across time.

Data analysis

The normalized path length for all trials was calculated using MATLAB 2017b (MathWorks). Repeated-measures ANOVAs were run in SPSS Statistics 25 (IBM) to determine the effect of goal location switches and session on the log-normalized path length during the Build-Up and across the three different types of Updates. Within-subject factors were goal location, update type, session and trial. The only between-subject factor was training 2 d/ week (group 2) versus training 3 d/week (group 1). If sphericity was not given, the Greenhouse–Geisser correction was used.

Results

The HexMaze

The HexMaze is arranged as six regular densely packed hexagons, forming 12 two-way and 12 three-way choice points (nodes) 36.3 cm apart, in total spanning 2 1.9 m (Fig. 1A). Gangways between nodes were 10 cm wide and flanked by either 7.5- or 15-cm-tall walls. Maze floor and walls were white and opaque, with local and global cues applied in and around the maze to enable easy spatial differentiation and good spatial orientation, leading, overall, to a complex, integrated maze. During training, food was placed in one of the nodes and the animal had to learn to navigate efficiently from different start locations to the goal location. **To measure performance in this maze, we divided the taken path of each trial by the shortest possible path** (Fig. 1B; for comparison of different performance parameters see below; see also Fig. 9). To eliminate the resulting skewness (skewness, 3.33), we used the log of the normalized path (skewness, 0.72). The reason for the skew of the data is that;30% of the trials are direct runs (paths), resulting in values of 1 and 0 (without and with log, respectively), and animals cannot perform better than a direct run (i.e., there is a ceiling effect and maximum values for best memory performance). Thus, no normal distribution can be achieved with this type of data. Using the log decreases the skew and allows for use of GLM in analysis. However, the data without log (see last section in Results; see also Fig. 9) show the same learning curves and effects. Each session lasted 30 min per animal, resulting in 25–35 trials per session with each trial starting from a different location within the maze (Fig. 1C). **Evaluation of the performances of only the first trials of the sessions measures long-term memory performance**, and during critical sessions (e.g., the second session of a new GL), to measure long-term memory after one session learning, this first trial was used as a probe trial where the food reward was not present for the initial 60 s to control for olfactory cues. In contrast to the first-trial evaluation for long-term memory, looking at the performance over all trials gives a measure of the overall working memory and navigational performance within the environment.

Animals went through the following two phases of training: Build-Up and Updates. In the **Build-Up, the animals should create a cognitive map of the maze environment; in contrast, during Updates, stable performance is achieved,**

and they should be simply updating the cognitive map. These two phases also differed in the frequency of GL switches: during Build-Up, the GL remained stable for five and more sessions, while during Updates a change occurred every three sessions (see also below). Different Update types were performed, including barriers in the environment (Bar), changing the goal location (Loc), and doing both (L + B; Fig. 1D). Five cohorts (coh 1–5) of four animals each were trained in the maze (Fig. 1E). Coh 1 was a pilot cohort to establish maze size, food deprivation, and other parameters, and is not included in the data. Group 1 (coh 2 and 3) was trained three times a week, while group 2 (coh 4 and 5) was trained two times a week. The GL was switched during the BuildUp every five to seven sessions (GL1, seven sessions; GL2, five of six sessions; GL3–5, five of seven sessions) to test when rapid updating could occur. Faster switches were initially avoided, to help shape the behavior of the animal. In the first trial of the day, animals would not find food at the last presented location for both the first session of a new GL as well as probe trial days (e.g., always the second session of a new GL); thus, these sessions were interleaved with normal training sessions with food present in the first trial at the last known location to avoid the animals learning the rule that food is initially not provided.

After 12 weeks of Build-Up, all groups were tested in the Updates, where a change (given by the different Update types) was introduced every three sessions. The sequence of the different Update types (Loc, Bar, L + B) was counterbalanced across repetition and cohorts. Further, the GLs were also counterbalanced across animals within a cohort as well as across cohorts. To ensure that the identity of individual GLs did not account for learning effects over time, the sequence was reversed between cohorts (e.g., GL1 of the first animal in coh 2 would be GL5 of the first animal in coh 3).

Overall performance for each group across time can be seen in Figure 1E. **Different learning effects were found** as highlighted in individual paths (Fig. 1F,G, Movies 1, 2, 3, 4, 5, 6, 7, 8, 9), as follows: on the first trial of the first training day of the Build-Up, the animals show random movement through the maze and just by chance find the GL (Fig. 1F, Movies 2, 4). On the next day at the first trial, some but not all animals already show more goal-directed behavior (Fig. 1F, Movies 3, 5). In contrast, during the Updates on the first trial of a new GL, the animals still show random exploration, since the goal location is unknown, but are then more likely to show memory effects and goal-oriented behavior in earlier trials of session 1 (Fig. 1G, Movies 6, 8). And in the succeeding session of the Updates most animals had already shown more goal oriented navigation to the reward location on the first trial (Fig. 1G, Movies 7, 9).

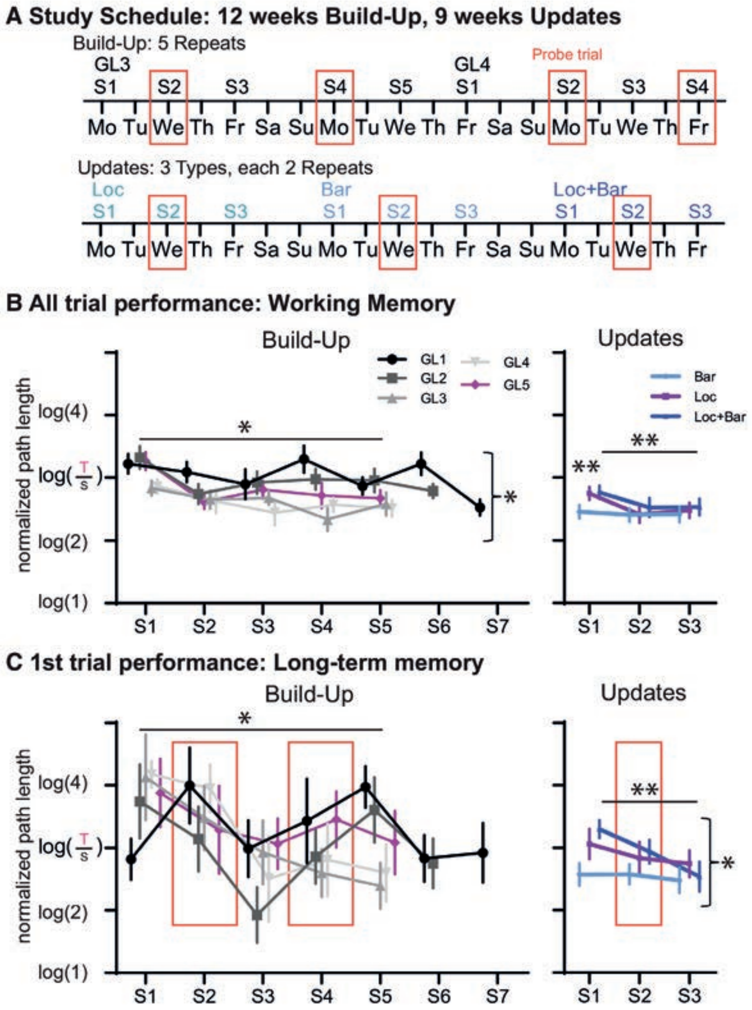


Figure 2 HexMaze Performance Group 1 3 days per week training: **A**. Shows schedule examples for the Build-up and Updates. Orange boxes indicate days with probe trials (no food for 60s of the first trial). **B**. Performance across all trials (including first trial) measures general working memory/navigational performance within the environment. During Build-Up there was a significant effect across session and across the five goal location (GL) switches. In contrast, during Updates, only if a location switch was involved in the update (Loc/L+B), performance was worse during the first session of the change and an improvement across sessions is visible. **C**. Performance on the first trial of each session measures the ability to remember the GL from 2 to 3 d ago. During the Build-Up long-term memory improved across sessions. During the Updates there was an improvement across sessions as well as a difference between types with larger changes in the environment (linear from Bar to both L+B) leading to worse performance. This is especially noticeable in session 1 for Loc and L+B switches where the goal is initially unknown, whereas for a Bar update only an adaption of the route is involved. Single asterisks indicate $p<0.05$ and double asterisk stand for $p<0.01$. Error bars are SEM. The number in brackets of the log is the relative length of the path taken by the animal (taken path T/ shortest path S) with 2 indicating that the path was twice as long as the shortest possible path.

Building and updating the map

To formally investigate the effects seen in the individual paths, we analyzed group-level performance in more detail. Group 1 (total, n= 8) was trained Monday/ Wednesday/ Friday (Fig. 2A, example study schedule) and during the Build-Up showed a significant improvement in navigation to the GL (all trials, which includes the first trial) across sessions as well as across GLs (GL1–5; session: F(4,28) = 6.2, p= 0.001; GL: F(4,28) = 3.3, p= 0.026; interaction, F(16,112) = 1.4, p= 0.15). For both session and GL, the linear contrast was significant (session, p= 0.027; GL, p= 0.043; Fig. 2B). In the Updates, the animals overall performed better than in the Build-Up (F(1,7) = 8.2, p= 0.024) and continued to show a significant improvement of performance over the three sessions (session: F(2,14) = 12.9, p= 0.001; linear contrast, p= 0.005). Additionally, there was an effect of Update type (Bar, Loc, L + B) as well as a type session interaction: in contrast to the Update types with location changes, animals already performed well in session 1 of the barrier updates (type: F(2,14) = 3.5, p= 0.058; with linear contrast across Bar, Loc, and L + B, p= 0.027; interaction: F(4,28) = 2.6, p= 0.059; orthogonal comparison session 1 Bar vs Loc/L + B, p= 0.01). During the first trial of each session, the animal had to rely on long-term memory (2–3 d between sessions) to navigate to the current GL. To minimize olfactory cues (e. g., chocolate smell and markings), the maze was cleaned with alcohol between animals, further on critical sessions (e. g., second session after a change to test for one-session learning), and no food was present in the maze for 60 s during the first trial. These probe trials were performed in sessions 2 and 4/5 or 6 during the Build-Up and in session 2 during the Updates (Fig. 2A). **Across sessions, long-term memory improved independent of the GL during the BuildUp** (Fig. 2C; session: F(4,28) = 4.0, p= 0.01; linear contrast, p= 0.056; GL: F(4,28) = 0.4, p= 0.77; interaction: F(16,112) = 1.1, p= 0.34). **In the Updates, long-term memory increased across sessions as well as differed between Update types** (session: F(2,14) = 3.7, p= 0.053; with linear contrast, p= 0.009; type: F(2,14) = 3.7, p= 0.052; with linear contrast across Bar, Loc, L + B, p= 0.028; interaction: F(4,28) = 0.58, p= 0.68). Similar to the all-trials performance, in barrier Updates performance was better than in the other two types of Updates where the GL changed.

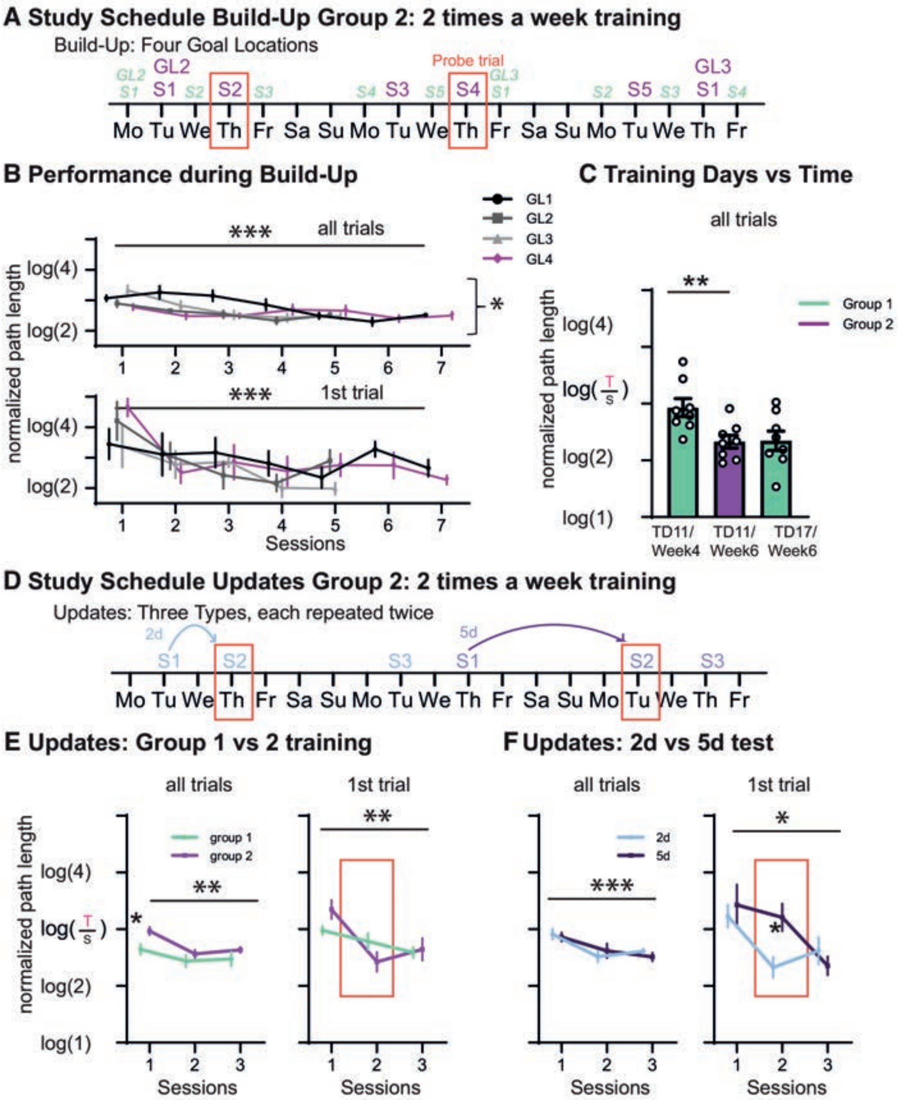
Time versus training

In contrast to group 1, group 2 (n = 8) were trained only 2 d/week, which resulted in **a shift between the training day and the time alignment between both groups** (Fig. 3A). As with 3 d/week training, 2 d/week training lead to an improvement in all-trials measurement across sessions as well as across GLs (GL1–4; session: F(4,28) = 18.3, p < 0.001; linear contrast, p < 0.001; GL: F(3,21) = 4.7, p = 0.011;

linear contrast, p = 0.044); further, in contrast to the 3 d/week training, there was a session GL interaction (F(12,84) = 2.7, p = 0.004) with a faster improvement across sessions in later GLs (Fig. 3B). Long-term memory (first-trial performance) improved across sessions (session: F(4,28) = 12.5, p < 0.001; linear contrast, p = 0.001), but there was no change from one GL to the next (p = 0.49), as also seen with group 1. Including both group 2 and group 1 in one ANOVA revealed a GL session training type interaction for all trials (F(12,168) = 1.9, p = 0.039), and for first trials it revealed a training-type main effect (F(1,13) = 6.7, p = 0.023) as well as a marginal session training type interaction (F(4,52) = 2.4, p = 0.066).

As one of the goals was **to evaluate whether general performance was determined by the amount of time that had passed in contrast to how much training the animals had received**, we included the same training day of group 1 and group 2 as well as the session of group 1 that corresponded to the same week of training as group 2 in a univariate analysis (F(2,21) = 5.253, p = 0.014; group 1: training day 11, session 4 of GL2, during week 4 and training day 17; session 4 of GL3, during week 6; group 2: training day 11, session 4 of GL2, during week 6). These specific sessions were chosen, since only then did the same session number (here, session 4) occur at the same time in weeks as well as the day within the week across groups; thus, it was the only training day that could compare time versus training overall but could still control for the amount of training to the current goal location as well as how long ago the last training session was performed. Group 2 performed in a similar manner to group 1 when compared with how much time had elapsed, but was significantly better than group 1 with the same amount of training (Fig. 3C). **Thus, performance in the HexMaze was more dependent on the time period in which the animals had been exposed to the maze and not how much training or exposure itself was involved.**

To further validate whether this also applies to the previous knowledge effects, we focused as a next step on the Updates (Fig. 3D). Both the all-trial as well as first-trial measure showed an improvement across sessions (F(2,28) = 9.5, p = 0.001; with linear contrast, p = 0.005) as well as a marginal session training interaction (F(2,28) = 3.1, p = 0.06), but did not expose an effect of training amount (p = 0.87; Fig. 3E). Only during the first session did group 2 perform worse than group 1 (p = 0.01). Thus, despite the decreased amount of training, rapid updating was still possible, indicating that **the creation of a cognitive map is dependent on time, not on training**.


A Study Schedule Build-Up Group 2: 2 times a week training
Build-Up: Four Goal Locations
GL2 S1 S1 S2 S2 S3 S4 S3 S5 S4 GL3 S1 S2 S5 S3 GL3 S1 S4 GL2
Probe trial
Mo Tu We Th Fr Sa Su Mo Tu We Th Fr Sa Su Mo Tu We Th Fr
B Performance during Build-Up
GL1
GL2
GL3
GL4
*** all trials
*
*** 1st trial
log(4)
log(2)
normalized path length
1 2 3 4 5 6 7
Sessions
C Training Days vs Time
all trials
**
Group 1
Group 2
log(4)
log(T/s)
log(2)
log(1)
normalized path length
TD11/ Week4
TD11/ Week6
TD17/ Week6
D Study Schedule Updates Group 2: 2 times a week training
Updates: Three Types, each repeated twice
2d
5d
S1 S2 S3 S1 S2 S3
Mo Tu We Th Fr Sa Su Mo Tu We Th Fr Sa Su Mo Tu We Th Fr
E Updates: Group 1 vs 2 training
F Updates: 2d vs 5d test
all trials
1st trial
group 1
group 2
2d
5d
**
*
***
log(4)
log(T/s)
log(2)
log(1)
normalized path length
1 2 3
Sessions

< **Figure 3** HexMaze group 2 performance 2 days a week training: **A.** Shows schedule examples for the Build-up. The schedule for group 2 is shown in purple and for group 1 in light green, illustrating the resulting shift in alignment of training days and time. Orange boxes indicate days with probe trials (food not present for 60s of the first trial). **B**. Performance across both all-trials measurement (general working memory/navigational performance within the environment) and first-trial measurement (long-term memory). We found a significant improvement in performance across sessions for both measures and additionally across GL and GL X session interaction for all trials. **C.** To compare two days per week with three days per week training, we included the corresponding training day as well as session according to time of group 1 and compared these with the performance of group 2. It is important to note, that the performance depended on how much time had elapsed since first exposure to the maze (weeks), not how much training the animals had received (TD is training day). **D**. Show examples from the study schedule of the Updates. With two days per week a natural alternation of two- and five-day gaps ensued during the Updates**. E.** Comparing only the 2d Updates of group 2 with the Updates of group 1 (also 2d gaps) showed only an Update difference during the first session. **F.** Plotted is the performance during Updates for group 2 for both the two- and five-day delays. One session of training only led to significant long-term memory that lasted two not five days whereas two training sessions did indeed lead to a five-day memory persistence visible in the third session (2d condition for session 2). The single asterisk stands for $p<0.05$, the double asterisk for $p<0.01$ and the triple asterisk for $p<0.001$. Error bars are SEM. The number in brackets of the log is the relative length of the path taken (taken path T/shortest path S) by the animal with 2 indicating that the path was twice as long as the shortest possible path.

The 2 d/week training schedule also allowed us to investigate **how many sessions are necessary for memory persistence** as the training schedule naturally alternated with 2 and 5 d gaps between sessions (Fig. 3D). While one session was sufficient for the animals to remember where the food was located in the first trial 2 d later, this memory did not last 5 d (Fig. 3F). However, after two sessions of training (Fig. 3F, 2 d condition), the animals did remember the GL in the third session (5 d after the second session; session: $F(2,14) = 8.1$, $p = 0.005$; with linear contrast, $p = 0.016$; interaction session delay: $F(2,14) = 3.6$, $p = 0.054$; delay overall, $p = 0.34$). In contrast, general navigational performance (an all-trial measure) did not show a difference between the two delays (interaction, $p = 0.24$; delay, $p = 0.9$; session: $F(2,14) = 34.7$, $p < 0.001$; with linear contrast, $p < 0.001$).

Three phases of map learning

Combining the data from groups 1 and 2, let us delve further into different phases of map learning. The main difference between the learning phases is **how quickly the animals can adapt their performance to new information** (e.g., a new goal location). First, all-trial performance was evaluated and separated for the four goal locations during Build-Up and the different Update types, and each of these for sessions 1, 2, and 3 (sessions 3–5/7 for BuildUp, only session 3 for Updates since no other sessions were run). This analysis highlights three phases of learning (Fig. 4A; GL/UP: $F(6,90) = 4.7$, $p < 0.001$; session: $F(2,30) = 40.1$, $p < 0.001$; GL/UP session interaction: $F(5.4,81.6) = 2.8$, $p = 0.018$). When learning the first goal location, the animals need three and more sessions to reach good performance (phase 1). In contrast, when learning the second goal location, the animals already perform better at the second session (phase 2). Finally, during GL4 and the Updates, the animals already perform better in the first session but also have additional gains to the second session (phase 3). Importantly, already in the first few goal locations during Build-Up the animals reach their best possible performance in the later sessions. The difference to the Update phase is that during Build-Up it takes more sessions to reach that optimal performance level. Once the animals reach the Update phase, performance is stable. Therefore, the different phases in map learning are expressed in how quickly they can adapt to new goal locations and are not confounded by a general, continuous increase in performance.

To further focus on changes in the first two sessions across the different learning phases, the first and last trial of session 1 of a change and the first trial of session 2 are plotted for the different goal locations in the Build-Up and Updates (Fig. 4B-E). The first trial of session 1 was consistently high across all phases with the exception of the barrier updates, reflecting the fact that only in that Update type was the current goal location known and that the first trial did not represent a search for the new goal location ($F(1,112) = 6.5$, $p < 0.001$; orthogonal comparison barrier vs other, $p = 0.0018$). The final trial of session 1 was also quite consistently stable across all phases, emphasizing that gains because of within-session learning also remained similar across phases ($F(1,112) = 0.1$, $p = 0.96$). **The main difference among the three learning phases can be seen in the first trial of the second session, reflecting long-term memory after one-session learning** ($F(1,112) = 3.6$, $p = 0.017$; orthogonal comparison Build-Up and Updates, $p = 0.0037$). There were gains from the very first goal location to subsequent goal locations during BuildUp but even more gains during the Updates, highlighting the stepwise increase in long-term memory performance over the different learning phases. These gains are also reflected when comparing performance on the last trial of session 1 to the first trial of session 2 (Fig. 4E). In the first goal location of Build-Up, this metric is positive, reflecting worse performance after the 24 h break, while during the other goal locations of Build-Up it is approximately zero, showing that they sustain their final performance level across the offline period. In the Updates, negative values are seen (one-sample t test to 0: $t(71) = 4.2$, $p < 0.001$), which shows that they perform even better at the first trial of the second session compared with the final trial of the first session, thus showing an offline gain in performance.

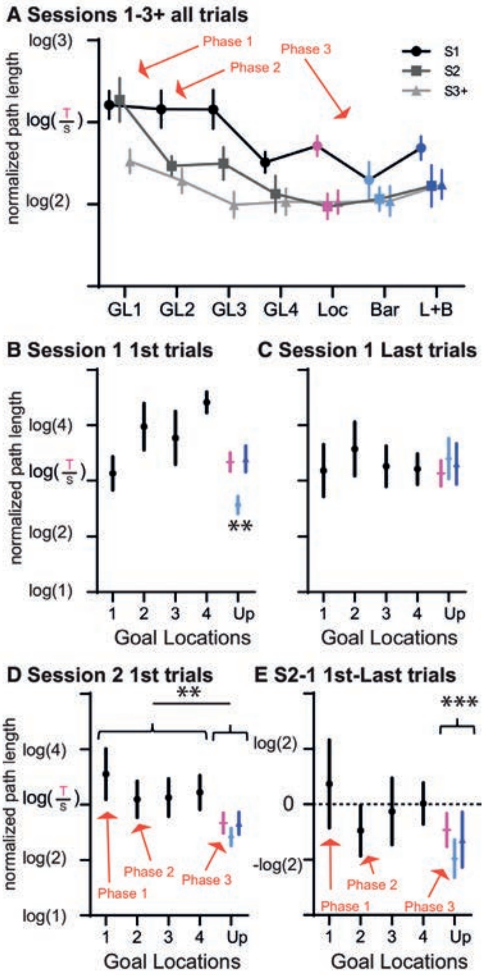
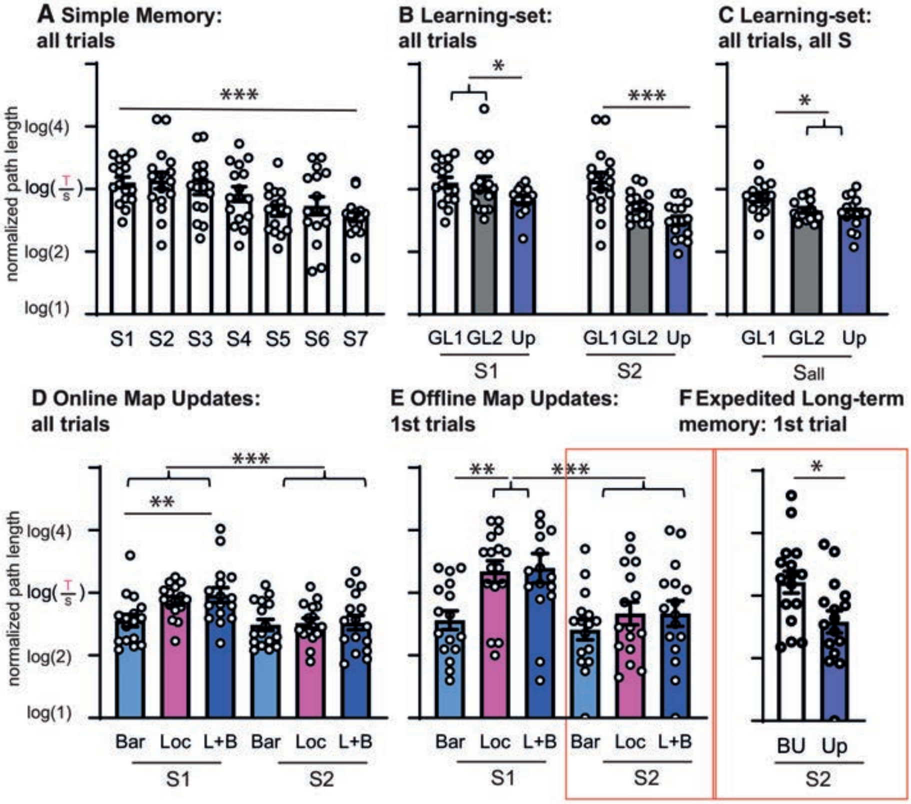


Figure 4 Three Phases of Map learning**: A.** Plotted are all trials separated for the four goal locations (GL) during Build-up as well as the different Update types with separate lines for first session, second session and third session onwards (for Build-up its session 3-5/7, for Updates just S3 since no further sessions were run). Three learning phases are noticeable: learning the first goal location, learning the second goal location with better session 2 performance and in the Updates with already good session 1 performance. Next the first trial of sessions 1 (**B**.), the last trials of sessions 1 (**C**.), the first trials of sessions 2 (**D**.) and the change from the last trial of sessions 1 to the first trials of sessions 2 (**E**) are shown for the different goal locations during Build-up (GL1-4 as well as the different update types. The first trial performance during session 1, when the goal is unknown, first became worse in GL 2-4 in comparison to GL1 most likely due to animals first navigating to the old goal location. Only in the Barrier updates (light blue) was performance better than in all other GL and updates, since the location did not change. At the end of session 1 (last trial) there is no difference between the different GL and updates. The three phases of learning are again noticeable in the first trial of session 2, reflecting long-term memory after one session training. This showed a step-wise function, improving in GL2-4 in contrast to GL1 and improving even more during the updates. The same is reflected in the difference values presented in E (updates one-sample t-test to 0 t_{71}=4.2 p<0.001).



In sum, there seems to be three phases in map learning: **(1) learning the new goal location; (2) learning the second goal location 2 weeks later, when performance gains close to optimal performance are already seen in the second session but are not yet expressed in long-term memory (first trial of the second session); and finally, (3) after 12 weeks, when performance gains are already expressed in the first session to a new goal location and also translate to long-term memory effects with good performance at the first trial of the second session**. This analysis also helps to distinguish among task–rule learning (e.g., "I need to run to a goal location"), maze learning (maze layout and surrounding cues), and goal learning (where in the maze is the food). In Figure 4E, the amount of training for one goal is controlled for, thus excluding the general effect of goal learning (for each data point, the amount of exposure to the current goal is the same: one session). The general task should be learned by the animal by GL2 or at the latest by GL3 (by then the animal learned that goal locations can change). Thus, only maze layout learning can explain the additional benefit seen in the Updates.

< Figure 5 Previous Knowledge effects**:** In these panels we highlight some previous knowledge effects **A.** Plotted is the whole session performance for the first GL during the Build-up. The significant session effect reveals a performance increase dependent on experience indicating a more efficient working memory/navigational performance. **B.** Plots the performance for the first two sessions of the first two GLs during the Build-Up, as well as Updates (averaged for all types). Already for the second GL (three weeks since training start) a significant increase in performance (decrease of path length) is seen in the second session in comparison to the first session. This overnight (offline) performance increase is comparable to the increase found after seven sessions for the first GL. This may represent a more efficient consolidation and updating effect but is only expressed in the whole session average (not long-term memory present in the first trial, see Fig. 2 and 3). During the Updates, this performance increase is already visible in the first session with additional offline gains found in the second session. This three-step performance gain is reminiscent of a learning-set effect (Harlow 1949). **C.** Considering all sessions, we find that animals already reach overall plateau performance by the second GL. **D.** Zooming in on the performance during the first and second session during the Updates, another previous knowledge effect is revealed across the different Update types. The barrier (Bar), goal location (Loc) and combined updates (L+B) differed in their overlap of previous knowledge (or need for updating that knowledge) which influenced how well they performed (all-trial) in the first session. **E**. Shows the same effect but now for only the first trials. Only in the presence of a goal switch did performance in the first session decrease. However, by the second session this performance difference was gone, revealing that one session is sufficient for the memory update. Finally, **F.** depicts the performance of only the first trial of the second session during the Build-up and Updates (only Loc and L+B) where long-term memory (2-3 d) after one session learning to a new GL improves from Build-up to Updates. Thus, it seems once a cognitive map is established, only one session training leads to better long-term memory performance. Orange boxes indicate that the trial was utilized as a probe trial meaning food was not present for the initial 60 s. The single asterisk stands for p<0.05, the double asterisk for p<0.01 and the triple asterisk for p<0.001. Error bars are SEM. Data taken from both group 1 and 2. The number in brackets of the log is the relative length of the path taken by the animal (taken path T/shortest path S) with 2 indicating that the path was twice as long as the shortest possible path.

Previous knowledge effects

Different effects of previous knowledge could be observed in the resulting data, so, next we will focus on specific sessions and trials to highlight some of these effects. The simplest effect is already seen in the first GL during the Build-Up, where a significant **session effect indicates that each session benefits from the experience of the previous session** (groups 1 and 2: n = 16, F(6,90) = 5.6, $p < 0.001$; Fig. 5A). This simple learning effect, while often not considered as a previous knowledge effect, does affect session performance and, thus, must be considered even in experiments that just focus on each session individually, as seen in most electrophysiological experiments (Roux, Hu et al. 2017, Lopes-dos-Santos, van de Ven et al. 2018, Michon, Sun et al. 2019)

The second previous knowledge effect can be evaluated by **how well an animal can navigate within an environment and how fast this navigational capability can be adapted to a new goal as soon as it has learned a specific task**. Here, this was tested at every GL switch from the beginning of the Build-Up to the end of the Updates (groups 1 and 2, n = 16). Including the first two sessions of the first two GLs during the Build-Up as well as during the Updates (averaged across all types) revealed three distinct steps (Fig. 5B; session: F(1,15) = 12.6, p = 0.003; GL: F(2,30) = 8.3, p = 0.001; interaction: F(2,30) = 3.9, p = 0.031). For the first GL, performance does not increase from the first session to the next, but, as seen in Figure 5A, a performance improvement develops over seven sessions. After the first GL switch (GL1 to GL2), performance decreases to the level of performance during the first session of GL1. However, a significant improvement is exposed already for the second session of GL2 (3 weeks after training start). Finally, as a third step, we find that these improvements occur in any first Update session, including additional gains in the second Update sessions (12 weeks after training start). **These effects are visible across all-trial performance measurements and are likely a result of a mix of learning set effects** (Harlow 1949) **as well as of an effect of increased knowledge of the maze layout.** When averaging the performance across all sessions (Fig. 5C; groups 1 and 2, n = 16), animals overall had already reached plateau performance at the second GL switch during the Build-Up.

By focusing in more detail on the first and second sessions during the Updates alone, we can consider the amount of information animals need to incorporate during the Updates (groups 1 and 2, n = 16). We found a significant main effect of session and an interaction between session and Update types (session: F(1,15) = 26.1, p < 0.001; type: F(2,30) = 2.9, p = 0.072; interaction F(2,30) = 6.3, p = 0.005). The follow-up test revealed that **within session 1 the amount of novel information that needs to be integrated into the existing map affects the within session online performance** (just a barrier, just a new location, or both; linear contrast in S1, p = 0.003; Fig. 5D). However, this difference is eliminated by the second session, indicating that the information had been completely incorporated during the offline period.

As a final step, we tested for the enhancement of long term memories by comparing the same two sessions but only including the first trial. Similar to the all-trial performance measurement, the first session performance was worse for conditions including a GL switch (Loc and L + B) compared with just a barrier switch, but this difference disappeared by the second session (Fig. 5E; groups 1 and 2, n = 16; session: F(1,15) = 14.4, p = 0.002; type: F(2,30) = 7.2, p = 0.003; interaction: F(2,30) = 1.2, p = 0.3). Finally, to investigate whether this enhancement of long term memory after one-session learning was missing initially during Build-Up, the first trial performance during the second session of the Build-Up was compared with the first trial during the second session of the Updates (only Loc and L + B). This revealed a significantly better long-term memory in the second session in the Updates compared with the Build-Up (Fig. 5F; groups 1 and 2: n = 16, t(15) = 2.1, p = 0.049). To confirm this effect with a different performance parameter, we counted the number of crossings for the new goal location, the previous goal location, and two control nodes (one in the inner ring of the maze, one in the outer ring) during this trial, since, because it was a probe trial, no food was present during the first trial of each second session. As can be seen in Figure 6, animals crossed both the current and last goal locations significantly more often than the control nodes starting with the second goal location, and an additional increased number of crossings were seen during the Update phase (groups 1 and 2 GL2-4 and Loc Update for full model, n = 16; node: F(3,45) = 22.3, p < 0.001; GL2-4/Loc: F(3,45) = 10.7, p < 0.001; interaction: F(9,135) = 2.0, p = 0.044). Interestingly, **this analysis also highlighted that animals did retain the memory of the old goal location after a goal location switch, since they tended to go more often to both the current, new goal location as well as the last goal location compared with control nodes**.

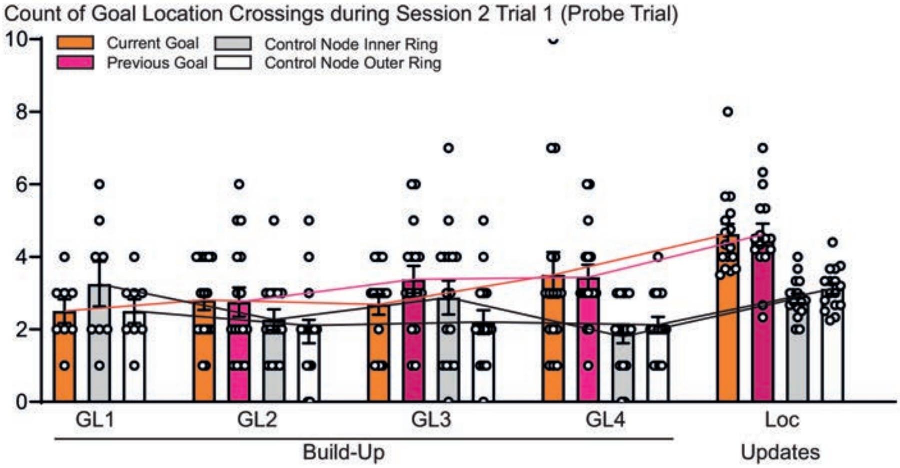


Figure 6 Probe trial analysis (each session 2 trial 1): Across the goal location switches during Build-Up and the during the Updates an increase in the number of crossings could be seen for both the current and previous goal location in comparison to the two control nodes (groups 1 and 2 GL2-4 and Loc for full model, n=16, node $F_{3,45}$=22.3 p<0.001, GL2-4/Loc $F_{3,45}$=10.7 p<0.001, interaction $F_{9,135}$=2.0 p=0.044)

How updates affect path length

To characterize how the Updates themselves affect path length, the path lengths (in terms of the number of nodes) for the shortest path and the taken path are shown in Figure 7, and the normalized path length (log of taken/ shortest path) is shown as used in the other figures for both the final trial before an update (usually, a session 3) and the first trial of the Update. If a barrier was included (Bar and L + B Update), there was a significant change in the shortest possible path (Bar: t(15) = 4.39, p = 0.001; L + B: t(15) = 3.69, p = 0.002), indicating that the inclusion of barriers does change the overall map geometry in the maze. However, the taken path only showed a significant change if the goal location was changed (taken path: Loc, t(15) = 3.29, p = 0.005; L + B, t(15) = 3.77, p = 0.002; normalized path: Loc, t(15) = 3.20, p = 0.006; L + B, t(15) = 1.83, p = 0.087). This emphasizes again that only the inclusion of barriers did not affect the performance of the mice and that the animals could rapidly adapt to this change, as was also seen in Figure 5.

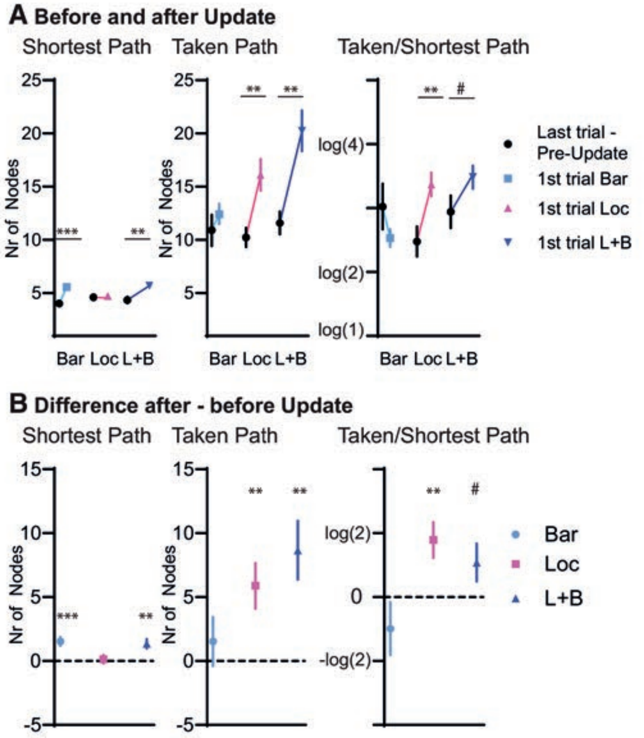


Figure 7 Changes due to Updates**:** Shown in **A**. always the last trial before an update (S3 of previous condition) and the first trial of the update and in **B**. the difference values (subtraction) for these. From left to right the shortest possible path, the taken path and the relative path is presented. If barriers were included (Bar and L+B) the shortest possible path would increase from the previous trial. But only if location was changed (Loc and L+B) did the taken path increase, for the Bar update the taken path only increased by the same amount of the shortest path (2 nodes). Interestingly, due to the change in shortest path the relative change (taken/shortest) actually decreased in the Bar update. The # stands for p=0.087, the double asterisk for p<0.01 and the triple asterisk for p<0.001 for **A** paired t-tests and **B** one-sample t-test to 0. Data taken from both group 1 and 2. Error bars are SEM. The number in brackets of the log is the relative length of the path taken by the animal (taken path T/shortest path S) with 2 indicating that the path was twice as long as the shortest possible path.

Within-session learning

To measure within-session learning, and to enable comparison across different phases of learning, trials were binned into trial blocks with trial 1, trials 2–10, trials 11–20, and trials 21–30. This was done for S1–3 of the first goal location and goal locations 2–5 in the Build-Up as well as in each Update type (Fig. 8). Since for the first goal location very few animals managed 20 trials in each session, for the overall analysis we included only up to 20 trials. There was a significant effect of training phase, session, trial block, as well as interactions between training phase and session, training phase and trial block, as well as between session and trial block (phase: $F(4,60) = 12.6$, $p < 0.001$; session: $F(2,30) = 21.2$, $p < 0.001$; trial block: $F(1.3,18.8) = 8.9$, $p = 0.001$; phase session: $F(8,120) = 3.7$, $p = 0.001$; phase trial block: $F(4.2,63.1) = 2.6$, $p = 0.041$; session trial block: $F(2.5,37.5) = 5.0$, $p = 0.008$). For the first goal location, neither session nor trial block showed a significant effect ($p = 0.39$; Fig. 8A) in contrast to the subsequent goal locations of the Build-Up, during which each factor as well as the interaction showed a significant effect (session: $F(2,30) = 30.8$, $p < 0.001$; trial block: $F(1.6,23.4) = 13.2$, $p < 0.001$; session trial block: $F(3.4,50.5) = 7.8$, $p < 0.001$; Fig. 8B). **This emphasizes that while the first goal location did not show strong within session learning during these first three sessions, for the subsequent goal locations during Build-Up the main learning occurred between trial 1 and the next trial block during session 1 and trial 1 of sessions 2 and 3 started lower but additional within-session improvement could be observed in the next block**. During the Updates of Loc and L + B, a linear improvement during session 1 was seen across trials, and now performance was sustained to session 2 and 3 with no strong additional gains from the first trial to subsequent trials. Thus, Loc showed significant effects of session and trial block but no interaction (session: $F(2,30) = 15.3$, $p < 0.001$; trial block: $F(1.9,29.3) = 6.5$, $p = 0.004$; session trial block, p. 0.79; Fig. 8D), and for location and barrier Updates the interaction became significant as well (session: $F(2,30) = 6.4$, $p = 0.005$; trial block: $F(3,454) = 4.0$, $p = 0.013$; session trial block: $F(6,90) = 3.6$, $p = 0.003$; Fig. 8E). In contrast, the performance of barrier Updates started trial 1 of the first session well and remained stable, resulting in no significant effect of any factor or interaction (session, $p = 0.07$; other, p. 0.2; Fig. 8C).

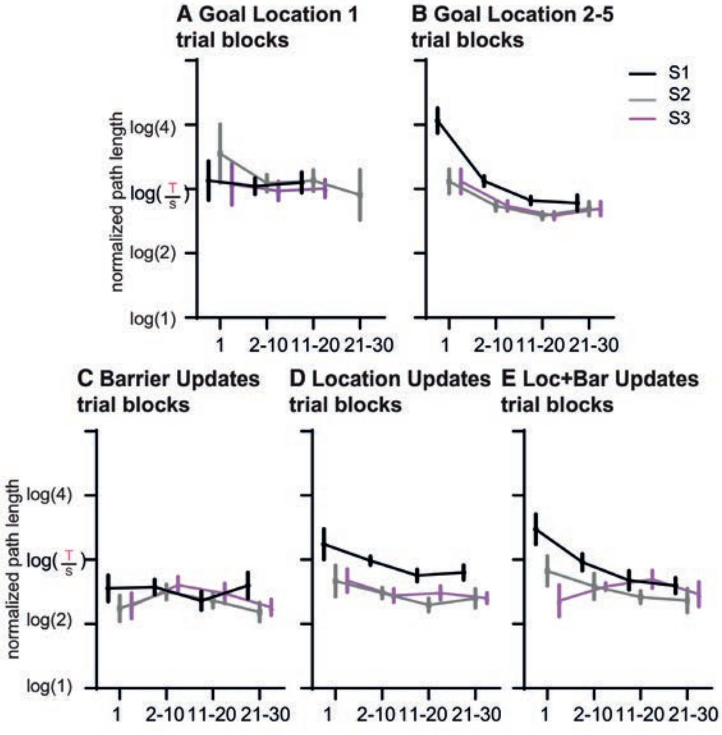


Figure 8 Within-session learning. **A–E**, The change in performance within session (trial 1 and then blocks of 10 trials) always across the first three sessions (S1–3), the very first goal location (**A**), averaged across the subsequent goal locations of the buildup (**B**), for barrier updates (**C**), for location updates (**D**), and for location and barrier (Loc 1 Bar) updates (**E**). The first goal location did not show strong within-session learning during these first sessions; in contrast, later on (**B**) the main learning occurred between trial 1 and the next trial block during session 1 and trial 1 of sessions 2 and 3 started lower, but additional within-session improvement could be seen in the next block. In the barrier updates, performance was starting trial 1 of first session well and remained stable. For the other updates, a linear improvement during session 1 was seen across trials, and now performance was sustained to sessions 2 and 3 with no strong additional gains from the first trial to subsequent trials. For statistics, see the main text. Data were taken from both groups 1 and 2. Error bars are the SEM. The number in brackets of the log is the relative length of the path taken by the animal [taken path (T)/shortest path (S)], with 2 indicating that the path was twice as long as the shortest possible path.

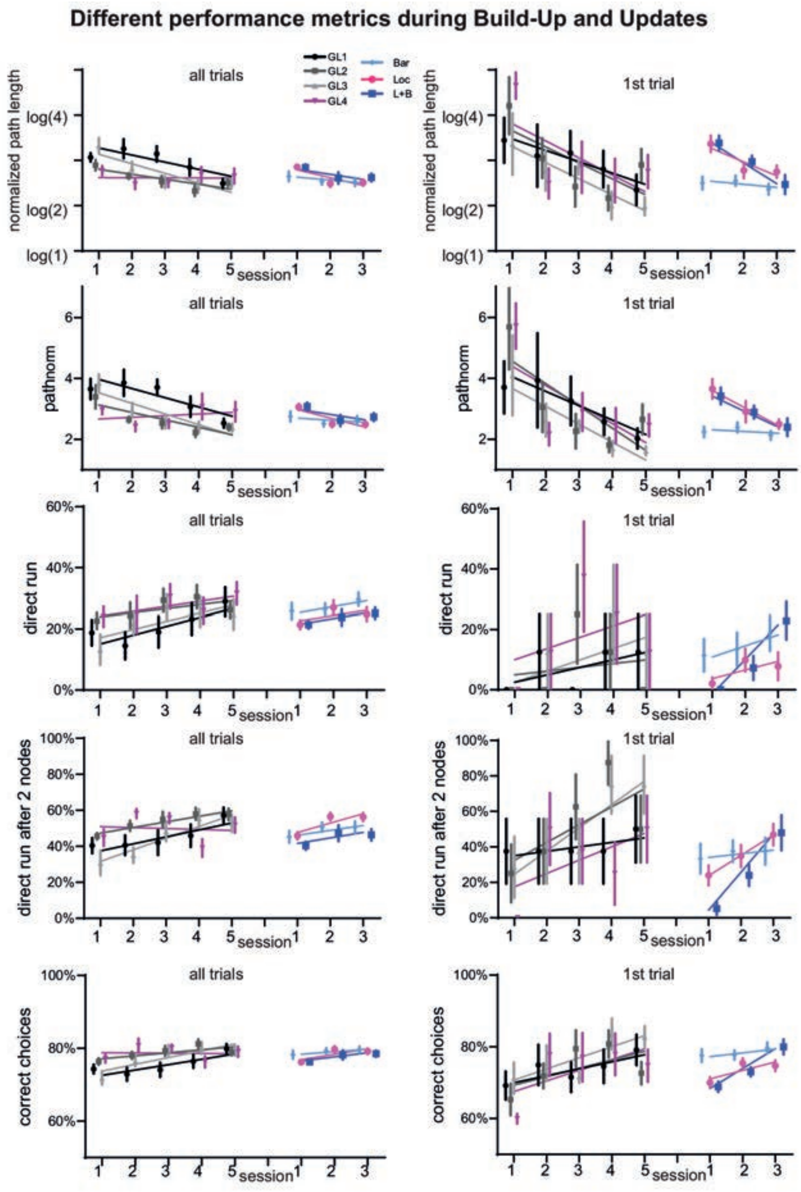


Figure 9 Different performance parameters during Build-Up and Updates. Shown are the log of normalized path length (top row, as used throughout manuscript), normalized path length, percentage of trials that were a direct run (second from top), percentage of trials that were a direct run after the second node since mice often would initially run in heading direction and then stop to consider where to go (third from top). As final parameter, we took for each node if the choice would bring the mouse closer to the food (correct) or not (incorrect) and created an average per trials across all traversed nodes. Each left all trials, right first trials for Build-Up (Group2) and Updates (all). Error bars are SEM. Lines are polynomial fits. The same effects seen in the log of the normalized path length can also be seen in the other parameters.

Other performance parameters

In the present analysis, we focused on using the normalized path length (the taken path in number of nodes divided by the shortest path), and since these values showed a strong skew, we used the log thereof to enable using GLM. However, other parameters, such as the normalized path length without the log transformation, the percentage of trials that were a direct run, the percentage of trials that were a direct run after the second node (since mice often would initially run in a heading direction and then stop to consider where to go), and the percentage of correct choices (Fig. 9), could also be extracted from our dataset. For the percentage of correct choices, we analyzed for each node whether the choice would bring the mouse closer to the food (correct) or not (incorrect) and created an average per trial across all traversed nodes. As can be seen in Figure 9, **the same effects seen in the log of the normalized path length can also be seen in the other parameters**. And while only 30% of the trials showed a direct run from the starting location, 60% of the trials showed a direct run two nodes after the starting location. In the accompanied data table, we share the raw data of all trials with all these different variables that can be used by others for further, more detailed investigations into spatial learning in mice.

Discussion

In the present study, we aimed at developing a new rodent task that enables the **investigation of map learning on memory encoding and consolidation**. More specifically, we tested how mice build up and update knowledge of a large spatial map and how their navigation abilities change over time. We could show that mice learn this complex spatial map in the following three main phases: (1) learning the initial goal location, (2) faster learning after 2 weeks when learning a new goal location, and then finally (3) a third phase after 12 weeks during which they express one-session learning leading to long-term memory. The data from the HexMaze allow the investigation of many different aspects of spatial navigation and memory. Here, we focused on previous knowledge effects on performance and learning. These effects ranged from simple day-to-day performance increases to effects reflected by offline consolidation and online learning. Initial application reveals that this task can be used to test different aspects of memory while simultaneously controlling for difficulty of learning across each phase in training: from the buildup of knowledge to updates testing both across-session as well as between-session performance development. The data from the 30,000 trials are supplied with this article and can be used for many more investigations and analysis of spatial navigation in mice. In the accompanying article (Vallianatou, Alonso et al. 2021), the data were used to model the strategies used by mice in the task. How do we build up and use knowledge of a large spatial environment or map? And how will experience in a maze shape new learning? Previous knowledge will affect behavior and learning (Harlow 1949, Bartlett and Bartlett 1995, Tse, Langston et al. 2007), and, thus, needs to be considered when applying any particular training paradigm. To test how mice learn a map, we used a large spatial environment, more naturalistic in its complexity. Mice were trained to find a food location from different starting points in a maze, thereby enforcing allocentric learning to one fixed goal location per session over two training phases: Build-Up (12 weeks) and Updates (9 weeks). During the Build-Up, the goal location was kept constant for five to seven sessions before switching to a new one, while during the Updates switches occurred every three sessions. The difference between the Build-Up and Update phases is characterized by how quickly new information could be incorporated into the spatial map and thus influence the navigational behavior of the mice. Three different types of updates were introduced during this final phase: including barriers blocking certain paths, changing the goal location, and including both new barrier locations and new goal locations. Across all phases, memory effects were revealed, reflected by performance increases from one session to the next (measured in the normalized path lengths). Further, four previous knowledge effects modulating performance and learning of the spatial map are highlighted. Thus, we could show how different spatial map knowledge properties are developed stepwise over learning and could identify three main phases of learning.

Highlighting some previous knowledge effects

The simplest and most obvious previous knowledge (or memory) effect of the spatial map is already visible in the first few sessions of the Build-Up where **navigation to the invariable goal location becomes more efficient from one day to the next**. This simple memory effect is what most rodent memory tasks would capture [e.g., using a radial arm maze (Jarrard 1995) or a watermaze, testing reference memory (Morris, Garrud et al. 1982)]. While one could argue whether this simple spatial memory effect is a “previous knowledge” effect, it is important to consider it as its simplest form: knowledge gained in previous training days affects performance the succeeding day.

The second previous knowledge effect of learning a spatial map is found when comparing the performance for the very first goal location with the performance after the first and other goal location switches. Already the second goal location exposed a significant improvement in overall navigational performance to the goal location within the known map during the second session compared with the first, thus resulting in a different learning curve across sessions when comparing with the performance for the very first goal location. This change characterizes the second phase of general map learning. This effect is then enhanced once again during the Updates as performance improvement is already present in the first session and is maintained from the first to second session as well. However, this initial effect during the second goal location did not yet translate to good performance on the first trial of the second session; thus, no one-session updating leading to long-term memory was seen this early in learning. This is reminiscent of the learning set effect (Harlow 1949). The results obtained in the HexMaze indicate that this **learning-set effect can be expressed in three phases: (1) naive, (2) gains after offline consolidation, and (3) online as well as offline gains in the final stage**. However, it remains unclear whether this is the result of the animals learning the rule (there is one constant food location) or the general spatial map, but most likely it is a mixture of both.

The third previous knowledge effect on spatial map updating is tied to the third phase of the learning set effect (corresponding to online gains) and is present across the different Update types**: the amount of new information incorporated into the map affected how rapid online learning could occur during the first session of each update**.

When only the general maze structure was changed (inclusion of barriers), the animals were able to rapidly adapt their routes to the goal and additional sessions were not needed to reach optimal performance. In contrast, when the goal location or both goal location and the maze structure (L + B) were manipulated, online learning was slower, resulting in a performance decrease during the first session (linear relationship with the number of elements changed). However, offline consolidation eliminated this effect and by the second session animals performed similarly for all Update types. This effect could potentially be linked to a schema or schema-like effect of the knowledge of the cognitive map. Considering the degree of change compared with the previously learned information could explain some differences in schema effects in previous rodent and human studies. In the original paired-associate task (Tse, Langston et al. 2007), the hippocampus was necessary during update encoding, and this hippocampal involvement was also observed in a similar human schema task testing for a recently acquired, simple schema (card–location associations; (van Buuren, Kroes et al. 2014). In contrast, during human schema tasks that involve long established, real-world schemas, the hippocampus tends not to be active, and instead the prefrontal cortex directly communicates with the other cortical regions (van Kesteren, Fernandez et al. 2010, Van Kesteren, Ruiter et al. 2012). **It would be tempting to speculate that there may be a gradient across the complexity or extent of an existing schema, which in combination with the amount of new information overlap, results in a shift from hippocampal to cortical involvement** (Alonso, van der Meij et al. 2020). (1) If no schema is present, the hippocampus is necessary for weeks to months; (2) if a simple schema is present, the hippocampus is necessary for memory encoding but new information becomes more rapidly hippocampal independent; and (3) if a complex schema is present, the hippocampus is not even necessary for encoding, similar to fast mapping [(Coutanche and Thompson-Schill 2014, Coutanche and Thompson-Schill 2015); but also see (Cooper, Greve et al. 2019)]. For a more detailed review of this concept, please see the study by Alonso et al. (Alonso, van der Meij et al. 2020).

The fourth previous knowledge effect of knowing the spatial map is reflected in long-term memory performance (first trial of each session) and is the critical difference between our Build-Up and Update phases, and thus is indicative of the third phase of spatial map learning. Initially, during the Build-Up, the animals show poor long-term memory (2–3 d) after one training session to a goal location; during the Updates, the consistent development of long-term memory is accelerated and detectable in the probe trials (critical trial for this is the first trial of the second session). Interestingly, counting the crossings of both the new as well as the last goal location revealed that animals retained the memory of the last goal location as well as learning the new one. **Thus, new information did not overwrite the old information**. However, one training session only led to a 2 d and not 5 d memory here in mice. For long-term memory to last 5 d in mice, two training sessions were required. This acceleration of consolidation has previously been linked to the schema effect (Tse, Langston et al. 2007), and therefore it could be speculated that the knowledge of the map may be linked to schema or schema-like effects.

The HexMaze also revealed interesting features of map effects in mice. First, **we are the first to show that the Build-Up of the cognitive map is dependent on time but not training or experience.** This was revealed by training animals either two or three times a week. When comparing these two training conditions, performance was more similar when aligned to time (weeks since start of training) than to the number of days already spent in training. Further, after the 12 week Build-Up with either 36 or 24 sessions of training, all animals showed rapid consolidation during the Updates, confirming the established cognitive map was independent of training amount. Thus, time dependency, and not experience dependency, indicates that the buildup of a knowledge network requires a remodeling of the network, which, importantly, occurs offline and for a certain time period and cannot be facilitated by a training increase. This is reminiscent of the massed versus spaced memory effect: massed training creates a stronger initial memory; however, spaced training creates a memory trace that lasts longer (da Silva, Bast et al. 2014, Nonaka, Fitzpatrick et al. 2017).

Schema versus learning set

Can a cognitive map, as tested in the HexMaze, be considered as a schema? There are many definitions of schema, as we recently reviewed (Alonso, van der Meij et al. 2020). Human schema investigations have used different types of schema from spatial maps of object–location pairs (van Buuren, Kroes et al. 2014); semantic concepts (van der Linden, Berkers et al. 2017); visual–texture combinations (Van Kesteren, Rijpkema et al. 2010), and movies (Van Kesteren, Fernández et al. 2010). In contrast, many rodent studies have used the term schema more loosely [e.g., to describe the first experience with a linear track (Dragoi and Tonegawa 2013) or a daily changing sequence of goal locations on a circular track (McKenzie, Robinson et al. 2013)]. Recently Ghosh and Gilboa (Ghosh and Gilboa 2014) summarized the following four key features of schemas: (1) an associative network structure, (2) based on multiple episodes, (3) a lack of unit detail, and (4) adaptability. The requirements are present in our task for testing a spatial map: the multiple extramaze and intramaze cues together with the maze layout represent the associate network structure; training takes multiple sessions or episodes; and we have shown adaptability in the Updates. However, we did not test the same animals in a similar maze with different extramaze cues. Further, animals could have used episodic memory of the last event/trial to solve the task, although by using different starting points in each trial we ensured that each trial did have a different path. At this point, it remains disputable whether the task does test extracted commonalities and shows a lack of unit detail. It is possible that the animals used specific features of the maze, rather than an abstract and general knowledge and therefore schema. Therefore, while it is tempting to speculate that in this task the map of the environment acts as a schema, currently there is not enough evidence for this. **What we could show is that knowledge of the map after 12 weeks of learning led to expedited long-term memory.** Expedited long-term memory has been argued to be a key feature of schemas (Ghosh and Gilboa 2014, Fernandez and Morris 2018, Alonso, van der Meij et al. 2020).

Another argument that spatial maps in general can be seen as schema is that they use the same underlying mechanisms. With place cells in the hippocampus and grid cells in the entorhinal cortex, we have learned about the basic building blocks for how the cognitive map is coded in the brain (McNaughton, Battaglia et al. 2006, Moser, Kropff et al. 2008). These same fundamental building blocks have been shown to then also be harnessed for nonspatial memory representation and associations between these (Behrens, Muller et al. 2018). **Therefore, in general, map learning can be the ideal model for us to understand how we build up as well as update our knowledge systems and therefore schemas**.

One criticism of schema tasks such as the paired-associated task is that usually pretraining on the schema and the updates differ in difficulty and cognitive load because the amount of items learned was differed in the build-up versus the update (Tse, Langston et al. 2007, van Buuren, Kroes et al. 2014), which could account for the rapid updating effect that is the hallmark of schemas. The advantage of our framework is that **during both the Build-Up and Updates only one goal location is presented for multiple sessions, thereby keeping the task difficulty constant**.

Another previous knowledge effect described in the literature is learning sets (Harlow 1949). The difference between learning sets and schemas is that learning sets describe learning a set of rules that can be applied to new information. This is in contrast with schemas that are an associated network structure that can accommodate new learning. **Our task-learning set would be the animal learning about the principle that there is one goal location within the maze that stays constant for a certain amount of time but then can change**. We believe that this effect can be seen when the animal is learning the first and second goal locations during buildup.

How the task can be applied

The three different phases in the HexMaze are optimal to apply to different types of experiments. For example, if the goal is to test classic reference memory, simply using the first seven sessions to the goal location is sufficient. In contrast, if the aim would be to measure neural correlates of navigation within an environment with many days of data for direct comparisons, training should first be to one goal location, but analysis would be applied from the second session of the second goal location onward when performance is stable over time (i.e., from the ninth training day). As a third application example, the investigation of offline memory consolidation would occur during the Updates as here, each change is comparable to the next (plateau performance). One key advantage of the HexMaze to many other rodent tasks is the following: because of the **naturalistic paradigm**, mice rapidly habituate to the maze (two 1 h sessions of habituation with all cage mates at once primarily for stress-free pickups with tubing) and do not require other pretraining/shaping.

One noticeable challenge in the behavior of the mice in the maze, is that they never reached perfect performance. Instead, even when a specific goal location was experienced for multiple sessions, the mice only performed perfectly with direct runs from start in 30% of the trials, which increased to 60% if you considered performance after the animals passed the first two nodes. This lack of perfect goal-oriented behavior from the starting location may be because of the difficulty of the task, but more likely is because of the nature of the species itself. In contrast to rats, mice move rapidly in bursts and show more shuttling and random movements, which is likely inert behavior to avoid predators (Jones, Paul et al. 2017), and even in known environments use random movement strategies (Gire, Kapoor et al. 2016). The prevalence of random movement patterns could be confirmed in the HexMaze by using a modeling approach to the data (Vallianatou, Alonso et al. 2021). **Instead of increased goal-directed behavior from the starting location, learning is expressed in increased foresight: the point of direct run to the goal location will move further away from the goal as experience with the maze increases**. However, importantly the modeling approach also confirmed that the behavior of the mice in the HexMaze is better than a random run through the maze once they learned the goal location (Vallianatou, Alonso et al. 2021). We are currently developing a rat version of the HexMaze and can confirm that rats show much more goal-oriented behavior in the maze than mice.

Conclusion

In sum, we have developed a flexible rodent task in which different effects of previous knowledge of a spatial map on navigational and memory performance, encoding, and updating can be investigated and both offline longterm memory and online navigational performance can be evaluated separately. We could show that mice learn this complex spatial map in the following three main phases: (1) learning the initial goal location, (2) faster learning after 2 weeks when learning a new goal location, and then finally (3) a third phase after 12 weeks to express one-session learning leading to long-term memory. We have highlighted different effects that can be seen in this very rich dataset with.30,000 trials, here focusing on the metric of normalized path length and previous knowledge effects. However, many more metrics such as binary choices at each node and the presence of direct runs are provided in the dataset as well. Thus, the dataset (Extended Data Fig. 1-1) can be used for many other applications and investigations into mouse navigation, as also seen in the accompanying article (Vallianatou, Alonso et al. 2021).

Further, the task itself will enable future studies investigating the principles of memory updates and the involved mechanisms. While we have not yet investigated whether the effect of rapid systems consolidation (hippocampal independency) is present in this task as well, we did find a behavioral rapid updating effect that is likely to be accompanied by the consolidation effect. Overall, brains are tuned to remembering things that are new, but how novel something is will depend on experience (Duszkiewicz, McNamara et al. 2019).

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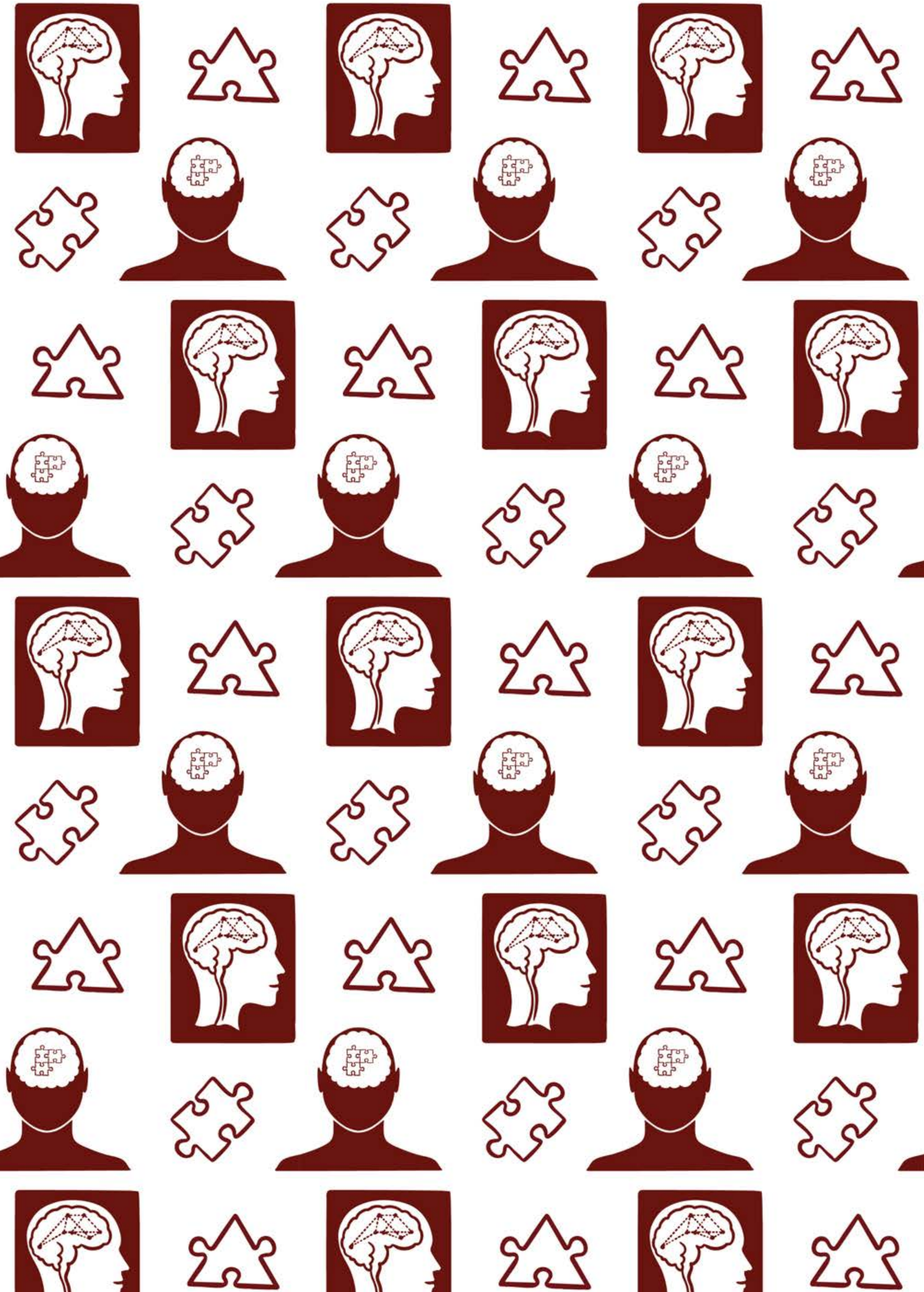

Chapter 3

Naïve to expert: considering the role of previous knowledge in memory

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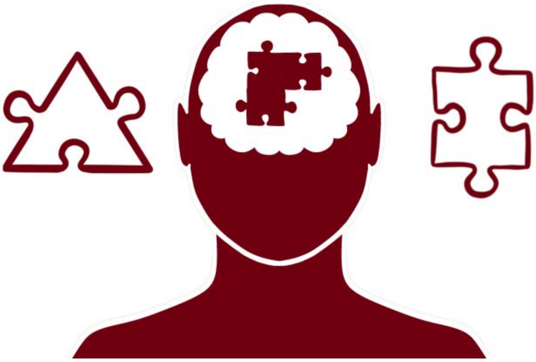


Abstract

In humans, most of our new memories are in some way or another related to what we have already experienced. However, in memory research, especially in non-human animal research, subjects are often mostly naïve to the world. But we know that previous knowledge will change how memories are processed and which brain areas are critical at which time point. Each process from encoding, consolidation, to memory retrieval will be affected. **Here, we summarize previous knowledge effects on the neurobiology of memory in both humans and non-human animals, with a special focus on schemas – associative network structures**. Furthermore, we propose a new theory on how there may be a continuous gradient from naïve to expert, which would modulate the importance and role of brain areas such as the hippocampus and prefrontal cortex.

Introduction

Once we have reached adulthood we rarely learn new information in isolation. Instead, most of what we experience will fit into what we know in some way or another. However, in most **non-human animal research the subjects are naïve to the world and have had very little experiences in life**. This is in harsh contrast to the adult human we are trying to model. This discrepancy is quite surprising since we do know that previous experience influences how new memories are processed (Bartlett 1932, Harlow 1949).

In general, **our memories tend to be stronger either when the encoded material 'fits' into our previous knowledge or when the information is completely novel** (van Kesteren, Ruiter et al. 2012, Fernández and Morris 2018). However, the mechanism underlying how these memories become long lasting is thought to be different for each case (for review please see Duszkiewicz, McNamara et al. 2019). Very novel experiences will lead to increased neuronal firing within the locus coeruleus, which releases dopamine into the hippocampus and strengthens the memory trace within this brain area (Takeuchi, Duszkiewicz et al. 2016, Genzel, Rossato et al. 2017). In contrast, if the new experience fits into what we already know, increased memory reactivations that occur later during Non-rapid eye movement (NonREM) sleep lead to consolidation of the hippocampal memory trace to the cortex (Genzel, Kroes et al. 2014, McNamara, Tejero-Cantero et al. 2014, Genzel, Rossato et al. 2017). In humans, the very novel side of the spectrum is most likely quite rare once we reached adulthood. In contrast, memory research in non-human animals will rarely be in the context of much pre-existing knowledge.

However, the mechanistic complexity does not stop there. Even within the realm of updating information that is easily incorporated into pre-existing knowledge, there seems to be a gradient. **The more something fits into your previous knowledge, the faster it can be incorporated into that pre-existing network.** It has been shown that schemas – associated network structures that encode knowledge – lead to acceleration of the systems consolidation process and thus, consolidation from the hippocampus to the cortex occurs in days rather than weeks (Tse, Langston et al. 2007). And again it is important to point out, that memory research in humans will range across the whole spectrum of some to extensive previous knowledge, while research in non-human animals involving any or even extensive previous knowledge is currently incredibly rare.

The concept of previous knowledge, and more specifically, the idea of memory schemas, is relatively old within human research (Bartlett 1932). However, these psychological concepts only began to be studied using animal models in 2007 (Tse, Langston et al. 2007). This seminal study by Tse and colleagues led to a renewed interest in the concept in both animal and human research, with a special focus on understanding the neurobiology of previous knowledge and memory schemas.

In this review, we will highlight different findings related to how previous knowledge affects memory consolidation in both humans and animals, and discuss possible roles for both hippocampus and cortical areas.

Previous knowledge and schemas

The term schema was defined in 1932 by Bartlett as an active organisation of past reactions or experiences, which would always be operating during a well-adapted organic response (Bartlett 1932). In a more current definition by Fernandez and Morris (2018), a schema is a "**framework of acquired knowledge, skills or attitudes implemented within a network of connected neurons in which memory traces of associated information have been stored that, when activated, can alter the manner in which new information is processed, including memory encoding, consolidation and retrieval**." Van Kesteren (2012), regarding human research, defines a schema as a pre-existing network of interconnected neocortical representations that affects the processing of new information.

In their review, Ghosh and Gilboa (2014) define schema as an **associative network structure, which is based on multiple similar experiences, but lacks unit detail and is adaptable**. As such, it expedites long-term memory at both encoding and retrieval levels. Further, schemas are sensitive to chronological relationships, hierarchical organization, cross connectivity and embedded response options (See Table 1).

Since we will summarize human and rodent studies in this review, it is important to understand that in humans most of what is learned is rarely completely novel once adulthood is reached; the many years of experience have created knowledge structures over time that can be harnessed for new learning. In contrast, in laboratory rodents, which start their lives in a non-natural, simplified environment of a home cage, are only subjected to what the experimenters may expose them to; their prior knowledge is very limited. Thus, by default, most human and non-human animal memory research will differ in the amount of previous knowledge. It can be incredible difficult to accumulate enough previous knowledge in rodents for it to count as a schema as defined by the above authors. Overall, in recent years, many rodent memory studies referred to schemas despite the fact that they would not fulfil the above-mentioned criteria but instead only comprise very little previous knowledge. Further, **many of the features described above are very difficult to test in rodents, in which for example, unit detail is almost impossible to assess since we cannot ask the rodent in which level of detail memories are present**. Instead, simple responses such as digging, swimming, or path finding are measured, which can be explained by different types of memory quality or memory process.

Previous knowledge will affect learning in many different ways and many sub-characterizations have been described in human psychology, such as expert knowledge (Bellezza and Buck 1988), schema (Bartlett 1932), scripts (Schank and Abelson 1977), and learning sets (Harlow 1949). These different types of previous knowledge are hard, if not impossible, to differentiate in non-human animal research, but we attempt to summarize memory effects across species in this review, we will refrain from using such specific terms and instead try to **refer to previous knowledge as an overall concept**.

Table 1

Authors	Definitions
Fernandez and Morris 2018	• Framework of acquired knowledge, skills or attitudes • Network of connected neurons • Memory traces of associated information • When activated, can affect the processes of memory encoding, consolidation and retrieval
van Kesteren, Ruiter et al. 2012	• Pre-existing network • Interconnected neocortical representations • Affects the processing of new information
Ghosh and Gilboa 2014	• Associative network structure • Based on multiple experiences • Lacks unit detail • Adaptable • Expedites long-term memory • Hierarchical organization, cross connectivity and chronological relationships • Embedded response options
Bartlett 1932	• Active organisation of past experiences • Always active during an organic response

Finally, previous knowledge can influence learning via different processes such as curiosity (Gruber, Gelman et al. 2014), attention (Kruschke 2006) and many others, but again since these are hard to discriminate in non-human animals and would also reach beyond the scope of this review, we will refrain from discussing them here (for other reviews see (Gottlieb and Oudeyer 2018)).

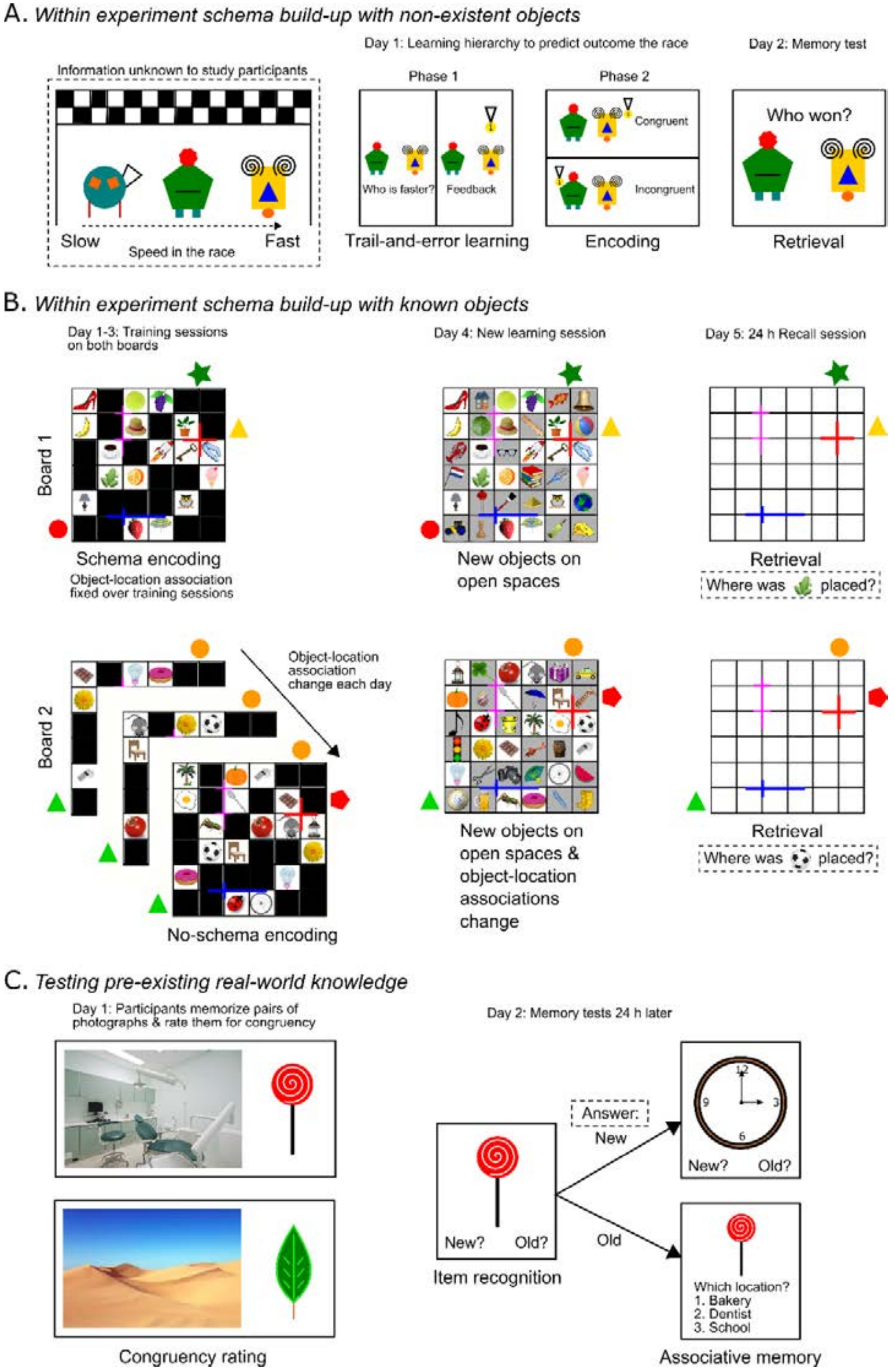
Previous knowledge studies humans

Tabula rasa or 'blank slate' refers to the concept that an individual is born without innate mental content and that **we thus have to gain all knowledge through experience**. Knowledge buildup throughout life requires coordinated activity between the hippocampus and medial prefrontal cortex (mPFC; (Sweegers, Takashima et al. 2014)), and is thought to facilitate the processing of new information, possibly by providing a structure into which the incoming information can be easily integrated.

During the last decade, a variety of **experiments measuring varying levels of prior knowledge** (i.e., from newly-learned information within the experiment, up to testing common, pre-existing world knowledge), have been investigated using human participants. These have tested, for instance, hierarchical rules about non-existent objects (Brod, Lindenberger et al. 2015) (see Fig. 1A), newly learned visual-spatial layouts (van Buuren, Kroes et al. 2014) (see Fig. 1B) similar to experiments performed in rodents (Tse, Langston et al. 2007, Tse, Takeuchi et al. 2011), rule-like associations between known objects (Zeithamova, Dominick et al. 2012, Preston and Eichenbaum 2013) and pre-existing real-world knowledge (van Kesteren, Rijpkema et al. 2014) (see Fig. 1C), in order to determine how new information is integrated into the existing knowledge network during the different stages of memory formation (i.e., encoding, consolidation, and retrieval).

> **Figure 1** Examples human schema studies

Overview of representative examples from human schema studies, ranging from using intra-experimental new schema build-up of non-existent objects to testing pre-existing real-world knowledge. **A**) Study design from Brod and colleagues. (Brod, Lindenberger et al. 2015) in which participants acquired a new schema within the experiment through trail-and-error learning about the outcome of a race between two "fribbles" (i.e., non-existing objects). After learning the hierarchy, participants learned both congruent (i.e., winner according to hierarchy) and incongruent (i.e., winner does not fit the hierarchy) pairs during the following encoding phase on which they were tested during the retrieval phase the next day. **B**) Study design from Van Buuren and colleagues (van Buuren, Kroes et al. 2014) in which participants over multiple days had to learn associations between known objects and their location on both a schema board (i.e., object locations were the same on each encoding day) and no-schema board (i.e., object locations changed during each encoding day) with the help of both intra and extra board cues. On the last encoding day (day 4), open spaces on both boards were filled with new objects. In addition, the objects on the no-schema board changed location again. On the retrieval day, participants were presented with an empty (schema/no-schema) board and had to retrieve the location of one of objects. **C**) Study design from Van Kesteren and colleagues (van Kesteren, Beul et al. 2013) in which participants had to memorize pairs of photographs portraying one known object and one real-world scene which were either congruent (i.e., they co-occur in the real world) or incongruent (i.e., they do not co-occur in the real world). The next day the participants were tested on their item recognition followed by an associative memory task.



A. *Within experiment schema build-up with non-existent objects*

B. *Within experiment schema build-up with known objects*

C. *Testing pre-existing real-world knowledge*

Though humans provide a wider range of cognitive skills than other animals, which can be used to investigate previous knowledge, it is more difficult to look at the dependency of particular brain regions as human studies are mostly observational (e.g., fMRI), though advances in interventional studies (e.g., transcranial magnetic stimulation, TMS) are being made (Berkers, van der Linden et al. 2017, Bovy, Berkers et al. 2020). This is in contrast to rodent studies, in which the effect of temporary and state-dependent inactivation (i.e., during encoding/consolidation/retrieval) on schema can be examined, in addition to the effects of lesions in specific brain regions. Moreover, most human studies use pre-experimental knowledge, and test item and non-spatial associative memory, whilst rodent studies use previous knowledge learned during the experiment related to, for example, item-location associations within a complex spatial layout. This means that, to date, **previous knowledge studies in humans and other animals differ substantially**. In the following sections we will look at the effects of previous knowledge on the different stages of memory formation and updating in humans, and since in humans research is quite extensive on the general topic of previous knowledge and can differentiate between different types of previous knowledge, we will focus on schema studies specifically and summarize the results of many different studies.

Effects of previous knowledge on encoding in humans

The encoding of new information can form both the basis for a new schema as well as adjusting or adding to an existing schema. Most human studies examine encoding of new information within pre-existing knowledge, so we will summarize here the effect of schema on encoding of new information in humans.

When incoming information can be directly linked to a pre-existing schema, the mPFC appears to be the main cortical node responsible for memory encoding (van Kesteren, Fernandez et al. 2010, van Kesteren, Beul et al. 2013), which it does through both strengthening of cortico-cortical functional connections and at the same time, by inhibiting the hippocampus (van Kesteren, Beul et al. 2013). The involvement of the mPFC in schema memory processing is furthermore supported by the results from studies using TMS during schema encoding (Berkers, van der Linden et al. 2017, Bovy, Berkers et al. 2020) and a study using the Deese–Roediger–McDermott (DRM) paradigm (Roediger and McDermott 1995) in patients with vmPFC lesions (Warren, Jones et al. 2014). On the other hand, hippocampal activity is increased and the hippocampus-mPFC connection is strengthened when incoming information is novel, and therefore inconsistent with existing schemas (van Kesteren, Beul et al. 2013, Bein, Reggev et al. 2014). This may be a strategy used to prevent new but inconsistent information interfering with the existing structured knowledge representations stored within the neocortical network.

The degree of hippocampal involvement during the encoding of new information seems to not only depend on the novelty of the new information, but also on the complexity of the schema in which the new information is to be encoded. Van Buuren and colleagues (2014), for instance, show that the hippocampus is involved when new information in a visual-spatial layout is to be integrated into a newly learned, and thus still simple, schema. Similarly, van Kesteren and colleagues (2018) show hippocampal involvement in translating previous spatial knowledge (i.e. a newly learned spatial schema) into new goal-directed behavior. On the other hand, Coutachan and Thompson-Schill (2014, 2015) show that Fast Mapping (i.e., the new to-be-encoded item is presented in the context of a known, real-world item during learning) can completely bypass the hippocampus, likely due to the complexity of the existing schema (but for controversy also see (Cooper, Greve et al. 2019)). Furthermore, while the encoding of schema-related events appears to be resilient to pre-encoding sleep loss, encoding of unrelated events is not (Alberca-Reina, Cantero et al. 2014). Alberca-Reina and colleagues (2014) suggest that sleep-loss-related encoding impairment of schema-inconsistent information is likely due to the fact that these memories require a higher level of hippocampal engagement.

In conclusion, although hippocampal-mPFC connectivity is reduced during the encoding of schema-related associations in humans, this type of encoding is not completely independent of hippocampal involvement. Thus far, **the amount of hippocampal involvement during encoding seems to mainly depend on the novelty or familiarity of to-be-encoded information and the complexity of the existing schema**. This means that, if the new information fits within an existing knowledge structure, more cortico-cortical connections are involved than in the case when the new information does not fit as well.

Effects of previous knowledge on consolidation in humans

Newly encoded memories need to be consolidated offline (e.g., during sleep) and integrated within existing knowledge structures to be able to persist over long time periods. **One idea is that memory reactivations during sleep enable the updating of the cortex (long-term memory) by the hippocampus (short-term memory buffer) via coordinated neuronal activity and/or reactivations** (Marr 1970, Marr 1971). Here we summarize studies that examined the effect of schema on memory consolidation in humans.

In general, consolidation requires context-guided retrieval of previously acquired memories to facilitate the integration the new memories within pre-existing knowledge. Integration of new information and thus, modification of the existing schema structure, is a continuous process. In the end though, it is necessary to reach an equilibrium that adapts schemas to be consistent with the external reality (Preston and Eichenbaum 2013). Only then will schemas be able to support novel inferences between indirectly related events (e.g., if A is linked to B and B linked to C, then A is also linked to C) and thus generalize towards new situations. Similar to encoding, the involvement and extent of involvement thereof, of certain brain regions during consolidation of new information, depends on how consistent this information is with already existing schemas or whether a new schema needs to be formed. **For instance, hippocampal-prefrontal interactions are sustained during the resting period following schema formation, whereas these interactions are fewer when the newly encoded information fits within an existing schema** (van Kesteren, Fernandez et al. 2010). In addition, offline reactivations of neuronal patterns in these brain regions, originally active during encoding, seem to facilitate the consolidation of newly-formed schemas (Preston and Eichenbaum 2013). Similar off-line hippocampal-cortical interactions have also been found during rest following an associative encoding task (Tambini, Ketz et al. 2010).

Depending on the amount of previous knowledge, and thus, the extent of the existing schema, the schema effect can arise immediately after encoding, as is the case for associative memories, or can only be seen after consolidation, as is the case for visual item recognition (i.e., a task that involves the hippocampus during encoding (van Kesteren, Beul et al. 2013)). Moreover, consolidation of schema-consistent information is resilient to sleep loss and to any kind of information interference after learning, while consolidation of schema-inconsistent information is quite vulnerable to both post-learning sleep loss and interference (Alberca-Reina, Cantero et al. 2014). The need for sleep-mediated consolidation seems to therefore depend on the type of learning and might thus be related to how well the newly acquired memory was integrated into the existing schema during encoding (Himmer, Muller et al. 2017). However, if sleep is present it may still contribute to consolidation of schema-related items. Hennies and colleagues (2016) taught subjects a new schema over a two-week period and showed that sleep-spindle density was correlated with decreased hippocampal activity at test for new schema-related items in contrast to non-schema items learned the day before.

Overall, the **current evidence seems to point towards the need of persistent, functionally-relevant hippocampal-neocortical crosstalk during consolidation** (van Kesteren, Fernandez et al. 2010, van Kesteren, Rijpkema et al. 2014) in order to form a new schema or update a freshly learned schema, while adding information to a longer, pre-existing schema seems to depend less on this interaction (van Kesteren, Fernandez et al. 2010).

Effects of previous knowledge on retrieval in humans

After offline consolidation, new information is integrated within the previous knowledge structure and is thus ready to be retrieved in the following day(s). In the following section, we summarize current knowledge regarding the effect of schema on memory retrieval in humans.

Whether a memory is properly retrieved after consolidation depends not only on whether the retrieval context provides enough information to recreate the encoding context, but also on whether this context and the associated memory form one unit (Brod, Werkle-Bergner et al. 2013). This means that the information present during the retrieval context needs to trigger the recombination of the representations of both the consolidated memory and the encoding context of that memory. However, it is not clear where in the brain the recombination of these neocortical representations might take place. Wagner and colleagues (Wagner, van Buuren et al. 2015) shed some light on this matter by showing that the angular gyrus plays an important role in converging distributed representations of rule-based schema components into one coherent memory representation. This converging role fits with the proposed other functions of the angular gyrus, namely involvement in cortical binding of information (Shimamura 2011) and the representation of memory content during successful retrieval (Kuhl and Chun 2014). Van der Linden and colleagues (2017) go even further and propose that for a visual schema-associated memory task, the schema information itself might to be stored in the angular gyrus. The mPFC seems to play a role in biasing retrieval towards schema-consistent memories (Preston and Eichenbaum 2013, Ghosh and Gilboa 2014), even for recently acquired schemas and when the time between encoding and retrieval is very short (Brod, Lindenberger et al. 2015).

Retrieval of inconsistent information, on the other hand, seems to rely on the lateral PFC via interaction with the striatum (suggested by (Scimeca and Badre 2012), shown in (Brod, Lindenberger et al. 2015)). Neither van Kesteren and colleagues, (2010), Brod and colleagues, (2015) nor van Buuren and colleagues (2014) found that hippocampal activity was reduced during the retrieval of both schema consistent and inconsistent memories. In addition, Brod and colleagues (2015) show that connectivity between the mPFC and hippocampus was not enhanced for the retrieval of incongruent compared to congruent information. In fact, the left hippocampus was involved in successful memory retrieval for both schema-consistent and schema-inconsistent memories, without significant difference between the two (Brod, Lindenberger et al. 2015).

Interestingly, Prull and colleagues (2015) show an age difference in retrieval but not in encoding of schema-inconsistent memories. Moreover, **schema effects appear to be more extreme in older adults and may be able to alleviate age-related deficits in memory** (*review in* (Umanath and Marsh 2014)). This seems to also be true for procedural memory, as Mueller and colleagues (2016) showed that prior motor experience does not only increase procedural learning but also has a protective effect against age-related decline for the consolidation of novel but related manual movements. In contrast, Badham and Maylor (2015, 2016) show that schemas can also have a negative impact on memory performance in older adults.

In summary, the hippocampus may not be necessary for retrieval but, when accessible (i.e. not lesioned or actively suppressed as possible in other animals), **may still contribute to the retrieval of schema-related information**. This means that, even after consolidation, the full expression of schemas may depend on (a perhaps low level of) continual hippocampal-prefrontal cortex interaction, possibly through a constant cycle of memory updating during retrieval (reviewed in (Preston and Eichenbaum 2013)). However, due to the fact that human studies are mostly restricted to observational methods like fMRI, instead of being able to utilize, for instance, brain lesions to study the necessity of specific brain regions in the retrieval of (schema) memories, it will be difficult to obtain a definitive answer on the exact involvement of specific brain regions during schema retrieval.

Summary previous knowledge studies in humans

A variety of schema types, including motor schemas in athletes versus non-athletes (Pereira, Abreu et al. 2013), word schemas (Takashima, Bakker et al. 2014), cultural schemas (Porubanova, Shaw et al. 2014), music or tonal schemas (Vuvan, Podolak et al. 2014), have been described in humans over the past years. Overall, **human neuroimaging studies converge with rodent studies in showing that the hippocampus and neocortex are complementary learning systems that interact during schema formation, consolidation, and retrieval**. However, the extent to which each brain region is involved depends on the to-be-encoded or remembered information as well as the extent of the existing schema. Furthermore, the range of the schema effect seems to depend on the task, type of memory, how much time has passed since learning (i.e., whether consolidation has taken place or not), and the extent to which the existing schema can be harnessed. As most of the above-mentioned studies test consolidation and retrieval of schemas over relative short timescales (i.e., shortly after learning), future research should investigate the specific roles of the prefrontal cortex and hippocampus in these processes over time. Because examination of schema neurobiology in humans is mostly bound to observational studies (e.g., fMRI), we will need to rely on interventional studies (e.g., lesions of particular brain regions) in rodents to extend our knowledge on the exact role of each brain region involved in schema formation, consolidation and retrieval.

Previous knowledge studies in animals

By observing fluctuations of voltage or metabolites in human participants using fMRI, PET and other methods while performing schema related tasks, we can study dynamic interactions between brain areas. Research has suggested that during studies involving previous knowledge based tasks, the hippocampus is critically involved during encoding if it is a highly novel event, and less if the event fits into an already established extensive previous knowledge, such that a **gradient of hippocampal involvement is inversely proportional to the complexity of the existing knowledge network.**

Using animal research in combination with an ever-developing range of tools will allow us to take a step closer to understanding the neurobiological mechanisms involved in memory. From electrophysiological and imaging recordings to pharmacological and genetic manipulations, a combination of these tools with specific and complex behavioral protocols provides us with the power to measure and target specific neural types or areas and thus **move from observational to interventional methods that allow us to draw conclusions on causality and mechanisms**.

Behaviorally, designing a task to evaluate previous knowledge or even schema specifically at a rodent level requires creative thinking, since we cannot rely on preexisting world knowledge when working with laboratory animals. In a way, the advantage of having perfect control over the experience an animal has, actually makes developing previous knowledge paradigms more difficult in rodents that do not have “real-world” knowledge, which we can harness. Having **such tasks gives us the chance to further understand the anatomical connectivity and synaptic properties involved in the dynamics of schema formation and updating**.

As mentioned before, a schema was defined by Ghosh and Gilboa (2014), as an associative network structure, which should be based on multiple similar experiences, with a lack of unit detail and adaptable. As such, it facilitates long-term memory at both encoding and retrieval levels. However, the term schema in rodent studies has been used loosely for different concepts, which we will highlight next in this review. Many of the studies cited below, would not fulfill the strict definitions of a schema, thus especially in this part highlighting non-human animal research we will refer to previous knowledge instead of schema. Further, to emphasize the amount of previous knowledge present in each study, we will describe the articles and procedures more detailed than the previous human examples. **The presence of a previous knowledge facilitates encoding of new congruent elements as well as expediting retrieval, for which its structure is not static but constantly developing and updating with experience**. It quickly identifies similar patterns or situations anticipating outcomes, giving the chance to make the best choice based on experience.

But where can we find a previous knowledge structure? These memory representations and multimodal associations are thought to be found distributed along the neocortex, in the medial prefrontal cortex (mPFC), orbitofrontal cortex (Orb), retrosplenial cortex (RSC), and anterior cingulate cortex (ACC). These structures are involved in making an act of judgement/decision-making based on previous experiences. They are richly interconnected, giving them the power to judge, predict and learn (Skelin, Kilianski et al. 2019).

The ACC is connected to several cortical structures and the limbic system, and is involved in problem solving, making choices, anticipation and motivations. The RSC lies close to the hippocampus and visual areas, and is involved with imagining future events, episodic memory, and navigation. The Orb receives inputs from visual, taste, olfactory and somatosensory areas, as well as from the amygdala, and is involved in correcting behaviors related to reward or punishment. The prelimbic cortex (PrL) integrates a diverse range of information to perform a behavioral response and is involved in goal-directed behaviors, attention and working memory (Aston-Jones and Cohen 2005, Frankland and Bontempi 2005, Mao, Neumann et al. 2018).

Paired-associates schema in rodents

Even though the concept of previous knowledge and schema in psychology has been known since the 1930s (Bartlett 1932), seven decades later the interest in this memory process reemerged. **The seminal study by Tse and colleagues (2007) introduced the concept of schema to neurobiology and opened up**

the possibilities of understanding the molecular mechanisms involved. To date different interventional approaches have been used in the same task (i.e. the paired-associates task) and in this section we will explore the current findings in more detail.

In the paired-associates task (Tse, Langston et al. 2007) rats have to learn a map of six flavor-place associations, or paired-associates (PA), in a large event arena (1.5m X 1.5m). One training session consists of six trials, at the start of each trial, rats are given a flavored pellet and during this trial only one sandwell is rewarded with more of the same flavor pellets. The location remains the same throughout the experiment. For the second trial, a different flavor pellet is associated with a different sandwell location, and so on (see Figure 2A, middle). **Rats have to learn associations with flavors and spatial locations as elements, the map of the event arena and its sandwell locations as a relational network** (Figure 2A, left).

Hippocampal lesions (Tse, Langston et al. 2007) and pharmacological studies (Hasan, Kanna et al. 2019) show that the hippocampus is critical for the initial learning of the task, which takes place gradually over several sessions. Similarly, if ACC was inhibited (by lidocaine or demyelination) (Hasan, Kanna et al. 2019) animals could not learn the task, **suggesting that initial learning of a potential schema is dependent on both the hippocampus and cortico-cortical interactions to stabilize the memories, with the hippocampus being more important at an early stage**.

Since adaptability is a core feature of schemas, nine weeks into training the original PA map was updated by replacing two of the original PAs with new flavors in nearby locations (Fig. 2A, right). If by then rats had built a schema of the PAs, learning new associations should be faster. Indeed, **exposure to a single trial of the new PAs was enough for them to recall the updates 24 hours later** (Tse, Langston et al. 2007). For the memory to persist, hippocampal dopamine plays a critical role at the time of encoding, as shown by Dopamine DA1/DA5 receptor antagonists given at the time of encoding (Bethus, Tse et al. 2010), rats could correctly remember if they were tested 30 mins after exposure to new PAs but not 24 hours later.

Tse and colleagues (2007) further investigated hippocampal dependency at the stage of updating information. A group of rats were given hippocampal lesions either three or 48 hours following exposure to the new associations, revealing a gradient where three hours after encoding the memory still depended upon the hippocampus, but 48 hours later it did not. Later, when rats with hippocampal lesions were exposed to either new PAs or a completely new map, they could not learn the new associations, showing that **it was not the task itself that had to be learned, but the associations within a relational network** (Tse, Langston et al. 2007).

If retrieval of new information after a certain timepoint is no longer dependent on hippocampus, the memory should then rely on extrahippocampal structures. Measuring immediate early gene expression allows to evaluate, which brain regions are experiencing synaptic changes. Brains from experienced rats were extracted 80 minutes after being exposed to either the six original PAs, two new associations and four original PAs or a totally new map with six new PAs (Tse, Takeuchi et al. 2011). A test minutes before brain extraction showed that, for the original and updated PAs conditions, animals could recall the associations correctly, but not for the completely new map. While immediate early gene expression is always present, immunohistochemistry against Arc and Zif68 showed that when updating the two new PAs into the schema, there was a greater upregulation of immediate early gene expression during encoding in cortical areas PrL (Tse, Takeuchi et al. 2011), ACC (Wang, Tse et al. 2012), RSC, and hippocampal region CA1 (Tse, Takeuchi et al. 2011). In contrast, when rats had to learn a completely new map, brain regions that showed an increase in IEG activation were limited to CA1. In the same study, pharmacological manipulation of the PrL during the encoding of new PAs revealed that both synaptic transmission and NMDAR-mediated synaptic plasticity was required in the PrL for successful encoding. Additionally, Wang and colleagues (2012) used pharmacological disruption of the ACC and found that NMDAR-mediated plasticity was necessary in this region for the encoding of new PAs into a pre-existing schema. **Both studies indicate that parallel encoding is occurring in both the hippocampus and prefrontal cortex** (PrL and ACC).

However, Lesburgueres and colleagues (2011) showed that there is simultaneous immediate early gene activation in the hippocampus and neocortex even when there is no previous knowledge present, which means the emphasis should be that with existing schema the cortical tagging is larger in magnitude (Tse, Takeuchi et al. 2011). In Lesburgueres' study (2011), rats performed the social transmission of food preference paradigm, where animals learned by smelling another rat's breath whether food was safe to eat, and were then tested 30 days later. Increased of immediate early gene activation in orbitofrontal cortex was observed at encoding and inhibition of orbitofrontal cortex by N-methyl-D-aspartate receptor (NMDA) antagonists on the day of encoding resulted in no memory persistence, showing that **an early "tagging" of cortical networks is crucial for the formation and maintenance of memories even without previous knowledge**. A similar finding with increased immediate early genes expression after encoding in the prefrontal cortex was also shown in the watermaze paradigm (Genzel, Rossato et al. 2017).

The increase in immediate early genes goes hand in hand with cellular processes, such as synaptic transmission for which α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and NMDA receptors are directly involved. These receptors can be inhibited in the PrL and the ACC at different stages of schema acquisition (Tse, Takeuchi et al. 2011, Wang, Tse et al. 2012). **AMPA receptors are associated with general synaptic transmission, and they were needed for encoding and retrieval of the new PAs. NMDA receptors are associated with plasticity and long-term potentiation, and they were found to be critical for memory encoding but not retrieval** (Tse, Takeuchi et al. 2011, Wang, Tse et al. 2012). Furthermore, offspring from rats exposed to dioxins, which inhibit gene expression and NMDA expression in the prefrontal cortex, could not learn the PAs task at all (Kakeyama, Endo et al. 2014).

In summary, the paired-associates task opened the gates for understanding the molecular mechanisms underlying how previous knowledge affects learning and memory. Initial encoding of a potential schema is dependent both on hippocampal and cortical areas, such as ACC and PrL, but **once a schema is formed, retrieval is rapidly independent of the hippocampus**. Encoding of new information that fits within existing previous knowledge is dependent on an intact hippocampus, dopamine transmission around the time of encoding (necessary for memories to persist), and both NMDA and AMPA receptors, which are involved in long-term-potentiation and general transmission. Retrieval of information from schemas is not hippocampus dependent if at least 48 hours have passed since encoding.

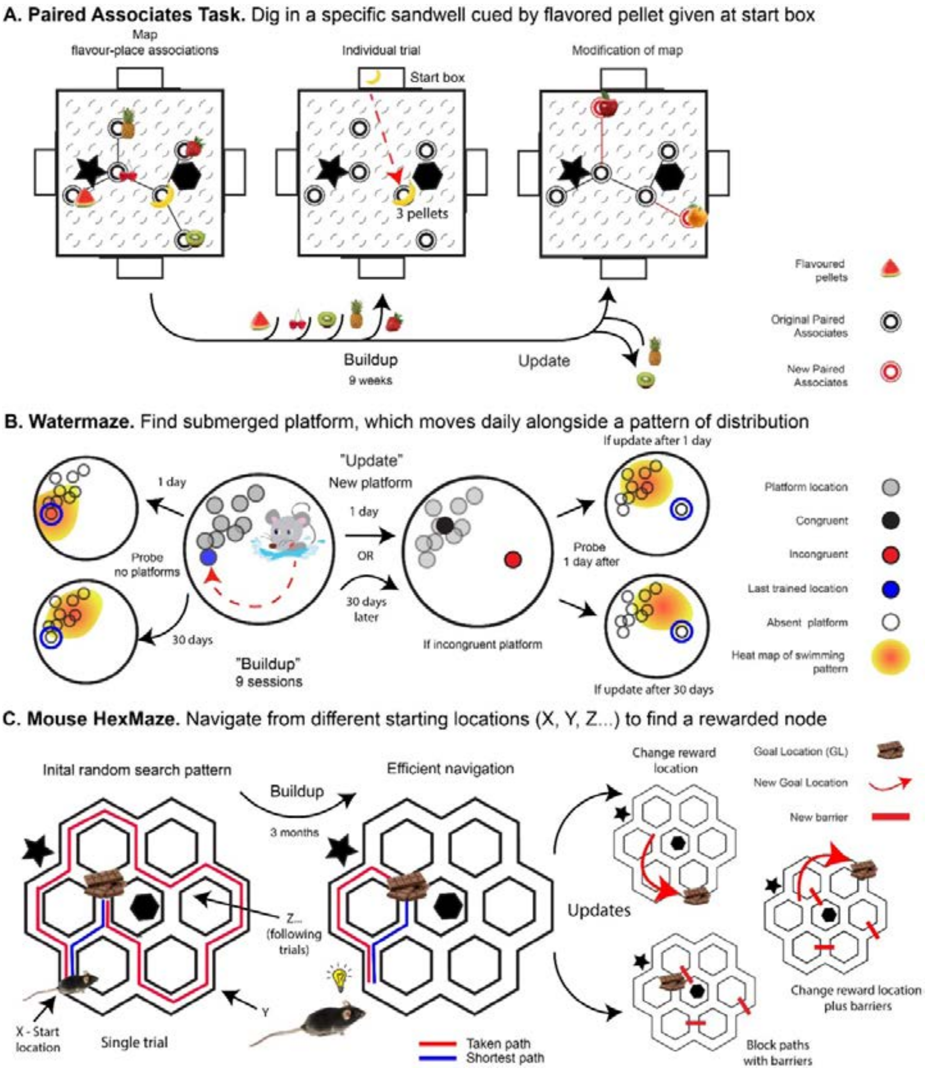


Figure 2 Examples rodent schema studies

Schematic overview of rodent paradigms. **A**) Paired Associates (PA) task. Event arena contains a 7x7 grid of potential sandwell locations from which a map of 6 sandwells associated to flavours is formed over time, as shown on the left arena. There are four start boxes around the maze, and intra- as well as extra-maze cues (star and hexagon). In a single trial, the animal is given a flavored pellet in the start box, as shown in the central arena, e.g., a banana pellet, and the rat has to dig in one out of 6 sandwells for more banana pellets (and repeated for the remaining 5 flavours). This is repeated during 3 months in a period denominated Buildup, where animals increase their performance over time, indexed by digging time in the correct sandwell and performing fewer errors when choosing the correct sandwell to dig in first. After this time an update to the flavour-place associations is made, seen as a change of two flavours in new locations, presented in the red symbols on the maze on the right. As discussed in the main text, this update can be learned within a single exposure. (Tse, Langston et al. 2007) **B**) Watermaze. Mice need to find a submerged platform within the circular pool, each day, 4 times a week. The platform location changes every day, and they were drawn from a statistical predefined distribution in space (grey circles). Animals were probed with no platforms present, one or 30 days after the end of their training or "Buildup". Swimming patterns were translated into a heatmap of average dwell time, as shown on the left of the figure. Animals that were tested one day after the buildup showed preference for the last presented platform, whereas those that were tested 30 days later showed preference for the overall distribution of the platforms. On the right: As an update, a new platform is introduced, which could be placed in a consistent position, as shown in the black filled circle, or in an inconsistent position, as shown in the red filled circle. This update can happen either 1 or 30 days after the original buildup training. If the inconsistent position is shown 1 day after the buildup, a probe trial conducted a day later shows the search pattern is more inclined towards the overall platform distribution. If this update happens 30 days after the buildup, a probe trial a day later shows a search pattern between the original distribution and the new platform (Richards, Xia et al. 2014). **C**) Mouse HexMaze. Animals navigate a big maze to find a rewarded location (GL). On the left the red trace shows a trial where a random path is taken by a naïve animal, until it reaches the chocolate reward. In one training session the mouse performs several trials, always from different start locations (X,Y,Z…). Performance is calculated by comparing the path taken to the shortest possible path (blue trace). The buildup of the task consists of 3 months, and over time the navigation improves. Later updates are introduced, where barriers can be added, the reward can be moved, or both. As discussed in the text, these updates can be learned in just one session. Intra and extramaze cues (star and hexagon) aid navigation in the maze. (Alonso, Bokeria et al. 2020)

Other tasks testing previous knowledge in non-human animals

Efforts have been made to develop other tasks utilizing schemas; in this section we discuss that these are **mostly related to cumulative experience** and represent general effects of previous knowledge and not schemas per se.

Schemas are based on multiple episodes and should not be detailed (Ghosh and Gilboa 2014). With this in mind a task was developed for mice to identify a pattern over time, however many other schema prerequisites were not included. In a watermaze based task (Fig. 2B, center-left), mice had to find a hidden platform below the water surface in a fixed place over four trials in one day, but the position of the platform changed slightly from one day to the next over nine training days, drawn from a statistically predefined distribution in space. Animals were later tested one day or 30 days after with no platform present (Richards, Xia et al. 2014). Mice tested at 30 days expressed a strong correspondence between search strategy and overall platform distribution compared to those tested one day later. The one-day group search pattern was more accurate with respect to the actual positions of the platforms with the final platform position dominating, while the 30-day group's search strategy was centered in the mean position of the platform distribution (Fig. 2B, left). This suggests that, **in presence of a long time period between encoding and retrieval, search patterns are driven by cumulative experience rather than specific events**.

A schema needs to be adaptable and to test this, in a variation of the same protocol, after the training of the distributed platforms, which we can call a "buildup", an update was introduced, either one or 30 days after the buildup. During the update the platform was placed either in a congruent or an incongruent position (Fig. 2B, center-right), congruent being within the mean distribution of previous daily locations and incongruent being far. Focusing in the incongruent platform update, a probe was done 1 day after, and the group where they had the update after 30 days, had higher prediction error than the one-day group, seen as a change in the search strategy (Fig. 2B, right). This strategy switch in the 30-day group was not seen when the mPFC was inhibited before being exposed to the last platform, **suggesting that the role of the mPFC in rapid consolidation may be limited to the learning of new incongruent information** (Richards, Xia et al. 2014).

Another way of evaluating cumulative experience is shown in the **Object Space Task** (Genzel, Schut et al. 2019), where rodents are exposed to a pattern of four possible object locations in an open field throughout the week, with one location that is stable across days whilst the others are shifted between the three other possibilities. Based on the natural tendency of rodents to explore novelty in presence of familiarity, exploration time of the object placed on the stable location should decrease over time. Across 20 trials in one week, a semantic-like memory is expressed with an extracted pattern of locations, which then guides their behavior towards exploring the object that was not in the stable location. However, one week is not long enough to suppose schema formation or semantic memories per se thus this task should rather be seen as simple, previous knowledge based task (Genzel, Schut et al. 2019).

A simpler form of previous knowledge can also just be the pre-exposure to the spatial environment in which learning should occur. Genzel and colleagues (2017) contrasted two different behaviors that can lead to memory persistence: post-training novelty and post-training sleep (Duszkiewicz, McNamara et al. 2019). **Post-training novelty should lead, via synaptic-tagging and capture mechanisms, to increased hippocampal cellular consolidation. In contrast, sleep allows for memory reactivations, and thus systems consolidation and integration into cortical networks** (Duszkiewicz, McNamara et al. 2019). Rats were taught two platform locations in the watermaze, one of which was followed by sleep while the other was followed by novelty exposure combined with sleep deprivation. In the probe trial one week later, rats remembered both platform locations but spent more time at the platform location followed by novelty. However, if animals were pre-exposed to the spatial layout and cues before training (with a dry-land inlay) this difference was abolished and now only having sleep after learning was sufficient for a strong long-term memory (Genzel, Rossato et al. 2017) perhaps due to the possibility of harnessing pre-existing cortical memory networks even though in this case they were clearly not complex schema representations.

Categorization of objects groups similar elements together, and this process can be seen as a form of semantic memory. A set of tests evaluating categorization of objects in mice (Creighton, Collett et al. 2019) showed that **mice could recognize categories of objects**. In a sample phase, mice were presented with two objects of the same category, and during a test phase they were presented with two novel objects, one belonging to the category presented during the sample phase and another unrelated object. Mice could recognize the familiar category over a short delay but not over a long one (30 min vs. one hour). If animals were pre-exposed to the category, they could discriminate the familiar object after long delays. This effect was lost under scopolamine (acetylcholine antagonist) if it was systemically administered before the test phase, which was expected since acetylcholine plays a role in memory and perception (Creighton, Collett et al. 2019).

Previous knowledge that facilitates encoding and retrieval on its own is not sufficient for classification of 'schema'. **By performing tasks with common features, certain features can be drawn from them, and can facilitate "learning how to learn" or learning set** (Harlow 1949). For example, training rodents in two similar tasks, the original watermaze, where throughout five days animals need to find a stable platform, and the Delayed-Matching-to-Place task, where the platform changes each day, throughout 26 training days (Ocampo, Squire et al. 2018). A commonality between these tasks is a circular water pool and a platform that needs to be found. If either of the two tasks is trained first, the second benefits from what was learned in the former. For early learning, the entire hippocampus is necessary, but the second task was not dependent on hippocampal region CA1, which is the main output pathway from hippocampus to neocortex (Ocampo, Squire et al. 2018).

Other studies refer to hippocampal schemas, without taking into consideration the enhancement of long-term memory, which is crucial in the definition of the schema effect, thus perhaps while using the term these do not test schemas as defined in the human literature. McKenzie and colleagues (2013), trained rats to find a water reward in circular maze, cued by a LED signal, and after these were learned, in a course of six days, they had to learn new reward locations that were spatially defined. By analysis of electrophysiological recordings in the CA1 region, they could show that by adding the non-cued reward sites gradually, hippocampal representations from the cued learning were modified to add the non-cued learning (McKenzie, Robinson et al. 2013). However, because long-term memory was never assessed, this study is most likely to be classified as an initially naïve learning where a potential schema is still hippocampus dependent.

Similarly, Dragoi and colleagues (2013) wanted to study place firing of cells in naïve animals, wild-types and CA3-NMDA receptor knock-outs. In this paradigm, mice were put on a linear track for two sessions on day one (novel), and similarly on day two (familiar), but on this second day the linear track was transformed to an L track by the addition of a perpendicular linear track (novel feature). By day three the L track would be familiar, after which a novel linear track was introduced for two sessions. Place cell firing stability was determined, and in the first novel-feature condition, place-cell stability was reduced in both the control and KO condition, and stability increased with experience. On the novel track at day three, only the KO mice had reduced place-cell stability, suggesting that NMDA receptors in the CA3 area are necessary when novel situations arise, and not when there is a previous knowledge network (Dragoi and Tonegawa 2013). And here again, while the term schema is used, it rather represents a very simple form of previous knowledge or experience.

These studies have all been performed in rodents, however, a recent study has used macaques. In this study (Baraduc, Duhamel et al. 2019), macaques were presented with both a familiar and a novel virtual maze sharing a common 'schema' (spatial map), yet differing in surface features, in which macaques had to search for food. Food locations were defined in relation to landmarks. During learning, a proportion of hippocampal neurons had firing rates modulated by task-related information in the novel maze, which matched that of the familiar maze in a manner suggesting that **these neurons abstracted spatial elements from the environment and encode space in a representation of a potential schema** (Baraduc, Duhamel et al. 2019).

Through repeated experiences, naïve animals can subtract patterns and categories which guide behavior and facilitate "learning how to learn efficiently" (i.e., learning set). Learning sets transform the strategy of adapting by trial-and-error to a reasoning-like strategy, involving hypothesis and insights. The time frame of most studies reviewed in this section were between one and six weeks, and in some cases the long-term memory was tested once, but additional tests were not performed. Thus in most cases above, it is hard to assess if they would fulfill the strict criteria of schema, instead most studies were testing the effect of previous knowledge in the most simplest form. An intact hippocampus appears to be essential for memory persistence in presence of novel situations, slowly disengaging as cumulative experiences start forming a relational cortical network.

The HexMaze for mice

In the effort to establish a different task to test for previous knowledge in rodents, the HexMaze was developed (Alonso, Bokeria et al. 2020). It is based on multiple episodes, is adaptable, and has the same cognitive load throughout.

In the HexMaze, mice learn to navigate a large gangway maze (Fig. 2C), where a chocolate flavored reward can be found in one of the 24 nodes. The goal location stays stable for several sessions. In a training day, the animal is placed repeatedly over many trials in different random nodes within the maze, from which the animal should navigate towards the food (Fig. 2C, left). The previous knowledge in this case is the map that they need to navigate using the environmental cues as reference points. **And how this previous knowledge affects new learning is tested by changing the goal location and measuring how quickly animals can adapt their behavior to this new information**. Performance is measured by the length of their navigational paths, as in the number of nodes the animal visited, in relation to the shortest path possible.

Initially, animals run around the maze, exploring randomly until they find the reward. This is the case in the first sessions in the maze, as well as each time a new goal location is introduced. As experience in the maze increases, so does performance level, as animals slowly learn to recognize their position based on the cues placed around the maze and choosing more efficient routes to the reward (Fig. 2C, middle).

Similar to the paired associates-task (Tse, Langston et al. 2007), the task consists of a build-up phase of three months, during which the location of the food changes every 7-5 sessions. This build-up phase is followed by a phase of updates (Fig. 2C right), where a change is introduced weekly. These changes could be a new goal location, adding a barrier, or both. Each training session consists of 30 min period during which the animal performs several trials (20-35). Due to the design of the task, **different types of previous knowledge can be tested in this paradigm**.

In the first three weeks of the build-up, during which the location of the reward stays stable, a gradual increase in performance was seen in each session, as mice gained experience in navigating the maze. If the goal location changed, performance initially dropped to the same level as when the animal was first introduced to the task, but by the second session following this goal location change, the overall performance was better compared to the second session of the previous goal location (Alonso, Bokeria et al. 2020). However, in this build-up phase, long-term memory (48 h) still took multiple sessions to develop. Because there were several trials per session, **the first trial served as a test for long-term memory, whilst the overall performance indicated working memory in addition to efficient navigation through the maze**. Whilst the overall performance increased during the second session of the second goal location, this was not the case for the first trial.

During the updates changes to the maze were made weekly. By the first session of the first update, the performance was already significantly better than the first session of the build-up. Furthermore, performance continued to improve throughout the week. This performance gain from build-up to updates to sessions throughout the update are reminiscent of a learning set (Harlow 1949). However, since the updates are of three different kinds, changing goal location, adding a barrier, or both, it can be shown that the rate of learning differs depending on the amount of overlapping information. For example, adding a barrier would be the easiest condition to learn, since the goal location remains the same, while if both location and barrier change, the conflicting information is greater. This was evident by a drop in performance on the first trial of the first session following an update when the goal location changed. However, by the following session, performance improved at the same level for all conditions, showing that **one session was enough for the memory update and long-term memory** (Alonso, Bokeria et al. 2020).

Additionally, we found that the build-up phase was not dependent on amount of training, but rather on time, that is, by training mice three times a week versus twice a week. **The increase in performance depended on the amount of time that had passed since the beginning of the experiment and not in how many times they were trained per week** (Alonso, Bokeria et al. 2020).

With a flexible task like this one, effects of previous knowledge on memory, encoding, updating and retrieval can be evaluated independently. Currently, we are also developing a HexMaze for rats (4 m x 9 m), in which the same spatial structure will be used but four times the size.

Summary previous knowledge studies in non-human animals

Clever behavioral tasks allow us to understand how previous knowledge affects learning in subjects with no “real world” knowledge. From the watermaze to the paired-associates task, they fulfill all or some but usually not all of the criteria that makes a schema: an associative structure, based on multiple episodes, not detailed, and dynamic (Ghosh and Gilboa 2014). Different paradigms focus on different memory levels, from naïve learning and prediction error based on cumulative experience, to long-term memory persistence and in rare cases the schema effect.

We have had a closer look at the gradient in which the hippocampus and neocortex depend on each other to form and sustain long lasting memories, mainly due to the advantage of interventional techniques, such as lesions, pharmacology and invasive recording methods (e.g., electrophysiology), that allow us to try and decipher how individual cells communicate with each other.

To support coherent long-lasting memories, there must be a developing dynamic between the hippocampus and the neocortex. The hippocampus is essential for acquiring novel experiences, both at an early stage of memory build-up and during the updating of memory structures. Simultaneous synaptic activity, seen as immediate early gene activation, is critical at both the hippocampus and neocortex during encoding**. The period of time during which a new event depends upon the hippocampus diminishes with the amount of the previous knowledge of that experience, and once a schema is present, system consolidation is greatly accelerated.**

Theories on the role of the hippocampus and prefrontal cortex in memory

Classic systems consolidation theory states that memories are initially encoded in a whole-brain network but only the hippocampus trace is sufficient for retrieval (Frankland and Bontempi 2005). But over time (weeks/months/years) cortical connections become reinforced in offline consolidation processes so that later on they are sufficient for retrieval (McClelland, McNaughton et al. 1995, Squire, Genzel et al. 2015). The transformation theory expanded on this concept and suggested that this type of systems consolidation would also lead to a change in the type or quality of memory: from hippocampal episodic or event memories to abstracted, gist-like memories in the cortex (Nadel and Moscovitch 1997, Moscovitch, Cabeza et al. 2016). However, **both theories had not yet proposed a special role for the prefrontal cortex or considered previous knowledge in any significant manner until recently** (McClelland 2013). In light of many recent findings, various new theories on how both the prefrontal cortex and the hippocampus could play a role in memory have emerged. In this next section we will highlight these different theories.

The SLIMM theory (schema-linked interactions between medial prefrontal and medial temporal regions) is based on human schema results and **proposes that the medial prefrontal cortex functions as resonance detector to recognize information that fits into pre-existing networks**. Once activated the mPFC then suppresses hippocampal activity during memory encoding, which would not occur when the information is very novel and does not fit into what we know (van Kesteren, Ruiter et al. 2012). Thus, the former would be immediately encoded into the cortex whilst the later would be encoded in the hippocampus.

In contrast, Eichenbaum **suggested that the hippocampus organizes memories within the context in which they are experienced and the medial prefrontal cortex would be relevant to retrieving the context-appropriate memories** (Eichenbaum 2017). During encoding, the context-cues would first be fed from the hippocampus to the mPFC and this information would be fed back to the hippocampus during retrieval to bias the hippocampal network to the appropriate context.

A slightly different view is that the hippocampus creates a rapid binding and encoding of all events as they occur, as an automatic, day-to-day recorder (Wang and Morris 2009) serving as an index or pointer to information coded in the cortex (Buzsáki and Tingley 2018, Skelin, Kilianski et al. 2019). However, most of these impressions would not last but fade away overnight**. Memories that would be tagged as salient would be consolidated to the cortex and the prefrontal cortex would take over the binding function of the hippocampus for memories that are related to established cortical networks** (schemas) (Wang and Morris 2009, Squire, Genzel et al. 2015, Genzel and Battaglia 2017).

Some more recent theories move beyond the concept of the hippocampus as a “memory” area. Barry and Maguire (2019) highlight the fact that most evidence for the hippocampus being involved in memory comes from naïve animals and only looking at very short time scales. They argue that with the rapid turn-over of synapses (average life of 10 d) in the hippocampus, a memory would not last very long there. Instead, the role of the hippocampus in memory would be defined by the process occurring within. More specifically, **they propose that the hippocampus is critical for scene construction, that is, creating our inner movie** (Barry and Maguire 2019). Thus, the hippocampus would reconstruct remote memories in the absence of the original trace by assembling consolidated neocortical elements into a spatially coherent scene. This would be facilitated by the mPFC. Evidence for this idea comes from patients with hippocampal lesions, in which imagining the future – a task that requires scene reconstruction – is just as affected as the recall of episodic memories.

The scene construction theory proposes that the hippocampus continuously constructs and anticipates scene representations beyond our immediate sensorium. In this context, a scene is a naturalistic 3D spatially coherent representation of the world typically populated by objects and viewed from an egocentric perspective. Scenes represent the fundamental components of unfolding mental events, whether recalling autobiographical memories, navigating through environments, forecasting plausible futures, or creating novel scenarios, all domains in which hippocampal-damaged patients are impaired (Barry and Maguire 2019).

Another recent proposition on the role of the hippocampus also emphasized the general properties of the hippocampus. **Instead of coding for space and time, both components of episodic memories, the hippocampus would be a general sequence generator** (Buzsáki and Tingley 2018). And whatever information is fed into the system – the "whats" – would be coded in the cortex and mapped onto content-free pointers in the hippocampus. Thus, activating the hippocampal sequence would lead to the retrieval of the sequence of experience. Further, in the hippocampus, self-organized activation during offline states would be constrained by existing attractor manifolds, or maps, and may be biased toward particular mapped locations by salient experience, which would result in the appearance of experience-specific replay (Swanson, Levenstein et al. 2020). Similarly, the impact of sharp-wave-ripple-associated reactivation on downstream regions, that would function as second readers, would not be a simple transfer of hippocampal representational content. Rather, the response of downstream regions would depend on a transformation function, defined by both the feedforward and local circuit architecture, as well as the 'listening state' of the downstream region (Swanson, Levenstein et al. 2020).

In sum, the concept of schema as well as other more recent findings in memory research, has induced a plethora of new theories on what the mPFC and hippocampus does mechanistically in memory. **Most of these theories move beyond the idea that memories are simply "stored" in the hippocampus and then "transferred" to the cortex,** and instead consider which physiological mechanisms or processes the hippocampus is involved in.

From naïve to expert: a new theory of previous knowledge

Whilst the theories mentioned above do consider how previous knowledge influences how we encode and consolidate memories, it is often seen as 'either-or' phenomena. Instead, **the amount of previous knowledge tested should be considered as a gradient, which can range from none in naïve situations to very extensive**, as is often the case in much human cognition. In the following section, we propose such a gradient schema theory and how it would influence which brain areas are needed during encoding, consolidation and retrieval (Fig. 3).

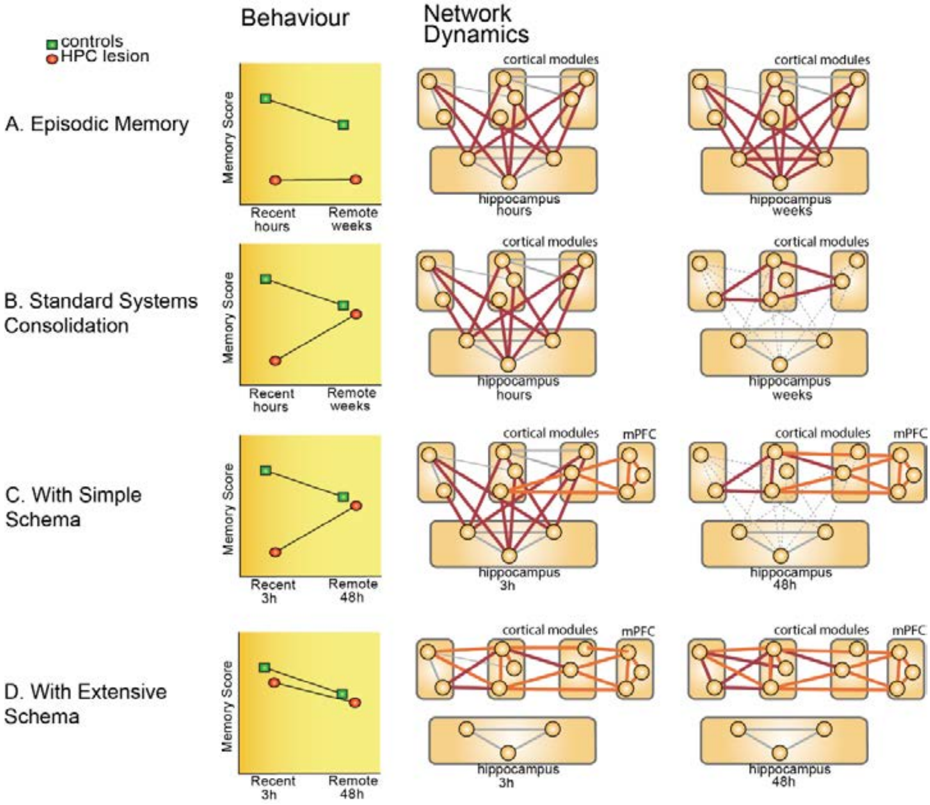


Figure 3 From naïve to expert: a new schema theory

How critical the hippocampus is for memory encoding and retrieval would depend on the type of memory and how much experience encoded in cortical networks can be harnessed. **A**) For very novel and unique events that will be retained in form of episodic memories, the hippocampus would always be involved. **B**) New memories that are consolidated to abstracted, gist-like memories the hippocampus would be involved during encoding and hippocampal independency at retrieval would take weeks to years. These types of memories are described in standard systems consolidation theory. **C**) In contrast, if new memories are congruent with pre-existing knowledge, but this knowledge is still quite new and forms a more simple schema, the same gradient of hippocampal involvement during encoding and hippocampal independency during retrieval is seen but now sped up. Memories can be hippocampal independent after a few days, perhaps with sleep as a crucial factor during the consolidation period. **D**. Finally, if new memories are congruent to large, extensive schemas the hippocampus can already be bypassed during encoding and memories directly stored in cortical networks.

Most animal research on memory would be placed on one side of this gradient, with new memories only able to rely on very little (if any) previous knowledge that animals had acquired during, for example, habituation or shaping periods in training. How unique the new event or experience is would then influence how these memories are consolidated and the outcome of this process (Duszkiewicz, McNamara et al. 2019).

Very unique, emotionally-arousing experiences would lead to increased initial cellular consolidation in the hippocampus resulting in a longer lasting hippocampal memory trace for these event memories (Fig. 3A) (Duszkiewicz, McNamara et al. 2019). The hub-like anatomical position of the hippocampus would allow it to orchestrate a wide range of cortical and subcortical networks during memory retrieval and thus link more detailed aspects of a given experience that are represented in distributed neocortical modules (Skelin, Kilianski et al. 2019). In this way, activity in the hippocampus can trigger the reactivation of neocortical patterns resulting in the retrieval of a memory in more detail and together with the scene-reconstruction properties, would thus always be necessary for the retrieval of episodic, detailed memories (Moscovitch, Cabeza et al. 2016, Barry and Maguire 2019, Skelin, Kilianski et al. 2019). **These types of memories would be very rare in adult humans**, due to the amount of previous knowledge influencing everything that is newly learned.

New memories that can rely only on very little previous knowledge but are not as unique or emotionally arousing as the memories mentioned above, would be consolidated to the cortex over time. However, in **this process they would lose their episodic detail and instead only salient information would be retained in a gist-like quality** (Moscovitch, Cabeza et al. 2016). These types of memories would depend on hippocampal activation during retrieval for weeks to months and only consolidate very slowly to cortical networks (Fig. 3B). Most current animal memory research would be operating on this level.

In an intermediate phase, some previous knowledge encoded in cortical networks is present, which can already be retrieved without the hippocampus. In this intermediate phase, **updating of these cortical networks would still need the activity of the hippocampus during encoding, as well as following offline periods of sleep, to enable a slow updating of the cortical networks**. However, this updating would be more rapid (e.g., days instead of weeks) since less cortical changes are needed than in the naïve animal (Fig. 3C). The paradigms used by Tse and colleagues and van Buuren and colleagues are examples of this case (Tse, Langston et al. 2007, van Buuren, Kroes et al. 2014).

On the other end of the spectrum, **when a lot of previous knowledge is present in a more complex cortical network, the hippocampus would not even be needed or at least needed much less for the update process.** This phenomenon of rapid, cortical consolidation can be seen in 'fast-mapping', when new information is presented in the context of previous known information (Fig. 3D). Studies harnessing real-world knowledge as schema would be operating at this level. Overall, most naturalistic human learning would be represented either on this or the previous level of the gradient.

In sum, here we propose a new memory theory in which the extent of previous knowledge influences the extent to which the hippocampus is involved in encoding, consolidation, and retrieval. **Overall, both levels would remain a continuous gradient with more extensive previous cortical networks leading to less dependence of the memory on the hippocampus and a faster shift from hippocampus to cortex as necessary memory structures** (Genzel 2020).

What does the hippocampus do?

Since the famous hippocampal lesioned patient H.M., **the hippocampus has been viewed as the brain area associated with memory**. The subsequent discovery of place cells in this brain structure initially supported the idea of the hippocampus being a critical memory brain area especially for spatial memories. However more recent findings do not really fit into this concept and have made many researchers rethink what and how the hippocampus really contributes to memory. In this section, we will propose how the hippocampus could contribute to memory.

One of the first and still most influential ideas on hippocampal function was proposed by David Marr in 1970s (Marr 1970, Marr 1971). He proposed that the hippocampus would be the 'fast learner' and with its increased plasticity would store memories as they occur. **This 'fast learner' would act as an intermediate buffer and during offline periods, especially during sleep, would slowly update the 'slow learner'** (cortex) via memory reactivations. Wilson and McNaughton (1994) and many others later showed evidence for these memory reactivations, supporting this idea.

However, some **recent findings have made us question if the hippocampus really 'contains' or stores memories**. For example, it has been shown that place cells, that were thought be invariant encoders of space, contain much more information that just location and in certain task situations will encode for other elements, such as time elapsed and not space. Further, Tanaka and colleagues (2018) combined classic, electrophysiological place cell recordings with the engram tagging technique (Josselyn and Tonegawa 2020) and showed that, surprisingly, it was not classic place cells that showed plasticity-related changes in the form of immediate early gene expression. These, and other findings, have led researchers to propose new theories of hippocampal function, as either a sequence generator (Buzsáki and Tingley 2018, Swanson, Levenstein et al. 2020) or to enable scene reconstruction (Barry and Maguire 2019) as covered in the previous section.

We would propose that the hippocampus would have multiple functions that could differ between encoding and recall. **During encoding, it would serve as the 'fast learner' and intermediate memory buffer**. However, this function would usually be very short lived. In the adult animal or human, with abundant real-world previous knowledge acquired during childhood and adolescence, this buffer function would only be needed for hours and last mainly for the first few nights after the experience. After a night of sleep with reactivations playing the new memories into the cortex reinforcing those connections, the hippocampal memory trace would quickly disappear due to synaptic renormalization during sleep (see also (Navarro-Lobato and Genzel 2019) for more detail). The hippocampus would be ideal for this role as memory buffer, due to its ability to generate sequences that can be used to quickly map on associations between different 'whats' that can be fed to 'secondary readers' downstream (Buzsáki and Tingley 2018, Swanson, Levenstein et al. 2020).

During memory retrieval, the scene reconstruction properties of the hippocampus would become more important (Barry and Maguire 2019). Perhaps the reason that episodic memories are 'dependent' on the hippocampus, is that, to be able to experience memory retrieval as a vivid memory re-experience, you need the scene reconstruction properties. However, as mentioned above, every night during sleep the hippocampal trace would be renormalized and thus this trace would disappear perhaps not completely but mostly over time. With most memories in the real-world, this would occur in a few nights and with more salient memories a longer time may be required.

When an animal is totally naïve to an experience and cannot harness cortical, previous-knowledge networks, the hippocampal trace would be necessary for a longer time period but still undergo deterioration over time. The dopamine signal from the locus coeruleus would facilitate this hippocampal persistence of memory trace (Duszkiewicz, McNamara et al. 2019).

Thus, when recalling a memory, **the type of retrieval experience would depend on how much of the hippocampal memory trace is left**. If no hippocampal memory trace is left, the hippocampus would have more difficulty in reconstructing the scene. Consequently, you would retrieve a sense of familiarity without explicit experience of recalling a memory (i.e. remembering the event of learning); this would be the case for classic semantic memories (Fig. 4A). On the other side of the gradient, if the hippocampal memory trace is still mostly intact, scene reconstruction would be faithful to the original experience and retrieval would come in the form of correct, episodic recall (Fig. 4C). You would become aware of the memory and the past event and thus have more direct recollection of the encoding event instead of just a sense of familiarity. The most interesting case would be when a partial but not complete trace is present in the hippocampus (Fig. 4B). Then the pattern completion properties of the hippocampus would come into play and the hippocampus would still try to reconstruct the scene for episodic-like recall. However, in absence of the complete, original trace, the memories would become increasingly vulnerable to inaccuracy and distortion, as often can be observed in 'flashbulb' memories of unique events (Barry and Maguire 2019).

One implication of this hypothesis is that **semantic memories** (and thus perhaps statistical regularities) **would be recalled more faithfully if no or less of a hippocampal trace is present, in contrast to if a partial trace is present that could generate memory distortions**. And since the hippocampal memory trace would decay over time, it would follow that semantic memories would be expressed better after longer time periods.

In sum, we propose that the hippocampus is not simply a brain area for storing memories. **Instead, its computational properties as a sequence generator, pattern completer, and scene reconstructor can explain its involvement in memory encoding, consolidation, and retrieval.**

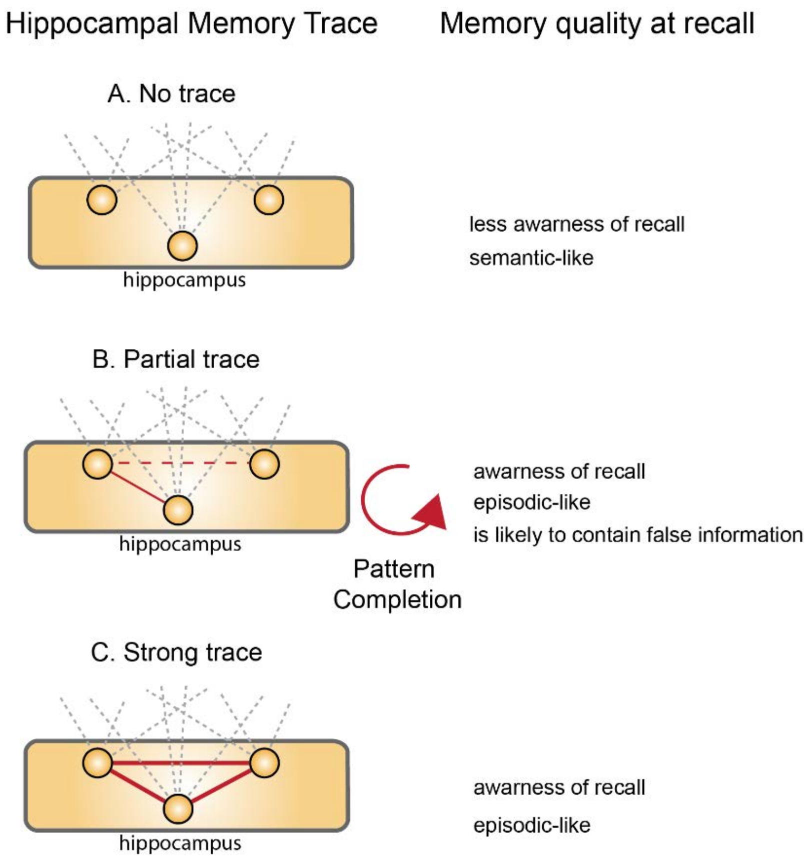


Figure 4 Possible hippocampal function

How would the hippocampus be involved in memory retrieval? **A**) If no hippocampal memory trace is left, memory retrieval would be fully dependent on cortical networks. This would result in less awareness of recall i.e. more a sense of familiarity not explicit recall and a classic semantic memory. **B**) If a partial trace is left in the hippocampus, the properties of this brain area would lead to pattern completion therein. Thus, more awareness at recall and episodic-like quality but the memory would also have a higher likelihood of including false information. **C**) Finally, if the hippocampus would still contain a strong, complete trace, it would contribute to awareness of recall with episodic-like quality that in this case is still faithful to the original experience.

Conclusion

Most of what we learn can be put in context of what we already know. In this review, we have summarized the existing research on neurobiology of previous knowledge, especially in relation to schemas even though in non-human animal research it can be hard to define if a schema in contrast to simpler forms of previous knowledge is truly present. Based on these findings, we proposed a novel theory on how involvement of brain areas can shift from hippocampus to cortex depending on the level and amount of previous knowledge. **When going from naïve to expert, the hippocampus loses its critical function during encoding, and cortical areas become more independent**.

Building up such cortical networks will usually occur early in the life-span of a human or any other animal. Perhaps this would also relate to why our cortical networks are more plastic when we are younger and once past adolescence **most learning would occur within the context of accumulated world-knowledge decreased cortical plasticity in the adult is likely important to protect our pre-existing knowledge** and avoid catastrophic interference when learning something new.

While much has been learned since the concept of previous knowledge and schemas was brought up as a concept in the 1930s (Bartlett 1932) and then picked up in neurobiology in 2007 (Tse, Langston et al. 2007), **many open questions remain.** For example, how exactly is previous knowledge in the cortex updated? What is the importance of sleep and therein reactivations occurring? Further, we currently focus on hippocampus and cortex as memory structures, but how do other brain areas contribute? The nucleus reuniens has been shown to be critical for long-term memory persistence (Barker and Warburton 2018, Ferraris, Ghestem et al. 2018, Mei, Logothetis et al. 2018, Troyner, Bicca et al. 2018, Wagner, van Buuren et al. 2019) but could it be critical for schema updating as well? And how do different connections between the hippocampus and prefrontal cortex contribute to this process? There is a direct connection from ventral hippocampus (Jay and Witter 1991) but also indirect pathways via reuniens (Barker and Warburton 2018, Ferraris, Ghestem et al. 2018, Mei, Logothetis et al. 2018, Troyner, Bicca et al. 2018, Wagner, van Buuren et al. 2019) and medial prefrontal cortex (Olafsdottir, Carpenter et al. 2017), but which pathways relate to which role remains unclear.

It is critical for memory researchers to consider the amount of previous knowledge and especially for studies done on non-human animals to venture more into this domain. We are trying to understand human cognition, which rarely does not harness world-knowledge. Further, when it comes to real-life application of our results, we have to consider implications on education in the young with less previous knowledge as well as adults who have more (Ruiter, van Kesteren et al. 2012, van Kesteren, Rijpkema et al. 2014). For example, we could show in the HexMaze that the time since first exposure is more critical for build-up of previous knowledge than the amount of training an animal has received (Alonso, Bokeria et al. 2020). This stands in opposition to the current preferred practice of “cramming” right before an exam as seen in many high-school and university students. Instead, students should space out their learning over longer time-periods, if they want to create long-term knowledge instead of just a short-term memory.

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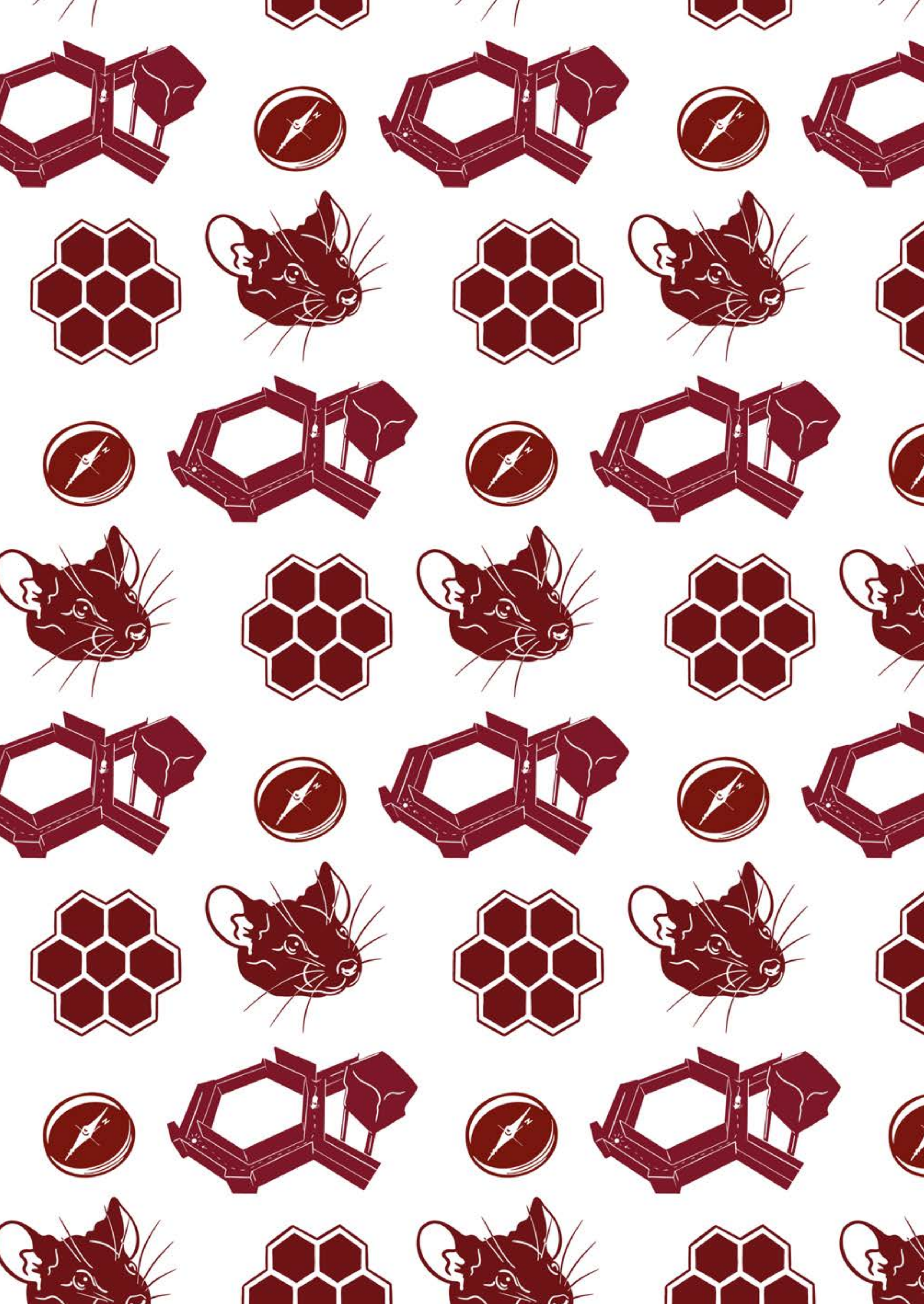

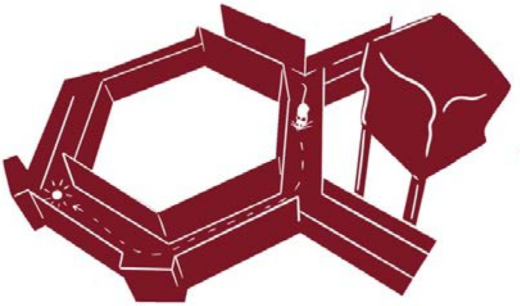

Chapter 4

Schema-induced shifts in mice navigational strategies are unveiled by a minimal behavioral model of spatial exploration

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Abstract

Shifts in spatial patterns produced during the execution of a navigational task can be used to track the effects of the accumulation of knowledge and the acquisition of structured information about the environment. Here we provide a quantitative analysis of mice behavior while performing a novel goal localization task in a large, modular arena, the HexMaze. To demonstrate the effects of different forms of previous knowledge we first obtain a precise statistical characterization of animals' paths with sub-trial resolution and over different phases of learning. The unveiling of a multiplexing of time scales regulating the improvement of performance shows the emergence of a flexible representation of the task. We then use a generative mathematical model of the animal behavior to isolate the specific contributions to the final navigational strategy. **We find that animal behavior can be accurately reproduced by the combined effect of a goal-oriented component, becoming stronger with the progression of learning, and of a random walk component**, producing choices unrelated to the task and only partially weakened in time.

Introduction

The problem of learning and especially of the integration of new information into an already existing knowledge structure is at the center of the effort to understand brain functioning (Alonso, van der Meij et al. 2020). When using rodent animal models, such problem has often been addressed in the context of spatial navigation and map learning (Wang and Morris 2010, Richards, Xia et al. 2014). Animals knowledge about the environment and the degree to which they can acquire new information can be linked to their ability to easily navigate to specific locations and flexibly adapt to changes in the environment (Behrens, Muller et al. 2018). Nevertheless, the characterization of the effects of learning has been mostly restricted to simple tasks with limited spatial and temporal complexity, focusing on isolating specific components of the learning process with highly-controlled paradigms. Indeed, the difficulties in precisely monitoring the animal behavior pose one of the major limiting factors in the development of more comprehensive experimental paradigms (Fonio, Benjamini et al. 2009). **Here we aim at filling this gap by providing a quantitative framework for the description of navigational strategies, expressed by mice while completing a spatial task**.

Assessing the effects of the accumulation of learning on the performance in a spatial orientation task requires the combination of two elements. On the one hand **the complexity of the task should be high enough to allow for the expression of rich behavioral patterns and of different grades of information acquisition** (Benjamini, Fonio et al. 2011) Disentangling the different components informing animal choices requires providing animals multiple options over a sizable spatial and temporal interval. Such condition is also a requirement in the interest of understanding animal behavior in its naturalistic setting (Tchernichovski, Benjamini et al. 1998). Wild rodents experience will include an articulate system of burrows together with the surrounding layout, a situation that can only be captured in the laboratory by studying spatial learning in larger, more complex environments (Wood, Bauza et al. 2018).

As a consequence of the **richer behavioral repertoire accessible to the animal**, successfully tracking the evolution of task-related abilities requires the deployment of specific quantification tools, aimed not only at measuring task performance but also the specifics of animal behavior that accompany it (Dvorkin, Benjamini et al. 2008). Such tools should also provide a link between observable changes in the animal choice patterns and shifting navigational strategies underlying such choices (Ruediger, Spirig et al. 2012, Gehring, Luksys et al. 2015).

In this study we use a novel navigational task, featuring a goal-localization paradigm with extended spatial and temporal dimensions, the HexMaze (Figure 1, top). Mice learn to locate a reward location in a larger, modularly structured maze providing precise control over animals' paths. Testing animals over a long temporal period, and after a modification of the environment as either we introduce a novel reward location or alternatively we place a set of barriers to block some paths (Figure 1), we look at the effects of previous knowledge on their performance and on their ability to flexibly incorporate novel information. We are thus able to track different contributions to the observed animal behavior, including those linked with information encoding, memory consolidation and schema acquisition (for a complete description of learning dynamics on the maze see (Alonso, Bokeria et al. 2021)). To test the effects of such components **we apply statistical analysis to obtain a fully-characterized picture describing the evolution of animal choices with sub-trial resolution**. Importantly, such detailed phenomenological description of trial-by-trial behavioral profiles is then complemented with a generative model of task trajectories based on a mathematical description of task completion process (Figure 1).

This modeling approach unveils a limited set of principles guiding animal choices. **Our results show how animal behavior can be faithfully reproduced by a minimal mixture of random walking and goal-directed runs over limited distances**. The relative importance of these two components over the different phases of learning (initial goal-location acquisition, consolidation over multiple sessions, schema update in coincidence with environmental modifications) not only provides a concise characterization of animal approach to the task, but mirrors the emergence of task-specific memory constructs, offering a direct quantification of learning induced patterns of behavior. We find that, although we can observe an increasing amount of knowledge about the task and the maze structure being incorporated by animals, their performance never really converges to be completely goal-oriented. Instead we can measure the influence of a consistent random component, interfering with optimal task performance, and being only partially reduced with increasing familiarity to the task. Persistence of such task-independent activity could be a product of exposing animals to an expanded task complexity, effectively giving them increased freedom to diverge from task completion, but we also consider the possibility of it reflecting mice specific idiosyncratic behavior, possibly triggered by the specie propensity to hyperactivity (Jones, Paul et al. 2017). In both cases, further application of our modeling approach is likely to provide further insight on the diversity of behavioral approaches linked to different cognitive demands and different species.

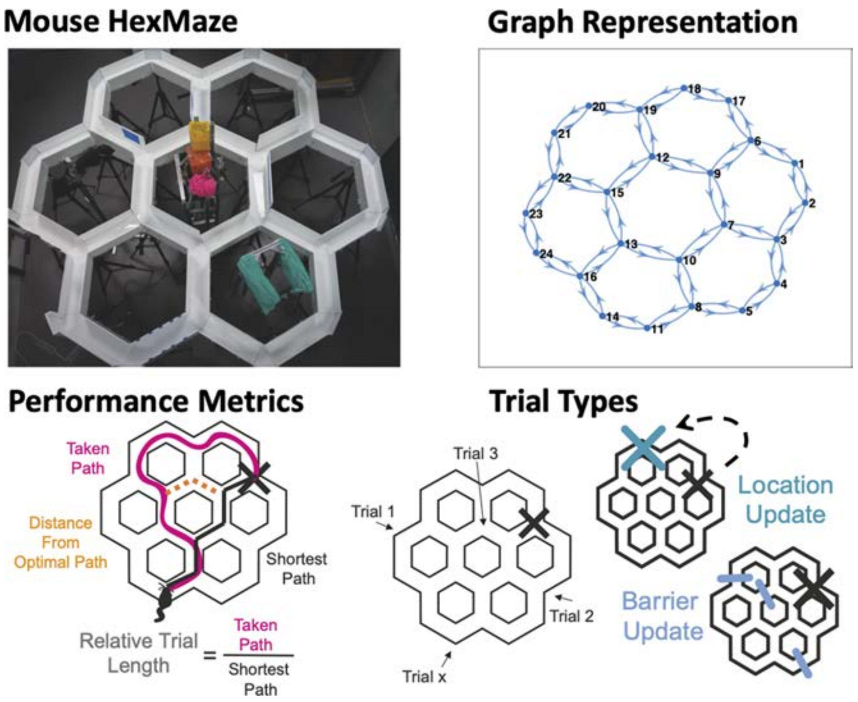

Figure 1 HexMaze Structure and Experimental Paradigm**. Top**: View of the maze (left) and its graph representation used in the analysis (right). **Bottom Left**: Two main performance metrics are used. 1) Relative Trial Length is the length of the paths taken by the animal divided by the shortest possible path to the goal location. 2) Distance from Optimal Path is the distance of the animal position at any time from the closest point of the shortest path. **Bottom right:** During training, animals started each trial from a different location and had to navigate to a fixed Goal Location. After the animals had acquired the general maze knowledge during the Build-Up, Updates were performed with inclusion of new barriers (Barrier Update) or new goal locations (Location Update)

Methods

Subjects

Five cohorts of four male C57BL/6J mice each (Charles River Laboratories) aged two months at arrival, were group-housed in the Translational Neuroscience Unit of the Centraal Dierenlaboratorium (CDL) at Radboud University Nijmegen, Netherlands. They were kept at a 12 h light/ 12 h dark cycle and were before training food deprived overnight during the behavioural testing period. Weight was targeted to be at 90% to 85% of the animals' estimated free-feeding weight. All animal protocols were approved by the Centrale Commissie Dierproeven (CCD, protocol number 2016-014-018). The first cohort (coh 1) was used to establish general maze and task parameters and are not included in this dataset.

HexMaze

The HexMaze was assembled from 30 10 cm wide opaque white acrylic gangways connected by 24 equilateral triangular intersection segments, resulting in 36.3 cm distance center-to-center between intersections (Fig. 1A). Gangways were enclosed by either 7.5 cm or 15 cm tall white acrylic walls. Both local and global cues were applied to provide visual landmarks for navigation. Barriers consisted of transparent acrylic inserts tightly closing the space between walls and maze floor as well as clamped plates to prevent subjects bypassing barriers by climbing over the walls. The maze was held 70 cm above the floor to allow easy access by the experimenters.

Behavioral Training

After arrival and before training initiation, mice were handled in the housing room daily for 1 week (until animals freely climbed on the experimenter) and then habituated to the maze in two 1 h sessions (all four cage mates together) with intermittent handling for maze pick-ups (tubing (Gouveia and Hurst 2017)). Mice were trained either on Mondays, Wednesdays and Fridays (coh 1-3) or Tuesday and Thursday (coh 4+5). Per training day (session) each mouse underwent 30 min of training in the maze, resulting in up to 30 trials per session (Table 1). The maze was cleaned with 70% ethanol between animals (later clean wipes without alcohol to avoid damaging the acrylic), and to encourage returning in the next trial, a heap of food crumbles (Coco Pops, Kellogg's) was placed at a previously determined GL, which varied for each animal. GLs were counterbalanced across animals, as well as within animals across GL switches. E.g. one out of four animals, and one out of four GL per animal would be located on the inner ring of the maze while the others were on the outer ring (to shape animal behaviour against circling behaviour). Start locations for each day were generated based on their relation to the GL and previous start locations (locations did not repeat in subsequent trials, at least 60% of the trials had only one shortest path possible, first trial was different to the last and first trial of the previous session and locations had at least two choice points distance to each other as well as the GL). On average 30 start locations were needed per day per mouse, which were generated the day before training. After the mouse reached the food and ate a reward, the animal would be manually picked up with a tube, carried around the maze to disorient the mouse, and placed at the new start location. All pick-ups in the maze were done by tubing (Gouveia and Hurst 2017). After placing the animal at the start location, the experimenter quickly but calmly moved behind a black curtain next to the maze to not be visible to the animal during training trials.

Training consisted of two blocks: Build-Up and Updates. During probe sessions (each second session of a GL switch and additionally in Build-Up GL1: S6, GL2: S5, GL3-5 S4) there was no food in the maze for the first and ninth trial of the day and each time for the first 60 s of the trial to ensure that olfactory cues did not facilitate navigation to the GL. After 60 s food was placed in the GL while the animal was in a different part of the maze (to avoid the animal seeing the placement). All other trials of the day were run with food at the GL. Probe trials and GLs switches were initially minimized, to help shape the animal behaviour. In the first trial of the day, animals would not find food at the last presented location for both the first session of a new GL as well as probe trial days (e.g. always the second session of a new GL); thus these sessions were interleaved with normal training sessions with food present at the last known location in the first trial of the day to avoid the animals learning the rule that food is initially not provided.

To measure the animals' performance, the actual path a mouse took was divided by the shortest possible path between a given start location and the GL, resulting in the log of normalized path length (Fig. 1B) and functioning as a score value. Given a sufficient food motivation and an established knowledge-network of the maze a mouse should navigate the maze efficiently. A score of 0 indicated that the mouse chose the shortest path and navigated directly to the goal. On average, animals would improve from a 3 times to 1.5-2 times longer path length than the shortest path, corresponding to 0.4 and 0.2-3 log values. Random walks through the maze are estimated with a model to result in a 4 times longer path (0.6 in log). The normalized path length of any first trial of a session was used to measure long-term memory since training sessions were two to three days apart.

First trial of the second sessions (probe trials) of each goal location in Build-up and Update phase were watched to score the number of times that animals crossed their current and previous goal location as well as the amount of time they dwelled there. As a control, same method was applied to two other nodes, one on the inner ring and the other on the outer ring of the maze. These nodes were selected in such a way that they were not close to each other and to the goal locations, with at least three gangways between them. Further, to control a false positive result, nodes that were in the way between goal locations were not chosen as a control

Food motivation was ensured by restricting access to food for 12 h to 24 h before training and confirmed by both the number of trials ran each day as well as the count of trials during which the animal ate food at the first encounter with the food in each trial. If animals were not sufficiently motivated, the count of both would decrease. Additionally, animals were weighted three times a week and the average weekly weight was ensured to not fall below estimated 85% free-feeding weight, which was adapted for the normal growth of each animal across time.

Behavior Analysis

The structure of the HexMaze experimental setup was reproduced as a directed graph with node numbering corresponding to the experimental one. Animal trajectories were thus described as sequences of visited nodes on this graph (Figure 1, top).

When measuring the distance of the animal location from one of the nodes in the optimal path, one has to consider the possible presence of multiple shortest paths of equal length connecting the start location with the goal. To take into account this source of ambiguity, for each trial, we computed the optimal path between the two locations using the HexMaze graph with weighted edges. The shortest path was computed multiple times each time on a different graph, first initialized with uniform weights and then adding small random noise to the value of every edge. In this way, in the presence of alternative and equivalent paths, the noisy weights would lead to the selection of either of the existing ones on a random basis. By collecting all the nodes happening to be described as belonging to a shortest path we thus obtain a list of all the nodes to be considered when computing the distance of the animal from the optimal path.

The experimental trials are divided as following: each condition (Build-Up, Location Update or Barrier Update) comprises 3 sessions. For each session we analyze separately the first trial and then the following trials in groups of 10 until trial number 31.

Distance from optimal path curves fitting

We used the following function to parametrize the animal performance.

$$\mathrm{F} = \mathrm{A} * ((\mathrm{N}(0, \mathrm{L1}) - \mathrm{N}(0, \mathrm{L2}))/\mathrm{Z})$$

The difference of Gaussian functions (N) is normalized (Z) so that its maximum value is equal to. Therefore, A then controls the peak value of fit, while L1 and L2 its descending and ascending length, respectively. The fit is performed by optimizing the values of A, L1 and L2.

Mean and variance of the different measures for each condition were evaluated using 50-fold bootstrapping.

GL shuffling analysis

The specificity of the results for goal-directed behaviour was tested by randomly assigning each trial with a random GL drawn from any of those used in the experiment.

We generated surrogate data by randomly shuffling GLs across all trials from a particular trial block used in the analysis. Behavioral performance analyses were then repeated using the newly assigned GL as the target location for the optimal path and for the evaluation of the relative trial length (RTL). In cases in which the mice trajectory for a particular trial did not include the surrogate GL, we assigned by default an RTL of 10.

Simulations

The simulations are performed as following: we create a virtual HexMaze as a directed graph having the same structure of the real one. At every time step the virtual mouse moves from one node to an adjacent one. We do not allow trajectory reversals, so the node visited at the previous time step is not taken into account as a target. The start and goal locations are the same as those used in the experiment. Each run consists then in a sequence of nodes visited by the mouse and the run eventually ends when the animal reaches the goal. We augment the size of the modelled data by simulating multiple independent runs (n=50) for each experimental trial.

The movements of the virtual animal are generated according to an algorithm with two components: Random Search and the Foresight. The Random Search part consists in a procedure to select which node the animal is going to visit next and is meant to approximate an optimal search strategy. While performing Random Search the animal randomly picks the next node among the available ones. On top of this we introduce the possibility for the animal to take long diagonal runs that take it to another section of the maze. These diagonal runs are initiated with a probability η at any time step. If a diagonal run is initiated, then a node is randomly picked among those in the outer ring and at a distance of at least 3 steps from the current position of the animal. The mouse then uses the following time steps to reach this target along the shortest available path. Once the target is reached, the random node selection is resumed. We use different simulations to vary the value of η. Decreasing the value of this probability makes the search strategy approximate more and more a purely random walk through the environment. Higher values introduce a larger amount of "optimality" as they allow the animal to more quickly leave an already explored area.

The Foresight component on the other hand represents the ability of the animal to anticipate the location of the goal when getting within a certain distance from it. It is therefore aimed at representing the effect of experience and an increasing knowledge of the environment and of visual cues. At every step, we draw a random number from an exponential distribution with mean F. $p(x) = \frac{1}{F} e^{-\frac{x}{F}}$ If the shortest path from the animal location to the goal node is smaller of this number, then the animal takes a direct path to the goal and the trial is over. Also in this case we run different sets of simulations varying the value of F. F=0 corresponds to an animal with no ability to remember the position of the goal from its current location, unless by running directly over it. As F increases the chances for the simulated mouse to detect the goal from some distance increase. Eventually a very large F would reproduce a goal-directed behavior.

For each set of parameters, we measure how well the statistics of the simulated runs reproduce those obtained from real animal behavior. To do so we use a combination of measures: 1) relative trial length (as the ratio between the actual length of the trajectory and the length of the shortest path between start and goal location); 2) maximal distance from the optimal path reached during the trial; 3) amount of time spent in the external ring of the maze vs. the internal ring. For each of these quantities we compare the distribution obtained from the experiment to the one generated simulating the trajectory of the animal according to a specific set of parameters. We measure the distance between the two distributions with Kolmogorov-Smirnov statistics. Therefore for each set of experimental trials we obtain how well the statistical properties of the animal behavior can be reproduced by a certain choice of the model parameters.

The experimental trials are divided as following: for each condition (Build-Up, Location Update or Barrier Update) we used the first 3 sessions. During Build-Up more sessions were run for each goal location, however here we focus on the first 3 to be able to compare it with the Update phase. For each session we analyze separately the first trial and then the following trials in groups of 10 until trial number 31.

Statistical analysis

Sample size of the data available and used for each behavioral condition is reported in Table 1. Simulated data samples correspond to the same numbers multiplied by 50. Throughout the paper, bootstrap estimates of mean and SD of different measured quantities are obtained from n = 50 resampling with replacement. Whenever mentioned, KS test is meant to be two-samples. Reported non-significant differences were all associated with a p. 0.1 and a size effect,0.08. Minimal n m/(n 1 m) ratio was equal to 50.

Code accessibility

The code/software described in the paper is freely available online at https://github.com/fstella/HexMaze_Behavior Analysis and also as the Extended Data 1. All analysis and simulations were performed using custom MATLAB code.

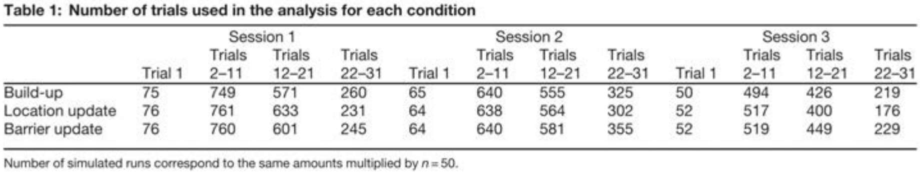

Table 1: Number of trials used in the analysis for each condition

	Session 1				Session 2				Session 3			
	Trial 1	Trials 2–11	Trials 12–21	Trials 22–31	Trial 1	Trials 2–11	Trials 12–21	Trials 22–31	Trial 1	Trials 2–11	Trials 12–21	Trials 22–31
Build-up	75	749	571	260	65	640	555	325	50	494	426	219
Location update	76	761	633	231	64	638	564	302	52	517	400	176
Barrier update	76	760	601	245	64	640	581	355	52	519	449	229

Number of simulated runs correspond to the same amounts multiplied by $n = 50$.

Results

The HexMaze Experiment

The HexMaze is arranged as six regular densely packed hexagons, forming twelve two-way and twelve three-way choice points (nodes) 36.3 cm apart, in total spanning 2 m x 1.9 m (Fig. 1, Top). Gangways between nodes were 10 cm wide and flanked by either 7.5 cm or 15 cm tall walls. Maze floor and walls were white and opaque, with local and global cues applied in and around the maze to enable easy spatial differentiation and good spatial orientation; overall leading to a complex, integrated maze. During training food was placed in one of the nodes and **the animal had to learn to navigate efficiently from different start locations to the Goal Location (GL)**.

Animals went through two phases of training: Build-Up and Updates. In the Build-Up the animals should create a cognitive map of the maze environment; in contrast, during Updates, stable performance is achieved and they should be simply updating the cognitive map. **These two phases also differed in the frequency of GL switches: during Build-Up, the GL remained stable for five and more sessions, while during Updates a change occurred every three sessions** (see also below). Different Update types were performed: including barriers in the environment (Barrier Update) and changing the goal location (Location Update) (Fig. 1, bottom). Number of trials used in the following analysis are reported in Table 1.

Characterization of animal behavior in the HexMaze

We first set out to quantify the time-evolution of animal behavior. The structure of the HexMaze allows for an efficient tracking of the animal choices, as its behavior is easily described by the sequence of visited maze nodes. Therefore, in the following we will focus on this descriptor to measure different aspects of the animal performance during the experiment. Clearly, **the main element to be taken into account when analyzing behavior is the ability of the animal to efficiently localize the reward and reach it through a path as short as possible** (Figure 1).

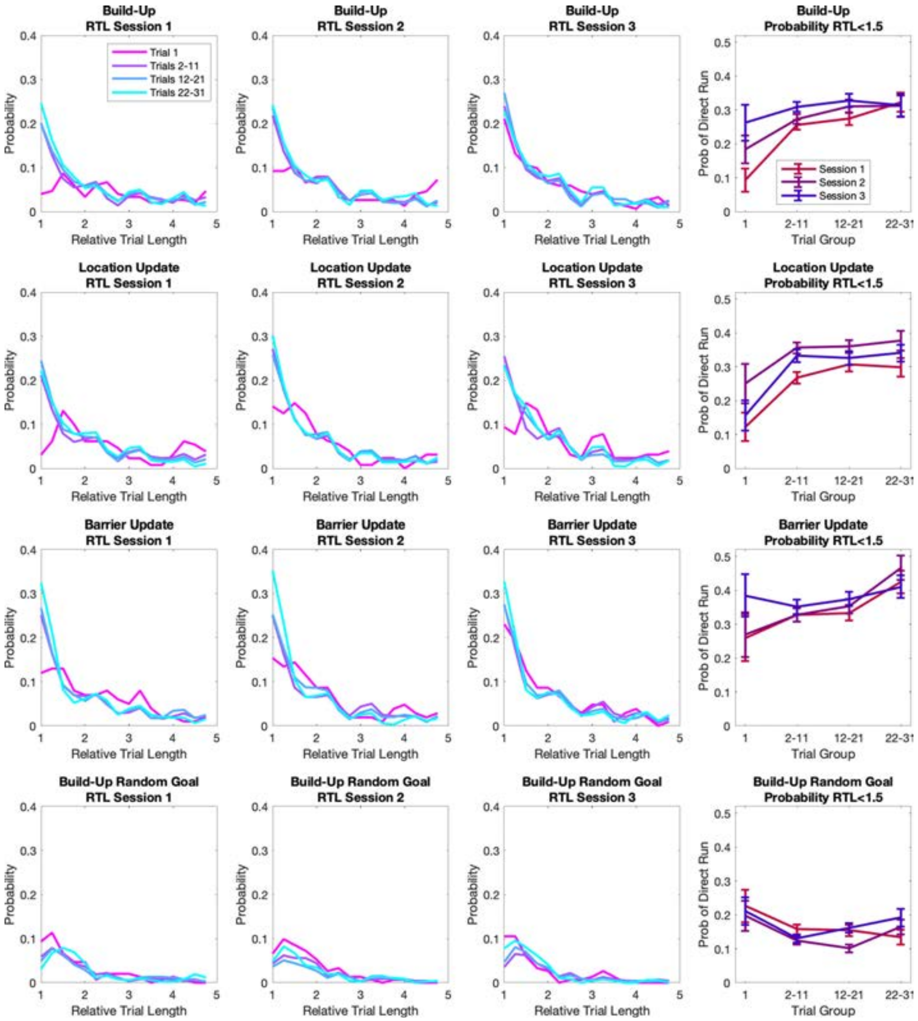

Figure 2 Animals are progressively more likely to take shorter paths to the goal**.** Distribution of relative trial lengths (RTL) for different trial groups and sessions. For each session, trials from different animals were grouped in four categories: first trial only, from second to 11th trial, from 12th to 21st trial, and from 22nd to 31st trial. RTL = 1 corresponds to perfect trial. Last column, Probability of RTL, 1.5 (optimal trial) over time. Last row, RTL computed after randomly shuffling the Goal Locations across trials. The absence of learning-induced changes indicates their specificity for goal-directed behavior. Error bars show STD computed by 50 bootstraps.

A perfectly optimal, goal-oriented behavior, would imply that after a necessary learning transient the trajectories selected by the animal would progressively converge toward the shortest available given the start location and the reward one. By measuring the ratio between the length of the actual path (measured in terms of number of nodes visited before reaching the goal) and the length of the optimal path (Relative Trial Length = RTL), over a certain amount of trials, one would then expect to observe this distribution to be more and more skewed towards the value of 1, corresponding to the animal actually following the optimal path (Figure 2). Indeed what we find is a progressive increase in the percentage of trials with a low ASR score, within each session, across sessions and also for each of the experimental conditions (Figure 2, last column**). Multiple effects point to an actual presence of learning and to a growing awareness of the maze structure and goal location in the animals**. First, during Build-Up not only the score improves within each session, but animals consistently do better in the first trial of a new session compared to the previous one. Then, while the score goes back to pre-learning values at the beginning of the Location Update, when a new unknown goal is introduced, it reaches its asymptotic value faster compared to Build-up. And finally, the insertion of barriers has only a very limited effect on the animal performance. At the same time the hypothesis of over-wise, totally committed mice is challenged by the fact that although increasing in time, the probability of a perfect (or almost perfect) run remains substantially below 1 over the entire arc of the experiment, even after the animals have been repeatedly exposed to the maze and to a specific reward location. The trial relative length distribution shows a long tail of values larger than 1 (Figure 2, columns 1 to 3), indicating that the animal choices are far from corresponding to a purely optimal, goal-oriented strategy.

One way to further characterize the degree to which the mouse behavior is goal-directed is to measure how far its trajectory would steer away from the optimal path joining the starting location to the reward**. For each trial and for each node visited by the animal during the trial, we then compute the Distance From Optimal Path (DFOP), that is, the distance between the visited node and any of the nodes comprising the optimal path**. For each trial we then obtain a measure of 'stray' over time, providing a profile of the animal approach to the goal (Figure 3). When averaged over different set of trials, this profile shows a bump shape, quantifying the amount of deviation from optimal behavior, showing an increase at the beginning of the trial and eventually converging again towards the correct path. These curves provide us with different information about the animal trajectories over the course of the experiment: i) the amount of 'stray', that is the average maximum distance from the optimal path, ii) the average length of the trials and iii) how fast the animal will go back to the correct path after straying away in the beginning. We can extract this information from the data by fitting a parametrized function to them. We use a normalized difference of Gaussians (see Methods) that provides us an excellent approximation of the experimental curve shapes (Figure 4, top row). This fit depends on 3 parameters: 1) The maximum height; 2) The amount of steps to reach the maximum and 3) The amount of steps to go back to the optimal path. Separately plotting the value of these three parameters, obtained by fitting the data from different stages of learning we can identify the different components contributing to the overall change in performance (Figure 4). In fact, we observe how the maximum height and the descending scale show a gradual decrease with time, consistently with the improving performance of the animal (Figure 4, first and second column; build-up within session decrease maximum height: Welch’s t test first session p = 10e-51, second p = 10e-29, third p = 10e-28, descending scale: Welch’s t test first session p = 10e-40, second p = 10e43, third n.s. Build up first trial decrease maximum height: Welch’s t test first vs second session p = 0.025, second vs third p = 10e-8, descending scale: Welch’s t test first vs second session p = 10e-20, second vs third p = 10e-31. Location update second session first trial build-up vs location update, maximum height: Welch’s t test p = 0.033, descending scale: Welch’s t test p = 10e-11). Again, also these two parameters show an overall trend across all the phases of the experiment although their evolution is not monotonous, but rather has a seesaw shape because of the partial rollbacks happening between the last trials of one session and the first trial of the following one. At the same time, the ascending phase is not significantly affected by learning, showing that while the animals progressively strayed less and eventually took more direct runs to the goal, they nevertheless maintained a comparable amount of undirected behavior in the first part of the trial. Together with the previous analysis, our quantification of the animal behavior, shows how the navigation of mice in the HexMaze, can be described as a combination of learning-based choices (evident in the progressive improvement in all goal-related metrics) and of a persistent non-optimal component, keeping the overall behavior away from perfect performance even for late sessions and trials.

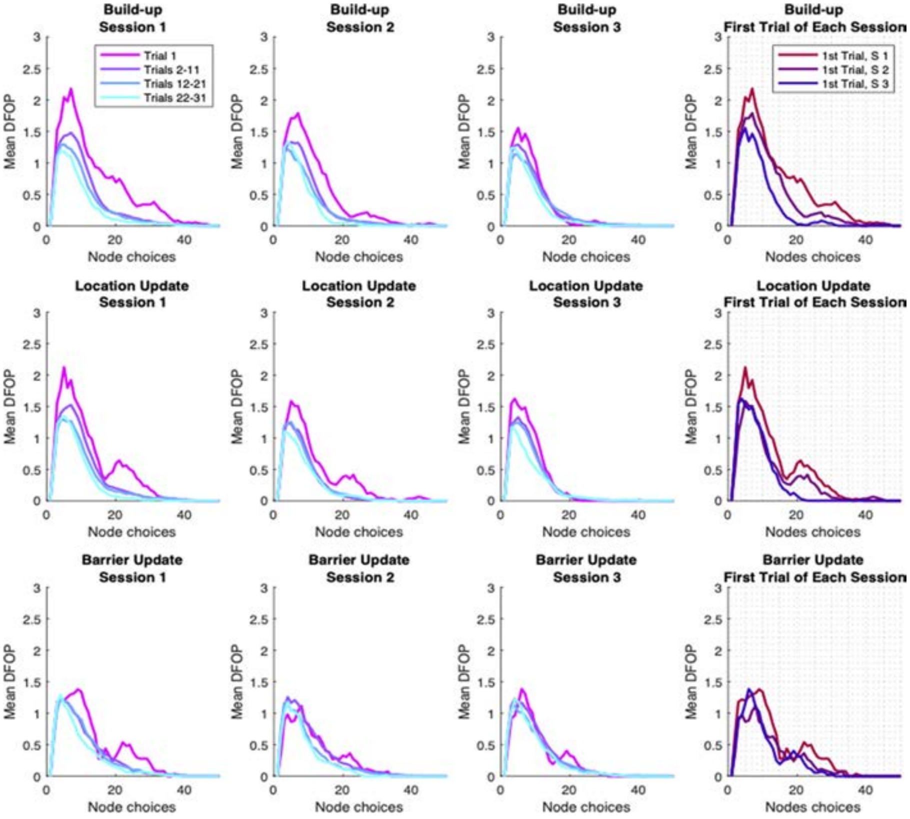

Figure 3 Quantification of animal trajectories departure from optimal path. Distance from optimal path (DFOP) over time for all trial groups and sessions. Last column panels, Comparison of the first trial for the three sessions. The effects of learning can be seen in the progressive reduction of the distance within each session. Additionally, DFOP decreases on the first trial of every successive session. Once a new Goal Location is introduced, convergence to asymptotic performance is faster than during initial learning. Finally, the insertion of barriers has only limited effects on behavior.

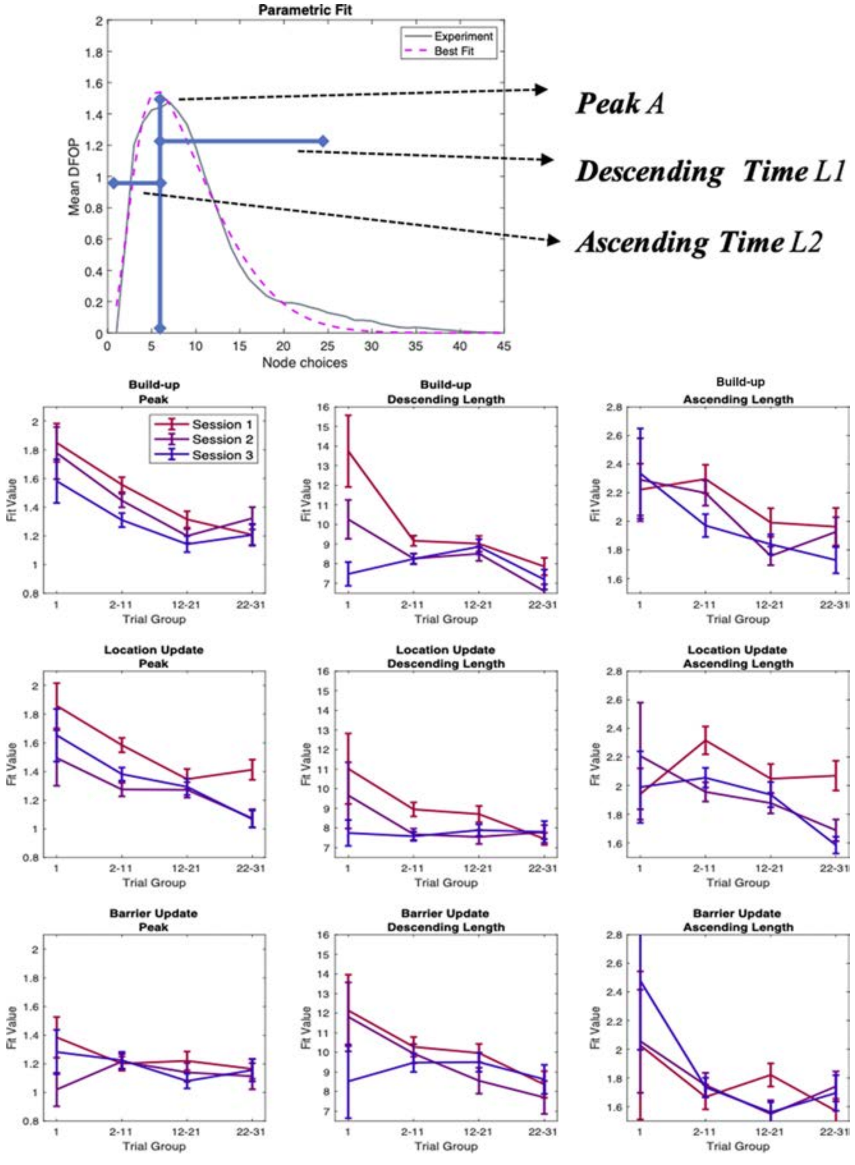

Figure 4 Learning sharpens animal performance by progressively reducing trajectory Distance from optimal path (DFOP). Results of the parametric fit of the curves in Figure 3. Top row, Example of the obtained match between experimental curves and the parametric fit. Bottom, value of the fit parameters over time for all conditions and sessions. Error bars STD from bootstrapping. This fit allows us to quantify: (1) the amount of "stray," that is, the average maximum distance from the optimal path; (2) the average length of the trials; and (3) how fast the animal will go back to the correct path after straying away in the beginning. Maximum distance and descending length show a decreasing modulation over time: within one session, across sessions, and during Goal Location shift, consistently with learning effects. Descending length shows instead no significant improvement, in line with a persistent influence of a random component on behavior.

Minimal Mathematical Model Describing Animal Choices

We then asked whether such results could be reproduced by a simplified model of the animal behavior. We simulate the trajectories produced by a virtual agent navigating the same HexMaze used in the experiment as it searches for the reward location. In these simulations the mouse moves in the environment selecting the next node to visit according to a set of predefined rules. We define these rules as a combination of a random walk through the environment and direct goal-runs based on the knowledge of the reward location. **Crucially, the model depends on only two parameters, η, the probability of taking a long diagonal run while randomly moving through the environment; and F, determining the probability that the animal will at any time start to run directly towards the reward location** (a quantity that for this reason, we named **Foresight**). We thus simulate the animal behavior using different combinations of these 2 parameters and compare the obtained statistics with those collected during the real experiment. The comparison is based on quantifying the distance between the distribution of relative trial lengths in real and virtual trajectories.

We find that our simple behavioral models very accurately approximate the animal strategy in every part of learning. For each set of trials, we find a combination of η and F that makes the distributions not significantly different ($p>0.1$, as evaluated using Kolmogorov-Smirnov statistics) (Figure 5).

We thus consider the (η, F) pair that minimizes the distance between experimental and simulation statistics for a specific set of trials (Figure 5). This pair of values was taken as best describing the behavioral characteristics of the animal navigation through the maze. **The evolution of these values in time provides us with a measure of the effects of learning on animal's behavior**.

First, we find that the best value of η is not significantly affected by the progression of learning, and that it remains confined to 0 across the entire experiment. Therefore, learning does not change the properties of random movement across the maze, and indeed this movement pattern appears to be largely unstructured, being captured by a simple sequence of random turns. Crucially we find that the **simulated trajectories obtained with parameters optimized to fit the trial length distribution also reproduced other statistical features of the animal behavior**. In fact, both the distribution of maximal distance from the optimal path, and that of average time spent on the inner vs. outer ring of the maze were captured by our simulations (comparison between behavior and model: KS $p>0.1$, all effect sizes <0.07; Figure 6).

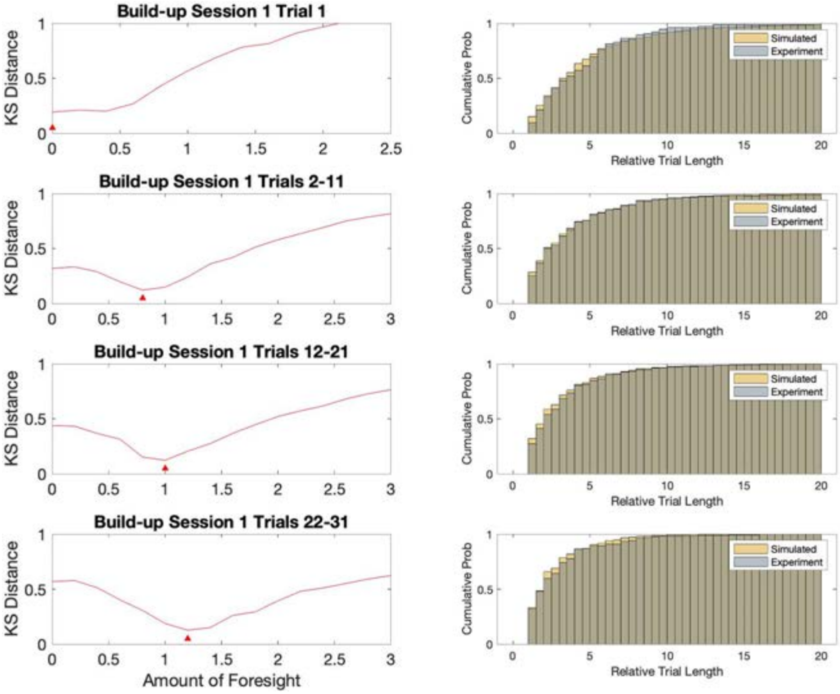

Figure 5 A minimal mathematical model reproduces the main properties of animal behavior. Model fitting to experimental data. Left column, Simulated-experimental Kolmogorov-Smirnov (KS) distance for a range of tested foresight values. Red triangles indicate location of best-fit F value for different sets of trials. Right column, Comparison of cumulative distributions for different trial groups and corresponding simulation results with best F value. All shown data are from build-up phase.

Studying the time evolution of the model parameter we first determine that the best value of η is not significantly affected by the progression of learning, and that it remains confined to 0 across the entire experiment. Therefore, learning does not change the properties of random movement across the maze, and indeed, this movement pattern appears to be largely unstructured, being captured by a simple sequence of random turns. On the other hand, the effects of learning are instead reflected in the evolution of the best value for foresight (Figure 7). As shown in the figure, its value starts at 0 for the first trial of the build-up phase, compatibly with an animal with no knowledge of the reward location and only randomly moving across the maze. **F then progressively increases with the accumulation of trials, indicating a growing awareness for the location of the reward, its relationship to visual cues and possibly for the geometrical structure of the maze itself.** Interestingly, foresight increase is significant (KS $p < 0.05$ comparing model distributions) both across trials within one session (single lines in the plot) and across the first trial for each session, indicating a nonmonotonic increase in performance (as we already found while analyzing the animal trajectories), reflecting a drop in performance between the end of one session and the start of the next one. What sort of conclusions can be drawn from the model results about the animal behavior in the maze? Even at late stages of the build-up phase, the foresight value remains relatively low, never raising above a value of 2. This limit points to a significant presence of random walking even for mice that have completed a substantial number of trials. **They appear to initiate goal-directed runs only when in close proximity to the reward and only rarely from the very beginning of the trial.**

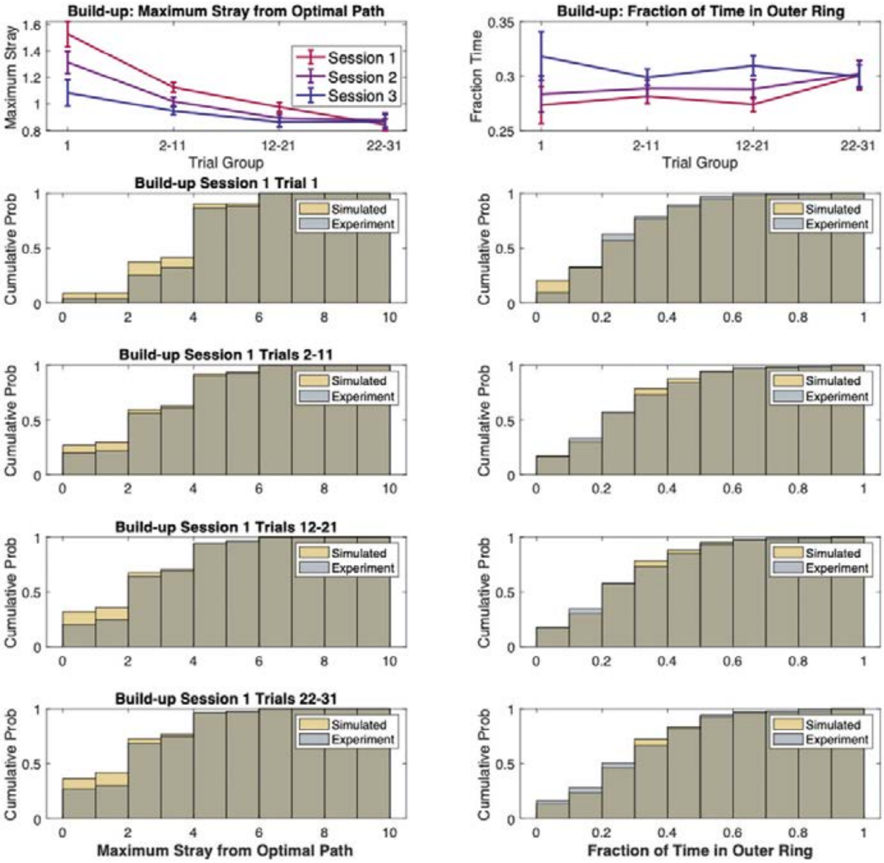

Figure 6 Further behavioral features matched by model fits. Top row, Evolution of the maximal DFOP and the fraction of time spent in the maze outer ring for the build-up phase. Rows 2–5, Same data as Figure 5. Using the same parameters obtained from fitting RTL distributions, the model also reproduces other aspects of animal behavior such as the distribution of maximal distance from the optimal path (left) and the distribution of relative time spent in the outer or inner ring of the maze on each trial (right). Real and model-based distributions are non-significantly different for every experimental phase and trial group (KS, all $p > 0.1$)

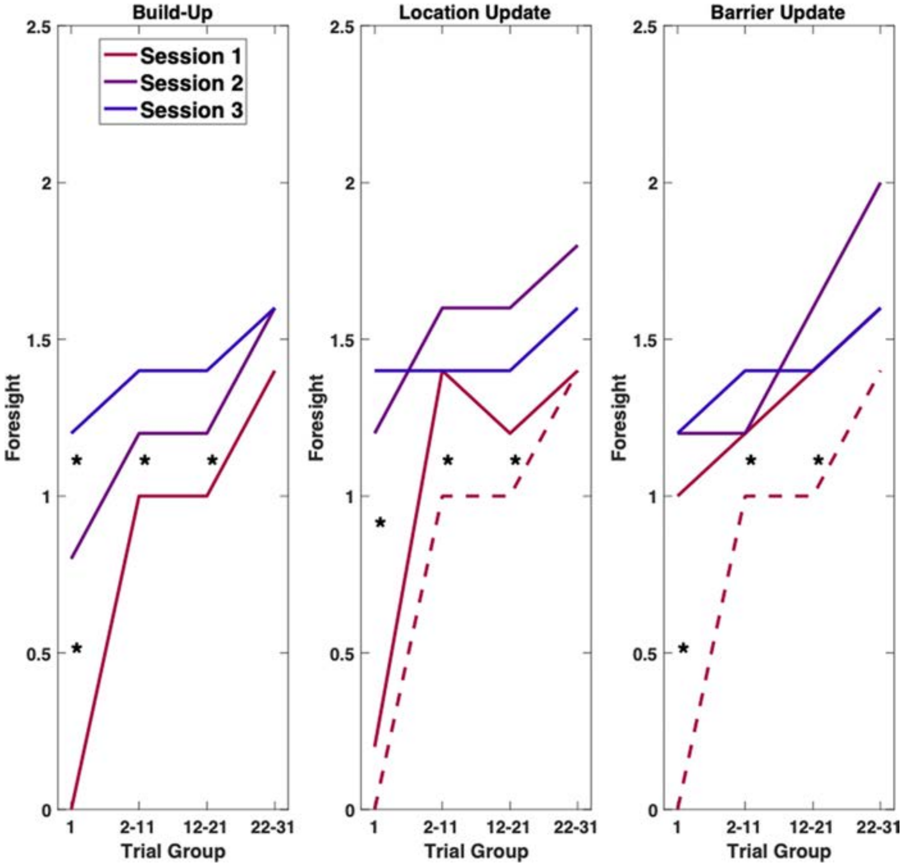

Figure 7 Modelling of animal behavior shows the accumulation of spatial information over the course of the experiment. Foresight Evolution: Best-fit values of Foresight for different experimental phases, sessions and trial groups. Our model reproduces the different phases of learning identified from behavioral analysis. The Foresight quantity appears to increase over the course of a session and with the accumulation of sessions. Goal location change is followed by a return to pre-learning values, but successive increase in faster than during initial task learning. Also in terms of inferred navigational strategy, the insertion of barriers in the maze has only limited effects.

Should the persistence of random behavior be taken as proof of a failure of the animal to build a complete “cognitive-map” of the maze? We can partially address this issue by looking at the performance of the model in different experimental conditions. Taking the Location Update phase, we see how Foresight is again close to 0 for the very first trial, consistently with the presence of a novel reward location. Nevertheless, in the following trials, the value of F increases at a significant faster rate compared to the build-up phase (KS $p<0.05$). **The mice are thus able to quickly integrate the new information into the knowledge they accumulated**

in the previous build-up sessions. This effect is not limited to the first update session but can be seen in a rapid saturation of F in the following session, although again its value does not grow beyond 2.

Similarly, using Barrier Update sessions, when barriers are added to the maze, while maintaining the reward locations stable, the behavior of the animal appears to be at the same level of the build-up trials from the very beginning of this phase. **Improvement in the goal-directed behavior can be still seen within one session, but no significant difference can be observed between sessions**. The relatively low impact of barrier introduction is consistent with animal strategy being dominated by a random walk and only affected by the presence of the goal when in the proximity of it.

Discussion

The HexMaze provides the ideal setting to characterize animal spatial cognition as it combines the availability of options to express complex behavior, with the possibility to precisely monitor and quantify animal navigational choices (Alonso, Bokeria et al. 2021). In particular, in this set of experiments we leverage on its structure to study the development of goal-directed behavior in mice learning to localize a reward location. We first provide a statistical characterization of the animal navigational patterns as they traverse the maze, by comparing them to the path expected from optimal goal-oriented behavior. Our results show a clear effect of learning in mice, as they progressively tune their trajectories to reach the reward in a shorter time and visiting less nodes on the maze. We find that their trajectories are less likely to stray away from the optimal path, and that eventual detours are shorter lasting. Indeed, we can show that all of the trajectory quantifiers evolve according to a superposition of different time courses: i) they steadily improve on the first trial of each session; ii) their improvement over the course of a session becomes faster in later sessions; iii) the rate of improvement is enhanced after a novel reward location is introduced during the Update phase in contrast to the Build-Up phase; iv) introducing path-blocking barriers in the maze has only a very limited effects when the reward location is already familiar to the animal. **Such sharpening of trajectories over the course of the experimental paradigm is in direct agreement with the presence of different forms of previous knowledge, all contributing to enhance the animal performance in the task** (Gire, Kapoor et al. 2016). The emergence of an allocentric representation of the maze, the linking of specific cues to the proximity of the goal location, the strengthening of memory encoding and consolidation, can be all considered to affect the measured properties of navigational patterns.

At the same time our measures also bring to the foreground how mice behavior remains significantly distant from a purely optimal one. Mice never develop a completely goal-oriented pattern of movements, as a consistent part of their choices on the maze appears to be independent of the goal location. To identify the nature of this layer of non-optimal behavior we develop a computational model of HexMaze navigation producing virtual animal trajectories based on specific generative principles. This minimal mathematical model indicates how two components are sufficient to reproduce, with little parameter tuning, most of the statistical properties of mice real behavior. As expected from the previous results we find that the first of these components is an increasing awareness of proximity of the goal location, whose effects are felt further and further away from the goal with the accumulation of learning. **Goal-directed runs stemming from this component are however combined with a basis of purely random choices that remains present throughout the experimental paradigm** (Thompson, Berkowitz et al. 2018).

One could find rather surprising this persistent neglect of the task requirements, leading to mice spending a considerable amount of time exploring portions of the maze distant from the goal, even when other behavioral measures indicate their awareness of its actual location. **It is possible that such tendency could have been hidden in other experimental paradigms by the lack of options available to the animals, as they were given very little possibilities to show behavior not related to the task**. In the context of a larger spatial arena the balance between “exploratory” and “exploitative” behavior (Jackson, Fatima et al. 2020, Wilson, Bonawitz et al. 2021) might shift sensibly toward the former, leading to an increase in undirected behavior. We are also aware that this pattern of behavior could be specific to mice. In fact, random exploration might be a consequence of mice hyperactivity (Jones, Paul et al. 2017) and their reluctance to consistently focus on the accomplishment of a specific task. A tendency that in the present case could be further exasperated by the absence of sheltered locations in the maze, an element that has been shown to be of great relevance for these animals’ sense of security. With this in mind, it is not too far out to expect rats to perform very differently in this same experimental setting, which is currently under investigation. In previous investigations, Jones et al (Jones, Paul et al. 2017) could show that rats and mice differed in levels of baseline activity measured as shuttle rate during inter-trial intervals; mice shuttled two to three times as frequently as rats. Species differences in behavioural ecology may underlie this difference as for example mice needing to move rapidly when outside burrows in order to minimise predation risk. They tend to use bursts of speed to run from a more sheltered position to the next. Applying our modeling approach to another experimental condition will allow us to extend it to include novel behavioral components, and might open the way to turn the current descriptive approach into a predictive one, producing different strategy combinations depending on the external context.

Regardless of the final explanation of this finding, we acknowledge how these results have significant consequences for the interpretation of the neural correlates of goal-directed navigation. Place cell activity in the rodent hippocampus has been shown to organize in sequential "sweeps" linking the current location of the animal to that of one or more goal locations, when the animal is asked to take a decision (Wikenheiser and Redish 2015). Similarly, it has been proposed that sequences of activated place cells during a Sharp Wave Ripple bear information about future navigational paths (Pfeiffer and Foster 2013). Our results, demonstrating the **co-existence of goal-oriented behavior with a substantial amount of random choices when testing mice in a more naturalistic experimental setting, suggests that such neural episodes might be circumscribed both in time and space, and might play a more limited role when considering animals with a richer set of behavioral options.**

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Interlude

Sex and Menstrual Phase Influences on Sleep and Memory

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Author contributions: A.A. and A.G wrote the first draft and made the figures.
All authors edited the final version

Abstract

This review highlights the effect of sex differences in sleep mediated memory consolidation and cognitive performance. In addition, the role of menstrual cycle and the fluctuating level of sexual hormones (mainly estrogen and progesterone) is stressed. The **literature indicates that sex hormones mediate and orchestrate the differences observed in performance of females in comparison to males in a variety of tasks and can also be related to how sleep benefits cognition.** Although the exact mechanism of such influence is not clear, it most likely involves differential activation of brain areas, sensitivity to neuromodulators (mainly estrogen), circadian regulation of sleep and temperature, as well as modification of strategies to solve tasks across the menstrual cycle. With the evidence presented here, we hope to encourage researchers to develop appropriate paradigms to study the complex relationship between menstrual cycle, sleep (its regulation, architecture and electrophysiological hallmarks) and performance in memory and other cognitive tasks.

Introduction

Sex is a variable known to influence diverse aspects of brain and behavior, but surprisingly sex differences and their consequences are often overlooked and are considered negligible by many researchers. Instead, **rodent and human studies regard male and female subjects as simply equal and opt to include only males, thus females end up being underrepresented in neuroscience research** [1]. In this review in addition to general sex differences, we also discuss how a specific aspect of sex differences (the menstrual cycle) may exert influences in sleep and learning in the context of sleep's contribution to cognition. We start with the general effects on sleep, continue with effects on cognition and memory, and finish with studies that include sleep, learning and memory. Within each section we describe sex driven differences as well as menstrual phase differences, focusing in common results across human and rodent studies. While rodent studies present with the opportunity for controlled designs (I.e. administration of hormones and gonadectomization) and limiting the influence of confounders such as self-reported sleep and cultural bias; they rely on behavioral measures as proxy of the subjacent cognitive skill, requiring extra efforts to clarify a particular strategy taken by the individual to solve an experimental task. Human studies are often weak in control and can only capture snapshots of the desired variable studied, which can be an important limitation in a phenomenon of repetitive nature such as menstrual cycle. Both types of studies offer a window into the fluctuating influence of menstrual cycle and sex hormones in the functioning of female individuals.

Influence of sex on sleep

Sex differences can be anatomical or functional [2] and express themselves both at volumetric and connective levels [3]. Those differences emerge early on during development and are later reinforced by exposure to sex steroids [4]. **Circulating gonadal hormones exert their effects not only on secondary sexual features, but also most brain regions express oestrogen and progesterone receptors**. These brain regions include sleep regulating areas such as the ventrolateral preoptic [2,5] median preoptic and suprachiasmatic nuclei, lateral hypothalamus [3], as well as areas involved in cognition and memory such as the prefrontal cortex [6] and the hippocampal formation [7,8].

Sleep presents itself in a complex form, featuring behavioural (for instance, diminished response to stimuli), as well as physiological changes compared to wakefulness (such as brain activity that displays distinct patterns of neuronal firing). **In most animals it is possible to differentiate between two main sleep stages: Non-Rapid Eye Movement Sleep (NREM) and Rapid Eye Movement Sleep (REM)** [9]. NREM is distinguishable by its higher amplitude, slower waves relative to wake; while REM displays a more similar brain-activity pattern to wakefulness, with low amplitude and high frequency waves [10] (Figure 1A). Muscle atonia and the characteristic eye-movements (similar to saccades) that give name to the state are also a hallmark of REM.

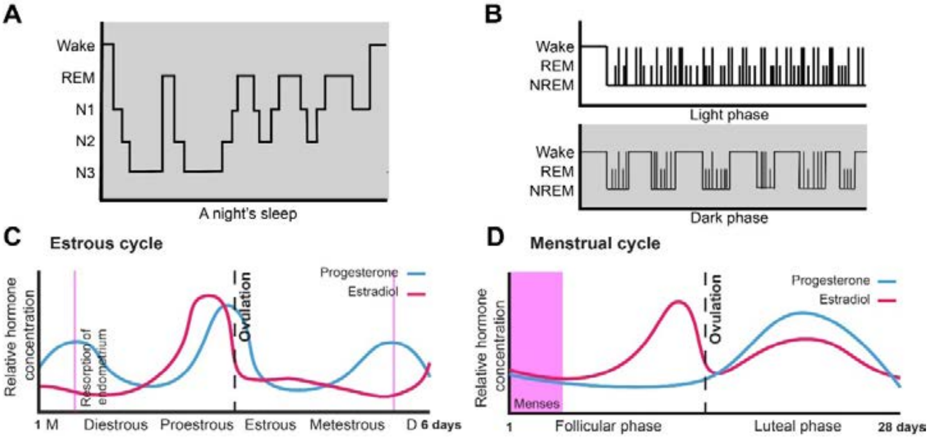

Figure 1. Diagrams of the menstrual cycle and sleep patterns in humans and rodents**. A** Sleep pattern in humans. N1 is the transition state into sleep, characterized by drowsiness and a low arousal threshold. N2 is a deeper sleep than N1, during which breathing and heartbeat decrease. It is also in this state when sleep spindles can be observed. N3 also known as slow wave sleep is characterized by a predominance of delta oscillations, with the EEG signal showing slow frequencies and high amplitude waveforms, believed to be a regenerative period during sleep. REM sleep or paradoxical sleep is characterized by an EEG state of high frequency and low amplitude oscillations, similar to wake, but with complete muscle atonia. The line depicts transitions between states throughout the night, with REM episodes becoming longer as the night progresses. **B** Sleep patterns in rodents. These animals sleep the most during the light cycle, where they have numerus short bouts of sleep and wake throughout the day. During their active phase, they also sleep, with bouts of short sleep wake transitions followed by a wake period, back to the sleep bouts. **C** Estrous cycle in rodents, divided in diestrous, proestrous, estrous and met estrous for an average of 5 days. During pro estrous a peak of estradiol followed by a peak of progesterone give place to ovulation. Rodents do not menstruate, instead they experience a resorption of the endrometrium, marked as magenta lines. **D** Menstrual cycle in humans is divided into follicular phase and luteal phase (luteal phase), with an average length an average of 28 days. On the last days of the follicular phase, estradiol increases, followed by a spike of luteinizing hormone which drives ovulation. The corpus luteum remaining secretes progesterone, and when both progesterone and estradiol are low, the uterine lining detaches along with the unfertilized egg, with the menses marked as a magenta box

Rodents

When studying sleep in rodents it is important to note that, contrary to humans, they are most active during the dark phase, and their sleep pattern is polyphasic, with **several bouts of sleep during both light and dark phases** of the day (see figure 1B).

Studies on spontaneous sleep show that female mice have less total sleep and daily NREM compared to males [11–13] and during the dark phase, males exhibit more wake-sleep shifts, while females have longer bouts of wakefulness [12]. REM is also decreased in female rats [13] and mice [11]. **Gonadectomy in mice of both sexes eliminates the differences seen in total spontaneous sleep and NREM**, as well as in delta power and sleep fragmentation, which implies that the differences in current hormones levels are responsible for these variations in sleep and not general developmental differences. Ovariectomized rats show decreased REM and total sleep time during the light phase, compared to castrated males [14]. When treated with stable levels of 17β-oestradiol or oestradiol and progesterone, they show increases in wake and decreases in REM and NREM during the dark phase [15–17].

Studies on recovery sleep after sleep deprivation in mice show for both sexes decreased NREM the first two hours of recovery sleep, followed by an increase during the first half of the dark phase, but this rebound is greater in females [13]. Female rats also showed an increased NREM rebound during recovery sleep as well as increased delta power compared to males [18]. Studies on gonadectomized rats with hormone implants show that during recovery sleep oestradiol and progesterone promote REM during the light phase and reduces NREM delta power (1-4Hz) during the dark phase [16,19]. **Gonadectomy on mice of both sexes eliminates most of the differences seen in recovery sleep after sleep deprivation** [13].

To further study the effect of sex chromosomes in sleep, the core four genotype mouse model [20] allows to have females and males with either XX or XY chromosomes. This is a transgenic line that manipulates the presence or absence of the *Sry* gene, which determines testes development and is normally found on the Y chromosome. By knocking *Sry* out of the Y chromosome, they can obtain females XY, and by knocking *Sry* into the X chromosome, they can obtain males XX. Studies in this line showed that **genetic sex does not influence sex differences in spontaneous sleep, but rather has an impact in recovery sleep**, where XY females had more total recovery sleep and more NREM than XX females at the midpoint of_the dark phase, suggesting that increased sleep propensity is linked to the Y chromosome. In gonadectomized animals, after sleep deprivation the dissipation of delta power was quicker in XX males than XX females [12], indicating that in the absence of female gonadal hormones, the advantage that females had in recovery sleep compared to males is lost.

Humans

Overall **female humans display more consolidated sleep (less awakenings), shorter sleep latencies, lower percentage of light sleep, than aged-matched males** [3,21] and this is consistent for different age groups and experimental assessments of sleep (laboratory vs home sleep-recoding conditions). Paradoxically, women report consistently worse subjective sleep quality than men. Females tend to describe their sleep quality as poor due to nighttime disruptions, insufficient quantity and long sleep latencies. Women also claim suffering from insomnia in a greater proportion than men [22,23], and this disruption grows stronger as women age (with menopause onset) [24].

Concerning circadian regulation of sleep, **women seem to have shorter and earlier rhythms of temperature change and melatonin secretion** (induces drowsiness and sleep) under a normal schedule [3]. It has also been documented that women go to bed and wake up earlier than men [25] Since often women extend the time before going to sleep to after the melatonin peak, this could be related to their complain of longer sleep onset latencies [23] due to the missing of the optimal time-window for sleep onset. However, under a 28-hours desynchrony paradigm (nine and half hours sleep and 18 and half hours wake), women show lower accuracy and exert more effort in cognitive tests after prolonged wakefulness compared to men [26].

In terms of the features of brain oscillatory events during sleep (i.e. power of electroencephalography signal and its frequency) Carrier and colleagues [27] showed in a sample of men and women between 20 and 60 years, that **women display higher power in diverse physiological frequencies considered during sleep** (delta: 0.1-4Hz, theta: 4-7Hz and alpha: 8-12Hz and spindles 14-16Hz). Spindles are a prominent feature of NREM sleep and have been associated, with intelligence and learning capabilities [28,29]. A difference in spindle power between the sexes has been consistently described [30] and may be related to the variations in spindle topography [31]. For instance, a study by Huupponen and colleagues [32] found that women had a higher percentage of spindles in the left frontal electrode (Fp1-A2) and men displayed more spindles in the occipital electrodes (O2-A1).

However, it is not completely clear whether this is due to lower skull thickness in women, since skull thickness could influence surface electroencephalography power and thus spindle detection [33].

Regarding slow wave activity (SWA), a low frequency (2-4Hz) activity observed in NREM and a proxy for sleep pressure, men and women do not show differences in power; **but the proportion of SWA was more strongly regulated by circadian rhythms in women than in men**. As for age effects on sleep, mid-age women display greater percentage of SWA than same-age men [27] and SWA/delta decline is less severe in women than in men [24,25]. In the same direction, women display greater SWA rebound after sleep deprivation, which has been interpreted as women accumulating a greater sleep-debt than men and making them more sensitive to sleep loss due to work-shifts and more propense to accidents [4].

Interim summary

In sum, **across both rodents and humans, sleep differs between the sexes for variables such as sleep architecture and its circadian regulation**, as well as features of electrophysiological events characteristic of sleep (for instance, spindles and slow wave activity differ in their power and frequency). However, in terms of observed sleep quality, the direction of the difference seems to be opposite among rodent findings and human studies, since the latter point towards better sleep- quality in females not seen in female rodent models.

Influence of menstrual cycle on sleep

After puberty females experience on a regular basis changes in the reproductive system that allow for pregnancy to occur, known as the menstrual cycle. It allows oocytes to mature and prepares the uterus by thickening the uterine lining. Different hormones produced by the hypothalamus and the gonads orchestrate the different stages of the menstrual cycle; however, **these hormones not only affect the reproductive system but also the nervous system**. In this section we will highlight how the menstrual cycle influences sleep on both humans and rodents.

Rodents

In rodents the estrous cycle lasts around five days and is divided in proestrous, estrous, metestrous and diestrous. Proestrous occurs the day before ovulation, with the highest concentration of progesterone and oestradiol, the highest peak is at the end of the light phase for oestradiol, and at the start of the dark phase for progesterone. Estrous is ovulation day, with oestradiol and progesterone starting to decay; during metestrous and diestrous, oestradiol starts to increase gradually, with a small increase in progesterone. In most mammals, after ovulation there is a luteal phase, however in rodents, only if the female has been involved in sexual behaviors the corpus luteum secretes progesterone [34] (see figure 1C).

Mice show little change in sleep distribution during the estrous cycle, with variations between different mouse strains, but **overall limited differences in estrous cycle**. For instance, in the C57 strain there is decreased REM during the night of proestrous compared to diestrous [35], followed by an increase the next day [18]; this phenomenon is also seen in Sprague-Dawley rats [14]. On proestrous night, rats show an increase in wake and decrease in NREM compared to estrous [26]. As for sleep architecture, proestrous females had a higher number of short duration wake bouts compared to estrous, metestrous and diestrous [18]. These differences are dependent on light/dark cycles. During the light phase, REM sleep is not affected by the estrous cycle, but in dark phase REM is inhibited during proestrous and estrous [14]; this has also been shown in ovariectomized rats with oestradiol replacement (which tries to mimic proestrous night) having decreased REM and NREM only during the dark phase [19].

Humans

In women menstrual cycle can vary from 24 to 35 days, with an average of 28 days and is divided in: i) a follicular phase, during which the ovarian follicles develop and mature, and blood shedding occurs, ii) an ovulation phase in which the mature egg is released and leaves the fallopian tube and iii) a luteal phase characterized by the formation of the luteal corpus (uterine lining) [33]. On the first day of menses the hormones regulating the cycle are low (progesterone, estrogen luteinizing hormone, follicle-stimulating hormone). In contrast, during the luteal phase follicle stimulating hormone and estrogen rise; as the non-fertilized ovule degenerates, the production of hormones drops again [36]. Sexual steroid hormones levels and the feedback for their cyclical regulation depend on the central nervous system. The nature of the release of such substances is recurrent and pulsates across the cycle, and therefore their effects are transient in nature [37] (Figure 1D).

Sex differences in reported sleep quality begin in the adolescence, with the onset of sexual maturation (menarche in women) [23]. As previously mentioned, subjective sleep quality is lower in women, but appears as specially affected before the menses [38] and women (mainly those affected by pre-menstrual disruptive symptom [36]) report insufficient and fragmented sleep [30]. Yet insomnia reports from women extend across all phases of the menstrual cycle [23], but **insomnia worsens during the premenstrual phase (mid-luteal phase) and is linked to women's anxiety and perception of life-disruption due to the menses** [3,23]. Paradoxically, oral contraceptives do not seem to influence subjective sleep quality [6].

Nonetheless, the International Classification of Sleep Disorders (ICSD-10) includes a category of menstrual sleep related disturbances: *menstrual-related hypersomnia* [2], which is described as consistent episodes of hypersomnia that begin one week before menses. In line with this, subjective daytime sleepiness was rated higher in the luteal phase by a group of women who were freely allowed to take 10 min naps in a 24 hours day period. Slow wave sleep (a key component of NREM) was also more frequent during luteal phase compared to the follicular phase. **Both hypersomnia and insomnia seem to be linked to abnormal temperature variation due to higher levels of progesterone and oestrogens** [6].

Body-temperature is also highly dependent of the hormone level and menstrual phase. Men and women are most similar in the circadian regulation of the body temperature in the follicular phase of the latter [38]. Body temperature in women seems to be at its lowest during the periovulatory days, due to the high level of oestradiol. In the luteal phase body temperature increases due to high levels of progesterone. During the night temperature falls, but during the luteal phase, night-temperature shows a reduced amplitude decay, which has been attributed to the progesterone counteracting the hypothermic action of melatonin [6]. Wright and colleagues [39] studied the relationship between phase of the menstrual cycle, use of oral contraceptives and circadian regulation on alertness and body temperature in a constant routine paradigm. They did not find an effect of oral contraceptives or phase of the menstrual cycle in circadian phase, however during the luteal phase women showed higher levels of alertness (measure with the *Psychomotor Vigilance Test*) and this is related to lower day-time temperature. Melatonin levels were similar in luteal phase and follicular phase, but higher along night hours for women using oral contraceptives, who also displayed higher temperatures.

Sleep continuity and sleep efficiency measured though polysomnographic recordings of healthy women, remain stable across the different phases of the menstrual cycle [30]. Individual percentages of sleep stages are also comparable across phases. Only a shorter sleep latency and decrease percentage of REM sleep during the luteal phase are documented, but this effect appears only on the first sleep cycle [36]. These changes seem to be linked to the mentioned raise in body temperature that takes place during this phase [40]. **As for the women using**

hormonal contraceptives, they have been associated with lower percentage of Slow Wave Sleep (SWS), shorter REM sleep onset latency and increased REM sleep efficiency in the active phase of the contraceptive method and this compared with women [3]. SWA and SWS do not appear to be sensitive to the influence of menstrual cycle, however a transient increase during the first sleep-cycle was evident in a study by Driver and colleagues, and it was attributed to temperature increase during the luteal phase [40]. Spindle activity is reported as higher in women during their luteal phase [41], concretely their duration, number and spectral frequency increases during mid-luteal phase, especially after the first cycle of sleep [40]. The upper spindle frequency (14 to 16hz) appears as particularly sensitive to menstrual fluctuation. Ishizuka and colleagues [42] report spindle frequency reaching a high peak three days before menses and a valley 18 days before the period. Plante and Goldstein [43] propose that this luteal enhancement of spindles can be attributed to higher levels of progesterone, as their sample of women taking the progestin drug Medroxyprogesterone acetate, displayed increased spindle density, power and amplitude in the spindle band (11-16Hz). In this study menstrual phase was not controlled. This effect was explained as progesterone being responsible for an increased binding in the reticular thalamic nucleus to GABA receptors, which may enhance spindles role in brain inhibition [4,36].

Interim summary

Regarding effects of menstrual cycle in sleep, findings are weaker than expected in both humans and rodents. **In rodents, changes seem to be more related to sex, than to hormonal variation with females spending more time in wake and less in NREM during the light phase**. One exception is the observed decrease of REM on proestrous night and rebound of REM on the following estrous day in rodents. In humans, effects of menstrual cycle in sleep are intertwined with body-temperature changes across menstrual phases. The intersection of human and animal studies indicates that progesterone reduces arousal, while estrogen increases the availability of norepinephrine, decreasing the time in REM sleep [6] These findings are consistent with the documented anxiolytic/sedative effect of estrogen and progesterone in animal models [6,44] and the reported decrease in sleep quality during the luteal phase and the frequent sleep disturbances displayed during menopause (due to the sudden drop of progesterone) [23].

Sleep's role in cognition and specially the consequences of its deprivation have been extensively studied in literature. Sleep is known to influence cognitive abilities [10,45], including working memory, planning and hence, it is expected that **sleep differences in both sex and menstrual cycle will also affect learning and memory**.

Effects of sex on cognition

As mentioned before, **sex differences seem to permeate the organization of brain [8,46] and even the mechanisms behind the observed cognitive performance in certain tasks**, such as synaptic plasticity (in the long- and short-term) and activation of other molecular pathways (neurotransmitter´s release, gene expression and transcriptional factors) [47]. It is difficult to distinguish between the cause of such differences, since sex hormones, sex chromosomes and environmental factors interact to enable cognition and behavior. In this section the most relevant findings regarding the differential performance of sex, in both humans and rodents in cognitive performance are described.

Rodents

Studies of gonadal hormones and sex differences in learning have shown mixed results, probably because sex studies do not usually discern between the different stages of the oestrous cycle in females. Differences in hormone levels can alter the strategies used by rodents to perform different tasks. These differences in cognitive strategy seem to emerge after puberty in rats [48], one potential reason why the role of sexual hormones seems to be central. In general, as with humans, male rodents are attributed to have better spatial learning, which is expressed in better spatial working and reference memory [49,50]. In the skilled reaching task (Figure 2A), in which rats have to reach with their paws through a small opening for a food pellet, females had better performance during acquisition of the task compared to males. In a discriminative fear-conditioned to context task (Figure 2B), in which the rat has to associate a context with a foot shock, Long Evan females showed longer freezing time than males, showing an increased emotional memory to a particular context [51].

There are many factors to consider, apart from sex, when trying to compare these different findings, such as age, strain, and variations in the tasks themselves [52], as well as the housing conditions (enriched environments, physical activity, handling). At the same time, when studying the menstrual phase influences, studies introduce a large variation in timing, route, length and dosage of hormone administration.

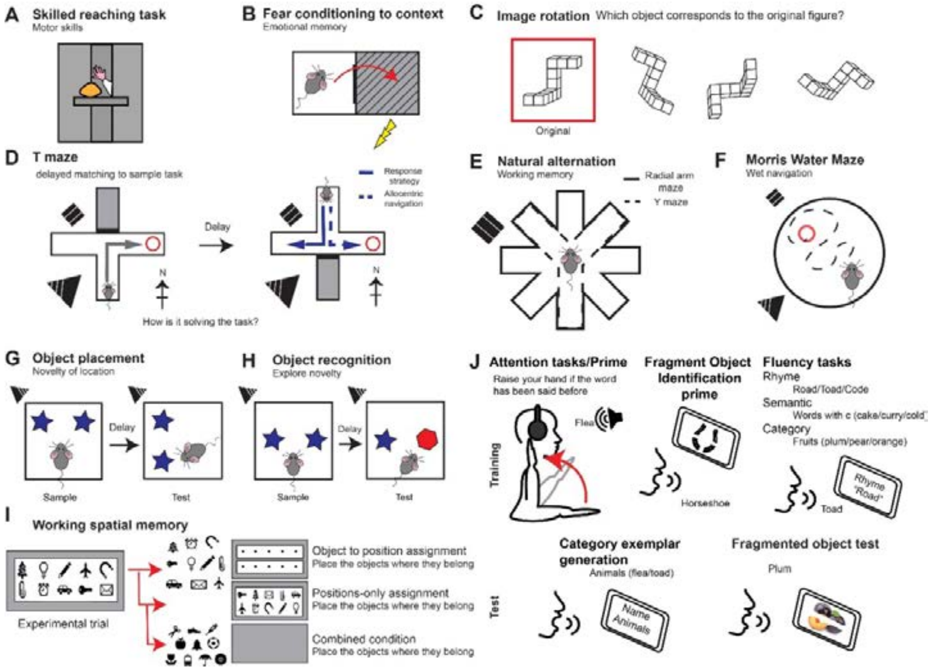

Figure 2. Examples of tasks used for evaluating learning in humans and rodents

A. Skilled reaching task, the animal has to learn to reach through a small opening, grasp a food reward, and retrieve it. Tests fine motor skills. **B.** Fear-conditioning to context, animal is placed either in the light box, or in the contiguous dark box, each paired with a specific aroma aswell. Both boxes are connected but the passage is blocked once the animal moves to the contiguous context, where a foot shock is given. After a day delay, a probe is conducted, where the animal is exposed to the shock context, and the freezing time is measured, or the animal is placed in the non-shock context and the latency to enter the shock context is measured. **C.** Example of item in an Image rotation test. A classically used task to measure spatial cognition. **D** Delayed matching to sample task. During several trials the rewards stays in the same arm, and the animal has to turn towards the east of the room, or turn right to get it. There are extra maze cues. During the probe, the maze is turned 180 degrees, or in this case, if using a plus maze, the lower arm is cut off and the animal now starts from the north. If the animal, during the trials used an egocentric or response strategy, it would turn right to reach the goal. If on the other hand, the animal had been using the cues to navigate towards the reward, it would have been using allocentric navigation. **E** Natural alternation. Either on the Y maze (dashed lines) or the radial arm maze (black line), animals need to remember in which arms had they been recently. Natural alternation between arms is counted. **F** Morris water maze, animal is placed in circular pool with opaque water, surrounded by extra maze cues (black square and triangle), where they have to swim to find a submerged platform (red line). The dashed line represents the swimming pattern of the rodent to reach the platform, from which distance and latency are measured. **G** Object placement, animal is presented in a box with two identical objects during a sample trial, with extra maze cues, and exploration time of the objects is measured. During the test trial, one of the objects is moved to a different position. **H** Object recognition. Animal is presented in a box with two identical objects during a sample trial, and exploration time of the objects is measured. During the probe one of the objects changes identity but not position. **I** Working spatial memory test as described in Postman et al. [53]. Contains a trial stimulus showing ten objects that disappear and a test, in which objects are to be reordered as per three conditions: object to position assignment (original position marked by a dot), positions only (organize the objects in space), and the combined condition (organize the object without spatial cue). **J** Implicit memory tests, such as the Fragmented object identification (middle) uses perceptual priming and Category exemplar generation test (right) uses semantic categories, reported in Maki and colleagues [76].

Humans

Numerous studies [46,47,53] point towards **men and women consistently performing differently in memory tasks according to the prevalence of verbal vs. spatial processing of information**. For instance, women on average perform better in word recall and recognition, story recall, name recognition and object (including faces) recognition in complex settings. These results do not seem to be explained by verbal fluency, because they remain stable even when this factor is controlled for [54]. On a different note, women perform on average worse than men in an object position memory task, even when verbal interference (repetition of meaningless syllable) was used [53]. However, the crucial element appears to be whether information can be verbally described, which gives an advantage to women (in these studies, menstrual cycle phase is typically overlooked) [54]. Also, the need to perform mental rotation of objects (see Figure 2C) also seems to be critical, since women from puberty on seem to display diminished performance compared to age-matched males [55].

In contrast to navigational and spatial reasoning tasks, **in verbal activities** (fluency, recall of wordlists and categorical naming) **women on average appear to surpass men's performance** [46,47,53,54]. However, the origin of such differences is controversial, since performance divergence appear early in childhood (before sexual maturation) and persist after menopause and seems to be more related to divergent brain activation (more lateralized in women) than to the effect of sex hormones [46]. This verbal advantage has been connected to more precise episodic memory in women [54–56]. Thus, episodic memory and particularly memory involving emotional arousal, seems to be favoured in women and this advantage has been attributed to the dimorphic activation of the left amygdala in women (presumably concentrating in context details) compared to right amygdala activation in men (focusing on gist encoding) [46].

Besides physiological/structural differences between the sexes to explain the observed performance, men and women can differ in the strategy used to resolve experimental tasks (just as seen in rodents). For instance, **in navigational memory, it has been reported that women rely more on egocentric cues while men incline towards allocentric references**. Solving strategies in turn will affect which brain structures are involved, and in turn different brain areas can be more or less impacted by sex hormones [54]. Concretely, as per animal evidence [57], **estrogen level has been pointed out as factor modifying cognition by enhancing plasticity in key areas s**uch as the hippocampus and prefrontal cortex [58], in female but not in males [8].

Interim summary

In both humans and rodents**, a higher performance is credited consistently to male individuals in tasks related to spatial navigation, and a higher performance to females in tasks related to domains other than spatial**. The factors contributing to such observed advantages are not clear and range from anatomical, and functional differences in the brain (mainly due to estrogen level), to confounders such as stress and experimental manipulations. For instance, experimentally instilled stress before a navigational spatial task affect women but not men, independent of cycle phase [59]. In women the presence of language and semantic processing gives another dimension to the differences in cognitive performance between sexes. As the range for variation for sex-dependent performance is wide, in the next section we will focus on cognitive tasks that show differences in females across the menstrual phase.

Influence of menstrual phase on cognition

In this section we will summarize the influence of the menstrual phase on memory first in rodents and then in human subjects.

Rodents

Depending on the type of information needed to solve a particular task, the presence of gonadal hormones will favour or deter cognition. In tasks involving working memory, such as T maze delayed matching to sample task (Figure 2D), which can be solved either by a place strategy (allocentric navigation/always turning towards a location in the room) or a response strategy (egocentric navigation/always turning to the right), disambiguation of strategy use can be determined by rotating the maze 180 degrees, or as shown in the figure, with a plus maze and starting from a different position within the maze. **Females during proestrous are more likely to exhibit a** *place strategy***, and during estrous** *response strategies* [59], both in appetitive (food reward) and aversive wet navigation tasks (submerged platform) [60]. Similarly, in ovariectomized rats treatment with oestradiol while performing a plus maze, in which place and response strategies can be evaluated, oestradiol favoured place strategies and decreased performance when response strategies were required [61].

In the Y maze [59] or radial arm maze (Figure 2E), where **working memory is measured by alternation, performance during estrous was decreased compared to other stages of the cycle**. Testing alternation in the T maze shows that mice during proestrous had more alternations than mice in diestrous [62]. In ovariectomized rats, performance during early acquisition in the radial arm maze was decreased compared to sham operated females [63], and treatment with oestradiol improved performance [64]. Similarly, in a water radial arm maze in which platforms were removed (which increases the working memory load as the trials advance) ovariectomized rats treated with oestradiol made fewer mistakes during the latest trials [65]. In contrast, for wet navigation tasks such as the classical Morris water maze (Figure 2F) [66], in which performance is measured by path length and latency to find a submerged platform, rats at proestrous show decreased performance during acquisition [67] and later trials [68]. In mice, however, it seems that it is during estrous that they have worse performance [69]. Ovariectomized rats, treated with oestradiol and oestradiol plus progesterone decreased their performance [70]. In a variation of this navigational task, in which there is a cue hanging above the platform (a beaconing strategy) for each trial the platform location and the cue would move to a new position together, rats during proestrous exhibited better performance during acquisition than female rats in estrous or diestrous [68]. Ovariectomized rats, treated with progesterone and oestradiol plus progesterone decreased cue-guided task performance [70].

In object placement (Figure 2G) and object recognition (Figure 2H) tasks, in which performance is evaluated as exploring time of novel versus familiar locations or objects respectively, both rats [71] and mice performing object recognition during proestrous and estrous show better discrimination of the novel object than in diestrous or metestrous [62,72], and discrimination during proestrous is better than estrous [62]. Rats performing the object placement task showed discrimination for the novel placement only during estrous [71]. Ovariectomized rats treated with oestradiol immediately after the sample trial showed at test trial better discrimination of the displaced or novel object. This response was dose-dependent (object placement task needed higher concentration than object recognition task, which fits into proestrous improving place and estrous improving response strategies). Ovariectomized rats without hormone treatment showed worse performance in the object placement task than in the object recognition task, and treatment with oestradiol given 30 mins before (or right after sample trial) improved discrimination in both tasks [73]. For further details in ovarian hormones regulating object recognition and object placement tasks see [74].

Humans

It has been claimed that **women's advantage in tasks in which they outperform men (verbal) intensify during the luteal phase of the cycle when female hormones are of their highest levels**. Contrarily, during women's menses, with low levels of female hormones, their performance drops in those tasks [41]. Men and women are more similar in spatial—centered tasks during women's menses [75]. Classically, spatial memory and spatial cognition tasks have been used to study the change in performance for women during the different phases of their cycle. In their study, Postma and colleagues [53] showed that women displayed difference in performance only in the absolute (fine grain) positioning of objects (see an example in figure 2I) with respect to men and this difference was obvious when women were in their menses. The intake of oral contraceptives did not exert significant effects on position accuracy for this sample. Effects in women were not related to levels of testosterone during the menstrual phase, but more likely to the level of estrogen.

A study by Maki and colleagues [76] showed that **performance in an implicit memory task (based on** *Category Exemplar Generation***: an example primes future word production) was better for women in the luteal phase compared to the follicular phase**. On the other hand, *Fragmented Object Identification* (a perceptual implicit test) showed greater priming effect in the follicular phase compared to midluteal phase (see figure 2J). Moreover, priming effect in the perceptual modality exerted carryover effects when first encountered during the follicular phase relative to the retest during luteal phase. These results were highly correlated with estradiol level in the participants.

In terms of recollection of emotional memory (stimuli causing positive or negative arousal), Bayer and colleagues [44] found that women during their follicular phase displayed increased detail and contextual recall (48 hrs. after encoding) of negative images compared to luteal phase and to positive and neutral images, which were not affected by menstrual phase. These differences were also correlated to decreased hippocampal (and in a portion of the accumbens) activation during the luteal phase in comparison to the follicular phase. There is also a reported differential lateralization of the amygdala during encoding of negatively arousing images, with women showing greater activation in the left amygdala and men showing the opposite pattern [77], but this asymmetry has also been reported for encoding happy faces and seems to be related to activation of other brain areas [78]. Convergently**, the amygdala appeared to be activated with preference for encoding emotional memory in women during the luteal phase**. Interestingly, Bayer´s neuroimaging results seem to support the idea of a shift in strategy for dealing with cognitive tasks during the different phases of the menstrual cycle, with a focus on arousing emotional aspects during follicular phase (linked to anterior hippocampal activation and accumbens) and a more semantic approach in the luteal phase (with preferential activation of posterior hippocampus).

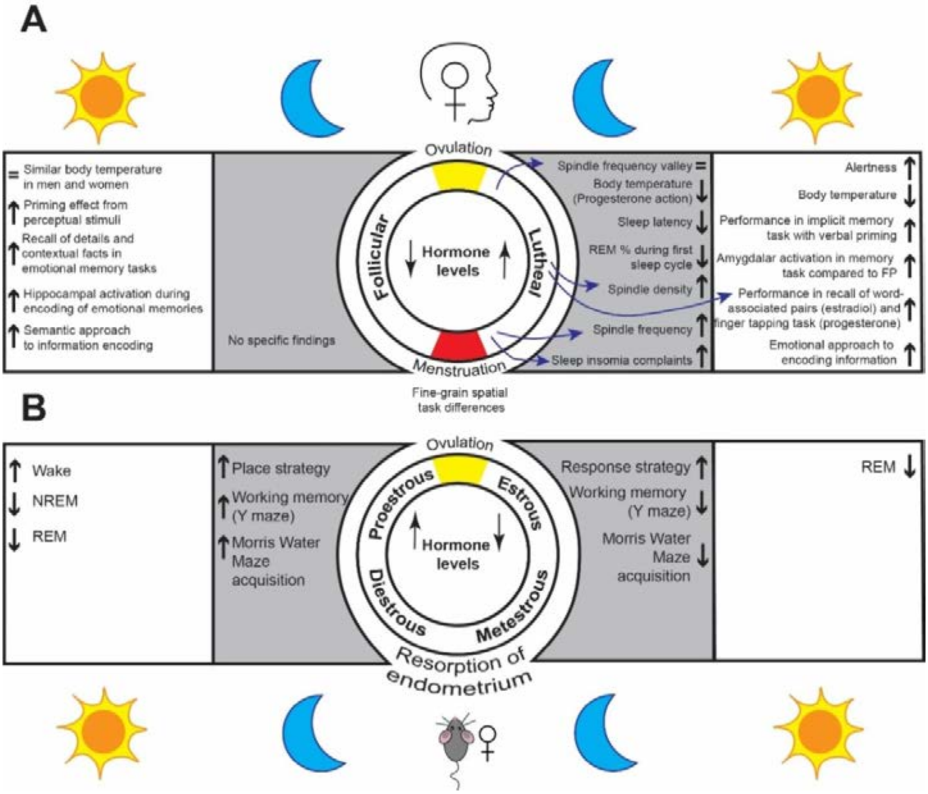

Figure 3. Graphic summary of main findings in sleep and cognitive performance during the menstrual/estrous cycle in women and rodents.

A. Main findings in humans for differences during menstrual phases in cognition and sleep. **B.** Main findings in rodents for differences during menstrual phases in cognition and sleep. Only the results for Proestrus (on the left) and Estrous (on the right) are shown. The sun represents the day or light phase and the moon the night or dark phase.

Interim summary

Hormonal level changes during the menstrual cycle seem to have an effect on the performance of both female humans and rodents. **Those changes can be related to differential activation of brain areas implicated in cognition and emotion (for instance amygdala and hippocampus), as well as use of cognitive**

strategies to resolve tasks during different phases of the cycle. Overall estrogen seems to facilitate memory encoding and recall during experimental tasks, and its effects seem to be related to its action in neuronal spine density, neurogenesis, connectivity and ultimately plasticity in key brain areas as the hippocampi [for a detailed review on this topic see the work of Hyer and colleagues [57] and Romeo et al. [8]). A summary of these effects can be seen in Figure 3. The effects of progesterone are less clear, and research is needed to see if has complementary or opposite effects to estrogen [8,44].

Sex differences in sleep, learning and memory

In this section we will highlight the findings in **studies that take into account both sleep and cognitive performance in relationship to sex and menstrual cycle changes in human and rodent models**. It is important to underscore the need for studies in this topic to better understand how these differences affect cognitive processes.

Rodents

REM sleep deprivation affected the performance in the Morris water maze of females more than of males [79], and ovariectomized rats had worse performance compared to intact females [80]. In the same page, in a fear conditioning to context task (Figure 2B), NREM and REM sleep deprivation before and after acquisition of the task, affected males more than females [81]. Rats were exposed to two contexts, one of which would be associated with a foot-shock. On a test 24 hours later, the latency for the animal to enter the foot-shock context is measured. Under control conditions males had longer latencies than females before entering the shock section, but under sleep deprivation, it was females that showed a longer latency compared to males, showing that females express a stronger fear memory than males under sleep deprivation conditions.

In the object recognition task there were no sex differences after sleep deprivation [81], but there was a main effect, in which the discrimination index dropped for both sexes. They also showed that after sleep deprivation, at hippocampal and cortical level there was an elevated concentration of kynurenic acid (a metabolite of tryptophan degradation) known to affect cognition, on males but not females. This effect was lost when males were gonadectomized. **When corticosterone levels were measured, these were higher in females than males following sleep deprivation** [81].

Humans

Sex differences related to sleep-dependent performance in cognitive tasks are reflected in a small number of experimental results in humans, but there is not an integrated picture of the relationship. These findings are described next.

McDevitt and colleagues [82] reported that differences in memory consolidation after a day-nap are observed for perceptual learning in a task to discriminate targets' motion direction. In this task, women showed a more generalized improvement, extending to target-direction and visual field locations not trained before sleep; while men only improved their performance for the trained motion direction. In women these findings were independent of whether the nap contained or not REM sleep. According to the authors, **a candidate mechanism behind those differences could be the differential level of ACh hippocampal release in males and females (a fact observed in rodents but not confirmed in humans), since women in their luteal phase display an increase in this neurotransmitter that would facilitate memory performance**.

Koriyama and colleagues [83] found that in relation to the circadian time for encoding aversive stimuli (violent scenes from a picture), women displayed greater accuracy in the recognition of the scenes after they have passed their habitual *sleep-onset-periods*, even when men performed better in their pre-*sleep -onset-periods*. Also, women recognized aversive scenes less accurately than neutral scenes if tested in their pre *sleep-onset-periods*, so women display a circadian (perhaps sleep-pressure-related) sensitivity to memory of aversive stimuli, while men display a stable pattern across time.

As mentioned in a previous section, sleep deprivation appears to have differential results in men and women. Interestingly, Binks, Waters and Hurry [84] found that sex had an effect on the IQ test measure of their sample. **Sleep deprived (36 hours) females scored slightly higher than sleep deprived males on the WAIS-R tes**t, no pre-sleep deprivation scores were obtained from participants. Unfortunately, the authors do not discuss the implications of such finding, but it seems to contradict the claim that sleep deprivation affects women more severely in their cognitive performance [4], as well as social let-jag impacts their academic performance more harshly [85].

A study into the differences for male and female ex-users of cocaine (in recovery/ abstinence) [86], allowed to see that **women are not sensitive to sleep disturbances caused by abstinence** (low sleep efficiency) as were men. Also importantly, women performed in a version of the motor sequence task, 24 hours after learning it, as well as control participants and significantly better that male-ex users. Female controls were not different in skill performance or sleep parameters than control males. The phase of the menstrual cycle was not controlled in this study; however, the authors speculate that estrogens may exert protective effects on the sleep of female ex-users.

Interim summary

By observing the differential effect of sleep deprivation on memory tasks performance in males and females, the sex dimorphism consequences in sleep-dependent learning can be tackled. From these studies it seems that under many circumstances, **females perform better compared to males after sleep deprivation.** As animal studies revealed, this could be related to elevated corticosteroid levels, a neuromodulator related to enhanced encoding under acute paradigms of stress. Also, in purely sleep dependent tasks (visual memory and emotional memory) females seem to benefit more from sleep than males, these findings will be revisited in the next section.

Menstrual phase influence on sleep and its relationship with memory

As highlighted in the previous section, sleep dependent learning displays differential features in female and males, in the present section the causes attributable to variation in females due to the menstrual cycle and the implicated hormones are described.

Rodents

Very few studies take into account both sleep and learning when looking at differences between sexes, and even less when looking at differences within the menstrual cycle. Cordeira et al. found that sleep deprivation negatively affected performance in Object recognition in mice during proestrus and estrous [72].

Humans

Sleep-dependent memory consolidation is one of the fields where the influence of sleep on cognition has offered abundant evidence [87]; it is surprising that relatively few studies have explored the possible dimorphisms in this area. Below the few studies that exist are highlighted.

Sleep-dependent memory consolidation exhibits differences in women across the menstrual cycle. Genzel and colleagues [41] demonstrated that women performed at the same level as men, during their mid-luteal phase in an associated-word-pairs and finger-tapping tasks. Overall women in their luteal phase in general, independently of the wake-sleep condition assigned to them, outperformed women in their follicular phase. The Nap-dependent performance increase, in both the motor and declarative tasks, was more noticeable in the luteal phase of participants compared to the follicular one. Furthermore, this nap-related-enhancement was correlated with increased spindle activity in both men and women, but specifically with progesterone level for the motor task and oestradiol level for the verbal one.

In a different type of sleep-related memory enhancement study, women did not display the effects of odor-sequence memory cueing (a well stablished paradigm in the study of sleep effects on memory consolidation) during different phases of their menstrual cycle [88]. **Overall women were unaffected by the experimental manipulation, in comparison to men, for whom exposure to encoded odors during sleep resulted in performance enhancement**. The fact that specific menstrual cycle effects on the observed outcomes for the female participants cannot be further explored, due to lack of experimental control of this variable, underscores the need to consider menstrual phase and the influx of external sex hormones (due to oral contraceptives) when it comes to describe the effects of sleep on memory consolidation. Since the effects of sleep can oscillate according to the momentary level of progesterone and estrogen, the high estrogen time-window for performance enhancement due to sleep (and spindle) increase could perhaps explain the differences across samples obtained in research.

Oral contraceptives have been shown to influence cognitive task performance and memory results. For instance, Genzel and colleagues [89] showed in a sample of females that memory performance for both verbal (word-pairs) as well as motor (finger tapping) tasks was beneficially influenced by a delay in recall (whether it was filled with wake or nap time) and was comparable across active or rest weeks of oral contraceptives intake. This finding has been observed across numerous populations (for a review see Stickgold [90] and Diekelmann, Wilhelm & Born [87]), but has been rarely studied in women using oral contraceptives. Furthermore, women taking contraceptives are rarely divided between the active and rest phase and by type of method used. The maintenance of the off-line consolidation effect across contraceptive weeks can be attributed to higher levels of estrogens (specially compared with the one of women not taking oral contraceptives) but not to an additional influence of sleep on memory consolidation.

Interim summary

There is a lack of studies regarding the role of menstrual cycle in the relationship between sleep and its effects on learning, and concretely on memory consolidation. From the human studies it seems plausible that different hormones affect sleep-consolidation based on the type of information being dealt with (motor or verbal, for instance). For this purpose, studies in women using oral contraceptives or suffering from irregular hormonal levels (i.e., menopause) are key to disentangle the relationship between sexual hormones and system consolidation of information in the brain.

Conclusions

The studies presented in this review underscore the fact that sex hormones, and subsequently the menstrual cycle in females, influence sleep and cognition in a variety of ways not fully comprehended. Among those areas we find an intersection of brain anatomical differences, activation changes in those areas, cognitive strategies to deal with a task and brain plastic changes. Overall, the results of the studies reviewed here point towards not only an effect of sex and hormones on sleep and cognition separately, but also at a differential effect on the interaction of sleep and cognition. Those effects are expressed for instance as a change of strategy in spatial navigation task in both female humans and rodents (from allocentric to egocentric), in the fluctuating role of amygdala and hippocampi in encoding and consolidating stimuli in the different menstrual phases, as well as in the changes in sleep architecture and body temperature control according to hormonal level (estrogen and progesterone). **The main purpose of this review was to highlight the importance and the need to consider sex and its significances into any research endeavor.** Instead of seeing sex as a confound or a variable to control for, it can be approached as an opportunity to study the role of hormones in cognition, sleep and their interplay. Since in females influence of sex hormones is more pronounced due to the cyclical variation on their level, this offers a natural milieu for the study of the impact of those substances in cognition, memory and behavior and the influence that sleep —as a complex mechanism— exerts on them.

Innovative methods to study the relationship between sleep and cognitive performance across the phases of the menstrual cycle need to be applied, since the literature reveals that correlational and subjective measures do not seem to capture the complexity in the changes that the female brain undergoes due to the developmental and cyclical exposure to sex hormones. These sex-dependent and hormonal fluctuation variations in learning and cognitive performance can be studied as mediated by other processes, from which sleep seems to be a topic especially prolific and promising. **Incorporation of female samples, accompanied by standardization of normative criteria for their parameters, into sleep research is urgently needed.**

Among some of the topics to be tackled is the exact role of sex steroids in sleep regulation, and its complex relationship with temperature oscillations in the mammal body. **Animal studies are key to disentangle the relationship between physiological factors in sleep regulation and sleep behaviors**, and especially to separate them from cultural or psychological traits.

Based on the above, we consider that a multi- level approach to the exploration of the variables involved in the relationship between menstrual cycle, sleep and cognitive performance needs to be taken since physiological/molecular, genetic, anatomical, behavioral as wells as circadian components are simultaneously and interactively likely to produce the observed changes in cognitive performance.

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Conflict of interest

The authors of this review certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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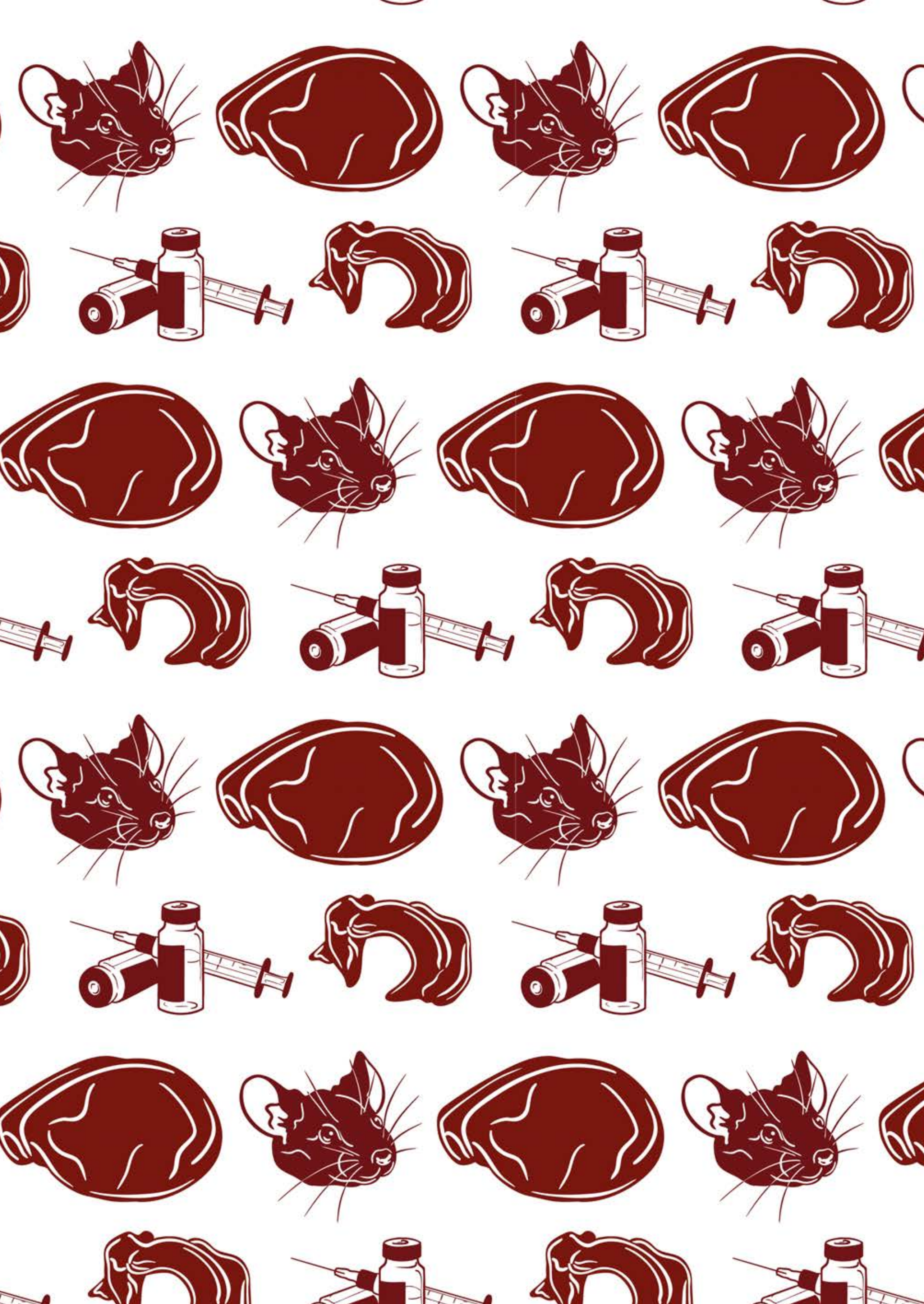

Chapter 5

Pharmacological and molecular techniques in the mouse HexMaze

Abstract

Previous knowledge networks accelerate memory consolidation, but the exact mechanisms remain unclear. To gain a deeper understanding of the contribution of different brain regions, we employed the mouse HexMaze task. This task comprises various learning stages, and animals can rapidly integrate new information once a prior knowledge network has been established. Through pharmacological manipulations inhibiting key areas such as dorsal CA1 regions of the hippocampus, the prelimbic cortex and the retrosplenial cortex, we show little hippocampal or cortical dependency for overall navigation, however we show that **during the schema phase CA1 is critical for encoding new congruent memories, accompanied by fast systems consolidation 48 hours later.** Immunostaining analysis of active cells in two learning stages showed a large overlap, implying that the initial memory engram is recruited for encoding of new schema-congruent memories.

Introduction

Understanding memory and its processes is a topic that has intrigued scientists since the late 1800s (Ebbinghaus 2013). **Schemas or previous knowledge networks are associative cortical frameworks which enable rapid consolidation of new information**. They have been described in humans since the mid-1900 (Harlow 1949) as complex memory structures, built over multiple experiences and continuously adapting. In a way, they are opposite to episodic memories, as they lack unit detail, they exist within a range and have no predetermined boundaries (Ghosh and Gilboa 2014). Human studies have shown that after encoding, the more congruent the information is with previous knowledge, there is a decreased functional coupling between hippocampus and medial prefrontal cortex, while retrieving congruent information showed an increase in functional coupling between medial prefrontal cortex and other cortical areas (Van Kesteren, Ruiter et al. 2012, van Kesteren, Rignanese et al. 2020). In rodents, immediate early gene studies have shown increased activity in the prelimbic cortex after encoding congruent information compared to incongruent (Tse, Takeuchi et al. 2011). Nevertheless, non-human animal studies are relatively scarce and challenging to develop, though they have started to emerge (Tse, Langston et al. 2007, Baraduc, Duhamel et al. 2019) (for a more extensive literature review see chapter 2 (Alonso, van der Meij et al. 2020)).

Memory consolidation denotes the transformation of labile memories into enduring cortical structures, a process traditionally believed to unfold over weeks, months or even years. In 2007, **Tse et al challenged prevailing memory consolidation theories by demonstrating that systems-level memory consolidation could occur within a of 48-hour window when new information is congruent with prior knowledge** (Tse, Langston et al. 2007). They employed the paired associates task, where rats learned associations between flavors and locations over several weeks, specific to a particular context. Once the animals achieved stable performance, two associations were replaced with new ones while keeping the remaining pairs. Notably, these newly added associations, congruent with the initial learned structure, were acquired after just one session. Lesioning the hippocampus 48 hours following the acquisition of these new associations revealed successful retrieval, indicating completed consolidation and the ability to recall the memory without hippocampal involvement (Tse, Langston et al. 2007). Nevertheless, the process of encoding such new information into the memory structures remained reliant on the hippocampus. A subsequent study replicated these results, employing local lidocaine infusions (Hasan, Kanna et al. 2019).

Next, neocortical structures, including the prelimbic cortex (Tse, Takeuchi et al. 2011) and anterior cingulate cortex regions (Wang, Tse et al. 2012) were subjected to inhibition to investigate their roles in memory updating and retrieval in the paired associates task. Findings indicated that these **neocortical areas played essential roles in both encoding and retrieval processes**. Experiments targeting NMDA receptors, crucial for synaptic plasticity, revealed the necessity of plasticity in prelimbic and anterior cingulate cortex exclusively during encoding, not retrieval. Furthermore, local lidocaine infusions in the anterior cingulate cortex reaffirmed the necessity of these brain regions for successful memory encoding (Hasan, Kanna et al. 2019). These results strengthen the concept of faster cortical encoding in the presence of previous knowledge.

The distribution of a memory network can be described as an engram. An engram represents an ensemble of cells that co-activate and encompass all facets and values of an experience, which can span the whole brain (Tonegawa, Liu et al. 2015). One method to visualize the engram involves the use of immuno-staining for activity- or plasticity-related proteins, commonly referred to as immediate early gene studies. Imaging immediate early gene expression facilitates engram visualization by demonstrating heightened expression of relevant neural markers in discrete brain areas (Balcerek, Włodkowska et al. 2021). In the context of the paired associates task, distinct activation profiles of prelimbic cortex, anterior cingulate cortex, retrosplenial cortex, CA1 and barrel cortex were characterized through the imaging of plasticity related proteins Arc and Zif 268 (Tse, Takeuchi et al. 2011, Wang, Tse et al. 2012). The conditions under examination encompassed the retrieval of original associations, the encoding of two new flavor-place associations within the same context, and the encoding of an entirely new set of flavor-place associations. During the retrieval of original associations, there was an increased expression of Arc in the anterior cingulate cortex. Conversely, **when encoding new associations within the established context, both plasticity-related proteins exhibited heightened expression in CA1, prelimbic, anterior cingulate and retrosplenial cortices** (Tse, Takeuchi et al. 2011). Moreover, a correlation of activity between the anterior cingulate cortex and CA1 during the retrieval and updating of this memory structure was observed (Wang, Tse et al. 2012). When encoding a wholly new set of associations, augmented expression of Arc protein was observed in the anterior cingulate cortex and CA1 region of the hippocampus. These findings further substantiate the involvement of the hippocampus in memory encoding and retrieval, even after memory consolidation has taken place, however it is not completely necessary during retrieval if the memory has already attained cortical stability.

Using the output obtained in the previous study, a functional connectivity study was carried out (Takeuchi, Tamura et al. 2022). For this, the correlation between brain areas was analyzed, to evaluate if the expression pattern of IEG products in one brain area was associated with another brain area. When analyzing correlations for all conditions together, three strong correlations were observed, one consisting of hippocampus CA1-CA3, another one of prelimbic, anterior cingulate and agranular retrosplenial cortices, and another one of orbitofrontal and retrosplenial cortices. A strong correlation was seen among DG-CA1-CA3, and among midline necortical regions prelimbic, anterior cingulate, and agranular retrosplenial cortices. A cluster analysis based on how different the counts are among brain areas, identified **DG, CA1, CA3, prelimbic, anterior cingulate and retrosplenial cortices as a functional network in control of schema updating.**

While the phenomena of accelerated consolidation has been established, the exact mechanisms are still unknown. Different hypothesis propose that new information is being directly encoded in the cortex (Van Kesteren, Ruiter et al. 2012, Coutanche and Thompson-Schill 2014), while others that an early hippocampal engram is stablished and cortical cells are "tagged" and later recruited (Tonegawa, Morrissey et al. 2018). In this present study, I employed the HexMaze task, a goal reaching task with extended spatial and temporal dimensions, designed to investigate prior knowledge in mice (Alonso, Bokeria et al. 2021). The animal subjects underwent extensive training over several months, and their performance was characterized across various learning stages, starting from the initial day of training and spanning up to 6 months later, as elaborated in chapter 2. **To investigate the brain area dependence in the context of spatial memory I employed pharmacological inhibition, offering a reversible method to temporarily silence specific brain regions**, albeit without specificity for cell types. Furthermore, **to understand the extent of such a memory network, immediate early gene imaging was performed to observe cell-resolution expression of activity**. Notably, I extended this investigation beyond a single event per animal, employing two timepoints, made feasible through the targeted recombination of active populations (TRAP2 method (DeNardo, Liu et al. 2019)), a methodology currently limited to mice. This allowed me to compare the initial engram with cell populations active later on the task, and evaluate overlapping of cells across these two events.

I found little to no dependency on hippocampus, prelimbic and retrosplenial cortex for initial memory formation when locally inhibiting these areas. Three months after the start of training, encoding new related memories proved to be hippocampal dependent, followed by rapid systems consolidation 48 hours later. Engram analysis suggested a distributed memory network by showing increased expression of cFos progressively over time in most regions studied, but specially in prelimbic cortex. A large overlap of cells recruited during an initial memory engram with cells was used to encode new related memories two or twelve weeks later.

Methods

This study encompasses two distinct experimental approaches: one involving the inhibition of discrete brain areas and the other utilizing immunohistochemistry against neural markers. In total, 115 male C57BL/6J mice (Charles River Laboratories) and 39 male cFosTRAP2xRosa mice (bred in-house) were employed to conduct this comprehensive investigation.

Pharmacological manipulations

Subjects

83 male C57BL/6J mice (Charles River Laboratories), aged two months upon arrival, were used. The animals were group housed within the University (Nijmegen, Netherlands). They were kept on a 12-h light/dark cycle, and provided with various forms of cage enrichment, including a running wheel, plastic igloo and nesting material. During the period of behavioral testing, mice underwent overnight food deprivation, with their weight targeted to be within the range of 90% to 85% of their estimated free-feeding weight. All animal protocols were rigorously reviewed and approved by the Centrale Commissie Dierproeven (protocols #2016–014-018, #2016–014-034, and #2020-0020-009).

HexMaze

The HexMaze was constructed using 30 opaque white acrylic gangways, each measuring 10 cm in width, interconnected by 24 equilateral triangular intersection segments. This arrangement resulted in center-to-center distance of 36.3 cm between intersections (Fig. 1A). Gangways were enclosed by either 7.5- or 15-cm-tall white acrylic walls. Visual landmarks, both local and global cues were placed to aid navigation (Fig 1B). To prevent mice from bypassing barriers by climbing over them, transparent acrylic inserts were clamped above the barriers. The maze was positioned 70 cm above the floor to facilitate access for experimenters.

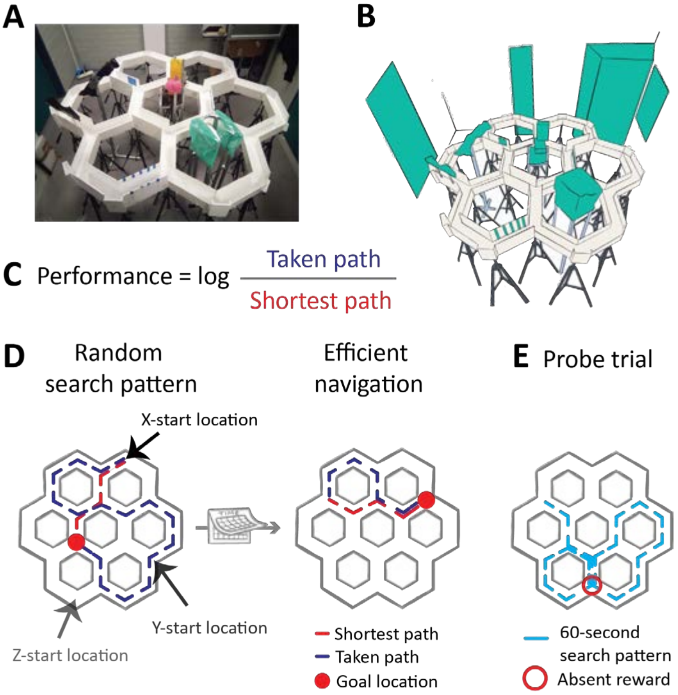

Figure 1 HexMaze task. **A** Picture of the behavioral setup. **B** Schematic of the Hexmaze, showing in green intra and extra maze cues **C** Performance measure for navigation **D** Explanation of the task, initially animals randomly wander within the maze (blue dashed line) until they reach the rewarded goal (red circle). Red dashed line shows the shortest possible path from X-start location to the goal location. Y and Z-start locations represent the following semi-random nodes from which animals begin new trials, and there are as many as possible within the 30-minute session. Over time taken paths become more efficient **E** Memory test. Red empty circle represents where the food reward should be, but its absent during the first 60 seconds of training. Light blue dashed line represents the taken path of the animal during the trial, noting that it crosses the goal location three times before food is added.

Video acquisition and tracking

Two USB cameras (model C270, Logitech) positioned 2.1 m above the gangway plane with an overlapping field of vision (FOV) provided full coverage of the arena and reduced obstruction of vision by maze walls. Image data (15 frames/s, 800 x 600 square pixels per camera) was acquired on a low-end consumer PC (Ubuntu version 19.04, AMD Ryzen 2200G processor, 8 GB RAM) with custom Python scripts (Anaconda Python version 3.7, OpenCV version 4.1.0) at controlled brightness and exposure levels.

Images were immediately compressed and written to disk for offline analysis. In parallel, online tracking was applied for feedback to the experimenter and adjustments of the paradigm. Briefly, for each camera view a mask was generated at the beginning of the experiment based on the contrasting brightness of the maze and experimental room floor. This arena outline mask was applied to new frames, and a foreground mask was generated using the OpenCV MOG2 background estimation implementation (Zivkovic and van der Heijden, 2006). The resulting foreground mask was cleaned, and the centroid for the largest detected foreground object in a tracking search window was calculated as the putative location of the mouse in the maze. The location was smoothed over time using a Kalman filter, interpolating occasional occlusions by the maze walls and similar detection failure modes. The detected location was mapped to the closest node, and visually presented to the experimenter as well as logged for offline path analysis.

Synchronization between cameras for offline analysis was enabled by presenting a blinking LED (1 Hz, 50% duty cycle) in the overlapping FOV of both cameras. Experimenters could indicate start and offset of trials using a remote presenter (model R400, Logitech).

Behavioral training

Upon arrival and prior to the initiation of training, mice underwent daily handling in the housing room for one week. This handling continued until animals freely climbed on the experimenter, as illustrated in the videos available at https://www.genzellab.com/#/animal-handling/). At the end of each handling session a couple of reward pellets were placed in the cage for animals to familiarize to the rewards. Subsequently, the mice were habituated to the maze in two sessions, the first one during which all cage mates were introduced together, followed by a 30-minute solitary habituation. Intermittent handling for maze pickups, specifically tubing (Gouveia and Hurst 2017), was incorporated, and the food rewards could be found scattered randomly within the maze.

The training regimen for the mice followed either a two or 3 days per week schedule, with sessions occurring on Monday, Wednesday and Friday or Tuesdays and Thursdays. Each training day consisted of a 30-minute training period, allowing for up to 30 trials per session. To maintain cleanliness and prevent damage to the acrylic structure, the maze was cleaned using alcohol-free cleaning wipes. Additionally a pile of food crumbles (Coco Pops by Kellogg's) was placed at a predetermined goal location, which varied for each individual animal.

The selection of Goal Locations (GLs) was counterbalanced across animals and within animals across GL switches, one of four GLs per animal would be located on the inner ring of the maze, while the remaining GLs were positioned on the outer ring. This strategic arrangement was implemented to shape animal behavior and mitigate circling behavior tendencies.

For each training day, start locations were generated based on their relation to the GLs and the previous start locations. Notably, start locations were carefully designed to avoid repetitions across subsequent trials. Furthermore, at least 60% of the trials featured only one shortest path to the GL. The first trial of each day differed from both the first and last trial of the previous session. Additionally, start locations were selected with a minimum distance of two choice points from each other and from the GL. On average, each mouse required approximately 30 start locations for a given day, and these locations were generated one day in advance of the training sessions.

Following the mouse's successful retrieval of a food reward and its consumption, the animal underwent manual pickup using a tube. Subsequently, it was transported around the maze to induce disorientation, and then placed at a new start location (Fig. 1D). All pickups within the maze were executed using the tubing method (Gouveia and Hurst 2017). After placing the animal at the designated start location, the experimenter swiftly, yet calmly, moved behind a black curtain situated adjacent to the maze. This measure was taken to ensure that the experimenter remained concealed from the view of the animal during training trials.

It is noteworthy that each cohort of mice was subjected to interactions with multiple experimenters, encompassing both female and male experimenters. Distinct cohorts were supervised by separate sets of students. Furthermore, each mouse underwent a habituation process with each experimenter prior to their participation in the maze training.

On each training day, the animals were transported to the training room no less than 20 minutes before the commencement of the training sessions.

During probe sessions, which occurred on each second session of a goal location switch and additionally during the Build-Up phase (specifically, GL1 in session 6; GL2 in session 5, and GL3-4 in session 4), a specific protocol was implemented. In these sessions, no food was present in the maze for the initial 60 seconds of the first trial of the day. This measure was taken to eliminate the possibility of olfactory aiding navigation to the GL. Subsequently, after the initial 60 seconds had elapsed, food was discreetly placed in the GL while the animal occupied a different section of the maze. This ensured that the animal did not witness the food placement.

For all other trials conducted on probe trial days and during GL switches, food was consistently available at the GL. Probe trial and GL switches were initially minimized to help shape the animal behavior and prevent the animals from forming the expectation that food would not be initially provided.

To assess the animals' performance, the actual path traversed by each mouse was divided by the shortest possible path between a given start location and the goal location. This computation resulted in the calculation of the log of normalized path length (Fig. 1C), which served as a performance score value. The logarithmic value was used due to a skewness in the data, shown in chapter 4. A mouse with adequate food motivation and a well-established knowledge network of the maze should navigate the maze efficiently. A score of 0 denoted that the mouse had chosen the shortest path and navigated directly to the goal.

On average, animals displayed improvement, transitioning from path lengths approximately three times longer than the shortest path to path lengths ranging from 1.5 to 2 times longer, corresponding to log values path length than the shortest path, corresponding to 0.4 and 0.2–0.3, respectively. A more refined random walk model, encompassing random choices at each node and occasional long diagonal runs, is explored in greater detail in chapter 4 of this thesis (Vallianatou, Alonso et al. 2021).

During the probe trials conducted within each goal location in the Build-Up and Update phases, careful observation was employed to record the number of instances in which animals crossed both their current and previous goal location, along with the duration of dwell time at these locations. In order to maintain a control condition, a similar methodology was applied to two distinct nodes within the maze – one situated on the inner ring and the other on the outer ring. The selection of these control nodes was conducted with precision, ensuring that they were sufficiently distant from each other and the GLs, with a minimum of three gangways separating them.

Furthermore, nodes that lay in the direct path between GLs were intentionally excluded from consideration to minimize the potential for false-positive results.

To guarantee that the animals were sufficiently motivated, access to food was restricted for a period ranging from 8 to 24 hours prior to training sessions. This level of motivation was validated by monitoring both the number of trials conducted each day and the count of trials in which the animals consumed food during their initial encounter with it in each trial. A decrease in either of these counts would indicate reduced motivation. Additionally, the animals' weight was assessed two to three times per week, with the aim of ensuring that the average weekly weight did not fall below an estimated 85% of their free-feeding weight. This adjustment was made to accommodate the natural growth of each animal over time.

Pharmacological interventions

Two distinct groups, each adhering to different training schedules, participated in local inhibition protocols. The Build-Up group underwent these protocols within the initial 3 months of training, while the Update group engaged in them after three months of training had transpired. The surgical implantation of cannulae occurred one week prior to the commencement of infusions.

During the surgical procedure, animals were anesthetized and securely placed within a stereotactic frame. Bilateral guide cannulae were meticulously implanted in specific brain regions, namely the hippocampus area CA1 (coordinates: AP -1.94, ML ± 0.1, DV -1.0), retrosplenial cortex (coordinates: AP -2.54, ML ± 0.4, DV -1.2), or prelimbic cortex (coordinates: AP +1.9, ML ± 0.4, DV -1.4). The level of isoflurane anesthesia was maintained at 2% throughout the procedure, and local lidocaine was administered on the skull and ears to minimize discomfort. A recovery period of one week was allocated post-surgery.

In order to manage postoperative pain, mice were provided with carprofen water as an analgesic (Carprofen 50 mg/ml, 0.3 mL in 300 mL of water). This medication was administered two days prior to surgery and continued for two days following surgery. To ensure adequate hydration, daily monitoring of water levels was carried out to confirm that the animals remained well-hydrated.

To locally inhibit excitatory activity, the AMPA antagonist, 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris Laboratories), was employed. This drug, along with a vehicle solution, was administered via bilateral internal cannulae, extending 0.5 mm beyond the guide cannulae. A volume of 1 uL per hemisphere of CNQX, diluted in artificial cerebrospinal fluid (aCSF), or exclusively aCSF as a control, was infused 30 minutes prior to the training sessions. To ensure the complete diffusion of the liquid from the internal cannulae tips, the internal cannulae remained connected for an additional 3 minutes post-infusion.

Infusions were conducted once a week. During periods when the animal was not receiving an infusion, protective measures were taken to safeguard the cannulae.

This was achieved by affixing a dummy, identical in length to the guide cannulae, along with a screw-on cap.

Two distinct training schedules were implemented in this study. The Build-Up group, following the implantation of their cannulae, underwent training sessions three times a week. This training regimen consisted of a constant Goal Location (GL) for a total of 29 sessions. Pharmacological infusions were conducted on sessions 2 and 4, 8 and 10, 14 and 16, 20 and 22, and 26 and 28. This design ensured a three-session gap between successive infusions for each animal. Notably, each animal received infusions of both CNQX and artificial cerebrospinal fluid (aCSF) on different days (Figure 4).

Conversely, the Update group initially trained twice a week for the first 3 months of their training. Subsequently, following the implantation of their cannulae, they transitioned to a more frequent training schedule of three times a week. Pharmacological inhibitions were administered either on the day of the new goal location switch or on the probe trial day, 48 hours after the goal location switch (Figure 6).

Nissl staining

Following perfusion (described in the following section), brain tissues were subjected to cryosectioning at a thickness of 30 micrometers. These sections were subsequently mounted onto gelatin-coated slides and allowed to air-dry overnight at a temperature of 40 degrees Celsius.

The slides then underwent a series of ethanol washes: 10 minutes in ethanol 96%, followed by 2 minutes in each of the following ethanol solutions: 90%, 80%, 70%, and 50%. Subsequently, the slides were immersed in demi water for an additional 2 minutes. Next, the slides were placed in a 0.1% cresylfastviolet solution for 2 minutes, followed by a 5-minute rinse in demi water. Dehydration was achieved through sequential immersions in ethanol solutions of 50%, 70%, 80%, and 90%, with each step lasting 2 minutes. Subsequently, the slides were immersed in acidified alcohol, consisting of one drop of acetic acid per 50 mL of ethanol, for 2 minutes.

Finally, the slides underwent two ethanol 100% steps and two xylene steps. Entellan mounting medium was applied to the slides, followed by the placement of a coverslip. Microscopy images were subsequently captured using a light microscope to assess the precise placement of the cannulae (as shown in Figure 2).

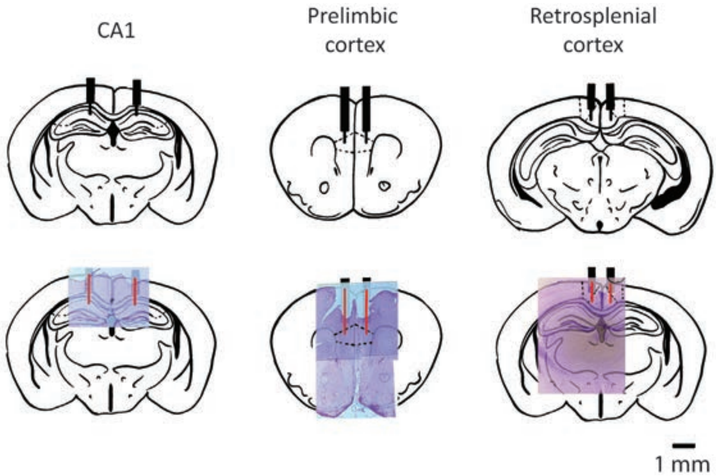

Figure 2 Target areas for cannulae placement (top row) and examples of Nissl staining showing bilateral implant placement (bottom row). Stereotaxic coordinates are: Hippocampus area CA1 AP -1.94 ML +/- 0.4 DV -1.0, Prelimbic cortex AP -1.94 ML +/- 0.4 DV -1.4, Retrosplenial cortex AP -2.54 ML +/- 0.4 DV -1.2 from Bregma. Note that in the nissl staining for retrosplenial cortex, a more anterior coordinate is being used. In red the length of the internal cannula is marked. Dash on the bottom right corner represents the size scale.

Data analysis

The normalized path length for all trials was calculated using MATLAB 2017b (MathWorks). Repeated-measures ANOVAs were run in SPSS Statistics 25 (IBM) to determine the effect of goal location switches and session on the log-normalized path length during the Build-Up and Updates. Within-subject factors were goal location, update type, session and trial. Bonferroni corrections were applied to multiple t-tests.

Immediate early gene assay

Subjects

A total of 41 male C57BL/6J mice, obtained from Charles River Laboratories, and 25 male cFosTRAP2xRosa mice, bred in-house, were employed in this experimental study. The reporter Rosa mouse line used was B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze/J}$ from Jackson Laboratory. Upon their arrival, all mice were approximately 2 months of age. These animals were collectively housed in the Translational Neuroscience Unit of the Centraal Dierenlaboratorium at Radboud University, located in Nijmegen, Netherlands. The housing environment provided a 12-hour light/dark cycle for the mice. Cage enrichment was included to enhance their welfare, consisting of amenities such as running wheels, plastic igloos, and nesting materials. During the behavioral testing phase, the mice were subjected to overnight food deprivation. Their weight was monitored and maintained within a range of 90% to 85% of their estimated free-feeding weight. All animal protocols adhered to ethical standards and received approval from the Centrale Commissie Dierproeven (protocol #2016–014-018, #2016–014-034, #2020-0020-004, and #2020-0020-009)

Behavioral setup, video recording and **behavioral training** were kept the same as during pharmacological interventions.

Experimental groups

To investigate active cell populations during different stages of learning, two strains of animals were used, c57/BL6J and cFosTRAP2xRosa. Within the wild type animals two groups of animals were used, GL1S1 and GL2S1. GL1S1 animals had previously been habituated to the maze, and were perfused 90 minutes after the end of their first training session. GL2S1 animals were perfused 90 minutes after their 8th training session, corresponding to the third week of training. Additionally each group was accompanied by homecage controls which shared the same regimen as the training animals, except the training section. They shared the same cage as training animals, were also food deprived, and would accompany their training cagemates to the training room.

In order to facilitate the observation of cell activity labelling at multiple time points, cFosTRAP2xRosa animals were utilized. The term 'TRAP' represents 'Targeted Recombination of Activated Populations,' a technique employed to permanently label active cells in vivo under the c-Fos promoter. The induction of the reporter gene tdTomato on the animals' second day of training (GL1S2) labelled all active cells within a 6-hour frame, by an intraperitoneal 4OH-tamoxifen injection. Mice were socially isolated one day prior the injection, and regrouped a day after. Two groups of animals, Build-Up and Update groups, were employed to visualize protein expression, specifically during the third week of training for the Build-Up group (GL2S1) and the 12th week of training for the Update group (GL6S1). Homecaged control animals were also included across all groups for comparative analysis, which experienced the same conditions as described above. An additional control group, the Task Specific control (TS) experienced training along the GL6S1 group, but on the final day of training were exposed to a novel square arena, and allowed to explore a set of 4 different objects during 30 minutes.

Table 1 Number of samples collected for immunohistochemistry

Group	n
Early learning Homecages	9
Early learning GL1 S1	16
Early learning GL2 S1	16
TRAP2 GL2 S1	7
TRAP2 GL6 S1	7
TRAP2 Task Specific Control	5
TRAP2 Homecages	6

Perfusion

Ninety minutes following the completion of training, the animals underwent transcardial perfusion. This process involved the sequential delivery of 10 mL of 0.1M PBS (Phosphate Buffered Saline) followed by 10 mL of 4% paraformaldehyde (PFA). Subsequently, the brain was carefully extracted and immersed in 4% PFA solution for a duration of twenty-four hours at a temperature of 4 degrees Celsius.

Following the fixation process, the perfused brains underwent a thorough washing procedure, involving three rinses with PBS 0.1 M. Subsequently, the brains were submerged in a solution of 30% sucrose (w/v) in PBS, complemented with 0.02% (w/v) Sodium Azide.

Once the brains had sunk to the bottom of the jar containing the PBS and sucrose solution, they were frozen with dry ice and stored at a temperature of -80 degrees Celsius.

Immunostaining

Brains were cryosectioned into tissue sections, each measuring 30 micrometers in thickness. These sections were subsequently subjected to a thorough washing step with 0.1M PBS before being blocked with a 2% solution of BSA (Bovine Serum Albumin) for a period of one hour.

For immunostaining, a primary antibody mixture comprising 0.02% BSA, PBS with Sodium Azide, 0.3% Triton X-100, and a combination of anti-cFOS (Synaptic Systems, cat. No 226 004) Polyclonal Guinea pig at a dilution of 1:1000 and anti-Arc (Synaptic Systems, cat. No.156 003) Polyclonal Rabbit at a dilution of 1:500 was employed. The tissue sections were incubated in this primary antibody solution for a duration of 72 hours while placed on a shaker at a temperature of 4 degrees Celsius.

Subsequently, the secondary antibody was prepared by combining 5% BSA, PBS with Sodium Azide, 0.3% Triton X-100, anti-rabbit Alexa fluor 488 at a dilution of 1:1000, and anti-guinea pig Alexa fluor 647 at a dilution of 1:1000. The sections were then incubated in this secondary antibody mixture, in the dark, for a period of 2 hours.

Finally, the sections were mounted in a manner that avoided direct exposure to light and covered with DAPI fluoroshield for preservation and visualization.

Microscopy

Thunder microscope from Leica was used for taking microscopy images of immunostained slides, at 10X and 20X. Below the details for each fluorophore.

Fluorophore	Excitation	Duration	Laser power
DAPI	395	50 ms	20%
Alexa 488	488 (thunder:470) + filter at 510 (for Arc)	100 ms	25%
Alexa 555	555	100 ms	25%
Alexa 647	640	100 ms	30%

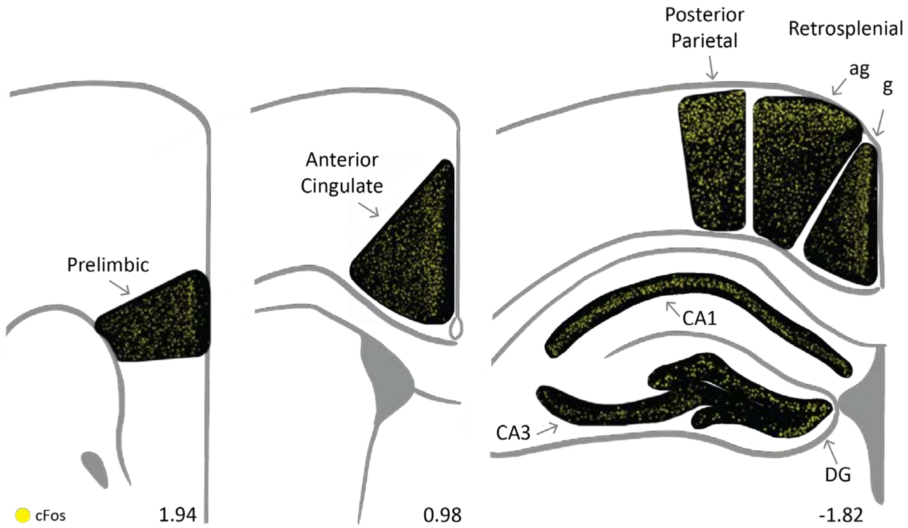

Figure 3 Examples of regions of interest (ROI) immunostained against cFos. There are no actual gaps between PPC, RSCxa and ag, for illustration purposes only. The bregma positions for each section is on the bottom right corner.

ROI cutting

From each microscopy image, specific regions of interest (ROIs) were meticulously chosen, encompassing areas such as the prelimbic cortex, anterior cingulate cortex, hippocampal regions including CA1, CA3, and DG, the retrosplenial cortex, and the posterior parietal cortex. A visual representation of one such slice, featuring the identified ROIs, is depicted in Figure 3.

Following the selection of ROIs, a machine-learning-based automated cell counting program known as Ilastik was employed. This program underwent a training process using 10 randomly selected images for pixel and object classification. It was trained to recognize cells and distinguish them from the background, giving an output of number of cells per ROI.

Data analysis

Counts were obtained as an output from a custom Ilastik script, and the area of each brain area was obtained from a custom ImageJ script. From these two outputs, density was calculated. Overlapping counts were obtained from a custom script.

Density values were then divided by the average value of the homecage groups, groups GL1S1 and GL2S1 by early learning homecages, and TRAP2 groups to TRAP2 homecages. Normalization was done per brain area.

Repeated measure ANOVAs, univariate ANOVAs and t-tests were run in SPSS Statistics 25 (IBM). Pearson correlation analysis were performed using GraphPad Prism. For selecting a cutoff point for p values, the values from GL1S1 were used as a reference, where the p value >0.001 corresponded to an r value between 0.723 and 0.735. This significance cutoff was then used for all the other correlations.

Venn diagrams were generated using www.meta-chart.com.

Results

Initial spatial learning

To assess the dependency of specific brain areas **during the establishment of a prior knowledge network**, the mouse HexMaze task was employed. Within this context, pharmacological inhibition was conducted separately in the hippocampus, prelimbic cortex, and retrosplenial cortex.

The Build-Up pharmacological group differed from the Build-Up presented in chapter 2, and from the Update pharmacological group. A key difference was that the goal location remained consistent throughout 29 training sessions, training three times a week, allowing for the evaluation and measurement of the timeframe needed for this type of memory become hippocampal independent. Inhibition occurred once a week, with a one-week interval between each pair of infusions, as illustrated in Figure 4A. Within each pair of infusions, every animal received either the drug or a vehicle, serving as a within-subject control.

When the CA1 region of the hippocampus was inhibited, specific effects on performance per session and an interaction for session*drug were observed (ANOVA session $F_{4,56} = 6.354$, **$p<0.001$**, treatment $F_{1,14}=1.117$, $p=0.308$, session*treatment $F_{4,56}=2.784$, **$p=0.035$**). Further examination with multiple t-tests, analysing each time point revealed an effect of the drug during sessions 2 and 4 ($t_{14}=2.372$, $p=0.033$). However, when applying the Bonferroni correction (alpha set to 0.01), **this result did not reach the threshold for significant difference** (Fig 4B, middle section).

Upon analysis of probe trials, an effect of session and an interaction of session*treatment was observed (ANOVA session $F_{4,56} = 10.254$, **$p<0.001$**, treatment $F_{1,14}=0.232$, $p=0.637$, session*treatment $F_{4,56}=2.929$, **$p=0.029$** (Fig 4B, right side). Subsequent multiple paired t-tests showed effects for the first two infusion sessions, and after applying a Bonferroni correction (alpha set to 0.01) this effect was confirmed (veh vs drug session 2 and 4 $t_{14}=3.141$, **$p=0.007$**, session 8 and 10 $t_{14}=0.2$, $p=0.845$, sessions 14 and 16 $t_{14}=1.344$, $p=0.2$, sessions 20 and 22 $t_{14}=1.266$, $p=0.226$, 26 and 28 $t_{14}=0.444$, $p=0.664$). These results indicate that **inhibition of the CA1 region of the hippocampus during the initial weeks resulted in decreased long-term memory expression for the goal location**. However, we noticed that the vehicles performed better than usual, compared to the Prl or Rscx vehicle groups, which might confound this result.

When the prelimbic cortex was inhibited, specific effects on performance were observed for session only (ANOVA session $F_{4,44}=23.048$ **$p<0.001$**, treatment $F_{1,11}=1.489$ $p=0.248$, session*treatment $F_{4,44}=0.349$, $p=0.843$) (Fig 4C, middle section). These results indicate that despite inhibiting the prelimbic cortex, animals still displayed memory expression of the location reward.

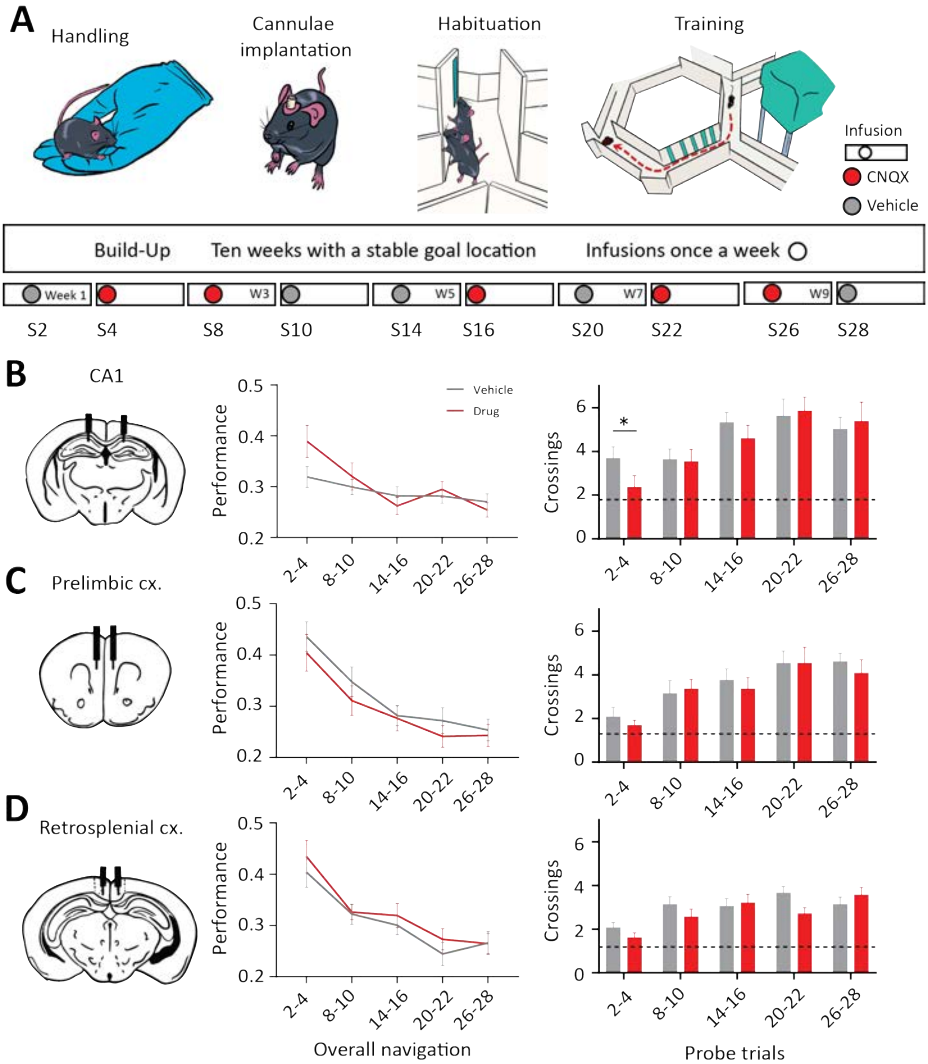

Figure 4 Pharmacological interventions during Build-Up phase. **A** Illustrations depicting procedures before and during HexMaze training. Below a timeline of the Build-Up group. Animals are handled for a week, followed by two habituation sessions, after which animals undergo surgeries to implant cannulas. One goal location is kept constant through all sessions, and infusions take place once a week on sessions 2, 4, 8, 10, 14, 16, 20, 22, 26 and 28. For every pair of infusions animals would receive vehicle and drug. Drug delivery sequence was counterbalanced across animals, and the sequence shown is just an example **B** Target area (left row), overall navigation performance (middle row) and probe trial performance (right row) after pharmacological inhibition of CA1 region of the hippocampus, of the prelimbic cortex **C**, and of the retrosplenial cortex, **D** Error bars correspond to SEM. Dotted line corresponds to the number of crossings of control nodes for both vehicle and drug conditions. Asterisks correspond to $p<0.05$.

Upon analysis of probe trials, only an effect of session was observed (ANOVA session $F_{4,48}$ = 11.497, **p<0.001**, treatment $F_{1,12}$=0.649 p=0.436, session*treatment $F_{4,48}$=0.31 p=0.87 (Fig 4C, right side). These results indicate that inhibition of the prelimbic cortex did not prevent animals from expressing long-term memory of the reward location.

When the retrosplenial cortex was inhibited, a specific effect was seen on performance for session only (ANOVA session $F_{4,56}$=16.956 **p<0.001**, treatment $F_{1,14}$=2.086 p=0.171, session*treatment $F_{4,56}$=0.243, p=0.913) (Fig 4D, middle section). These results indicate that despite inhibiting the retrosplenial cortex, animals still displayed memory expression of location reward.

Upon analysis of probe trials, only an effect of session was observed (ANOVA session $F_{4,56}$ = 11.019, **p<0.001**, treatment $F_{1,14}$=1.94 p=0.185, session*treatment $F_{4,56}$=2.313 p=0.069 (Fig 4D, right side). These results indicate that inhibition of the retrosplenial cortex did not prevent animals from expressing long-term memory of the reward location.

Across all brain areas, a trend appeared where vehicles performed better during sessions 2 and 4 than the treatment group. Thus, the data was combined and compared, analyzing only these two sessions for the three brain areas (Fig. 5), which showed an effect of brain area and treatment but no interaction (ANOVA BA $F_{2,80}$=8.801 **p<0.001**, treatment $F_{1,1=80}$=7.398 **p=0.008**, BA*treatment $F_{2,80}$=1.346, p=0.266). These results suggest that local inhibition of CA1, prelimbic and retrosplenial cortex negatively affected long-term memory expression of the reward location, but only during the first two weeks of learning, when animals are not yet performing optimally.

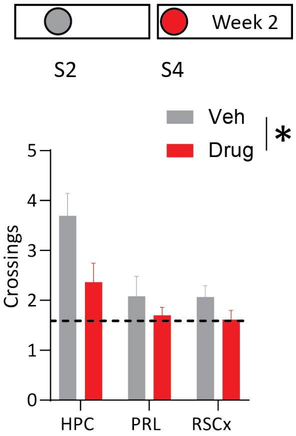

Figure 5 Probe trial measure of crossings of goal location for all three brain areas for sessions 2 and 4. Infusion of drug or vehicle was counterbalanced across animals, so that some had vehicle on S2, like shown on the example, or on S4, not depicted. Dashed lines represent the number of crossings of control node, for both vehicle and drug groups.

Overall, **pharmacological inhibition of the brain areas studied had a minimal impact on navigation and memory.**

Hippocampal dependency in schema updating for long-term memory

In the Update group, animals underwent four goal location switches over a span of three months. Afterwards, they received cannulae implants and were allowed a week for recovery before resuming training. New goal locations were presented on a weekly basis, involving either switching the goal location or changing the goal location along with the introduction of three barriers. **Infusions were administered weekly, either during encoding or retrieval sessions of new goal locations**.

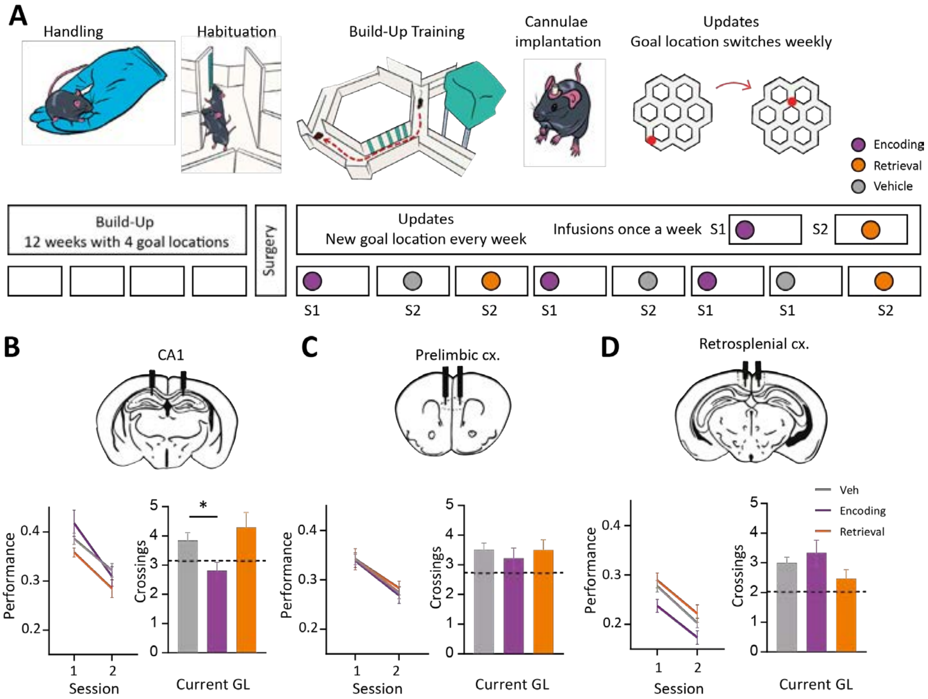

Figure 6 Pharmacological interventions during Updates. **A** Illustrations depicting procedures before and during HexMaze training. Below a timeline Update group. Animals are handled for a week, followed by two habituation sessions, after which animals train for three months. Animals undergo surgeries to implant cannulae, and new goal locations are introduced weekly. Infusions take place once a week, either on the day of map updating (Session 1), or 48 hours later (Session 2). Each animal received vehicle and drug on a counterbalanced design. **B** Target area (top), navigation performance (left side) and probe trial performance (right side) for animals that received drug on session 1 (purple), session 2 (orange) or that received vehicle on either session (gray) targeting hippocampal region CA1; **C** prelimbic cortex and **D** retrosplenial cortex. Error bars correspond to SEM. Dotted line corresponds to the number of crossings of control nodes for both vehicle and drug conditions. Asterisks correspond to $p<0.05$.

Analysis comparing performance in the update phase during vehicle infusion, drug infusion on encoding session, and drug on retrieval session in the CA1 region of the hippocampus only showed an effect of session (ANOVA Session $F_{1,109}$=33.161 **p<0.001**, treatment $F_{2,109}$=2.766 p=0.067, Session*treatment $F_{2,109}$=0.901 p=0.409) (Fig 6B). When analyzing treatment effect on the prelimbic cortex, again only an effect of session was observed (ANOVA Session $F_{1,113}$=21.195 **p<0.001**, treatment $F_{2,113}$=0.104 p=0.901, Session*treatment $F_{2,113}$=0.047, p=0.954). Lastly, when analyzing treatment effect on the retrosplenial cortex, specific effects were seen for session and treatment (ANOVA Session $F_{1,118}$=36.901 **p<0.001**, treatment $F_{2,118}$=5.959 **p=0.003**, Session*treatment $F_{2,118}$=0.1, p=0.905). **The results indicate that once the spatial map is established, inhibition of any of the three brain regions studied didn't impair navigational performance.**

Upon analysis of the probe trials, 48 hours after map updating, the hippocampus group showed an effect of treatment (ANOVA treatment $F_{2,64}$=3.459 **p=0.037**), however no effect was seen for treatment on the prelimbic cortex group (ANOVA treatment $F_{2,57}$=0.224 p=0.8) or the retrosplenial cortex group (ANOVA treatment $F_{2,59}$=1.781 p=0.177). One-sample t-tests comparing the number crossings between the different treatments and against chance level for the CA1 region of the hippocampus showed that the number of crossings was statistically different for vehicle and retrieval compared to chance. Chance is defined by the number of crossings for both vehicle and drug conditions of a control node (chance vs encoding t_{17}=1.092 p=0.29, chance vs retrieval t_{17}=3.296 **p=0.004**, chance vs vehicle t_{17}=3.631 **p=0.002**) and a Bonferroni correction was applied (alpha 0.016). An orthogonal comparison of treatments comparing the encoding group versus retrieval and vehicle groups revealed a statistical significance between them ($t_{34.457}$=2.302 **p=0.027**). **These results indicate that the rapid encoding of a memory is dependent on the CA1 region of the hippocampus during the update phase.**

Immediate early gene expression

Immunohistochemistry targeting the cFos protein, a recognized neural marker of cellular activity (Verbalis, Stricker et al. 1991), was conducted on two distinct mouse lines: the wild-type C57BL6J line and the transgenic TRAP2 line. The **TRAP2 animal model offers distinct advantages for visualizing the engram, primarily due to its capability for dual labeling within the same tissue capturing two distinct events separated in time**.

On the second day of training to the first goal location (GL1S2), in-vivo tagging of active cells was induced through the administration of a 4-OH tamoxifen injection

(Fig 7A). Subsequent immunostaining against cFos within the same brain results **in dual labeling, involving the TRAP2 signal from GL1S2 and the labeling of either the first session after the first goal location switch (GL2S1) or the first session after the build-up period is over (GL6S1)**. This innovative approach facilitates the quantification of overlapping cells, denoting those cells that exhibited activity initially on GL1S2 and were also active in the later stages.

The primary focus of this study revolved around the examination of cFos expression during the three stages of learning mentioned in chapter 2: the initial reward location (GL1), the second reward location (GL2), and the sixth reward location (GL6, Update Phase with expedited long term memory). These three stages can also be compared to reference memory, reversal learning, and schema memory, as will be discussed in length in chapter 6. The **specific brain areas under scrutiny encompassed the prelimbic cortex, anterior cingulate cortex, retrosplenial cortex, posterior parietal cortex, and hippocampal regions CA1, CA3, and DG.**

Previous experiments showed dependency on the prelimbic cortex during schema updating (Tse, Takeuchi et al. 2011). To see if my results replicated these findings, analyses of density of cFos positive cells for each brain area were evaluated for the three learning stages. An effect of **increased expression over time was observed for the prelimbic cortex** (ANOVA $F_{2,40}=3.803$ **$p=0.031$**)(ANOVA ACC $F_{2,37}=0.476$ $p=0.625$, RSCx $F_{2,41}=0.411$ $p=0.666$, PPC $F_{2,41}=0.464$ $p=0.632$, CA1 $F_{2,41}=0.131$ $p=0.877$, CA3 $F_{2,42}=0.747$ $p=0.48$, DG $F_{2,42}=2.338$ $p=0.109$) (Fig 7B).

The methodology used for tagging active populations in vivo allowed me to measure the overlap of active cells on the second day of training for GL1, to GL2 S1 and GL6 S1. An example of this overlap can be seen in Figure 8A, for three different brain areas. The analysis of all engram cells observed on the second day of training, when overlapped to S1 of GL2 and S1 of GL6, revealed a statistical difference between overlapping cells in animals navigating the HexMaze versus those engaged in a control task (ANOVA GL $F_{2,100}=5.065$ **$p=0.008$**, BA $F_{6,100}=1.133$ $p=0.349$, GL*BA $F_{12,100}=1.181$ $p=0.307$). An independent sample t-test showed a statistically significant difference between animals which performed the HexMaze task versus animals that performed the Task Specific control ($t_{167.957}=6.132$ **$p<0.001$**) (Fig 8B). Posterior independent t-test analysis comparing each brain area for GL1 and GL6 showed no differences between groups (Fig 8C). Taking into consideration the overall density of cFos positive cells in both events for all brain areas analyzed, showed a large overlap of cell identity (Fig 8D).

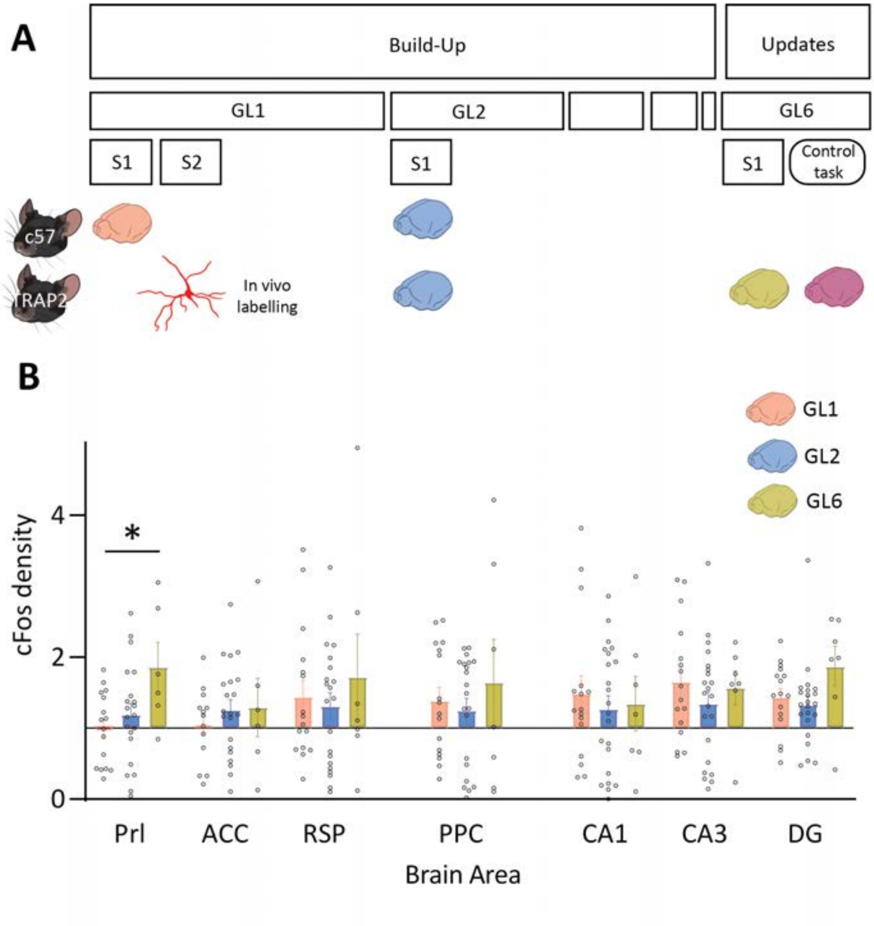

Figure 7 Immunohistochemistry results **A** Timeline of sample collection. Two animal strains were used, c57BL6j as depicted on the top row, and cFosTRAP2xRosa on the bottom row. TRAP2 animals received a 4OH-Tamoxifen injection on GL1S2 to induce the expression of tdTomato on cFos positive cells, depicted as a red neuron. Color coding of the brain indicates the timepoint of sample collection. **B** cFos density normalized to homecage across three different timepoints.

Network analysis of corticocortical and corticohippocampal connections conducted via Pearson's correlation analysis were used to **evaluate if the expression pattern of cFos in a particular brain region was associated with that in another brain region, when encoding information for GL1, 2 and 6** (Fig 9). Warmer colors indicate stronger positive correlation. The strongest correlations are shown as a thick solid black line on the brain schematics shown on the bottom row of figure 9.

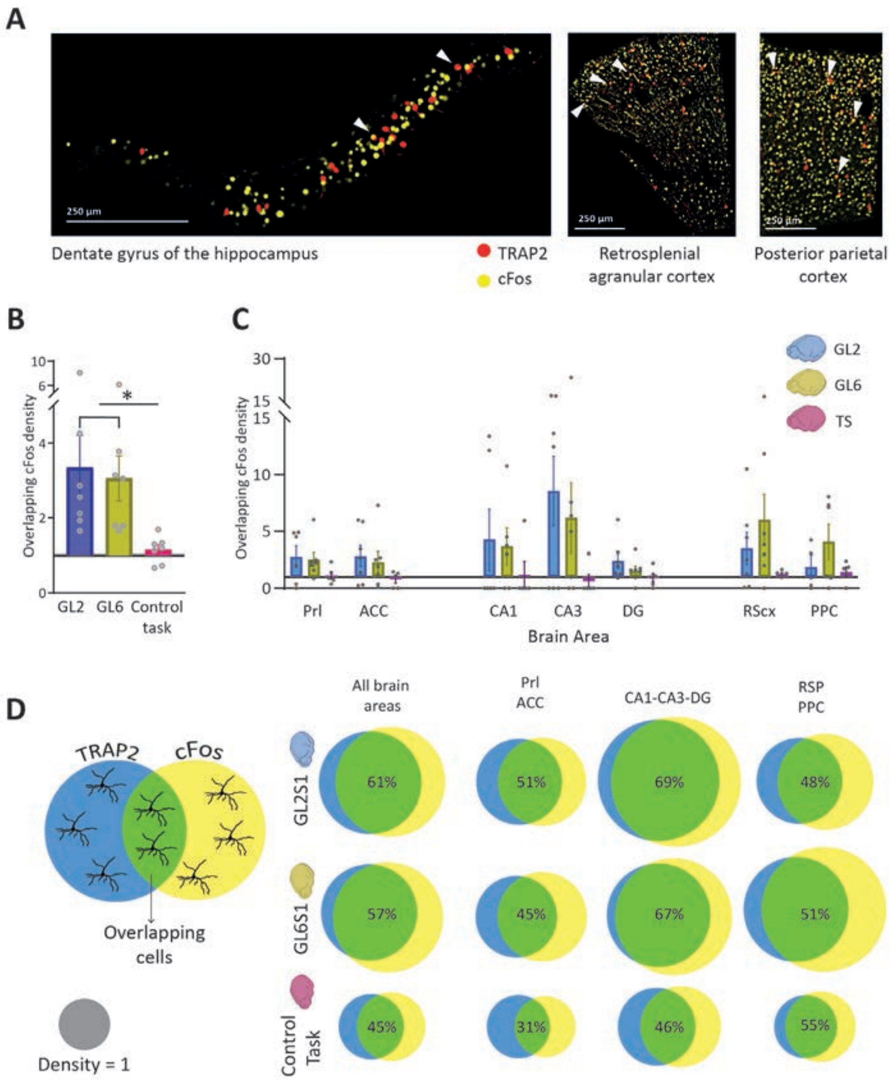

Figure 8 Overlapping cell populations **A** Microscopy examples of overlap of TRAP2 cells (red) and cFos (yellow). White arrowheads show examples of overlap **B** TRAP2 overlapping density of all cFos positive cells for GL2, GL6 and for the control group, which instead of performing in the mouse HexMaze, experienced a control spatial task for their last training session. **C** cFos density per brain area **D** Illustration depicting a double labeled brain tissue, with a TRAP2 signal from GL1S2 (blue), and a cFos signal of either GL2, GL6 or the control task (yellow). Overlapping cells are those that are active in both timepoints (green). Venn diagrams depict the density of stained cells with the radius length of the circle, and in the center of the overlapping section the percentage of overlapping cells are indicated, for all brain areas (left) or for grouped brain areas (right).

For the first day of encoding (GL1) the strongest correlations at cortical level are prelimbic cortex with anterior cingulate cortex and retrosplenial cortex with posterior parietal cortex, while cortico-hippocampal correlations are strongest for retrosplenial cortex with CA1 and CA3. At hippocampal level, the strongest correlations were among CA1 and CA3.

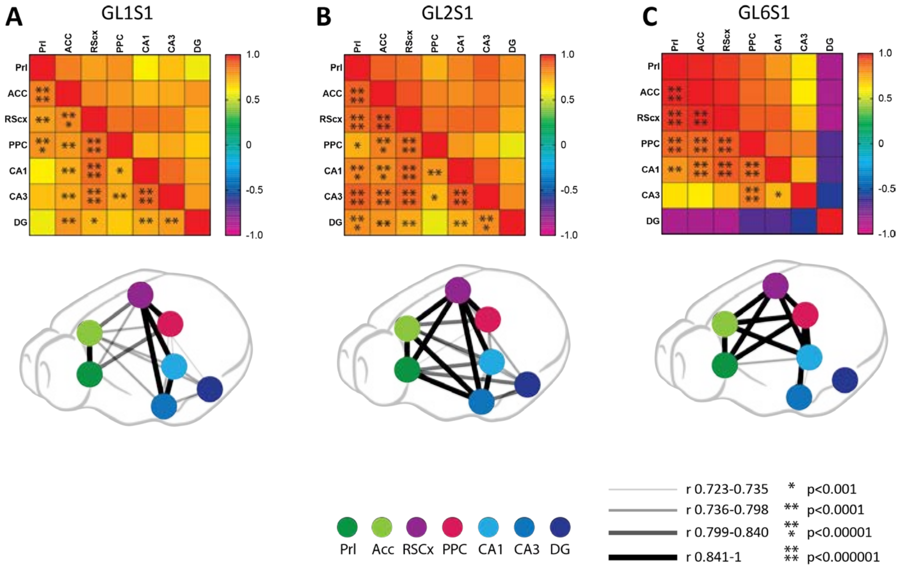

Figure 9 Network analysis. Pearson correlation of cFos density in each brain area against each other area, for GL1S1 **A**, GL2S1 **B,** and GL6S1 **C** Bottom row, representation of strength of connectivity between different brain areas

For GL2, correlations were strong among most brain areas. The strongest correlations at cortical level are prelimbic cortex with anterior cingulate cortex and retrosplenial cortex, anterior cingulate cortex with retrosplenial cortex, and retrosplenial cortex with posterior parietal cortex. Cortico-hippocampal correlations are strongest between prelimbic cortex and CA3, anterior cingulate cortex and CA3, retrosplenial cortex with CA1 and CA3. At hippocampal level, correlations were strongest between CA1 and CA3.

For GL6, cortical correlations were the strongest. Prelimbic cortex with anterior cingulate cortex, retrosplenial cortex and posterior parietal cortex, anterior cingulate cortex with retrosplenial cortex and posterior parietal cortex, and retrosplenial cortex with posterior parietal cortex. At cortico-hippocampal level, CA1 was correlated with anterior cingulate cortex, retrosplenial cortex and posterior parietal cortex, and CA3 with posterior parietal cortex. Important to note, at this stage of learning, there is a strong anti-correlation, as shown in the blue and purple colors, of dentate gyrus with all brain areas.

These results indicate an overall increased connectivity among the brain areas analyzed as learning progresses.

Discussion

This investigation aimed to understand the processing of new information within the context of prior knowledge. The roles of the hippocampus, prelimbic, and retrosplenial cortex were assessed in mice through navigation and memory tests in the HexMaze. Navigation and memory can be evaluated separately in this task, observing the length of the taken paths overall during a training session, and by observing the frequency of crossings of the rewarded location during the first training trial 48 hours after a location update. To study the role of these brain areas thought to be critical in mnemonic processing and navigation, local pharmaceutical AMPA antagonists were infused during different stages of the experience to temporarily inhibit all neural activity locally. Pharmacological inhibition of these brain areas demonstrated minimal impact**. Notably, hippocampal area CA1 was only essential for updating information in pre-existing knowledge networks, consistent with previous findings in rats** (Tse, Langston et al. 2007). Navigational performance remained unaffected by inhibition in any area, which was surprising as hippocampal dependency was expected due to the spatial nature of the task. Engram analysis via immediate early gene mapping of cFos showed increased expression in prelimbic cortex with experience, and overlapping analysis showed that either 2- or 12-weeks into training animals engage a large percentage of the same neurons as in the initial memory engram. Connectivity analysis suggested strengthening of the cortical network over time, maintaining engagement with CA1.

Despite the seemingly established link between the hippocampus and memory, some have argued that the role of the hippocampus in navigation may confound some memory effects. For instance, the watermaze seemingly never is hippocampal independent even after many weeks of training, but this may be due to the increased path-integration processes needed to solve such a swimming task. **Results from the HexMaze indicate that we can separate the mnemonic and navigational function of the hippocampus in this task**, since animals do not use computation of the direct path to the goal to solve this task. Thus we can highlight a special importance of the hippocampal CA1 field in updating previous knowledge networks.

Dissociation between memory and navigation

The functions of navigation and memory have been tested in various tasks, as discussed in chapter 1 of this thesis. Notably, the Morris water maze poses a paradox in memory consolidation theories. Regardless of how many weeks of training the animal has had, the hippocampus remains essential for platform localization. A well-consolidated location would be expected to become dependent on the cortex and independent of hippocampus. Despite this expectation, platform location was never successful in absence of the hippocampus (Morris, Garrud et al. 1982). This may mean that some spatial memories never become hippocampus independent or that a key feature of memory retrieval or memory expression remains dependent on the hippocampus. An important detail on memory expression pertains to the nature of the water-maze itself. During probe trials, rats must constantly swim in search of a platform that is not present. Rats cannot swim in place above where the platform should be; instead, they must keep moving and swim back towards the location, necessitating constant path computation. **In contrast, in a dry maze, animals might remain where the reward is expected, with no further need for navigation functions, relying solely on their memory.** In my results CA1 region proved necessary for encoding new information in the presence of previous knowledge, but not for retrieval 48 hours later, replicating findings in rats (Tse, Langston et al. 2007).

In Tse's studies prelimbic inhibition during encoding of new paired associates, or during retrieval of a newly learned association, resulted in task impairment. In contrast, in my experiments prelimbic inhibition during encoding yielded no discernible difference in behaviour. Interestingly, we did replicate findings at the engram level, where the **prelimbic cortex exhibited increased activation throughout the learning proces**s. Notably, increased cFos expression in prelimbic cortex over time has also been observed in watermaze experiments, increasing with the extended time elapsed between acquisition and testing (Barry, Coogan et al. 2016).

The **difference of prelimbic dependency between the Hexmaze and the paired associates task may be due to the inherent differences in the task.** In Tse's study, the associations to be learned occurred in a square event arena, which could be compared to object-words associations, which memories have been demonstrated to rely on prefrontal cortical areas, while remembering scene-words pairs reported increased connectivity between retrosplenial cortex and hippocampus (Cowan, Liu et al. 2020). The HexMaze task, with its array of gangways, features a more pronounced navigational component, which may render it more reliant on posterior cortices that support spatial maps, such as the retrosplenial and parietal cortex.

Navigation

Following an extended learning experience in a complex environment, hippocampal computations are essential for recording new changes to the environment, **however the navigating component of the task seems to not be complex enough for hippocampal inhibition to deter efficient navigation,** at least at the level of inhibition presented here.

Traditionally, the function of navigation and the ability to determine one's position based on salient landmarks have primarily been associated with the hippocampal region, supported by place and space representations (O'Keefe and Nadel 1979). **The hippocampus is known to be critical for path integration** and many spatial-coding functions have been identified in the hippocampus such as place cells, boundary cells, and object location cells. Place cells specifically represent a distinct location in the environment, while path integration continuously updates the location in reference to the environment, distance travelled, and speed (Eichenbaum, Dudchenko et al. 1999).

As previously mentioned, hippocampal lesions in rats performing the watermaze impaired allocentric navigation, while cortical lesions above the hippocampus did not (Morris, Garrud et al. 1982). However, these cortical lesions did impair flexibility in learning new locations (de Bruin, Sanchez-Santed et al. 1994). Retrosplenial lesions, on the other hand, impaired performance in the watermaze (Harker and Whishaw 2002, Czajkowski, Jayaprakash et al. 2014). It is essential to emphasize that the watermaze task is highly dependent on path calculation, driven by the animals strong motivation to find an escape from swimming. **In my results, nor prelimbic nor retrosplenial cortex inhibition impaired navigation within the testing environment**.

In the Build-up pharmacology group, even though animals have a fixed reward location during several weeks, perfect performance is never achieved even in the absence of the treatment. We previously showed that the mice’s navigational strategies are not purely goal oriented (Vallianatou, Alonso et al. 2021), as a model of their behaviour indicated that their movement patterns toward the goal involved two key elements: **random movements with a probability to perform long diagonal runs, and foresight.**

Foresight represents the animal's ability to anticipate the goal's location when nearing a specific distance from it. As learning iterations progress, foresight increases, indicating **that animals become more adept at directly running to the reward from greater distances away**. Animals recognize a certain location, enabling them to path integrate and locate the placement of the food. If the mice in my task exhibited less goal orientated behavior and consequently a reduced need of path integration, it would make sense that inhibition of the CA1 region wouldn't alter their navigational performance; instead, CA1 proves crucial for integrating new memories into the network.

While foresight reflects memory, **the random navigation component of behavior persists despite extensive training**. Navigation may rely on unconscious familiarity or reflect an evolutionary trait for survival, spanning the environment for potential threats. This behavior was also shown in a spatial navigation task where animals, despite knowing the reward location, spanned large areas of the maze throughout the session (Rosenberg, Zhang et al. 2021).

What could be happening is that after initial map learning, other brain areas may be guiding navigation. One potential brain area would be the striatum. **The striatum is part of the basal ganglia, and is relevant for skill learning and habits, engaged in goal-directed behaviors and strongly modulated by dopamine** (Penner and Mizumori 2012). It could be that after repeated instances of training, the movement of the mice through the maze could turn into a habit for them, next to the skill of efficiently navigating within the environment, instead of performing intricate path computations which would be hippocampal dependent. An alternative interpretation of the behaviour of this task is that the schema is reward-based in a familiar environment and is being solved by unconscious motor responses, adding a valence to the rewarded location. Further studies involving the striatum within the mouse HexMaze would help to further comprehend the neurobiology of navigational strategies.

Memory

To safeguard memory structures from interference, the assimilation of new information is a gradual process. However, if the new information aligns with one or more existing memory structures, the updating of the knowledge network occurs more quickly. In my task, during initial two weeks of build-up, the location is fixed, hence we would be testing reference memory, and the results from the probe trials showed a slight dependency on all areas studied. The effect seen was not large, maybe confounded by the animals not performing optimally at this stage. **After**

this period, local inhibition of the studied areas did not impair the long-term memory expression of the goal location.

Hippocampal lesioning also did not impair reference memory in the radial arm maze (Olton, Collison et al. 1977), suggesting that animals rely on **non-hippocampal strategies to reach their goal**. It is crucial to note that these animals were pre-trained before the lesion, so the development of the memory structure for solving the radial arm maze was not evaluated. On the other hand, lesioning the prelimbic region in the radial arm maze resulted in impairment, indicating the essential role of the prelimbic region for prospective response strategies in this context (Floresco, Seamans et al. 1997). In the case of the mouse HexMaze, hippocampal inhibition takes place while the animals are learning the task, which could explain the initial decrease in memory expression, contrasting with radial arm reference memory tasks where no such effect is seen, given that the task had already been learned.

Once the cognitive map of the task is believed to be established, **only the inhibition of hippocampal area CA1 during encoding of new goal locations in the update phase impaired memory expression.** This result indicates that a schema has been formed it still relies on hippocampus for rapid updating, as shown before in the paired associates task (Tse, Langston et al. 2007).

The use of double labeling technology in the TRAP2 mouse line allows the visualization of the initial original memory engram and contrasts it with a later learning point. **This analysis revealed that the original memory engram was recruited for encoding a new goal location later on, even many months later.** Previous fear conditioning studies testing remote memory (DeNardo, Liu et al. 2019) using TRAP2 animals have also shown overlap in the retrieval of memory in the prelimbic cortex. However, this overlap tends to favor neural populations that were active in later stages of retrieval rather than initial ones.

Functional connectivity studies conducted to assess whether if the expression pattern of activity-induced immediate early genes products in one brain area is associated with others revealed strong cortical correlations. The observed activity correlations during the build-up phase indicated a strong correlation between hippocampal areas CA1-CA3. **Throughout buildup and updates, a strong correlation was observed in prelimbic, anterior cingulate and retrosplenial cortical areas**. Similar results were found in a study evaluating plasticity in the paired associates task (Takeuchi, Tamura et al. 2022), showing a strong correlation of hippocampus CA1-CA3, another one involving prelimbic, anterior cingulate and anterior retrosplenial cortical areas, and another one involving orbitofrontal and posterior retrosplenial cortical areas.

During updating for the second (GL2) and the sixth goal location (GL6), a robust connectivity was observed among cortical hubs such as prelimbic, anterior cingulate, retrosplenial and posterior parietal cortical areas. At hippocampal level, dentate gyrus (DG) showed correlation only during GL2, but not during GL6. Similarly, during encoding of new associations in the paired associates task, a strong correlation was observed among midline necortical regions prelimbic, anterior cingulate and anterior retrosplenial cortical areas, while at hippocampal level, a strong correlation was seen among DG-CA1-CA3. **These network analysis reveal a high similarity with the paired associates task, further supporting the claim that grouped DG-CA1-CA3 and prelimbic, anterior cingulate and retrosplenial cortical areas are relevant hubs for schema updating**.

Conclusion

In conclusion, this exploration into the intricacies of memory, learning, and navigation sheds light on the dynamic interplay between various brain regions. The evidence presented underscores the pivotal role of the hippocampus in memory. As the cognitive map solidifies, mice appear to rely on non-hippocampal strategies for navigation, with the striatum potentially emerging as a central player. The strong functional connectivity observed among cortical hubs, such as prelimbic, anterior cingulate and retrosplenial cortical areas, during schema updating emphasizes their relevance in memory networks. While the paired associates task exhibits parallels in network dynamics, the unique features of the HexMaze highlight the intricate relationship between navigation and memory functions. However, as a key finding we could replicate that previous knowledge networks result in rapid systems consolidation. This nuanced understanding deepens our comprehension of the brain's adaptive mechanisms, providing valuable insights into the orchestration of memory and learning processes.

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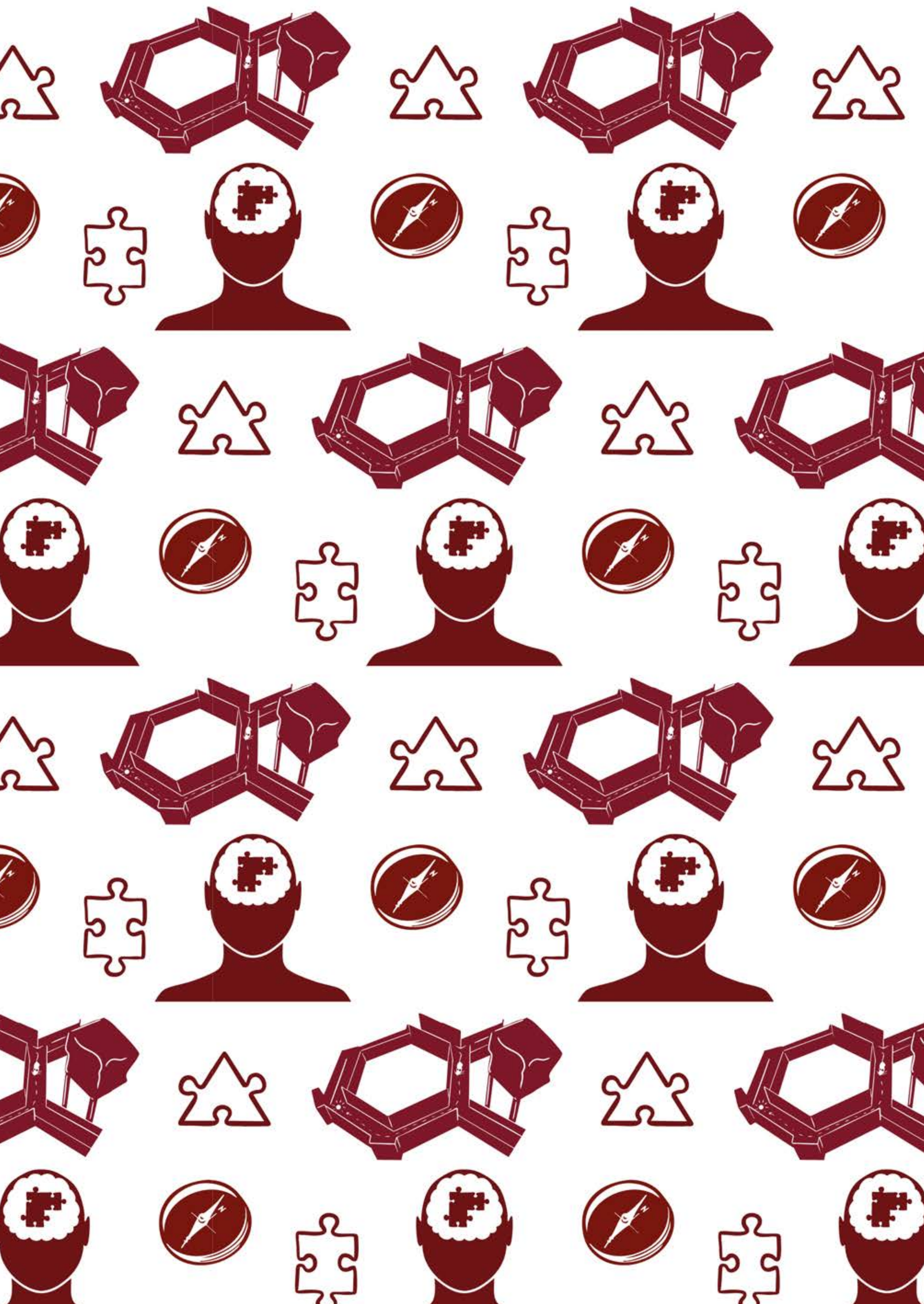

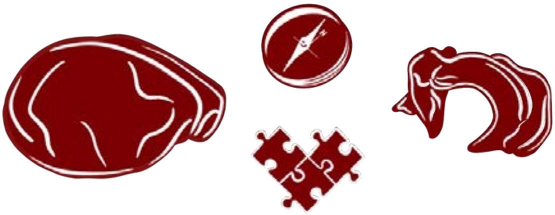

Chapter 6

General discussion

Summary

This thesis contributes to a further understanding of the neurobiology of schemas in memory consolidation. Schemas or previous knowledge networks are memory structures that benefit learning by expediting consolidation. In this thesis **I investigated which brain areas are necessary to build and update a previous knowledge network in the context of spatial memory**, as well as which brain areas are associated to encoding and retrieval of goal information in the presence of previous knowledge. Additionally, what task-solving strategies are being used by the animals in this particular maze.

The task is the mouse HexMaze, developed at the Genzel lab to investigate map learning in mice. **This goal-localization paradigm incorporates extended spatial and temporal dimensions allowing for the study of various memory types, including schema memory**. The HexMaze provides an ideal setting to characterize animal spatial cognition, as it combines the opportunity for animals to express complex behaviors, with the ability to precisely monitor and quantify their navigational choices.

I identified three main learning phases: (1) learning the initial goal location, (2) faster learning after 2 weeks when learning a new goal location, and (3) a third phase after 12 weeks during which the mice exhibited one-session learning leading to long-term memory. **Importantly I found that the gradual development of a mental map of the maze depended on the time elapsed rather than the number of experiences**. Mice that trained twice a week and those trained three times a week learned at the same pace.

In this set of experiments, we utilized the structure of the HexMaze to study the development of goal-directed behavior in mice learning to localize a reward. We statistically characterized the navigational patterns of the mice, comparing them to the optimal goal-oriented paths. **Our results demonstrated a clear learning effect: mice progressively optimized their trajectories, reducing the time and number of nodes visited to reach the reward**. Their paths increasingly conformed to the optimal route, with shorter and less frequent detours. The trajectory improvements followed distinct time courses: steady improvement on the first trial of each session, faster improvement in later sessions, enhanced improvement after introducing a novel reward location, and minimal impact from path-blocking barriers when the reward location was familiar. This trajectory sharpening supports the presence of different forms of previous knowledge enhancing task performance, influenced by allocentric representation, cue-linking to goal proximity, and memory strengthening.

This thesis proposes **a gradient schema theory, suggesting that the amount of previous knowledge influences the involvement of the hippocampus in memory processes**. Most animal memory research is positioned at the low end of this gradient, with new memories relying on minimal prior knowledge. Unique and emotionally-arousing experiences lead to strong, lasting hippocampal memories, while less unique events consolidate more slowly to the cortex, losing episodic detail over time. In intermediate phases, some prior knowledge allows for faster hippocampal updating of cortical networks. At the high end of the gradient, extensive prior knowledge results in rapid cortical consolidation with minimal hippocampal involvement. This theory posits a continuous gradient where increased prior knowledge leads to faster, more efficient memory processes shifting from hippocampus to cortex.

Using pharmacological manipulations I found that building up map learning in the context of spatial memory was not reliant on hippocampal region CA1, prelimbic nor retrosplenial cortex. **Once the memory structure was established, fast updating was a hippocampal-dependent event, followed by rapid consolidation, as retrieval 48 hours post encoding proved to be hippocampal independent**.

The prelimbic cortex was not necessary for encoding or retrieving goal information, as local inhibition did not impair performance in the task. However, the expression of activity-related proteins in the prelimbic cortex showed a gradual increase over time throughout the training period. The retrosplenial cortex was not critically necessary during early or late learning for navigation or memory, however, this region was engaged during task performance, as evidenced by increased expression of activity-related proteins. All three brain areas -the hippocampus, prelimbic and retrosplenial cortex- were part of a **distributed memory network**. Correlation analysis indicated the strength of connectivity among these areas as the training period progressed.

The overall performance of the animals, in terms of efficiency to reach the goal location, was measured by evaluating all trials performed in a single session. The memory aspect of the task was measured by the number of visits to the rewarded location during sessions in which the reward was absent for the first trial**. CA1 inhibition resulted in a memory deficit, but not in a navigational deficit during the updating period**. These results suggest that navigational and mnemonic functions of the hippocampus may be dissociated in this task.

A possible explanation on the lack of effect on navigation when inhibiting the hippocampus is that **the mice's behaviour within the task is not entirely goal-**

oriented. The strategy used to solve this task was mathematically modelled and revealed two main components driving behaviour, one reflecting learning of the environment and a strong random component which did not decrease over time. If animals are not perfectly planning their trajectories to the goal location, they may be using different strategies which rely on brain areas different to hippocampus. **Having a spatial task that does not depend on the hippocampus for navigation would be enormously beneficial for further dissecting complex semantic-like memory processes in rodents without considering navigation as a variable**.

This thesis began by introducing key areas such as the hippocampus and neocortex, followed by concepts like navigation and different memory theories. Next, the task was presented, identifying three phases of learning and introducing the concept of previous knowledge. Delving deeper, a literature review on schemas is provided in Chapter 3. From the behavioral output in Chapter 2, a mathematical model describing the navigation strategies of the mice was developed, highlighting that mice do not exhibit completely goal-oriented behavior. Only male mice were used in this research, and the reasons for this choice are discussed in the interlude, which highlights the influence of sex and menstrual phase on memory processes. Returning to the main thesis topic, Chapter 5 presents the manipulation of three key brain areas—CA1 area of the hippocampus, prelimbic cortex, and retrosplenial cortex—as well as an analysis of the encoding networks at three learning stages. Next, I will discuss the main findings of my thesis.

Three stages of learning

The human learning process associated with semantic memory is characterized by gradual development over extended periods (Squire, Genzel et al. 2015). Semantic memory structures store common experiences slowly built up over time and require repeated reactivations to induce and maintain plasticity**. By remaining plastic, these memory structures allow for new similar experiences to be quickly assimilated into the activated cortical network** (Basu and Siegelbaum 2015).

Spatial memory in rodents is normally used as a model for cognition, and to study memory, tests which require map learning are used. A cognitive map aids in the navigation, and motivated animals may use it to reach a reward faster (Tolman 1948). **I posit that mice in my task build a mental map of the experimental environment over several exposures, and use it to guide navigation towards a reward in an allocentric fashion**. Hungry, motivated mice expressed long-term memory by repeatedly visiting the rewarded location during probe trials, when the reward was absent. Abrupt changes in the reward location initially took a few re-exposures for the animals to adapt, but after three months in training, a single exposure to a new goal location was enough for the animals to express long-term memory on top of stable performance by the following session.

The experience from the first training session to three months into training can be dissected into three learning stages, and will be discussed at behavioral, histological and network levels.

Initial goal location learning

During this phase, animals required three or more training sessions to decrease the length of their paths to the goal. This is comparable to reference memory, where **navigation to a fixed location becomes more efficient day by day**, in this case, within session and on the first trial. This measure of memory through navigation has been evaluated repeatedly in tasks such as radial arm maze (Olton, Collison et al. 1977) and the water maze (Morris 1981).

Modelling of the animal behavior describes their movements based on two factors, a random component with a probability to take long diagonal runs, plus a learning component, which is the probability for the animal to perform a direct run to the goal from an ever increasing distance, named foresight. During build-up, foresight was at a level of zero for the first training trial of the first training session, but increased within and across sessions, implying that **animals are performing direct runs from further away as they become more familiar with the testing environment**.

The population of cells active during the first training day was identified by labeling cFos positive cells, an immediate early gene attributed to neural activity. The relative amount of cells active was not statistically different to control animals. There was a slight hint of increased expression in hippocampal, retrosplenial and posterior parietal cortex. Other reference memory studies show similar results, **with low levels of cFos expression overall on the first session** (Barry, Coogan et al. 2016). Unless something very novel happens, memories of the experiences of the day are encoded with a baseline synaptic weight (Duszkiewicz, McNamara et al. 2019). As these animals had been habituated to the environment prior their first training, a dopamine-driven novelty effect would be missing to drive a surge of expression of immediate early genes. Additional experiments are currently being run to capture what this effect would be like.

At network level, there was a **strong connectivity of the retrosplenial cortex with CA1, CA3 and posterior parietal cortex, as well as a strong connection among prelimbic and anterior cingulate cortex**, seen in correlation analysis of cFos expression. Similar results have been reported in the paired associates task (Takeuchi, Tamura et al. 2022), which required rats to learn a novel set of flavor-place associations, although they didn't observe prefrontal correlations.

The relevance of areas CA1, prelimbic and retrosplenial cortex during the first stage of learning was evaluated by local inhibition of AMPA receptors during sessions two and four. A slight decrease in long-term memory expression was observed when inhibiting hippocampus area CA1, prelimbic or retrosplenial cortex. CA1 inhibition caused an impairment in overall navigational performance during the initial week, however this result could be confounded by the control group, which performed exceptionally well.

In the water maze, hippocampal damage prevents animals from finding the platform location, no matter how much training the animals received prior their lesion (Morris, Garrud et al. 1982). **It is not known however if this effect is due to a navigational impairment or to a memory impairment**. Importantly the navigational load required for rats in the water maze differs greatly from the mice in the HexMaze during probe trials, as it is harder to stay in a particular location for rats while they are swimming. It is argued that rats would need to be constantly needing to path integrate as they continue swimming past the absent platform location (Benhamou 1997). Path integrations refers to the ability to navigate based on an initial reference and self-motion information (McNaughton, Battaglia et al. 2006). Others may argue that relevant spatial information is stored in the hippocampus itself, which would prevent for the memory to be expressed in its absence (Squire, Genzel et al. 2015). These results further support role of hippocampus in forming a cognitive map.

New goal location learning after two weeks

A sudden change in the reward location forced the animals to adapt their strategy from their past weeks. **After an initial session where performance dropped to the level expressed on day one, by the following session they took shorter routes**. However, when probed for long-term memory, they showed no preference to the location of the reward.

This learning stage can be compared to **reversal learning** in the water maze, where learning of a new platform location does not require the animal to learn the complete procedure all over again. Reversal learning refers to behavioral flexibility when there is a conflict in the expected outcome. A switch of the platform location is quickly learned by rats in the water maze, showing behavioral flexibility (Manning, Bradfield et al. 2021). This can also be interpreted as a reconsolidation process, where a memory is reactivated, switching into a labile state (Nader and Hardt 2009).

Histological analysis of cells expressing cFos on the day of the goal location switch showed a trend of slightly higher expression in cortical areas with a slight decrease in hippocampal areas, although not statistically different.

The use of in vivo labelling of active cells with the technique of TRAP2 (DeNardo, Liu et al. 2019) allowed me to test co-allocation of two memories, in this case, the second day of training (phase 1, second day after the first goal location presentation, GL1 S2) versus the eighth day of training (phase 2, first day of the new goal location presentation, GL2 S1). **I was able to show that a large fraction of the cells active on the initial memory engram were also active during the encoding the memory of the new goal location, implying that the initial memory structure is being used to encode new related memories, something never shown before in a spatial task**. Similar results have been shown in engram tagging experiments, comparing extinction and reconsolidation in fear expression research (Luft, Popik et al. 2024). Extinction of a fear memory by prolonged exposure to the conditioned stimulus is thought to be mediated by suppression from a different set of amygdala neurons, while reconsolidation by a brief exposure to the conditioned stimulus recruits the original engram and updates it, which is what would be happening when learning new goal locations.

A contextual fear paradigm study using TRAP2 animals (DeNardo, Liu et al. 2019), TRAPed cells on the day of encoding, or at three different recall timepoints, to later compare them to remote recall 28 days after encoding. They found that the cells TRAPed at later timepoints were more likely to be reactivated during the 28-day memory retrieval. In this case they compared retrieval processes instead of the encoding of new memories.

At network level, there was stronger correlation among most brain areas analyzed, with the exception of dentate gyrus that had weaker correlations. As the dentate gyrus is involved in formation of new spatial representations, pattern separation and differentiating subtle differences in the environment (Leutgeb,

Leutgeb et al. 2007), its relevance at this stage of the task would be decreased, as animals are becoming increasingly familiar to the testing environment.

Frontal and posterior cortices connectivity was also seen at this point. This could be indicating that an **extended network** has developed. The idea of a distributed memory network making memories more resilient to cortical damage (Fuster 1998) has also been tested with TRAP2 experiments. Tonegawa's group (Roy, Park et al. 2022) showed that a contextual fear conditioning memory activated several brain regions which had not been reported yet, and further optogenetic and chemogenetic manipulations of one or more of these regions elicited freezing behaviour. The more areas that were activated, the greater the response of memory recall.

I did not perform local inhibition on the day of encoding a new goal location in the build-up period, but I did inhibit sessions eight and ten in a group of mice which kept the same goal location for three months. At this stage, hippocampal, prelimbic nor retrosplenial cortices were not essential for overall navigational performance or long-term memory. These results suggest that as memory becomes more widely distributed, **disruption of a single hub is not enough to disrupt complex behavior**.

Twelve weeks after the start of training

In this stage, animals had already experienced multiple goal location changes, and **only needed a single session to reach stable performance after introducing a new goal location, also leading to long-term memory**, as observed during the probe trial by the persistence of the animal to visit the last introduced goal location.

To reach this point, the prior three months of build-up were important as a timeframe, rather than the frequency of the trainings. This was evidenced by comparing two groups with different training schedules, twice a week or three times a week. Already **by week 6, these two groups performed equally, even with a difference in the number of trainings sessions between these groups, although not yet optimally.** By the time the update phase started, both groups were performing optimally. This showed that the gradual build-up of a memory structure depended on time elapsed and not necessarily the number of experiences. This is reminiscent to findings in the water maze with a massed versus a spaced schedule. Massed means that there are very short intertrial intervals, while spaced, longer intertrial intervals. Massed training generated a strong initial learning, however spaced created a longer lasting memory (da Silva, Bast et al. 2014, Nonaka, Fitzpatrick et al. 2017) implying a difference of an initially strong episodic memory that fades if not reactivated, versus a stronger semantic memory built over time.

This stage of the learning process may be compared to schema memories, as tested with the paired associates task (Tse, Langston et al. 2007), where a set of flavour-place associations was used to evaluate memory performance. After a build-up period, two new paired associates were presented, and rats were able to recall these within 48 hours in absence of a functional hippocampus. However, they could not learn these new associations successfully if the hippocampal function was absent at the moment of encoding (Tse, Takeuchi et al. 2011). If the prelimbic cortex was damaged, memory expression was also impaired. **I was able to replicate rapid systems consolidation in the mouse HexMaze task, confirming that this novel task can be used to study schema memory**. In contrast, prelimbic inhibition did not impair the behaviour in mice performing the mouse HexMaze, which could be due to the different nature of these two tasks.

In Tse's study, the mental representation guiding navigation involved an association of flavour to a place within a square arena, while in the HexMaze a single location must be reached within a gangway arrangement. This could be compared to human studies where object-word pairs or scene-word pairs must be remembered. The object-word association would be similar to flavour in a place, while scene word would be comparable to recognizing spatial cues in respect to a goal. **In the human study they found that object-word pairs increases connectivity between prefrontal cortex and hippocampus, versus scene-word pairs increases connectivity between retrosplenial cortex and hippocampus** (Cowan, Liu et al. 2020, Navarro-Lobato and Genzel 2020). However, inhibiting the retrosplenial cortex did not impair navigation in the mouse HexMaze. This lack of effect could be due to the need of inhibiting larger portion of the retrosplenial cortex, or to the distributed nature of the memory by the time that the interventions take place.

The mathematical model at this stage showed no change in the chance of random diagonal runs. **The foresight component**, indicating the probability of performing direct runs to the goal location, with the exception of the first trial after the goal location switch, had a **steady strong influence on the behaviour**.

Immediate early gene analysis at this stage showed **elevated cFos expression in the prelimbic cortex, an area commonly associated with schema memory**, in humans (van Kesteren, Fernandez et al. 2010) and rats (Tse, Takeuchi et al. 2011). Overlap analysis comparing cells TRAPed on the second day of training to the cells that encode a new goal location three months into training, showed that a large portion of the original memory engram was recruited in all brain areas studied, with the exception of dentate gyrus. Again, it has never been reported that the initial memory engram is used for encoding a new related memory, three months after the first exposure to the maze.

The recruitment of most of the same initial memory engram 12 weeks later also provides evidence **against the representational drift theory** (Driscoll, Duncker et al. 2022), which posits that as weeks pass, correlations between neural activity and external variables "drift" or change.

Connectivity analysis showed a very **strongly correlated network of all brain areas studied**, leaving dentate gyrus completely out of the network. This disengagement of the dentate gyrus was also observed in another task of our lab, the object space task, in semantic-like memory processes (unpublished data), probably due to the a reduced need in detailed representations by this stage of learning.

The accelerated rate of learning new goal locations could be due to new related experiences being directly encoded into the cortex, or that the consolidation process is accelerated. To test this, inhibition took place on the day of encoding a new goal location or on the day of retrieval of a recently learned goal location. **CA1 inhibition on the day of encoding impaired long-term memory expression 48 hours later, but not overall navigation performance**, while inhibition on the day of retrieval had no negative effect on memory or navigation. Immediate early gene analysis showed that encoding of new related memories on later stages engaged the prelimbic cortex, favouring both ideas of accelerated consolidation and direct cortical coding. As discussed in chapter 3, a possibility is that hippocampal involvement depends on the amount of previous knowledge, a continuous gradient where increased prior knowledge leads to faster, more efficient memory processes shifting from hippocampus to cortex.

With the mouse HexMaze, these three memory types can be studied, which contributes to the need in research for testing more naturalistic memory processes, in the absence of fear or stress, and over extended periods. However, the behavior observed in mice is not completely goal oriented, as it was confirmed by quantitative analysis of behavior, compared to optimal goal-directed behavior. The model simulated the trajectories a virtual agent would take in the maze, to isolate the specific contributions to the navigational strategy. The results revealed a mixture of trials with directed runs to the reward, and trials with explorative walks. There is a goal-directed behaviour, accompanied by an interfering random component which does not decrease as learning progresses. By increasing the availability of choice, other non-task related behaviors may emerge, hence why the overall behaviour of the animals is not optimal. **Exploitation vs exploration behavior** has also been reported in foraging tasks (Jackson, Fatima et al. 2020) and in larger more complex labyrinths (Rosenberg, Zhang et al. 2021), where the goals of an animal extend beyond merely reaching food or water.

While the goal was to provide a more naturalistic testing environment for rodents, and the gangways of the mouse HexMaze may resemble the "rodent highways" on which rodents move when outside their burrows. As it is made from white acrylic and lacks any form of ceiling, it could feel like a threat to the animal, having no hiding or resting place. Perhaps if choice points were sheltered, a more goal-directed behaviour could be seen. Experimental mice have been reported to move quickly, suggesting less deliberate trajectories and continuously in movement (Jones, Paul et al. 2017), even in familiar environments (Gire, Kapoor et al. 2016). **The smaller size of mice, which makes them more vulnerable to predators, may have driven their evolutionary tendency to maintain erratic movements as a survival strategy**. In contrast, unpublished results from our lab, from the rat HexMaze, reveal differences between these two species. Rats, when placed in the maze, tend to pause and observe their surroundings (possibly exhibiting vicarious trial and error behaviour (Gire, Kapoor et al. 2016)) before commencing their movement. They are slower initially but, once on the correct path, can rapidly sprint toward the goal.

Initial experiments with the HexMaze included the use of barriers during the update phase to block certain pathways to evaluate flexibility. Changing the goal location plus adding barriers caused animals performance to drop to initial levels for the first trial, but improved performance from the second trial onward and across sessions. Only adding barriers and keeping the previous week's goal location did not negatively impact their performance. Further tests were not applied to the barrier only condition tested in chapters 3 and 4. **The lack of effect of barrier placement** in their navigational performance could be due to the animals strategy being heavily influenced by the random component, and only showing goal-directed behaviour when in the proximity of the goal. However, unpublished results of the rat HexMaze, also show little to no effect in behaviour when barriers are introduced, even considering that their behaviour is more goal-oriented than mice.

An **alternative interpretation** of the behaviour of this task is that once the cognitive map is formed, the navigational strategies are reward based (Sosa and Giocomo 2021) having dopamine as a strong neuromodulator. Both hippocampus and entorhinal cortex are strongly modulated by dopamine, in the sense of reward and also of novelty (Duszkiewicz, McNamara et al. 2019). It could be that there is a re-assignation of valences respect to the goal location which is driving navigation. It is likely that in the barrier conditions, only local changes in geometry are needed, instead of valence re-assignation.

Another complex spatial task, also made from hexagonal units, measured dopamine levels during decision making while foraging for rewards in a changing environment (Krausz, Comrie et al. 2023). They showed dopamine pulses at reward receipt, and a ramp-up when rats discover new available paths, but not when finding a newly blocked path, implying that dopamine is signalling some sort of error signal, beyond unexpected stimuli. In such complex environments such as this, dopamine conveys place values, updated by different learning strategies. To further evaluate the similarity to the mouse HexMaze, experiments measuring dopamine levels would be needed.

This novel goal-reaching task was evaluated at behavioural level, revealing three stages of learning. Importantly it allows to study schema memory in rodents, as I was able to show **rapid systems consolidation**. Pharmacological manipulations revealed a low impact in behaviour when inhibiting CA1 region of the hippocampus, prelimbic and retrosplenial cortices, indicating that the ability to navigate to reach a goal is not dependent on a finite brain structure. The ability to encode new memories however, is dependent on hippocampus even during the latest stage of the experiment. Histological analysis of the engram showed that animals recruited a large part of the initial memory engram to encode new task-congruent memories. Next I will discuss further the results in respect to the results found with hippocampal manipulations to try and further understand its function.

Hippocampal dependency on navigation and memory functions

Traditional memory consolidation theories posit that encoding and cortical consolidation would take an extended period of time, and that it would always involve the hippocampus, as semantic memories can also contain episodic elements (Moscovitch, Rosenbaum et al. 2005). With the mouse HexMaze, I was able to show that hippocampal area CA1 was necessary to encode a memory, no matter how well established a memory network was, but not necessary for retrieval 48 hours after. I was expecting to see a greater impairment in performance during build-up when the hippocampus was inhibited, however this was not the case. Instead, **a navigational task which seems to be relatively hippocampal independent, still needed the hippocampus for rapid updating during the schema phase**. While memory processes were altered by hippocampal inhibition, navigational abilities were not, suggesting a disconnect between the hippocampal area CA1's roles in memory processing versus navigation. This dissociation is evident when considering the specific functions related to task-solving processes, such as path computation and pattern completion, compared to memory functions.

It's worth noting the antiparallel with previous research involving the water maze and its continued reliance on the hippocampus even after weeks of training (Morris, Garrud et al. 1982), which also raises questions about the nuances of path computation and potential confounding factors (Squire, Genzel et al. 2015). In the water maze, when rats are probed, path integration is constantly required due to the need to keep swimming once passing the location of the absent platform, hence the hippocampus would be constantly engaged (Benhamou 1997). In the paired associates task (Tse, Langston et al. 2007), animals retrieve their reward by digging in the same spot, hence further path computation is not necessary, and recognition memory can suffice. In the case of the mouse HexMaze, once animals have experienced several food location changes, if the reward is absent they continue searching the maze, but return more often to where the reward was found in the prior session, as well as the location from the previous week. This evidences that **the memory of the new goal location is not overwritten, rather added onto the memory network**. If the hippocampus is inactive during the encoding process of a new goal location, mice are not able to express long-term memory during their probe, but once they are rewarded, for the rest of the session animals return to the rewarded node, evidencing an online improvement of performance, likely using working memory. It's important to note that this task engages various memory levels, ranging from temporal aspects like spatial working memory to more complex constructs like allocentric spatial memory and long-term memory.

The concept of path computation (i.e. path integration), which involves how animals navigate within an environment based on an initial spatial reference and self-motion information (McNaughton, Battaglia et al. 2006), can be assessed by examining their overall performance. In my research, a subtle decline in performance, particularly in the Build-Up group when inhibiting the hippocampal region, may indicate an initial reliance on cognitive map formation. There is also a small effect on long-term memory expression observed across all three brain areas studied. This effect may be confounded by the animals not performing optimally at this stage yet. As weeks pass and memory becomes stronger, the observed decrease in memory effect diminishes. During the update phase, 12 weeks into training, when a new goal location is introduced, the memory function becomes more critical. Inhibiting the hippocampus results in animals struggling to remember the exact location of the new goal, indicating the hippocampus's role in encoding, importantly **that rapid learning is a hippocampal dependent phenomenon**, but the memory is quickly stored extra-hipocampally as its inhibition doesn’t impede memory expression 48 hours later. On the other hand, the navigation function during the build-up period is initially slightly impaired when the hippocampus is inhibited, but by week three, inhibition of the hippocampus did not significantly impact their overall performance.

A possibility could be that **the navigational strategy as a motor function may be executed independently by the striatum**, while the memory component of encoding of new related memories remains hippocampal dependent. The striatum is a central hub for motor memory, routines, skills, and reward-based learning (Penner and Mizumori 2012). It integrates sensory, contextual and motivational information, enabling flexible navigational behaviors. Receiving inputs from all cortical areas, as well as from CA1/subiculum, the striatum is engaged in goal-directed behaviors and strongly modulated by dopamine. However, to conclusively answer this question we would need to perform further experiments involving striatum.

Conclusion

This thesis enhances our understanding of the neurobiology of schemas in memory consolidation by examining the brain areas involved in building and updating previous knowledge networks within the context of spatial memory. Using the mouse HexMaze, I identified three learning phases: initial goal location learning, faster learning after two weeks when a new goal location was introduced, and one-session learning after twelve weeks leading to long-term memory. CA1 hippocampal inhibition resulted in a memory deficit but did not affect navigation, suggesting a dissociation between navigational and mnemonic functions of the hippocampus.

This research proposes a gradient schema theory, where the amount of prior knowledge influences hippocampal involvement in memory processes. Extensive prior knowledge facilitates rapid cortical consolidation with minimal hippocampal participation. Findings validate the hypothesis that learning is flexibility, with increased plasticity allowing for adaptation and integration of new experiences; that schema memories span the entire brain, with distributed networks providing higher flexibility and robustness; and that accelerated consolidation in the presence of prior knowledge requires the hippocampus during encoding, but not for navigation. These insights emphasize the importance of rich experiential backgrounds in modeling adult human memory processes in rodents and demonstrate the value of the HexMaze for studying complex memory processes in mice over extended periods.

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Appendices

Summary

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Dankwoord

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Summary in English

This thesis contributes to a deeper understanding of the neurobiology of schemas in memory consolidation. Schemas, or previous knowledge networks, expedite learning by facilitating consolidation. **I investigated which brain areas are essential for building and updating these networks in the context of spatial memory**, and which are associated with encoding and retrieving goal information when prior knowledge is present. Additionally, I examined the task-solving strategies used by animals in a specific maze.

In the first chapter, the introduction starts by highlighting **anatomical properties** of the hippocampus and neocortex. Their **cognitive functions**, such as learning, memory and navigation are also introduced, followed by a summary on existing theories on systems memory consolidation.

The mouse HexMaze, described in detail in Chapter 2, was the foundation for the following experiments. The HexMaze is a behavioral task developed at the Genzel lab to investigate map learning in mice. **This goal-localization paradigm incorporates extended spatial and temporal dimensions, allowing for the study of various memory types, including schema memory**. The HexMaze combines opportunities for complex behaviors with precise monitoring and quantification of navigational choices, making it ideal for characterizing animal spatial cognition. The task involves allocentric navigation towards a reward, where reward locations and routes within the maze are periodically changed, forcing the animals to adapt their navigation strategies. I showed that mice are able to quickly adapt to reward location changes. On the first trial of the following session, animals express memory of the recently learned location, suggesting long-term memory gain by this phase.

I identified three main learning phases in the HexMaze: (1) learning the initial goal location, (2) faster learning after two weeks when learning a new goal location, and (3) a phase after twelve weeks during which mice exhibited one-session learning leading to long-term memory. The gradual development of a mental map of the maze depended on the time elapsed rather than the number of experiences, as mice trained twice and three times a week learned at the same pace.

To further comprehend the concept of previous knowledge or schemas, a literature review in Chapter 3 described the history and development of this topic. **We proposed a new theory based on a gradient of knowledge, where the more**

previous knowledge is available, the less critical the hippocampus is for memory encoding. This gradient schema theory suggests that the amount of prior knowledge influences the involvement of the hippocampus in memory processes. Most animal memory research is positioned at the low end of this gradient, with new memories relying on minimal prior knowledge. Unique and emotionally-arousing experiences lead to strong, lasting hippocampal memories, while less unique events consolidate more slowly to the cortex, losing episodic detail over time. In intermediate phases, some prior knowledge allows for faster hippocampal updating of cortical networks. At the high end of the gradient, extensive prior knowledge results in rapid cortical consolidation with minimal hippocampal involvement. This theory posits a continuous gradient where increased prior knowledge leads to faster, more efficient memory processes shifting from the hippocampus to the cortex.

In Chapter 4, a computational model was created to understand how mice navigate through the maze and their possible strategies. This model mimics behavioral data at different stages and provides a detailed analysis of the animals' performance within sessions. A simulated virtual agent navigates node by node in search of a reward, with the goal of taking the shortest path possible. The rules comprise two elements: the probability of taking a direct pathway to the goal location, named Foresight, and a random movement, with a probability of taking long diagonal runs. We statistically characterized the navigational patterns of the mice, comparing them to optimal goal-oriented paths. **Results showed a clear learning effect: mice optimized their trajectories over time, reducing the time and number of nodes visited to reach the reward**. Path improvements followed distinct time courses: steady improvement on the first trial of each session, faster improvement in later sessions, enhanced improvement with novel reward locations, and minimal impact from path-blocking barriers when the reward location was familiar. This trajectory sharpening supports the presence of different forms of prior knowledge enhancing task performance, influenced by allocentric representation, cue-linking to goal proximity, and memory strengthening.

An interlude on the effects of sex and menstrual phase on sleep and memory highlights why only male animals were used in this study.

In Chapter 5, pharmacological and molecular techniques are used in the mouse hexmaze to further comprehend the neurobiology of schemas. Using pharmacological manipulations, I found that building up map learning in the context of spatial memory did not rely on hippocampal region CA1, prelimbic, or retrosplenial cortex. **Once the memory structure was established, fast updating was hippocampal-dependent, followed by rapid consolidation, as retrieval 48 hours post-encoding proved hippocampal-independent**. The prelimbic cortex was not necessary for encoding or retrieving goal information, as local inhibition did not impair task performance. However, activity-related protein expression in the prelimbic cortex gradually increased over time during training. The retrosplenial cortex was not critical during early or late learning for navigation or memory, but was engaged during task performance, evidenced by increased activity-related protein expression. All three brain areas—the hippocampus, prelimbic, and retrosplenial cortex—formed a distributed memory network, with correlation analysis indicating strengthening connectivity among these areas over the training period.

In Chapter 6, an overview of the thesis is presented and the main results are discussed. Overall this thesis enhances our understanding of the neurobiology of schemas in memory consolidation by examining the brain areas involved in building and updating previous knowledge networks within the context of spatial memory. Using the mouse HexMaze, I identified three learning phases: initial goal location learning, faster learning after two weeks when a new goal location was introduced, and one-session learning after twelve weeks leading to long-term memory. CA1 hippocampal inhibition resulted in a memory deficit but did not affect navigation, suggesting a dissociation between navigational and mnemonic functions of the hippocampus.

This research proposes a gradient schema theory, where the amount of prior knowledge influences hippocampal involvement in memory processes. Extensive prior knowledge facilitates rapid cortical consolidation with minimal hippocampal participation. Findings validate the hypothesis that learning is flexibility, with increased plasticity allowing for adaptation and integration of **new experiences; that schema memories span the entire brain, with distributed networks providing higher flexibility and robustness; and that accelerated consolidation in the presence of prior knowledge requires the hippocampus during encoding, but not for navigation**. These insights emphasize the importance of rich experiential backgrounds in modeling adult human memory processes in rodents and demonstrate the value of the HexMaze for studying complex memory processes in mice over extended periods.

Samenvatting in het Nederlands

Deze thesis draagt bij aan een dieper begrip van de neurobiologie van schema's bij geheugenconsolidatie. Schema's, ofwel eerder bestaande kennissystemen, versnellen het leren door consolidatie te vergemakkelijken. Ik onderzocht welke hersengebieden essentieel zijn voor het opbouwen en bijwerken van deze netwerken in de context van ruimtelijk geheugen, en welke geassocieerd zijn met het coderen en ophalen van doelinformatie wanneer eerdere kennis aanwezig is. Bovendien bestudeerde ik de taakoplossingsstrategieën die dieren gebruiken in een specifieke doolhof.

In het eerste hoofdstuk wordt in de inleiding de anatomische eigenschappen van de hippocampus en neocortex belicht. Hun cognitieve functies, zoals leren, geheugen en navigatie, worden ook geïntroduceerd, gevolgd door een samenvatting van bestaande theorieën over systeemgeheugenconsolidatie.

De muis HexMaze, in detail beschreven in Hoofdstuk 2, vormde de basis voor de volgende experimenten. De HexMaze is een gedragstaak ontwikkeld in het Genzel-lab om kaartleren bij muizen te onderzoeken. Dit doel-lokalisatie paradigma omvat uitgebreide ruimtelijke en temporele dimensies, waardoor de studie van verschillende geheugentypen, inclusief schema-geheugen, mogelijk is. De HexMaze biedt mogelijkheden voor complexe gedragingen met precieze monitoring en kwantificering van navigatiekeuzes, wat het ideaal maakt voor het karakteriseren van ruimtelijke cognitie bij dieren. De taak omvat allocentrische navigatie naar een beloning, waarbij beloningslocaties en routes binnen de doolhof periodiek worden veranderd, waardoor de dieren gedwongen worden hun navigatiestrategieën aan te passen. Ik toonde aan dat muizen zich snel kunnen aanpassen aan veranderingen in beloningslocaties. Tijdens de eerste poging van de volgende sessie tonen de dieren geheugen van de recent geleerde locatie, wat suggereert dat langetermijngeheugen in deze fase is verworven.

Ik identificeerde drie hoofdleerfasen in de HexMaze: (1) het leren van de initiële doel locatie, (2) sneller leren na twee weken bij het leren van een nieuwe doel locatie, en (3) een fase na twaalf weken waarin muizen eenmalig leren wat leidt tot langetermijngeheugen. De geleidelijke ontwikkeling van een mentaal kaart van de doolhof hing af van de verstreken tijd in plaats van het aantal ervaringen, aangezien muizen die twee en drie keer per week werden getraind in hetzelfde tempo leerden.

Om het concept van eerdere kennis of schema's beter te begrijpen, beschrijft een literatuurstudie in Hoofdstuk 3 de geschiedenis en ontwikkeling van dit onderwerp. We stelden een nieuwe theorie voor gebaseerd op een kennisgradiënt, waarbij hoe meer eerdere kennis beschikbaar is, hoe minder cruciaal de hippocampus is voor geheugen codering. Deze gradiëntschema theorie suggereert dat de hoeveelheid eerdere kennis de betrokkenheid van de hippocampus in geheugenvormen beïnvloedt. De meeste diergeheugenonderzoeken bevinden zich aan de lage kant van deze gradiënt, waarbij nieuwe herinneringen afhankelijk zijn van minimale eerdere kennis. Unieke en emotioneel opwindende ervaringen leiden tot sterke, blijvende hippocampale herinneringen, terwijl minder unieke gebeurtenissen langzamer naar de cortex worden geconsolideerd en na verloop van tijd episodische details verliezen. In tussenfasen stelt enige eerdere kennis de hippocampus in staat om sneller corticale netwerken bij te werken. Aan de hoge kant van de gradiënt resulteert uitgebreide eerdere kennis in snelle corticale consolidatie met minimale hippocampale betrokkenheid. Deze theorie postuleert een continue gradiënt waarbij toegenomen eerdere kennis leidt tot snellere, efficiëntere geheugenvormen die verschuiven van de hippocampus naar de cortex.

In Hoofdstuk 4 werd een computationeel model gecreëerd om te begrijpen hoe muizen door de doolhof navigeren en hun mogelijke strategieën. Dit model bootst gedragsgegevens op verschillende stadia na en biedt een gedetailleerde analyse van de prestaties van de dieren binnen sessies. Een gesimuleerde virtuele agent navigeert knooppunt per knooppunt op zoek naar een beloning, met als doel de kortste weg mogelijk te nemen. De regels omvatten twee elementen: de waarschijnlijkheid van het nemen van een directe route naar de doellocatie, genaamd Vooruitziendheid, en een willekeurige beweging, met een waarschijnlijkheid van het nemen van lange diagonale runs. We karakteriseerden statistisch de navigatiepatronen van de muizen en vergeleken deze met optimale doelgerichte paden. Resultaten toonden een duidelijk leereffect: muizen optimaliseerden hun trajecten in de loop van de tijd, waarbij de tijd en het aantal bezochte knooppunten om de beloning te bereiken werden verminderd. Verbeteringen in het pad volgden verschillende tijdsverlopen: gestage verbetering bij de eerste poging van elke sessie, snellere verbetering in latere sessies, verbeterde verbetering met nieuwe beloningslocaties en minimale impact van pad-blokkerende barrières wanneer de beloningslocatie bekend was. Deze trajectverfijning ondersteunt de aanwezigheid van verschillende vormen van eerdere kennis die de taakprestaties verbeteren, beïnvloed door allocentrische representatie, cue-koppeling aan doel nabijheid en geheugenversterking.

Een intermezzo over de effecten van geslacht en menstruatiecyclus op slaap en geheugen benadrukt waarom in deze studie alleen mannelijke dieren werden gebruikt.

In Hoofdstuk 5 worden farmacologische en moleculaire technieken gebruikt in de muis HexMaze om de neurobiologie van schema's beter te begrijpen. Met behulp van farmacologische manipulaties ontdekte ik dat het opbouwen van kaartleren in de context van ruimtelijk geheugen niet afhankelijk was van de hippocampale regio CA1, de prelimbische of retrospleniale cortex. Zodra de geheugenstructuur was vastgesteld, was snelle bijwerking hippocampus-afhankelijk, gevolgd door snelle consolidatie, aangezien ophalen 48 uur na codering hippocampus-onafhankelijk bleek. De prelimbische cortex was niet noodzakelijk voor het coderen of ophalen van doelinformatie, aangezien lokale inhibitie de taakprestatie niet verstoorde. Echter, activiteit-gerelateerde eiwitexpressie in de prelimbische cortex nam geleidelijk toe in de loop van de training. De retrospleniale cortex was niet cruciaal tijdens vroeg of laat leren voor navigatie of geheugen, maar was betrokken bij taakprestatie, zoals bleek uit verhoogde activiteit-gerelateerde eiwitexpressie. Alle drie hersengebieden - de hippocampus, prelimbische en retrospleniale cortex - vormden een gedistribueerd geheugennetwerk, met correlatieanalyse die versterkte connectiviteit tussen deze gebieden gedurende de trainingsperiode aangaf.

In Hoofdstuk 6 wordt een overzicht van de thesis gepresenteerd en worden de belangrijkste resultaten besproken. Over het algemeen verbetert deze thesis ons begrip van de neurobiologie van schema's bij geheugenconsolidatie door de hersengebieden te onderzoeken die betrokken zijn bij het opbouwen en bijwerken van eerder bestaande kennisnetwerken binnen de context van ruimtelijk geheugen. Met behulp van de muis HexMaze identificeerde ik drie leerfasen: initiële doel locatie leren, sneller leren na twee weken wanneer een nieuwe doel locatie werd geïntroduceerd en eenmalig leren na twaalf weken leidend tot langetermijngeheugen. Inhibitie van de CA1 hippocampus resulteerde in een geheugenverlies maar had geen invloed op navigatie, wat duidt op een dissociatie tussen navigatie- en geheugenfuncties van de hippocampus.

Dit onderzoek stelt een gradiëntschema theorie voor, waarbij de hoeveelheid eerdere kennis de hippocampale betrokkenheid in geheugenvormen beïnvloedt. Uitgebreide eerdere kennis faciliteert snelle corticale consolidatie met minimale hippocampale deelname. Bevindingen valideren de hypothese dat leren flexibiliteit is, met toegenomen plasticiteit die aanpassing en integratie van nieuwe ervaringen mogelijk maakt; dat schemaherinneringen zich over de hele hersenen uitstrekken, met gedistribueerde netwerken die hogere flexibiliteit en robuustheid bieden; en dat versnelde consolidatie in aanwezigheid van eerdere kennis de hippocampus tijdens codering vereist, maar niet voor navigatie. Deze inzichten benadrukken het belang van rijke ervaringsachtergronden bij het modelleren van volwassen menselijke geheugenprocessen in knaagdieren en demonstreren de waarde van de HexMaze voor het bestuderen van complexe geheugenprocessen bij muizen over langere perioden.

Curriculum Vitae

Alejandra Alonso received her Bachelor's degree in Biochemistry in 2017 from Pontificia Universidad Católica de Chile. During her undergraduate studies, she completed a series of internships that shaped her research trajectory. Her first internship was conducted in a neuroanatomy lab, where she focused on labeling potassium channels within the dopaminergic system. For her second internship, she joined an electrophysiology lab, where she performed optogenetic manipulations alongside simultaneous electrophysiological recordings. This work culminated in her undergraduate thesis, which explored the optogenetic inhibition of somatostatin cells in the basal forebrain and their effects on cortical dynamics. The findings from this research were published and subsequently facilitated her admission to a doctoral program.

In 2018, Alejandra was selected for the prestigious M-GATE programme, supported by the Marie Skłodowska-Curie Actions, and commenced her PhD at the Donders Institute under the supervision of Dr. Lisa Genzel. Her doctoral research centered on investigating the influence of prior knowledge on learning and memory. Additionally, she collaborated on a calcium imaging project under the supervision of Dr. Francesco Battaglia. During the COVID-19 pandemic, she completed an online secondment at Dr. Hugo Spiers' lab, where she acquired foundational coding skills in MATLAB. The M-GATE programme also allowed her to establish an extensive professional network across the European scientific community.

Throughout her PhD, Alejandra mentored over 40 Bachelor's and Master's students, guiding them through both experimental projects and literature review internships. In recognizing the challenges of conveying complex scientific concepts, she developed a passion for scientific illustration. This interest led her to enroll in a Master's program in Creative Illustration at BAU, Barcelona, with the intention of pursuing a career in scientific illustration following the completion of her PhD. She is currently a Post doc at the Genzel Lab following up research related to this project, as well as continuing her work in illustration through her small business, A. elegans Design and Illustration.

Publications

Main author

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Co-author

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Manuscripts in preparation

“Molecular and pharmacological techniques in the mouse HexMaze” A. Alonso, L. v. d. Brand, A. Samanta, J. v.d. Meij, L. Genzel

Research Data Management

This thesis research has been carried out under the institute research data management policy of the Donders Institute for Brain, Cognition and Behavior. This research followed the applicable laws and ethical guidelines. Research Data Management was performed according to the FAIR principles. The information below details how this was achieved.

Ethical Approval

This thesis is based on the results of animal studies, which were conducted in accordance with the European, Dutch and local regulations on the basis of the DEC Projects 2016-014 and 2020-0020. The local Animal Welfare Body has approved the protocols for the present project 2016-014-018, 2016-014-034 and 2020-0020-009. This research was funded by M-GATE project which receives funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 765549, the Branco Weiss Fellowship Society in Science and European Union's Horizon 2020 Research, Innovation Programme under Grant Agreement 840704 (BrownianReactivation) and NWO Vidi Grant 62003105, which did not entail any financial interests of conflict.

Findability and Accessibility

The table below details where the data and research documentation for each chapter can be found on the Donders Repository (DR), as Data Sharing Collections (DSC). All data archived as a Data Sharing Collection remain available for at least 10 years after termination of the studies. This repository will be made available to the public once chapter 5 has been published. Data for chapter 2 and 4 is already available through the publications.

Chapter 2, 4 and 5	**DAC_626830_0005_506**

Interoperability and Reusability

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

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To my 40 students, who contributed immensely to the collection, processing, and analysis of data, thank you for your dedication. Teaching you not only tested my knowledge but also deepened my understanding, especially in explaining complex concepts simply. This experience inspired me to pursue scientific illustration, merging science and art to create illustrations that are both informative and visually appealing.

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After my contract ended and I returned to my home country, my former supervisor, Pablo, welcomed me back with open arms. Initially, I sought only office space to write my thesis, but I soon became involved in the lab again, eventually being hired as a guest researcher and offered a postdoc position. I am profoundly grateful for Pablo's support, as he played a crucial role in helping me secure my PhD position. I know I can always return to Chile and find a place in his lab.

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Donders Graduate School

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

Since 2009, the Donders Graduate School has grown into a vibrant community of highly talented national and international PhD candidates, with over 500 PhD candidates enrolled. Their backgrounds cover a wide range of disciplines, from physics to psychology, medicine to psycholinguistics, and biology to artificial intelligence. Similarly, their interdisciplinary research covers genetic, molecular, and cellular processes at one end and computational, system-level neuroscience with cognitive and behavioural analysis at the other end. We ask all PhD candidates within the Donders Graduate School to publish their PhD thesis in de Donders Thesis Series. This series currently includes over 600 PhD theses from our PhD graduates and thereby provides a comprehensive overview of the diverse types of research performed at the Donders Institute. A complete overview of the Donders Thesis Series can be found on our website: https://www.ru.nl/donders/donders-series

The Donders Graduate School tracks the careers of our PhD graduates carefully. In general, the PhD graduates end up at high-quality positions in different sectors, for a complete overview see https://www.ru.nl/donders/destination-our-former-phd. A large proportion of our PhD alumni continue in academia (>50%). Most of them first work as a postdoc before growing into more senior research positions. They work at top institutes worldwide, such as University of Oxford, University of Cambridge, Stanford University, Princeton University, UCL London, MPI Leipzig, Karolinska Institute, UC Berkeley, EPFL Lausanne, and many others. In addition, a large group of PhD graduates continue in clinical positions, sometimes combining it with academic research. Clinical positions can be divided into medical doctors, for instance, in genetics, geriatrics, psychiatry, or neurology, and in psychologists, for instance as healthcare psychologist, clinical neuropsychologist, or clinical psychologist. Furthermore, there are PhD graduates who continue to work as researchers outside academia, for instance at non-profit or government organizations, or in pharmaceutical companies. There are also PhD graduates who work in education, such as teachers in high school, or as lecturers in higher education. Others continue in a wide range of positions, such as policy advisors, project managers, consultants, data scientists, web- or software developers, business owners, regulatory affairs specialists, engineers, managers, or IT architects. As such, the career paths of Donders PhD graduates span a broad range of sectors and professions, but the common factor is that they almost all have become successful professionals.

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