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UNIVERSITY OF EDINBURGH

DOCTORAL THESIS

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**Investigating the role of  
hippocampal-cortical circuits in  
memory, and their dysfunction in a  
rat model of Fragile X Syndrome**

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*A thesis submitted in fulfillment of the requirements  
for the degree of Doctor of Philosophy*

*in the*

Deanery of Biomedical Sciences  
College of Medicine and Veterinary Medicine

2022



# Declaration of Authorship

I, Joe W. MOORE, declare that this thesis titled, “Investigating the role of hippocampal-cortical circuits in memory, and their dysfunction in a rat model of Fragile X Syndrome” and the work presented in it are my own. I confirm that:

- This thesis was composed by myself.
- The work presented was completed mainly by myself, and the contributions of others have been indicated below, and in the relevant Methods sections.
- The work has not been submitted for any other degree or professional qualification, except as specified.

For Chapter 2, all experimental work was conducted by Dr Elizabeth Allison and Julia Thomas, and half of the data were included in Dr Elizabeth Allison’s doctoral thesis. All data analysis was completed by myself, using pre- and post-clustering scripts written by Dr Daisy Arkell and Steven Huang, and GLMM scripts written by Dr Owen Dando. For Chapter 3, the initial surgeries and perfusions were performed by Dr Sarah Tennant, and a large proportion of the video scoring was performed by Ella Cronkshaw. For Chapters 4 and 5, experimental work and analysis were completed by myself, using freely available analysis scripts as indicated in the Methods.

Signed: \_\_\_\_\_

Date: 29/07/2022  
\_\_\_\_\_



# *Abstract*

Place cells in the hippocampus form an internal spatial representation of an animal's environment, which is thought to provide a basis for spatial and episodic memory. In order to form this representation, inputs from other areas of the brain, via the entorhinal cortex, must carry sensory information about the surroundings. The medial prefrontal cortex may also play a role, in organising information flexibly, and consolidating memories during sleep. The overall aim of this thesis is to study these brain networks and their function in spatial and episodic memory formation and consolidation, including assessing the role of specific connections and studying how network activity is affected in a model of intellectual disability.

A key property of hippocampal place cells is anchoring of their firing to distal and proximal landmarks. The first experiment aimed to test the hypothesis that the medial entorhinal cortex (MEC) is required for anchoring of hippocampal place cells to distal landmarks. Tetrodes were implanted into dorsal CA1 of the hippocampus and place cells were recorded from mice in which the MEC had been lesioned, and control mice that had undergone a sham surgery. The mice explored a platform surrounded by visual landmark cues which were rotated by 90° around the platform between sessions. The place cells in the control mice were shown to anchor to the visual landmarks, as has been demonstrated previously, whereas the place cells from the lesioned mice did not rotate with the visual landmarks. A rotation protocol using objects within the environment as cues showed that place cells in MEC-lesioned mice did rotate their fields to follow objects. These findings suggest that information about distal visual landmarks, but not proximal objects, may be integrated into the hippocampal spatial representation via the MEC.

The second experiment was designed to test the hypothesis that layer 2 stellate cells (L2SCs) in the MEC are required for episodic-like memory in mice. The MEC L2SCs form a major projection to the hippocampus, and are among the first neurons to be affected in Alzheimer's disease. Mice with inactivated L2SCs were compared with control mice injected with a GFP virus on performance in a series of spontaneous object exploration tasks. No significant differences between the groups were found in object recognition, or object association memory, but low virus expression as well as low subject numbers meant that it was difficult to make reliable conclusions from these results.

Fragile X Syndrome (FXS) is one of the most common inherited forms of intellectual disability. In the final experiment, a model of FXS, the *Fmr1* knockout (*Fmr1*<sup>-/-</sup>) rat was used to test functional connectivity between the hippocampus and medial prefrontal cortex (mPFC) as a potential mechanism that may cause spatial memory deficits. Tetrodes were implanted into both regions in wild-type (WT) and *Fmr1*<sup>-/-</sup> rats, and the local field potentials in each region were recorded as the rats explored a familiar environment, followed by a novel environment. *Fmr1*<sup>-/-</sup> rats showed increased mPFC gamma power, as well as increased coherence between the mPFC and hippocampus, across all of the sessions. During a period of sleep following each day of environment exploration, cortical spindles, delta waves, and hippocampal ripples were detected and interactions between them analysed. *Fmr1*<sup>-/-</sup> rats had fewer ripples during slow wave sleep than WT, and they were timed to a different phase of the cortical delta oscillation. Ripples were also less strongly modulated with the phase of mPFC spindle oscillations in *Fmr1*<sup>-/-</sup> rats. This evidence suggests discoordination of mPFC and hippocampal networks in *Fmr1*<sup>-/-</sup> rats that may underlie memory encoding and consolidation deficits.

The results presented in this thesis contribute to understanding how sensory information is integrated into spatial and episodic memories, as well as how organisation and consolidation of memories may be affected in a model of intellectual disability.

# *Lay Summary*

We constantly and spontaneously form memories of our surroundings that allow us to navigate in the world, and to remember past events and where they took place. For example, you might remember a birthday from your childhood, including the layout of the room, where furniture was placed, and family members who were there with you. This type of memory is known as episodic memory. The hippocampus is the main brain area involved in forming spatial and episodic memories, but it relies on connections with other areas to bring together and organise sensory information into a full memory, and then store it long-term. This thesis describes a series of experiments that aimed to investigate connections between the hippocampus and other brain structures, and how they support spatial and episodic memory.

In the first experiment, place cells were recorded in the hippocampus. Place cells are neurons which activate when you are in a particular place within an environment. These neurons form a map of the surroundings, telling you where you are. This map needs to be based on sensory information about the environment, like stable visual landmarks such as a tall building or tree. Here, place cells were recorded with electrodes in mice where another brain structure, the medial entorhinal cortex (MEC), had been removed. The key finding was that the place cell map could no longer use distant visual landmarks, but could use local objects within the environment. This suggests that the MEC is involved in integrating information about distant visual landmarks into the hippocampus, but not local object information.

The next experiment was testing the role of a specific population of neurons in the MEC that send inputs to the hippocampus. A virus was used to specifically shut down these cells in mice, and the mice were tested on tasks assessing their memory for objects, as well as combinations of objects and their context, their position, or their position within their context. Mice without these inputs to the hippocampus could successfully remember objects, and object positions. In a more complex version of the task which is dependent on the hippocampus and thought to be similar to episodic memory, the results were inconclusive.

Fragile X Syndrome is the most common inherited cause of learning disability and autism spectrum disorder, and can be modelled in rats by shutting down the gene

that causes the disorder in humans (*Fmr1*). In the final experiment, connections between the hippocampus and medial prefrontal cortex (mPFC) were tested in these rats, as a possible cause of memory problems in FXS. *Fmr1*<sup>-y</sup> rats were implanted with electrodes to record the electrical activity in both areas while they explored a new environment. Waves in the electrical activity coordinate neurons within brain areas, and allow interactions between different areas. The coordination of these waves appeared to be affected in the *Fmr1*<sup>-y</sup> rats, which may cause the memory problems seen previously.

During sleep, memories are replayed, strengthened, and transferred from the hippocampus to the cortex for long-term storage. This involves coordinated interactions between the hippocampus and cortex. After exploring the environments, recording took place while the rats slept. The *Fmr1*<sup>-y</sup> rats showed discoordination in the interactions between mPFC and hippocampus, which may further contribute to memory problems.

Overall, the results in this thesis provide evidence to help to understand how memories are processed in the brain, and how this may be affected by a learning disorder.

# *Acknowledgements*

Firstly, I would like to thank Emma – I could not have done this without your support, advice and encouragement, which were much-needed at many times during my PhD. As well as this, I have learned so much from you that I will take forward in my future career. I would also like to thank Jamie for his guidance as my co-supervisor.

Big thanks go to everyone in the lab, for being such brilliant people to work with over the years – thank you Noah, Łucja, Adrian, Paul, Anna and Tizzy. I would especially like to thank Daisy and Antonis for their help, advice and reassurance in my final couple of years, and Sarah and Brianna for their help early on in my PhD. I would also like to acknowledge all the undergraduate students who did projects with me, even if the experiments didn't work out! Thanks to everyone else who has helped out, including Patrick for building/fixing various things, and all the animal technicians – Lynn, Gordon, Richard etc., who did an amazing job of looking after all the rats and mice (whom I would also like to thank for being good little guys).

To my dad, Paul, thanks for teaching me about action potentials – it came in handy with this. Thank you to my mum, Sue, for always being there for me when needed, and to my siblings, Ben, Luke and Hannah, for being alright I guess.

Finally, thank you to all the friends who were there to listen to me despair when things were difficult, and kept me going through this process. To Calu – see, it was all worth it! I really can't thank you enough for everything you have done for me.



*Dedicated to Beryl (Hawkey) Moore*



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# List of Abbreviations

|                  |                                                                          |
|------------------|--------------------------------------------------------------------------|
| <b>ACC</b>       | anterior cingulate cortex                                                |
| <b>AMPA</b>      | alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor        |
| <b>ANOVA</b>     | analysis of variance                                                     |
| <b>AP</b>        | anterior-posterior axis                                                  |
| <b>CA1</b>       | cornu ammonis 1                                                          |
| <b>CA2</b>       | cornu ammonis 2                                                          |
| <b>CA3</b>       | cornu ammonis 3                                                          |
| <b>CI</b>        | confidence interval                                                      |
| <b>dIPFC</b>     | dorsolateral prefrontal cortex                                           |
| <b>dmPFC</b>     | dorsomedial prefrontal cortex                                            |
| <b>DREADDs</b>   | designer receptors exclusively activated by designer drugs               |
| <b>DV</b>        | dorsal-ventral axis                                                      |
| <b>dWPLI</b>     | debiased weighted phase lag index                                        |
| <b>EC</b>        | entorhinal cortex                                                        |
| <b>EEG</b>       | electroencephalogram                                                     |
| <b>FMR1/Fmr1</b> | FMRP translational regulator 1 (the gene affected in Fragile X Syndrome) |
| <b>fMRI</b>      | functional magnetic resonance imaging                                    |
| <b>FMRP</b>      | Fragile X mental retardation protein                                     |
| <b>FXS</b>       | Fragile X Syndrome                                                       |
| <b>GABA</b>      | gamma-aminobutyric acid                                                  |
| <b>GLMM</b>      | generalised linear mixed-model                                           |
| <b>IL</b>        | infralimbic area                                                         |
| <b>L2SC</b>      | layer 2 stellate cells                                                   |
| <b>LEC</b>       | lateral entorhinal cortex                                                |
| <b>LFP</b>       | local field potential                                                    |
| <b>LRT</b>       | likelihood ratio test                                                    |
| <b>LTP</b>       | long-term potentiation                                                   |
| <b>LTD</b>       | long-term depression                                                     |
| <b>MD</b>        | mediodorsal thalamus                                                     |

|              |                                |
|--------------|--------------------------------|
| <b>MEC</b>   | medial entorhinal cortex       |
| <b>MI</b>    | modulation index               |
| <b>ML</b>    | medial-lateral axis            |
| <b>MRI</b>   | magnetic resonance imaging     |
| <b>mPFC</b>  | medial prefrontal cortex       |
| <b>MTL</b>   | medial temporal lobe           |
| <b>NMDAR</b> | N-methyl-D-aspartate receptor  |
| <b>NR</b>    | nucleus reuniens               |
| <b>NREM</b>  | non-rapid eye movement         |
| <b>OC</b>    | object-context                 |
| <b>OFC</b>   | orbital-frontal cortex         |
| <b>OiP</b>   | object-in-place                |
| <b>OL</b>    | object-location                |
| <b>OP</b>    | object-place                   |
| <b>OPC</b>   | object-place-context           |
| <b>OR</b>    | object recognition             |
| <b>P</b>     | postnatal day                  |
| <b>PAC</b>   | phase-amplitude coupling       |
| <b>PFC</b>   | prefrontal cortex              |
| <b>PrL</b>   | prelimbic area                 |
| <b>PV</b>    | parvalbumin                    |
| <b>REM</b>   | rapid eye movement             |
| <b>SEM</b>   | standard error of the mean     |
| <b>SI</b>    | spatial information            |
| <b>SST</b>   | somatostatin                   |
| <b>SWS</b>   | slow-wave sleep                |
| <b>TRN</b>   | thalamic reticular nucleus     |
| <b>VIP</b>   | vasoactive intestinal peptide  |
| <b>vmPFC</b> | ventromedial prefrontal cortex |
| <b>WT</b>    | wild-type                      |

# **Chapter 1**

## **General Introduction**

Memory is a critical component of cognition, key to an organism's ability to learn about and understand its environment in order to survive within it. The hippocampus is an important brain area involved in the formation of autobiographical memories in the mammalian brain. Study of its function, particularly over the last half century, has advanced our understanding of the brain and how it stores information, but there are still questions to be answered. The brain is a highly complex interconnected system, and construction, organisation and storage of memories all require the hippocampus to interact with other regions of the brain. This thesis will focus on key interactions of the hippocampus with particular cortical regions that are involved in memory processing, including entorhinal cortex (EC) and medial prefrontal cortex (mPFC), as well as how such interactions are disrupted in a model of intellectual disability. This chapter will introduce the key literature and theory that led to the hypotheses and experiments presented in later chapters.

### **1.1 The hippocampus, cortex and memory**

The case of patient H.M. initiated research into the hippocampus and its role in memory. Patient H.M. had severe epilepsy that required resection of his medial temporal lobes (MTL) in order to control the seizures. Unfortunately, this left H.M. with a profound inability to form new memories of experiences (Scoville & Milner, 1957). Further study of H.M.'s abilities found some interesting deficits. In one test, he was asked to trace a star shape, staying within two boundaries, while only looking at his hand and the page via a mirror. This is a difficult skill, but it can be honed over days with practice. H.M. showed no problems with this task, improving

his performance each day; but, each day, he could not recall having done the task previously (Milner, 1962; Squire, 2009). Along with studies of other patients with MTL damage, this helped to show that there are different types of memory, leading to the distinction between declarative/explicit memory and non-declarative/implicit memory. While non-declarative memory includes long-term unconscious memory, such as motor skills and habit-formation, that do not require conscious recollection of experiences, declarative memory is a conscious recollection of facts or events (Cohen & Squire, 1980). It was the formation of new long term declarative memory that was affected in H.M. and other patients with MTL lesions.

H.M.'s memory impairments are often attributed to hippocampal damage alone, but the surgeon's original reports as well as more recent magnetic resonance imaging (MRI) noted that damage was not limited to the hippocampus. The lesion included the hippocampus and the anterior part of the parahippocampal gyrus, as well as the amygdala (Figure 1.1, Corkin et al., 1997). Other patients with more restricted and more extensive MTL damage have been compared in their memory abilities. More specific hippocampal damage causes less severe memory impairments than those seen in H.M., while patients with damage that extended to both the posterior and anterior parts of the parahippocampal gyrus have more severe memory impairments (Kirwan et al., 2008). Hence this highlights the importance of the parahippocampal gyrus in declarative memory.

Patients with more specific hippocampal damage appear to be largely spared with regard to semantic memory, memory for facts and knowledge, and impairments are more restricted to memory relying on contextual detail, including episodic and spatial memory (Vargha-Khadem et al., 1997; Tulving & Markowitsch, 1998; Bright et al., 2006). Episodic memory is the type of declarative memory for events. It involves the binding of multi-modal sensory information to form memory of the context and the content of experiences. Endel Tulving, who originally coined the term, defined episodic memory as autobiographical, containing information about "temporally dated events", and "temporal-spatial relations" between them (Tulving, 1972).

The less severe impairment of semantic memory with hippocampus-restricted damage suggested that this type of memory can be processed independently of the hippocampus, but the effects of larger MTL lesions suggested the importance of the parahippocampal-hippocampal network in declarative memory as a whole. It also

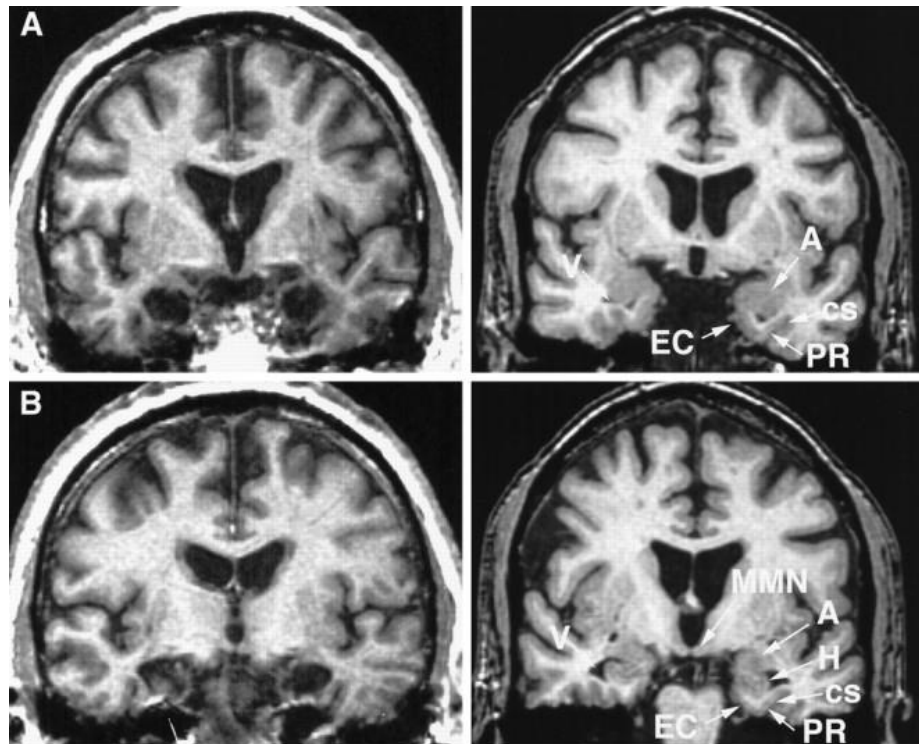


FIGURE 1.1: MRI scans from patient H.M. (left), showing resection of medial temporal lobe structures, in comparison to a control patient (right). EC = entorhinal cortex; H = hippocampus; A = amygdala; PR = perirhinal cortex; cs = collateral sulcus; MMN = medial mammillary nucleus (Taken from Corkin et al., 1997).

suggested that there is something unique about episodic memory that requires an intact hippocampus. Tulving and Markowitsch (1998) highlighted distinctions between semantic and episodic memory, including that episodic memory allows the recall of, and “mental time travel” to, previous experiences, it develops later in childhood than semantic memory, may be more vulnerable in old age, and may be more dependent on frontal cortical areas.

Patients with frontal lobe damage demonstrate that the contribution of this region to memory processes is subtler. The prefrontal cortex (PFC), particularly dorso-lateral PFC (dlPFC) in humans, has been implicated in encoding and retrieval of episodic memory. Damage to the dlPFC has been shown to cause impairments in recall of events, and errors in recalling the context in which information was learned (Janowsky et al., 1989; Mangels et al., 1996). Deficits in the temporal ordering of events have also been reported (Shimamura et al., 1995). It has been proposed that these impairments are due to an inability to regulate irrelevant competing information or memories during encoding and retrieval. This is illustrated

by paired-associate learning, in which subjects must learn a list of word pairs (e.g. “glass”-“table”), then must learn a second list in which words from the first list have different associates (e.g. “glass”-“eye”). Patients with frontal lesions show deficits in learning the second list, when information learned from the first list creates interference (Shimamura et al., 1995). While the role of the PFC is not completely understood, it appears to have an important role in the organisation of declarative memories, possibly binding information with its context, as well as preventing interference with previously learned information (Szczepanski & Knight, 2014).

Another finding from amnesic patients is that not only ability to form new memories, but also the ability to recall memories from prior to the onset of amnesia is typically impaired. However, while there are often deficits in recall of recently formed memories (from the months to years prior to amnesia) more remote memories are less likely to be affected by MTL damage. For example, H.M. had retrograde amnesia for declarative memories formed up to around 3 years before his surgery, but could recall early life memories, and patients with similar MTL damage showed similar patterns (Scoville & Milner, 1957; Penfield & Milner, 1958). Based on assessment of descriptions of episodic memories among patient groups, MTL or hippocampal damage causes deficits in recall of recent memories and leaves remote memory recall largely intact (Kirwan et al., 2008). This suggests that more remote memories are stored independently of the MTL, with the theory being that memories undergo a gradual process of distribution among different neocortical networks, known as systems consolidation (Frankland & Bontempi, 2005). However, there is some debate about the continual involvement of the hippocampus particularly in the recall of episodic memory. Some tests of patient H.M.’s remote autobiographical memories found that they were not very rich in contextual detail, and more like remembered facts (i.e. semantic) (Corkin, 2002). This, and imaging studies showing the continual activation of hippocampus during recall of remote memories, suggest that the hippocampus may always be required for episodic retrieval (Nadel & Moscovitch, 1997; Bonnici et al., 2012). Nonetheless, more remote episodic memories show greater activation of ventromedial PFC (vmPFC), suggesting that memories are gradually consolidated to cortical networks, and may show decreased reliance on hippocampal networks (Bonnici et al., 2012).

While important insights have come through the human case studies and imaging experiments described above, these ideas of hippocampal and cortical function in memory have been further developed in rodent studies. Although the rodent brain

differs from the human brain, especially in the extent of the cortex, the structure of the hippocampus is well conserved between mammalian species. And study of comparable cortical connections has shown the rodent brain to be a valuable model for understanding brain function.

## 1.2 The hippocampus

### 1.2.1 Hippocampal anatomy

In order to study the function of the hippocampus, and make comparisons between human and rodent hippocampus, it is important to understand its anatomy.

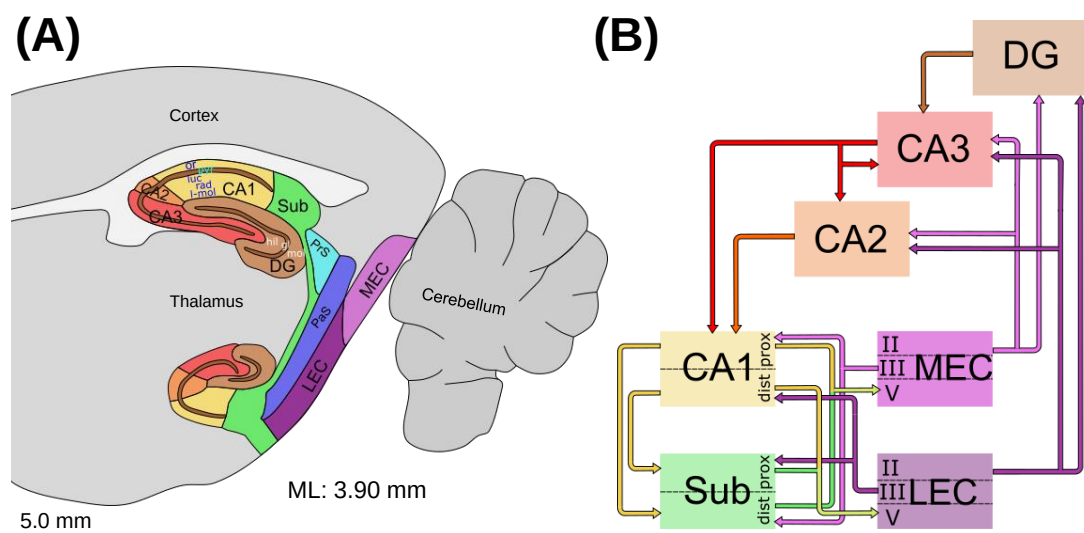


FIGURE 1.2: The rodent hippocampus and its medial temporal lobe connections. **(A)** A sagittal rat brain atlas image, showing dorsal and ventral hippocampus, consisting of dentate gyrus (DG, brown), CA3 (red), CA2 (orange), CA1 (yellow). The layers of the CA regions (or: stratum oriens; pyr: stratum pyramidale; luc: stratum lucidum; rad: stratum radiatum; l-mol: stratum lacunosum moleculare) and DG (hil: hilus; gl: stratum granulosum; mol: stratum moleculare) are also indicated. Parahippocampal regions shown include subiculum (Sub, green), presubiculum (PrS, light blue), parasubiculum (PaS, dark blue), lateral entorhinal cortex (LEC, dark purple), and medial entorhinal cortex (MEC, light purple). **(B)** Schematic of the main excitatory connections between the regions of the MTL. (Adapted from Nilssen et al., 2019)

The hippocampus, named for its similarity in shape to a seahorse, is a large elongated structure deep in the MTL. In rodents, it is C-shaped, just below the temporal

cortex; Figure 1.2A is a schematic of a sagittal section from a rat brain, showing the dorsal and ventral parts of this C-shape. The classic excitatory feed-forward pathway through the hippocampus, the ‘trisynaptic loop’, consists of projections from the entorhinal cortex (EC), via the perforant path, which synapse onto cells of the dentate gyrus (DG), which then send projections known as the mossy fibres to CA3, cells of which send their projections via the Schaffer collateral pathway to CA1. CA1 cells then project back to EC to complete the loop, as well as to the subiculum (Figure 1.2B) (Andersen et al., 2007). Besides their projections to CA1, CA3 cells have strong recurrent connections with other CA3 cells (Witter, 2007a). Furthermore, the EC projects directly to CA1. The projections from EC to the hippocampus will be discussed in a later section. CA2 has often been overlooked as a transitional region between CA3 and CA1 in the past, but recent research has identified important computational functions for CA2 (Dudek et al., 2016).

The principal excitatory cell types in the CA1, CA2 and CA3 regions are pyramidal cells, while the DG contains granule cells. Cells in all of the regions are arranged in a laminar structure. In the CA regions, the layers are named:

- *Stratum oriens*, which contains inhibitory basket cells, as well as the dendrites of pyramidal cells, where they receive projections from other pyramidal cells.
- *Stratum pyramidale*, is densely populated with the cell bodies of pyramidal cells, and some inhibitory interneurons.
- *Stratum lucidum*, only present in CA3, where mossy fibres from the DG project, then synapsing in *stratum pyramidale*.
- *Stratum radiatum*, contains Schaffer collateral fibres, as well as some interneurons that are also found in the more superficial layers.
- *Stratum lacunosum-moleculare*, also contains Schaffer collateral fibres, as well as perforant path projections.

A cell-free region known as the hippocampal sulcus separates the CA regions from the DG, which has its own laminar structure, consisting of:

- The *hilus*, containing many interneurons and the axons of granule cells projecting to CA3.

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- *Stratum granulosum*, containing the main excitatory cells of the DG, granule cells.
- *Stratum moleculare* contains axons from the perforant path and is where they synapse with dendrites of granule cells. (Figure 1.2B) (Andersen et al., 2007).

The different functions of these subfields has been demonstrated mainly from electrophysiological studies recording cellular activity from each. The dorsal part of the hippocampus is thought to be more involved with spatial memory, while the ventral hippocampus may be more involved in processing contextual and emotional information.

As well as projections from the EC, the hippocampus receives cortical input from the presubiculum and parasubiculum. Important inputs also arise from the septal nuclei, which will be discussed in a later section, the supramammillary area and hypothalamus, as well as brainstem nuclei, which provide dopaminergic, noradrenergic and serotonergic inputs (Andersen et al., 2007).

### 1.2.2 Place cells

Edward Tolman showed that rats could navigate to a goal location of a maze via different routes that they had not previously learned. He suggested that the rats may have an internal map that stored information about relations between different locations of the maze, allowing formulation of new routes (Tolman, 1948). Recording from electrodes implanted in the hippocampi of rats, O'Keefe and Dostrovsky (1971) found a cellular correlate of this "cognitive map", when they discovered place cells.

Place cells are neurons which fire action potentials when an animal occupies a particular region of an environment (O'Keefe & Dostrovsky, 1971; O'Keefe & Nadel, 1978). Much of what we know about place cells comes from recordings in rodents, using electrodes implanted into the dorsal hippocampus and allowing the animal to explore an environment freely. Single neuron activity can be recorded simultaneously with position tracking to determine the locations in which action potentials occur. Figure 1.3 (middle) shows the trajectory of a rat in a square arena represented by the black line, overlaid with red dots indicating where spikes from a

particular cell were detected. From this information, spiking positions are binned into pixels, and firing rate maps can be calculated (Figure 1.3, right). As in the example shown, place cells show characteristic fields where firing rate is greatly increased compared to the rest of the environment, where there is very little firing. Simultaneously recorded place cells tend to have fields that are quite evenly distributed throughout an open field arena, so place cell populations may together form an entire representation of a given environment (Muller & Kubie, 1987). Cells with overlapping fields will fire together to encode current position, known as a cell ensemble. Firing rate maps of simultaneously recorded place cells can be used to predict the location of an animal from firing rates of the cells at each time point during a recording, with greater numbers of inputted cells giving increased position accuracy (Wilson & McNaughton, 1993; Jensen & Lisman, 2000).

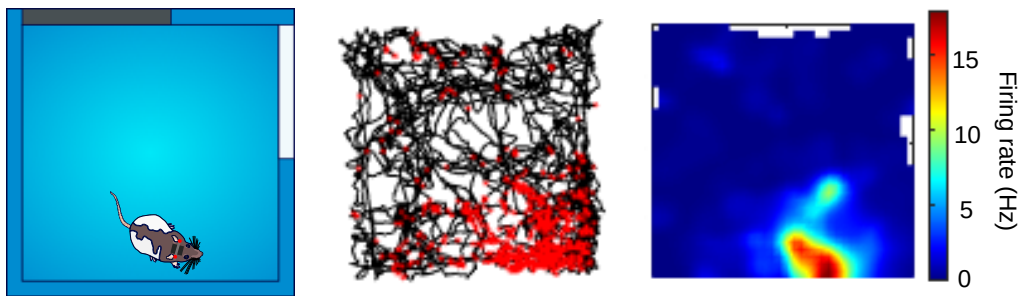


FIGURE 1.3: A place cell. Left: A rat running in an open field environment, with a black cue card on the north wall, and a white cue card on the east wall. Neurons in the dorsal hippocampus and position tracking, using LEDs on the rat's head, are recorded simultaneously. Middle: The black line represents the rat's trajectory over a session exploring the environment, red dots represent the positions where spikes from a single dorsal CA1 neuron were detected. Right: A firing rate map produced from the spiking of the neuron, showing high firing rates (warmer colours) only in the south east of the environment, forming a place field.

Place cell coding emerges rapidly in a novel environment, with location-sensitive activity occurring in hippocampal neurons during the first pass through. This then develops with experience, with more stable representations forming with longer exposures and multiple exposures over days (Thompson & Best, 1990; Frank et al., 2004). Furthermore, attention to behaviourally relevant features may increase this stability (Kentros et al., 2004). Therefore, place cell representations may form the basis of memory of an experience, which is gradually improved and updated with continued exposure and behavioural relevance.

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Place cell activity has been shown in the main excitatory neurons of all of the hippocampal subfields, including CA1, CA2, CA3 and DG (O'Keefe & Dostrovsky, 1971; Jung & McNaughton, 1993; Leutgeb et al., 2007; Lee et al., 2004; Mankin et al., 2015; Lu et al., 2015). There are subtle differences in their properties, some of which will be discussed in the following section.

There is strong evidence showing place cell activity as a conserved cross-species phenomenon in the mammalian brain. As well as rats, place cells have since been identified in mice, bats, chinchillas (McHugh et al., 1996; Ulanovsky & Moss, 2007; Muir et al., 2009), non-human primates (Ludvig et al., 2004; Hori et al., 2005), and in humans as they navigated a virtual environment (Ekstrom et al., 2003; Miller et al., 2013). There is even some evidence of place cells in the avian hippocampus (Payne et al., 2021). In addition, place cells do not simply represent a single location on a 2-dimensional map, with studies showing place cells with multiple fields in large environments (Park et al., 2011), and 3-dimensional place fields have been demonstrated in flying bats and rats climbing in a lattice structure (Yartsev & Ulanovsky, 2013; Grieves et al., 2020). This evidence suggests that the hippocampal place cell is an important part of representation of the external environment among a number of different species. Furthermore, a later section will discuss in further detail evidence for non-spatial correlates of hippocampal place cells, showing that their activity does not only represent environments, but may constitute more general representations of memory.

The behavioural relevance of the information provided by the hippocampus is evident from human patients such as H.M. The discovery of place cells in rodents led to development of behavioural tasks to test the effects of hippocampal lesions, and hence the loss of place cell information. One task to show this effectively was the spatial reference memory task in the Morris water maze, in which animals must swim in a pool of water and find a platform hidden just under the water's surface, gradually learning the platform's location over days of training. It was found that rats with hippocampal lesions were significantly impaired in learning to navigate to the platform, suggesting information provided by the place cell representation is important for navigation (Morris et al., 1982). A recent experiment provided more direct evidence that the activity of place cells is causally linked to spatial behaviour; place cells were recorded as mice navigated to a particular point on a virtual linear track, where they would lick to receive a reward. A subset of the recorded place cells coded for the reward position, and optogenetic stimulation of these place cells

at earlier points on the track caused mice to start licking, suggesting they may be perceiving that they were at the reward location (Robinson et al., 2020).

### 1.2.3 Hippocampal remapping

Through recordings of large numbers of place cells simultaneously, it was shown that the hippocampus can store and switch between different “maps” for distinct environments (Muller & Kubie, 1987; Leutgeb, Leutgeb, Barnes, et al., 2005). When two environments have very distinct sensory features, the majority of individual place cells will change the location of their firing fields within each. When only particular individual features of an environment are changed, such as wall colour, individual place cells may remain firing in the same location, but their firing rates may be modified. This modification of place cell firing in response to environmental changes is termed remapping.

Remapping is characterised by the observed modification of place cell firing. In cases where environments are very distinct, and a total change in firing locations of the observed place cell population is seen, it is described as global remapping (Leutgeb, Leutgeb, Barnes, et al., 2005). This likely represents the place cell representation recognising the two environments as distinct places that require distinct spatial maps in order to differentiate between them. For example, Muller and Kubie (1987) showed that moving rats from a circular arena to a square one elicited a change in the active neuronal population, with no relation between firing patterns in one environment to the other. This is illustrated by Figure 1.4A, showing a place cell which fires on opposite sides of two different environments with distinct floors, walls and cues. Many place cells will become inactive in one environment, such that the populations of place cells that are active in each are distinct, but overlapping (Thompson & Best, 1989).

The extent to which two place cell representations are different to one another appears to be dependent on how different two environments are. This can be unpredictable, as it appears to depend on the perception of the animal, with different rats showing different levels of remapping in the same conditions, and during different behaviours (Bostock et al., 1991; Kentros et al., 2004). More distinct spatial maps can also develop over time, becoming more distinct with more training exposures

(Lever et al., 2002). Furthermore, the observed remapping response is often heterogeneous among recorded place cells. Anderson and Jeffery (2003) recorded CA1 place cells in environments with different combinations of sensory features – the box could have white or black walls, with a lemon or vanilla odour. Using the four combinations of these, they observed a small proportion of cells that showed no change in location to any of the changes, some cells that changed their location or activity only for colour changes, and some for only odour. But most place cells showed complex responses across the colour and odour combinations, with many expressing specific activity patterns for certain combinations, becoming inactive, or changing place field location (Anderson & Jeffery, 2003).

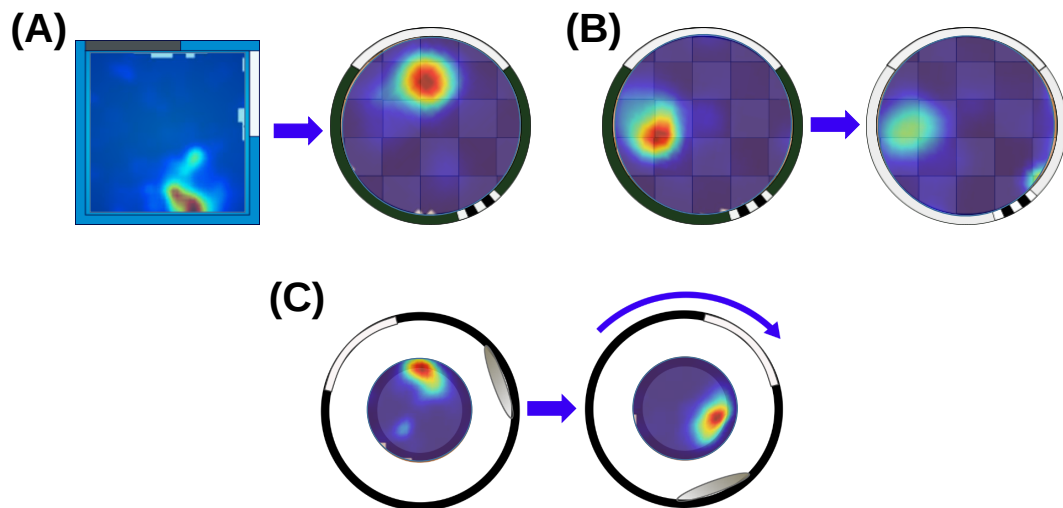


FIGURE 1.4: Place cell remapping and rotation. **(A)** Global remapping: a new environment leads to a change in a place cell firing field locations. **(B)** Rate remapping: changes in features of the environment can lead to place cells maintaining their firing field locations, but with changes in firing rate within the environment. **(C)** Rotation with cues: rotation of salient landmarks between sessions in an environment leads to place fields rotating with the cues.

Changing only certain features of an environment can also cause some place cells to remain firing in the same location, but with modification of place field firing rate, known as rate remapping. For example, changing only the wall colour can lead to very high correlation between the place cell firing locations in the two contexts, but with variations in firing rates (Leutgeb, Leutgeb, Barnes, et al., 2005). Figure 1.4B illustrates this – the place field stays on the left side of the arena, but has a lower within-field firing rate when the wall colour is changed. An experiment using an environment which could be morphed between a circular and square shape showed clear rate remapping between the two conditions among the recorded place cells.

Additionally, when rats experienced the environment in stages between the two conditions, firing rates showed a similar gradual change (Leutgeb, Leutgeb, Treves, Meyer, et al., 2005). This suggests there is also information about physical properties of environments, such as the colour, shape and odour, encoded in the place cell firing rate, as well as information about spatial location. A later experiment tested the effect of habituating rats in the square and circular environments in two separate locations. As expected, differently to when the square and circular conditions were experienced in the same location, global remapping was seen between the two conditions. When these rats were placed in the environment, and it was morphed between the two shapes in the same location, place cells still showed global remapping – in intermediate shapes the place cell representation was similar to the square or circle, depending on which it most resembled at that time (Colgin et al., 2010). This suggests that they were being encoded as two different places, rather than representing changes within the same environment.

Observation of place cell remapping in the different hippocampal subfields reveals an important distinction in their function. Recording of both CA3 and CA1 place cells in two environments with different coloured walls found that populations of CA1 showed higher correlations between the environments than those in CA3 (Leutgeb et al., 2004), indicating that CA3 place cells appear to show more distinct representations than CA1 across different environments (Alme et al., 2014). In the DG, place cells often have multiple spatial fields, and show large changes in firing in response to subtle environmental changes (Leutgeb et al., 2007; van Dijk & Fenton, 2018). As DG and CA3 provide major inputs to CA1, a mechanism of pattern separation was proposed, whereby the DG and CA3 disambiguate similar or overlapping memories to reduce interference between them. CA3 may store multiple representations for different environments, while the DG may represent the subtle changes between them (Colgin et al., 2008).

The behavioural relevance of place cell remapping has been shown through association of impaired remapping with impaired context discrimination in a memory task. McHugh et al. (2007) showed that mice lacking functional N-methyl-D-aspartate receptors (NMDAR) specifically in the DG had impaired CA3 place cell remapping between similar environments, in conjunction with poor discrimination between similar environments in a contextual fear conditioning task.

### 1.2.4 Cues and objects

Another feature that has been observed in place cells is their anchoring to particular environmental cues. Recording often takes place in environments with a cue card on the wall, which can serve as an orienting landmark. When this cue is rotated between sessions, the locations of place fields will rotate in register with the cue, and fire in the same position relative to it (O'Keefe & Nadel, 1978; Muller & Kubie, 1987; Fenton et al., 2000). Figure 1.4C shows a place cell which fires in the same location relative to visual cues placed on a curtain outside an arena. A sufficiently salient distal cue will cause the rotation of large proportions of place cells in the hippocampus. The importance of this anchoring to landmarks for behaviour has been demonstrated using behavioural tests during place cell recording. Manipulation of cues were found to change place field locations, concurrently with where the rats judged the position of a reward location to be (Lenck-Santini et al., 2001; Kubie et al., 2007).

Place fields can also be associated with objects or proximal cues within an environment. Experiments where objects are placed within the environment show that some place cells fire when the animal explores around particular objects (O'Keefe, 1976). When an object is removed, some of the cells will then represent the previous location of the object (Deshmukh & Knierim, 2013). Individual place cells within a population show diverse responses to cue manipulations. This is shown through experiments in which multiple different cues are manipulated simultaneously (Shapiro et al., 1997). Joint representation of distal landmarks and proximal cue reference frames was shown by use of a circular track with textured flooring, surrounded by visual cues outside the track; when the two types of cues were rotated in conflict with one another, place cell populations were split, with some following the proximal cues and others following the distal (Knierim, 2002; Yoganarasimha et al., 2006). There is also some evidence for place cell anchoring to odour cues (Zhang & Manahan-Vaughan, 2015), and tactile cues (Gener et al., 2013), when these are the most salient information.

Scaplen et al. (2014) aimed to specifically test the differences between different types of cue information, by using purely visual cues, projecting shapes onto the walls and floor of an environment. They found that projections covering a large section of the floor were sufficient to cause widespread CA1 place field rotation, but projections of smaller 'objects' did not cause the rotation of as many fields.

This may reflect the fact that, although objects are represented in CA1 place cells, they are less likely to form the basis of the map. This may be because object cues are seen as less stable and reliable for orientation in space.

Anchoring of place cells to visual information is not necessary for their spatial firing, as place fields can be maintained in darkness (Quirk et al., 1990; Markus et al., 1994). In the absence of external sensory cues, place cells may rely on path integration, the estimation of distance travelled through the use of self-motion information. Animals can navigate guided only by path integration mechanisms, although this can be difficult to test as rodents can reliably use odour cues, which are difficult to control (Etienne & Jeffery, 2004). Place cells are less precise and less stable in darkness, showing that visual input makes an important contribution to anchoring the hippocampal spatial map to the external environment in rodents.

### 1.2.5 Not-just-place cells

While the majority of research has focused on spatial firing in place cells, the original theory of the cognitive map was not just based on navigational memory, instead proposing a model for declarative memory as a whole (Tolman, 1948; O'Keefe & Nadel, 1978). This idea has been furthered by evidence that place cells do not simply encode information about current position, but show a number of different correlates, depending on the situation.

Staying within the spatial domain, place cells have been shown to represent directional information conjunctively with place under certain circumstances. For example, as rats run on linear tracks, different ensembles of place cells will represent the track during trajectories in the two different directions (McNaughton et al., 1983; Battaglia et al., 2004b). Directional coding by place cells in an open field has also been demonstrated in bats and rats (Ulanovsky & Moss, 2011; Rubin et al., 2014; Acharya et al., 2016), and may represent the relative direction of certain landmarks (Acharya et al., 2016; Jercog et al., 2019).

Another finding of recording place cells on linear maze tasks found different firing patterns depending on intended trajectory. On mazes with a central stem leading to junctions with multiple possible routes, once a rat has learned the maze well, place cells will often selectively fire before the junction depending on whether the

rat intends to take a particular direction (Wood et al., 2000; Ainge et al., 2007; Grieves et al., 2016). Additionally, before a maze task has been learned fully, during deliberation of which path to take at a junction, place cells in rats were shown to play out possible future trajectory sequences along the optional routes on the maze (Johnson & Redish, 2007).

There is also evidence that hippocampal cells can represent other non-spatial modalities that are relevant to behaviour (Eichenbaum et al., 1987). Wood et al. (1999) trained rats to dig in a sand well if its odour had been changed from the previous one presented, but not if it was the same. They found many hippocampal cells that responded to specific odours, independent of their spatial position. In a task where rats were trained to modulate the pitch of an auditory tone using a joystick until it reached a rewarded frequency, hippocampal cells were found to map the tone trajectory similarly to mapping of space; individual cells formed firing fields at particular pitches of the tone. Additionally, in this study, the researchers demonstrated that the same cells that had firing fields at particular tone frequencies also had place fields in a spatial environment (Aronov et al., 2017). Changes in firing rates of place cells within their spatial fields have also been shown to represent different tastes (Herzog et al., 2019).

Cells in the hippocampus have also been shown to represent a key feature of episodic memory: time. MacDonald et al. (2011) showed that cells mapped a time delay in a task, with individual cells firing maximally at particular time points within the delay period. Later studies have shown time coding to be independent of distance travelled, and could be used to estimate varying time delays (Kraus et al., 2013; Shimbo et al., 2021).

Other place cell studies have shown ‘social place cells’ that respond to other rats (Danjo et al., 2018). This evidence all shows that hippocampal function is beyond just tracking current position in an environment, suggesting place cells may be a more general unit of complex experiential memory.

## 1.3 Cortical-hippocampal circuitry and functional connectivity

The hippocampus is, of course, not anatomically or functionally isolated. The human studies described in the first section illustrate the importance of its connections with cortical regions. Studying its connectivity with the rest of the brain is important in understanding episodic memory. In order to form spatial or non-spatial representations within the hippocampus, sensory input is required. The main cortical input to the hippocampus comes from superficial layers of the EC, and is the pathway via which para-hippocampal cortices send sensory information to be integrated into the hippocampus. The hippocampus then sends outputs to the cortex, via the EC, and directly to other regions such as the mPFC. Since the experimental work of this thesis will focus on hippocampal connections with the EC and medial prefrontal cortex (mPFC), these will be the focus of the following sections. Firstly, their anatomy, physiology and connectivity with the hippocampus will be discussed, leading onto the role of the networks in memory.

### 1.3.1 Entorhinal cortex anatomy

The entorhinal cortex (EC) forms the main cortical input to the hippocampus, with multiple neuronal populations projecting through different pathways to the hippocampus. It also receives feedback projections from the hippocampus. A diagram of the main excitatory connections between hippocampus and EC is shown in Figure 1.5A. The EC is divided into two separate areas, the medial entorhinal cortex (MEC) and lateral entorhinal cortex (LEC). This section will mainly focus on the MEC but will contrast with the LEC where there are important distinctions.

The superficial layers of the MEC form its main input to the hippocampus. This projection is composed of two cell types across layers 2 and 3. These cell types are described by their morphological properties, termed stellate cells and pyramidal cells (Figure 1.5A). They can also be distinguished by protein expression, with stellate cells expressing Reelin, and pyramidal cells expressing Calbindin (Witter et al., 2017). In layer 2 of the MEC, these two cell types form a characteristic anatomical pattern, with stellate cells spread out throughout the layer, and pyramidal cells forming separated clusters within this. This pattern earned them alternative names

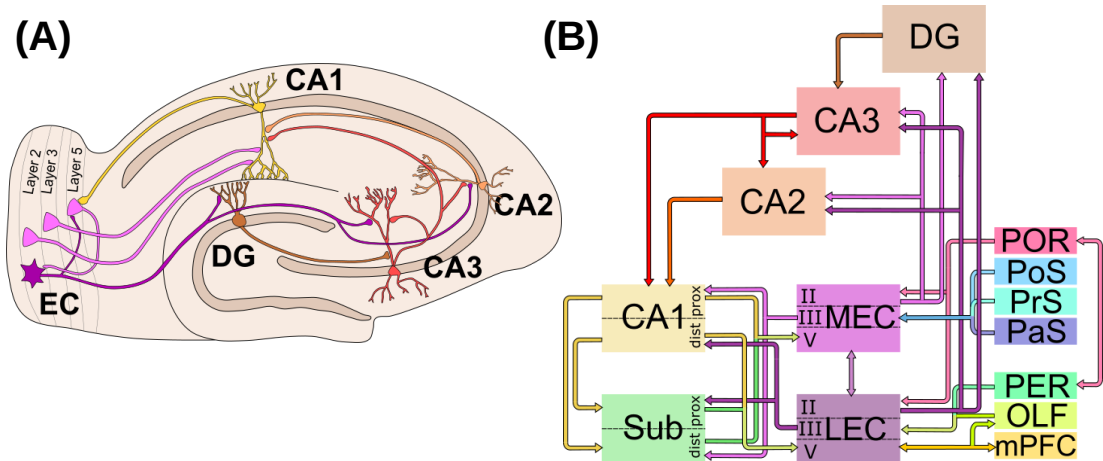


FIGURE 1.5: Entorhinal cortex-hippocampus circuitry. **(A)** The main excitatory connections of the entorhinal cortex and hippocampus. Stellate cells and pyramidal cells in the EC project to different regions of the hippocampus. The hippocampus projects back to the deeper layers of the EC. **(B)** Diagram showing some of the important connections of the EC. The LEC receives projections from the perirhinal cortex (PER), the olfactory cortex (OLF), medial prefrontal cortex (mPFC). The MEC receives projections from the presubiculum (PrS), parasubiculum (PaS), postsubiculum (PoS). Both MEC and LEC receive input from the postrhinal cortex (POR). It should be noted that many of these regions also project directly to the hippocampus (Adapted from Nilssen et al., 2019).

of ‘ocean cells’ for stellate cells and ‘island cells’ for pyramidal cells (Kitamura et al., 2014). As well as morphology, expression, and their placement within the layer, they also differ in their projections to the hippocampus.

Pyramidal cells project directly to CA1, via the temporoammonic path, where they synapse onto GABAergic inhibitory interneurons (Kitamura et al., 2014). Pyramidal cells are also present in layer 3 of the MEC. Projections of these neurons are spatially organised such that pyramidal cells in the dorsal part of the MEC project to dorsal hippocampus, and ventral MEC pyramidal cells project to ventral hippocampus. MEC pyramidal cells project principally to the proximal half of the CA1 cell layer, which borders CA2, while LEC pyramidal cells project to distal CA1, closer to the subiculum (Tamamaki & Nojyo, 1995; Witter et al., 2000).

The stellate cells, present only in layer 2 of the MEC, form the perforant path, projecting to the DG, CA3 and CA2 (Witter et al., 2017). They innervate excitatory cells in all regions, synapsing onto granule cells in the DG, and pyramidal cells in CA3 and CA2. This evidence originally came from the rat, and one study showed a lack of stellate cell projections to CA3 in mice, only showing DG projections (van Groen

et al., 2003); however, more recent evidence suggests it is also present in mice (Sürmeli et al., 2015). Stellate cells also project to pyramidal cells in the deep layers of EC (Sürmeli et al., 2015). There is a similar population of Reelin-expressing cells in the LEC, with similar hippocampal projections as MEC stellate cells, that have different morphology, and have been named fan cells instead (Tahvildari & Alonso, 2005; Vandrey et al., 2020). Similar to the pyramidal cell projections, there is a continued separation of DG terminals coming from the MEC and LEC Reelin-expressing layer 2 cells, with MEC projecting to the middle one-third, and LEC projecting to the outer one-third of the molecular layer (Witter, 2007b).

The deeper layers of the MEC contain pyramidal cells, which receive projections from CA1 of the hippocampus and the subiculum (van Haeften et al., 2003). Layer 5 cells in the MEC project to the superficial layers of MEC, as well as forming the main output to other cortical and subcortical structures throughout the brain (Witter et al., 2017). Layer 5 can be subdivided into 5a and 5b pyramidal cells, which have distinct protein expression patterns and connectivity (Ramsden et al., 2015; Sürmeli et al., 2015). Layer 5b cells are the targets of hippocampal projections, and themselves project to superficial MEC cells and layer 5a cells. Layer 5a cells form the major output of the MEC to other cortical and subcortical structures (Sürmeli et al., 2015; Witter et al., 2017).

Upstream of the EC, regions that project to the LEC and MEC are also partially segregated. The MEC is more strongly connected with the postrhinal cortex, post-subiculum and presubiculum, whereas the LEC receives more projections from the perirhinal cortex, olfactory cortex and mPFC (Burwell & Amaral, 1998; Nilssen et al., 2019). This may have implications for the respective functions of the two regions, with the MEC receiving input from more visual and spatial regions, and the LEC receiving input from more sensory associative regions. Although importantly, reciprocal connections exist between both the LEC and MEC, and the postrhinal cortex and perirhinal cortex (Burwell, 2000; Nilssen et al., 2019). Furthermore, re-analysis of anatomical data has suggested that the postrhinal cortex likely projects equally to the MEC and LEC (Doan et al., 2019; Nilssen et al., 2019) (Figure 1.5B).

### **1.3.2 Entorhinal cortex function**

An early experiment employing lesions of the entire entorhinal cortex in rats abolished the anchoring of hippocampal place cells to distal landmarks (Miller & Best, 1980), suggesting the entorhinal cortex may be important for the integration of sensory information into the hippocampal spatial representation.

Recording of superficial excitatory neurons in the medial entorhinal cortex (MEC) identified cells that were spatially modulated, that were categorised into a number of different functional cell types. The first of these to be defined were grid cells. Like place cells, grid cells show firing fields at particular locations, but grid cell fields regularly repeat throughout the environment (Fyhn et al., 2004). The fields of grid cells form a triangular lattice that tiles 2-dimensional environments. Different cells show different grid field sizes and spacing between fields – a dorsoventral gradient is seen, with smaller fields and less space between fields in cells recorded in the dorsal part, compared to those in the ventral part (Hafting et al., 2005). Grid cell populations are organised into ‘modules’, cells of which each show the same orientation and spacing between fields, but are shifted in location (Stensola et al., 2012).

Head direction cells have also been identified in the MEC. Head direction cells fire only when an animal is facing a particular direction, and had previously been found to occur in a number of other structures including the postsubiculum, which projects to the MEC (Taube et al., 1990). Head direction information is carried to the postsubiculum and MEC from other regions in which head direction cells have been observed. The head direction signal arises in connections between the dorsal tegmental nucleus and the lateral mammillary nucleus, which then projects to the anterodorsal thalamic nucleus, which projects to the postsubiculum (Taube, 2007). This is evidenced by the fact that lesions of upstream structures abolish the head direction firing of cells in downstream regions (Goodridge & Taube, 1997; Blair et al., 1998). Head direction cells are also observed in lateral dorsal thalamic nucleus and retrosplenial cortex, which are connected with other regions in the main head direction pathway (Taube, 2007).

Another type of cell that has been recorded in the MEC are border cells. Border cells show spatial fields which are located adjacent to, or at a set distance from, walls or edges of environments (Solstad et al., 2008). As well as the MEC, they

have been recorded in the subiculum and postsubiculum (Barry et al., 2006; Lever et al., 2009; Boccara et al., 2010).

A more recent study reported that a majority of cells recorded from the MEC show spatial firing that is more similar to place cells of the hippocampus than any of the previously mentioned MEC cell types, showing single spatial fields (Diehl et al., 2017). In addition, cells have been observed in the MEC which fire at a set distance and direction from an object or landmark (Høydal et al., 2019; Kinkhabwala et al., 2020; Andersson et al., 2021). Finally, speed cells show modulation of firing with running speed of the animal (Kropff et al., 2015). Furthermore, these cell functions are not completely distinct from one another – some cells exhibit head direction firing, grid firing, and speed modulation conjunctively, particularly in the deeper layers of MEC (Sargolini et al., 2006; Hardcastle et al., 2017). Interestingly, it has been shown that many grid cells in the superficial layers also encode head direction, with different directional firing preferences within each spatial firing field (Gerlei et al., 2020).

Discovery of these spatial cell types in the MEC initially led to the hypothesis that grid cells, along with the information from other spatially modulated cell types, may provide input to the hippocampus that is essential for place field formation (Moser et al., 2008). A number of computational models were developed to describe how this translation may work (Solstad et al., 2006; Savelli & Knierim, 2010; Giocomo & representation, 2011). However, evidence that place cell activity is intact, although less precise, with EC or MEC lesions (Miller & Best, 1980; Van Cauter et al., 2008; Hales et al., 2014) and that place cell activity in the hippocampus develops before grid cell activity in the MEC of juvenile animals (Langston et al., 2010), suggest that place cell activity may not be simply formulated from the combination of MEC spatial cell inputs.

Nonetheless, the MEC clearly provides an important contribution to spatial information coding by hippocampal place cells – many experiments have attempted to further specify what this may be. Like place cells, grid cells and other spatial cells in the MEC show changes in firing (similar to remapping) between different environments and in response to different environmental features (Fyhn et al., 2007; Diehl et al., 2017). Furthermore, the activity of cells in the MEC can predict the remapping observed in the hippocampus – environmental changes that induce rate remapping in the hippocampus do not induce drastic changes in grid cell firing, but

movement to a different environment where hippocampal global remapping is observed also induces reorganisation of the grid cell map (Fyhn et al., 2007). Unlike place cells, which respond heterogeneously to environmental changes, however, all grid cells show activity in all environments, with no changes in their firing in relation to one another (Hafting et al., 2005; Fyhn et al., 2007). A later study found that grid cells showed translation of firing fields in response to a change in contextual odour, that had previously been shown to induce partial global remapping in the hippocampus (Anderson & Jeffery, 2003; Marozzi et al., 2015). This, alongside experiments showing context-dependent firing by grid cells, and induction of artificial remapping of CA3 place cells through MEC manipulations, suggested that the MEC provides the hippocampus with information about the spatial context (Kitamura et al., 2015; Miao et al., 2015; Kanter et al., 2017).

Furthermore, grid cells, head direction cells, and border cells in the MEC show rotation with landmarks, similarly to place cells (Hafting et al., 2005; Solstad et al., 2008; Pérez-Escobar et al., 2016). Both grid cells and head direction cells show coherent responses to visual cue rotations, with consistent relational activity to one another (Taube & Burton, 1995; Hargreaves et al., 2007). In the absence of visual information, the regularity of MEC grid cell firing, and selectivity of head direction firing show strong degradation, indicating that visual information is an important factor for maintenance of spatial firing patterns in the MEC (Pérez-Escobar et al., 2016).

In describing the function of the MEC and its contribution to hippocampal spatial representations, it is useful to contrast with the LEC. Recordings in the LEC found cells that were much less spatially tuned than MEC cells in an empty environment (Hargreaves et al., 2005). However, LEC cells do show spatially-tuned firing patterns when rats are presented with objects in their environment (Deshmukh & Knierim, 2011). Cells represent the current positions of objects, as well as previous locations of objects after they have been moved (Tsao et al., 2013), showing a correlate of associative memory of object with location. Similar object-associated firing has been observed in cells of the perirhinal cortex, which projects strongly to the LEC as well as directly to CA1, although they perhaps carry less spatial information than those in LEC (Burke et al., 2012; Deshmukh et al., 2012). LEC cell firing patterns have also been shown to correlate with egocentric bearing relative to objects and boundaries of an environment – that is, the angle between the animal's head direction and an object (Wang et al., 2018). This is in contrast to

the observed cell types in the MEC, which tend to fire in the allocentric reference frame, independent of the animal's own relative head direction. This is exemplified by object-vector cells in the MEC, which fire in a set position relative to an object, independent of the direction in which the animal is heading (Høydal et al., 2019).

An experiment in which rats ran on a circular track with different textures on its floor, and surrounded with distal landmarks, showed that spatially-tuned cells in the MEC were more likely to anchor to distal visual landmarks, while LEC were more likely to anchor to the local information on the track (Neunuebel et al., 2013). The cellular correlates of the two regions led to the hypothesis that the function of the MEC was more involved in processing a global spatial representation – the “context” of an experience – while the LEC is more involved in processing of local features of an environment including objects – the “content” of an experience (Knierim et al., 2013). These are then combined into a complete representation in the hippocampus.

This hypothesis is supported by an experiment in which the DG, LEC and MEC were recorded simultaneously. The MEC and DG were synchronised during a task in which rats had to learn a rewarded spatial location, while the LEC and DG were synchronised while rats were performing a rewarded object discrimination task. Consistent with this, optogenetic disruption of the connection between MEC and DG led to impaired DG spatial firing and spatial memory, whereas disruption of the LEC connection to DG led to reduced object-associated firing in DG and impairment of the object task (Fernández-Ruiz et al., 2021). Earlier studies showed that MEC-lesioned rats are slower to learn the platform location in the Morris Water Maze, while LEC-lesioned rats can perform the task similarly to controls (Van Cauter et al., 2013; Hales et al., 2014; Poitreau et al., 2021). Additionally, changing the visual cues around the water maze after the rats had learned the platform location caused MEC-lesioned rats to perseverate to the old location, suggesting they had not used visual cues to navigate to the platform, perhaps using the more local information within the maze (Hales et al., 2014). This indicates that the MEC is important for navigation using a global sense of space. Furthermore, LEC-lesioned rats are impaired in associative memory tasks involving binding of objects with particular contexts, while MEC-lesioned rats are not (Van Cauter et al., 2013; Wilson, Watanabe, et al., 2013). This will be discussed in a later section, linked with the role the mPFC may play in memory tasks.

In addition to navigation using external cue information, another important function of the MEC is path integration. MEC lesions impair the ability to estimate distance travelled in real world in the absence of sensory cues (Jacob et al., 2017), and inactivation of MEC stellate cells impairs estimation of distance in virtual reality (Tennant et al., 2018). MEC lesions also caused a significant reduction in place cell stability when only self-motion information was available (Jacob et al., 2020).

Therefore, the EC provides important functions, broadly separated into spatial/context or non-spatial/content functions between the MEC and LEC, respectively. It is the role of the MEC in processing external information that will be explored in the first two experimental chapters of this dissertation (Chapters 2 and 3).

#### **1.3.3 mPFC anatomy**

Another important region connected with the hippocampus is the PFC. The structure of the frontal cortices is not as well-conserved between humans and rodents as the hippocampal formation. There has been much debate about where the equivalent region to the human PFC is in the rodent, or if it even exists. While comparisons cannot be made as directly between species, there are important similarities that still make the rodent a useful model for studying prefrontal function. The dlPFC in humans is often related to the rodent mPFC, due to similar functional correlates, although this remains controversial. The following sections will describe what is known about the anatomy and function of the mPFC in rodents, with a focus on its role in memory processes.

It was originally thought that rats do not have an area equivalent to human PFC, as they lack a granular area rostral to the motor cortex (Brodman, 1909). Rose and Woolsey (1948) used tracers to propose that the orbitofrontal region in rats could be considered as equivalent to human frontal cortex, because of reciprocal connections with the mediodorsal thalamic nucleus. This then became a defining feature for identification of prefrontal homolog regions (Uylings & van Eden, 1990). Originally proposed by Campbell and Hodos (1970), five criteria can be used to discuss cortical homologies in the rodent brain in comparison to the primate. These included the patterns and densities of connections, functional properties (electrophysiological and behavioural), distribution of neurotransmitters and receptors, embryological development, and cytoarchitecture (Uylings et al., 2003).

Mainly using the first of these criteria, characterising connectivity with the thalamus in particular, a number of regions were named as prefrontal cortex in the rodent. The rodent PFC is classically composed of the mPFC, orbito-frontal cortex (OFC) and agranular insular cortex. The mPFC is in turn divided into the prelimbic (PrL), infralimbic (IL) and anterior cingulate (ACC) areas, based on their connectivity with other structures (Ray & Price, 1992; Vertes, 2004). All of these sub-regions project strongly to the medial structures of the thalamus, including very dense reciprocal connections with the nucleus reuniens (NR) (Vertes, 2004). This division is a continual debate, however, and some evidence suggests a distinction between dorso-medial PFC (dmPFC) and vmPFC based on connectivity patterns, which splits the PrL area into two regions (Heidbreder & Groenewegen, 2003; Kamigaki, 2019). Similarly, recent analysis of mPFC connectivity studies in mice proposed a division into three broad, interconnected regions (Le Merre et al., 2021). Firstly, the dmPFC, which includes the ACC and is densely connected with sensory and motor cortices. Secondly, the vmPFC, which includes PrL, IL, and medial OFC, can be characterised by its strong connectivity with the limbic system, including ventral hippocampus, subiculum and perirhinal cortex. The lateral parts of the OFC constitute the vlPFC, and is defined by a combination of limbic and sensory input (Le Merre et al., 2021). For the purposes of this thesis, the mPFC will be defined as the PrL, IL and ACC, mainly focusing on the PrL.

A key difference between human and rodent mPFC is the lack of layer 4 cells in the rodent mPFC areas. The mPFC circuit contains both excitatory glutamatergic neurons, principally pyramidal cells in layer 2/3 and layer 5, and three main inhibitory GABAergic interneuron subtypes (Figure 1.6A). Pyramidal cells in layer 2/3 send projections laterally to other pyramidal cells, as well as to pyramidal cells in layer 5. Layer 2/3 pyramidal cells receive long-range projections from mediodorsal thalamus, hippocampus and amygdala, as well as neuro-modulatory inputs from the ventral tegmental nuclei, dorsal raphe nuclei, and locus coeruleus (Kuroda et al., 1996; Hoover & Vertes, 2007). Layer 5 pyramidal cells form the main excitatory output from the mPFC. They also receive similar long-range inputs as layer 2/3 pyramidal cells, but with reciprocal connections with subcortical and neuro-modulatory regions (Kuroda et al., 1996; Gabbott et al., 2005). There is some evidence for two subtypes of layer 5 pyramidal cells, distinguished by their dominant currents, and projections to either subcortical areas or contralateral cortex (Wang et al., 2006; Dembrow & Johnston, 2014).

Three subtypes of GABAergic interneurons have been identified in the mPFC, including parvalbumin-positive (PV), somatostatin (SST), and vasoactive intestinal peptide-expressing (VIP) neurons. Of these, PV interneurons are the most abundant and active, providing inhibitory input to pyramidal cells in all layers of the mPFC (Kuroda et al., 2004), and may play a key role in the excitation/inhibition balance within the mPFC. They receive excitatory input from the mediodorsal thalamus, and synapse directly with layer 5 pyramidal cells to regulate output (Ferguson & Gao, 2018). PV interneurons are fast-spiking and form somatically-targeted connections with large numbers of local pyramidal cells non-specifically, which gives the ability to influence network excitability through feedback and feedforward inhibition (Packer & Yuste, 2011; Kvitsiani et al., 2013; Ferguson & Gao, 2018). The importance of PV interneurons in cortical circuits has been shown through stimulation of thalamic inputs; excitation of pyramidal cells by thalamic inputs is followed by a strong hyperpolarisation due to activation of PV interneurons (Agmon & Connors, 1991; Delevich et al., 2015). This feedforward inhibition is important for temporal precision and specificity of excitatory inputs in cortical circuits, including the mPFC (Delevich et al., 2015). A model has been proposed for the connectivity of the mPFC circuit, emphasising the importance of thalamic input onto interneurons (Ferguson and Gao, 2018, Figure 1.6A).

#### **1.3.4 mPFC-hippocampal connectivity**

The mPFC receives inputs from many different brain regions, and sends projections to many different regions throughout the brain (Uylings et al., 2003; Heidebreder & Groenewegen, 2003; Le Merre et al., 2021). Its connectivity with the hippocampus may be important for certain aspects of memory (Eichenbaum, 2017). Three major pathways connect the mPFC and hippocampus, shown in Figure 1.6B (adapted from Eichenbaum, 2017). The first is a direct monosynaptic projection from CA1 to mPFC (shown in red). This projection mainly originates in ventral and intermediate hippocampus, axons terminating in all layers of mPFC and OFC (Swanson, 1981; Ferino et al., 1987). There is also some evidence for a direct connection from dorsal CA1 to the PrL area of the mPFC (Ye et al., 2017). A recent preprint has provided evidence for a monosynaptic projection in the opposite direction, from PrL to dorsal CA1 projection (Malik et al., 2021, preprint) (Figure 1.6B, purple).

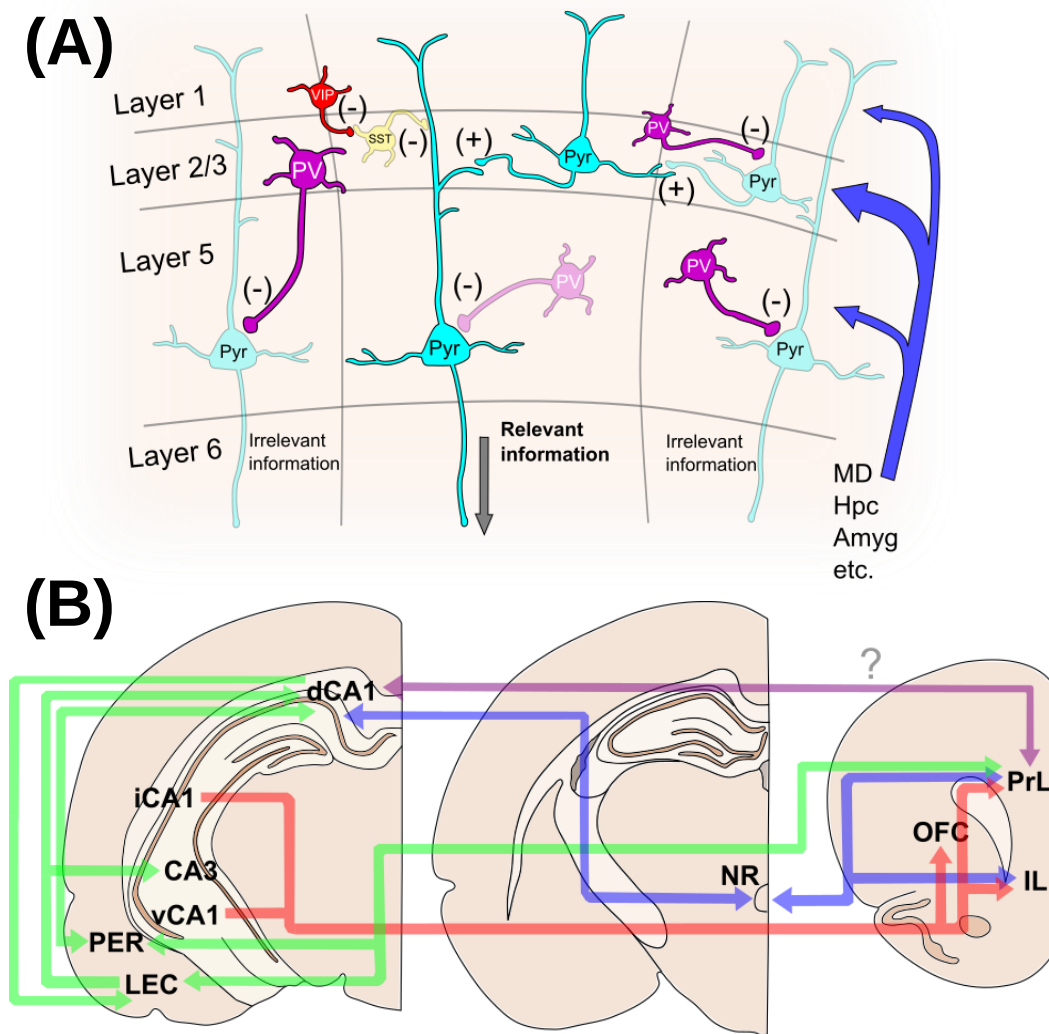


FIGURE 1.6: Circuitry of the medial prefrontal cortex and mPFC-hippocampus pathways. **(A)** Model of the cell types and their connections in the mPFC. Input from regions including mediodorsal thalamus (MD), hippocampus (Hpc) and amygdala (Amyg) arrive at all layers of the mPFC, exciting both parvalbumin-positive (PV) inhibitory interneurons and excitatory pyramidal (Pyr) cells. PV interneurons inhibit layer 5 neurons involved in transmitting irrelevant information to selectively output relevant information to other regions. Vasoactive intestinal peptide (VIP) and somatostatin (SST) interneurons also inhibit pyramidal cells (Adapted from Ferguson and Gao, 2018). **(B)** The main connections between the mPFC and hippocampus. The purple arrow shows possible direct connections between the pre-limbic area (PrL) and dorsal CA1 (dCA1). The blue arrows show the indirect connections between the PrL and infralimbic (IL) regions of the mPFC and dCA1, via the nucleus reuniens of the thalamus (NR). The red pathway shows direct projections from ventral (vCA1) and intermediate (iCA1) hippocampus to the PrL, IL and orbital-frontal cortex (OFC). The green pathway indicates the reciprocal connection between perirhinal cortex (PER) and lateral entorhinal cortex (LEC), which are both connected to regions of the hippocampus (Adapted from Eichenbaum, 2017).

The two other major pathways between the hippocampus and mPFC are indirect. As described above, this includes bidirectional connections between each area and the mediodorsal thalamus, particularly the nucleus reuniens (NR) in the thalamus (Vertes et al., 2007) (shown in blue in Figure 1.6B). The hippocampus has projections to and from the NR throughout its dorsoventral extent. In the mPFC, both the PrL and IL regions have bidirectional connectivity with the NR.

Another indirect connection passes through the para-hippocampal cortices (shown in green in Figure 1.6B). Bidirectional pathways exist between the PrL and IL regions of the mPFC and the perirhinal and entorhinal cortices, which in turn project to and from multiple regions of the hippocampus (as described above). Axons from the mPFC terminate in the superficial layers of the perirhinal cortex, and the deep layers of EC, in both the LEC and MEC (Burwell & Amaral, 1998; Apergis-Schoute et al., 2006). mPFC projections to the LEC are thought to be stronger than those to the MEC (Burwell & Amaral, 1998).

#### **1.3.5 mPFC function**

Initial theories of dIPFC function came from human patients with damage to the area, as well as primate research on the homologous region. Rodent studies then followed this with experiments showing similar effects of lesions, and correlates in electrophysiological activity. The functions perhaps most consistently linked to mPFC function include working memory and cognitive flexibility, processes often included under the broader concept of ‘executive function’.

As discussed at the beginning of this introduction, patients with damage to dIPFC exhibit impaired memory during conditions where it may be susceptible to interference, such as the paired-associate task. The PFC may therefore play a role in top-down organisation of memory encoding and retrieval (Szczepanski & Knight, 2014). Studies of non-human primates with dIPFC lesions found impaired working memory (Funahashi & Kubota, 1994), mostly using cue-response tasks where a stimulus is presented, followed by a delay period, then presentation of another cue, which is either the rewarded cue or a non-rewarded cue. Another important finding of such studies was that dIPFC neurons showed increased firing during the delay period, which appeared to be reduced by distracting stimuli (Fuster, 1973). A later study showed that dIPFC neuronal activity increased in the latter part of

the delay, and may reflect prospective coding for the rewarded cue (Rainer et al., 1999), suggesting the PFC may be involved in making predictions about future information. Furthermore, dlPFC neurons were found to encode task rules; monkeys were trained to either respond when two pictures were the same, or when they were different, depending on which rule was currently being rewarded. Activity of dlPFC neurons was found to be selective for these abstract rules (White & Wise, 1999; Wallis et al., 2001). Varying the location of the response cue showed that dlPFC neurons could also represent this spatial information (Rainer et al., 1998). This suggested that dlPFC neurons can adapt to represent diverse forms of information that are relevant to goal-directed behaviour (Duncan, 2001). The role of the dlPFC therefore, may be in selecting relevant information and removing the influence of irrelevant information.

Studies of the rodent mPFC show similar patterns to those of monkey dlPFC. For example, mPFC neurons show firing associated with particular phases of working memory tasks, as well as anticipatory firing in expectation of reward associated with a particular spatial location, similar to the prospective coding in primate dlPFC (Jung et al., 1998; Pratt & Mizumori, 2001). Early studies of the effects of lesions to the mPFC in rats found deficits in spatial working memory tasks and tests involving cognitive flexibility (Kesner et al., 1996; Ragozzino et al., 1999). Ragozzino et al. (1999) trained rats to learn the location of a reward that was initially associated with a particular response (turning left or turning right), no matter where they started on the plus-shaped maze. Upon successful learning, the rule was changed so that the reward was always in a particular place relative to the room (East or West). Rats with mPFC lesions, specifically to the PrL and IL, were impaired in learning the new strategy and inhibiting expression of the old one, but were able to switch between the two rules when both had been fully learned prior to the lesions, suggesting the PrL and/or the IL are important for adaptively learning new information rather than strategy-switching per se (Ragozzino et al., 1999; Rich & Shapiro, 2007). Furthermore, similar to the primate studies, electrophysiological recording showed rule-selective firing of neurons in the PrL and IL regions of the mPFC (Rich & Shapiro, 2009).

More recent studies have also shown flexible coding by mPFC neurons. In a study in which rats were first trained to perform three different actions sequentially on a diamond-shaped maze, and then were switched to perform the same actions in a

different context, and in the reverse order, ACC neurons showed very diverse responses to the actions. Some responded selectively to a particular action across both contexts and sequences, some were context- or sequence-specific, but the overall population showed responsivity to particular actions in each condition (Ma et al., 2016). This suggests the network was selecting the relevant behavioural output, through representation of the changing task conditions. In another experiment, calcium imaging was used to monitor activity of mPFC neurons as mice were trained to categorise visual stimuli into two groups based on a particular rule, and to respond by licking for the rewarded category (a go response) and to withhold licking for the unrewarded category (a no-go response). mPFC neurons showed no responsivity to the visual stimuli initially, but then developed category-selective and response-selective firing over the course of learning. In order to separate activity related to the response from activity related to the category of the visual stimuli, the two response options were next switched to left vs right licks rather than go/no-go responses. Following this response change, some neurons remained selective for particular categories (Reinert et al., 2021), indicating that activity in the mPFC can flexibly represent a variety of contextually-relevant information, and may provide the function of biasing neuronal populations downstream to select network activity that is suitable for goal-directed behaviour (Miller & Cohen, 2001).

Interneuron networks in the mPFC are important for maintenance and specificity of this activity. Firing of PV interneurons is modulated with attention during goal-directed tasks, and correlates with successful performance. This was shown using a serial reaction time task, in which mice were required to initiate the task, monitor an array of holes in which a cue would appear after a certain time period, then poke their nose into the hole in which the cue was presented. During the delay between the initiation and the cue, many mPFC cells classified as PV interneurons showed increased firing, and this effect was correlated with correct responses (Kim et al., 2016). Excitatory cells showed either decreased or increased firing in the delay, and increased suppression of excitatory output was also associated with successful performance (Kim et al., 2016). This suggests that the role of inhibitory firing in the mPFC may be to reduce the firing of neurons associated with irrelevant information that does not need to be attended, to increase specificity of the neuronal representation required to achieve the goal (Figure 1.6A). Moreover, another study investigated the effects of optogenetic activation of PV interneurons during extinction of a response associated with a particular cue when the cue was no longer rewarded. When PV interneurons were stimulated, the extinction of the response

was accelerated (Sparta et al., 2014). Inhibition of either SST or PV interneurons during a go/no-go cue-response task led to an impairment in performance that was mainly due to go responses to no-go cues (Kamigaki & Dan, 2017). Therefore, interneuron activity in the mPFC may regulate behavioural output through the suppression of inappropriate responses, which is important for flexibly adapting to new information. It is likely that this is important for suppressing interfering information during memory encoding and retrieval.

### 1.3.6 The hippocampus, EC, and mPFC in memory

The previous sections mostly considered the individual functions of the hippocampus, EC and mPFC, but it is important to examine their function in memory as an interconnected network. As described above, the mPFC and hippocampus are reciprocally connected via the EC (Figure 1.7B, green), as well as directly and via the thalamus (Eichenbaum, 2017).

A study by Navawongse and Eichenbaum (2013) investigated the effects of either mPFC or MEC inactivation on CA1 place cell activity during a context-guided memory task. In the task, rats were presented with two adjoined contexts with the same pair of different objects in each, the opposite object being rewarded in each context. Some CA1 place cells fired preferentially with rewarded object-context pairings as the rats learned the task. Bilateral pharmacological inactivation of mPFC, or of MEC, were sufficient to impair performance on the task, but had different effects on place cell activity. mPFC inactivation did not affect spatial firing of place cells significantly, but did result in reduced object selectivity of the cells. MEC inactivation, on the other hand, induced more unpredictable changes in CA1 place cell firing, with many place cells changing their spatial and object-context pairing selectivity, leading to reorganisation of the activity similar to remapping.

Studies investigating associative recognition memory using spontaneous object exploration tasks have also demonstrated the importance of the entorhinal cortex-hippocampus-medial prefrontal cortex network. Spontaneous object exploration memory tasks rely on the natural tendency of rodents to explore novelty in environments. In the simplest of such tasks, which tests recognition of objects (object recognition or OR task), rodents are presented with a pair of identical novel objects

and allowed to explore for a certain period of time. In the test phase, one of the objects is replaced with a different novel object that the animals have not seen before. If the rat explores the novel object for a longer time than the familiar object, it can be inferred that it had remembered the familiar object (Aggleton, 1985; Ennaceur & Delacour, 1988). This spontaneous object exploration task was adapted to test recognition of objects associated with particular contexts, or particular places, or particular places within contexts (Dix & Aggleton, 1999; Eacott & Norman, 2004) (Figure 1.7). The latter of these, the object-place-context (OPC) task, has been suggested as a model of episodic memory in rodents and, in support of this, is dependent on the hippocampus, while non-associative object recognition, as well as associative object-context (OC) and object-place (OP) memory are not (Langston & Wood, 2010). A more recent study found that the hippocampus was required for the OC task, possibly due to use of a longer delay between the sample phases and test phase (1 hour, compared to 2 minutes in Langston and Wood, 2010) (Barker & Warburton, 2020). The hippocampus is also necessary for a version of the task which tests non-associative memory for object locations (the object-location (OL) task) memory in which two identical objects are used, with one being displaced to a novel location in the test phase (Barker & Warburton, 2011). Similarly, the hippocampus is required for the object-in-place, in which four different objects are used, with two switching positions for the test phase (Barker & Warburton, 2011).

Lesions of mPFC, LEC, MEC or hippocampus do not impair object recognition (Mumby et al., 2002; Barker et al., 2007; Langston & Wood, 2010; Barker & Warburton, 2011; Van Cauter et al., 2013; Wilson, Watanabe, et al., 2013), but perirhinal cortex lesions do (Ennaceur et al., 1996; Norman & Eacott, 2005), suggesting this region is important for memory of specific objects. mPFC lesions, on the other hand, impair OiP and OPC, but not OL (Barker et al., 2007; Barker & Warburton, 2011; Barker & Warburton, 2020). This shows a role for the mPFC in recognition memory, perhaps particularly in instances where memory may be susceptible to interference, in instances where multiple associations must be made. The deficits may be caused by impaired disambiguation of the two sample phases in OPC, and impaired disambiguation of the multiple object-place pairings in OiP.

In terms of the EC, requirement on these tasks supports the hypothesis described previously, that the MEC is more involved in spatial context and LEC more involved in content and object representation (Knierim et al., 2013). Accordingly, MEC lesions or inactivations show disruption of OL tasks, where it is necessary to

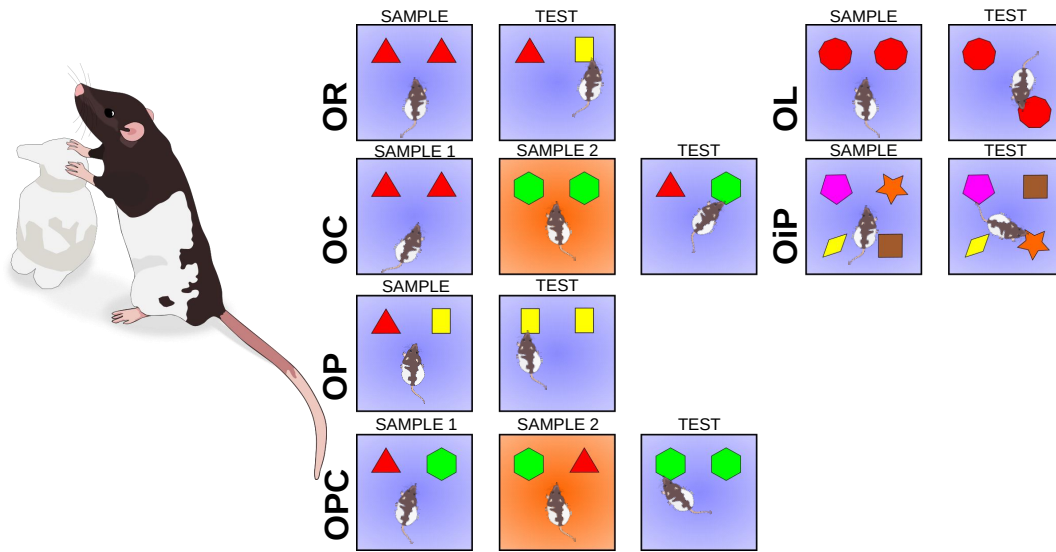


FIGURE 1.7: Spontaneous object association tasks. OR, object recognition: a rat or mouse explores two identical objects in the sample phase, then one object is replaced with a novel object in the test phase. OC, object-context recognition: two different pairs of identical objects are explored in each of the sample phases, then one of each pair is presented in the test phase, in one of the contexts. The object that has not been previously experienced in the test context is associated with novelty. OP, object-place recognition: A pair of different objects is presented in the sample, followed by a pair of one of the sample phase objects in the test. The object that has not been previously seen in its current position is associated with novelty. OPC, object-place-context recognition: Two pairs of different objects are presented in the sample phases, in opposite positions within different contexts. In the test phase, a pair of one of the objects is presented in one of the contexts; the object which has not been previously presented in its current position within the test context is associated with novelty. OL, object-location recognition: a pair of identical objects is presented in the sample phase, with one being displaced to a novel position within the environment for the test phase. OiP, object-in-place: four different objects are presented in the sample phase; two are switched in position for the test phase. The two objects that have been switched are associated with novelty.

remember the locations in which objects were experienced, but memory for object identity is not required (Van Cauter et al., 2013; Rodo et al., 2017; Tennant et al., 2018). In contrast, LEC lesions disrupt OC, OP, and OPC associative memory, but not OL memory (Wilson, Watanabe, et al., 2013; Kuruvilla et al., 2020). In each of these tasks, it is necessary to bind information about object identity with contextual and/or spatial information. These findings suggest that the MEC is important for forming an overall representation of space, and hence, memory of where objects are placed within it; whereas the LEC is important for binding of specific objects with the context in which they are experienced.

Experiments using a crossed disconnection lesion approach, in which a unilateral lesion of one structure is combined with a unilateral lesion of a different structure on the contralateral side of the brain (contralateral lesions) can be used to test the functional role of ipsilateral connections between the two structures (ipsilaterally-lesioned animals serve as controls). This technique has been used to show that ipsilateral connections between mPFC and LEC, and between mPFC and hippocampus are crucial for both OPC and OiP (Barker & Warburton, 2011; Chao et al., 2016; Barker & Warburton, 2020). Furthermore, specific inactivation of CA1-mPFC pathways has been shown to impair OiP and a variation of the task to test temporal order memory (Barker et al., 2017). The hippocampus-mPFC-LEC pathway has therefore been proposed as an important circuit for episodic memory.

One of the ways in which these circuits may interact is based on another of the pathways between the mPFC and hippocampus. The NR, which is reciprocally connected with both CA1 of the hippocampus and mPFC, is thought to synchronise activity of the two regions, which may facilitate interaction via direct, or other indirect, pathways (Eichenbaum, 2017). This comes from studies in which interactions between the mPFC and hippocampus have been shown to correlate with learning. Jones and Wilson (2005) first demonstrated that activity in the mPFC and hippocampus synchronises during the choice point of a T-maze spatial working memory task. The specific nature of this interaction will be discussed further in the following section on network oscillations. Interactions between the mPFC and hippocampus are associated with correct decision-making and increase as animals learn such tasks, including new rules on a strategy-switching version of the task (Jones & Wilson, 2005; Hyman et al., 2010; Benchenane et al., 2010).

The NR appears to be key to this synchronisation of activity. Firstly, trajectory-dependent firing has been observed in mPFC, CA1 and NR during a similar task, which is reduced in CA1 after NR inactivation (Ito et al., 2015). Another study showed that working memory, and synchronisation of mPFC and hippocampus was dependent on the NR, and further showed two phases of directed interaction between the two areas (Hallock et al., 2013; Hallock et al., 2016). In a delayed spatial working memory task, hippocampus was shown to influence mPFC during the delay period, while mPFC influenced the hippocampus during the decision, both of which were dependent on NR (Hallock et al., 2016). Place et al. (2016) showed similar directed interactions in a context-guided memory task, as described above.

Hippocampus-mPFC directed interactions were shown as the rats entered the context, followed by mPFC-hippocampus directed interactions as the rats sampled the objects (Place et al., 2016). This evidence suggests the influence of hippocampus on mPFC may be important for context or cue-guided learning, with mPFC then influencing hippocampus for retrieval of appropriate contextual memories (Spellman et al., 2015; Place et al., 2016). This influence of mPFC on hippocampal retrieval could be via selection of appropriate memories in the LEC, which has been shown to interact with the hippocampus during cue-guided memory tasks (Igarashi et al., 2014; Fernández-Ruiz et al., 2021), or potentially through direct interactions. Both of these directed interactions may be facilitated by synchronisation mediated by NR inputs (Hallock et al., 2016; Eichenbaum, 2017).

The studies described above indicate that the function of the mPFC is in retrieval of appropriate memories. However, other evidence suggests that it may also influence encoding of memories in the hippocampus. Blockage of NMDARs in the mPFC during the sample phase, but not during the test phase, of an OiP task impaired subsequent memory during the test phase, suggesting mPFC is required for the formation of the memory (Barker & Warburton, 2008). Guise and Shapiro (2017) analysed CA1 population prospective coding on a strategy-switching task, finding that inactivation of mPFC during learning of rules led to less distinct CA1 representations during learning. This suggested that mPFC activity during learning of the rules was important for separating CA1 activity to reduce interference between different rules (Guise & Shapiro, 2017). Thus, mPFC interactions with hippocampus appear to play a role both in the formation and retrieval of memories.

## **1.4 Network oscillations and mechanisms of connectivity**

The preceding sections reviewed the evidence that interactions between hippocampus and prefrontal cortex play a role in memory encoding and retrieval. Interactions between brain regions can be facilitated by coupling of the excitability of networks. This is thought to be achieved through synchronisation of oscillations in the electrical fields in communicating regions (Fries, 2005). All simultaneous currents in the brain summate to result in an electrical field; recording the electrical field of the brain across time shows rhythmic oscillations created by synchronised activity of

networks. The name given to this electrical field depends on how it is recorded; when recorded using scalp or skull surface electrodes, the overall electrical field generated from relatively large areas of the brain is referred to as electroencephalogram (EEG). When recorded from electrodes implanted within the brain, more local electrical field activity can be recorded, referred to as the local field potential (LFP). The main contributor to the LFP/EEG are synaptic potentials, and the amplitude or power of oscillations depends on the level of synchrony within the network or networks being recorded (Buzsáki et al., 2012).

##### 1.4.1 Theta and gamma oscillations

In the hippocampus, the dominant oscillation frequency is known as theta and is clearly discernible from the LFP during the awake state, especially during movement and attention. It was first reported in rabbits (Jung & Kornmüller, 1938), and its relationship to hippocampal neuronal spiking in rats was first reported by Buzsáki et al. (1983). In rats, theta is a 6-12 Hz oscillation, and is prominent as the animal moves through an environment (Vanderwolf, 1969; O'Keefe & Nadel, 1978). Figure 1.8A shows an example trace recorded from a freely moving rat, overlaid with a line showing the signal filtered in the theta range. This oscillation is clearly visible in the signal, showing around seven sinusoidal cycles in a second.

The original paper by Buzsáki et al. (1983) detected and characterised the “rhythmical slow activity”, defined as theta, and “hippocampal fast activity” at 25-70 Hz, which was later defined as gamma. In addition, it was found that spiking of individual neurons occurred at particular phases of these oscillations, supporting the idea that the function of network oscillations may be to orchestrate the activity of ensembles of neurons within the network. This may provide the function of discretising information processing into separate cycles of oscillations.

One demonstration of this is place cell theta phase precession. As a rodent runs through a place cell's firing field, the spiking of that cell will be timed to a particular phase of the ongoing theta oscillation. As the rat enters the field, the cell will fire at a particular phase of the theta oscillation, then as the rodent moves through the field, the cell's spikes are timed to progressively earlier phases (Figure 1.8B) (O'Keefe & Recce, 1993). Therefore, the timing of the place cell spikes relative to the theta oscillation contains information about position within the place field

(Jensen & Lisman, 2000). Extending this to populations of place cells, the temporal order of firing of cells within a theta cycle carries information about the spatial order of firing fields; cells firing at late phases have fields ahead of the animal, while those firing at earlier phases will have fields behind the animal (O'Keefe & Recce, 1993; Skaggs & McNaughton, 1996; Gupta et al., 2012). This can be used to encode the sequences of place cells that are ahead of the animal, and may be used to plan future trajectories (Johnson & Redish, 2007).

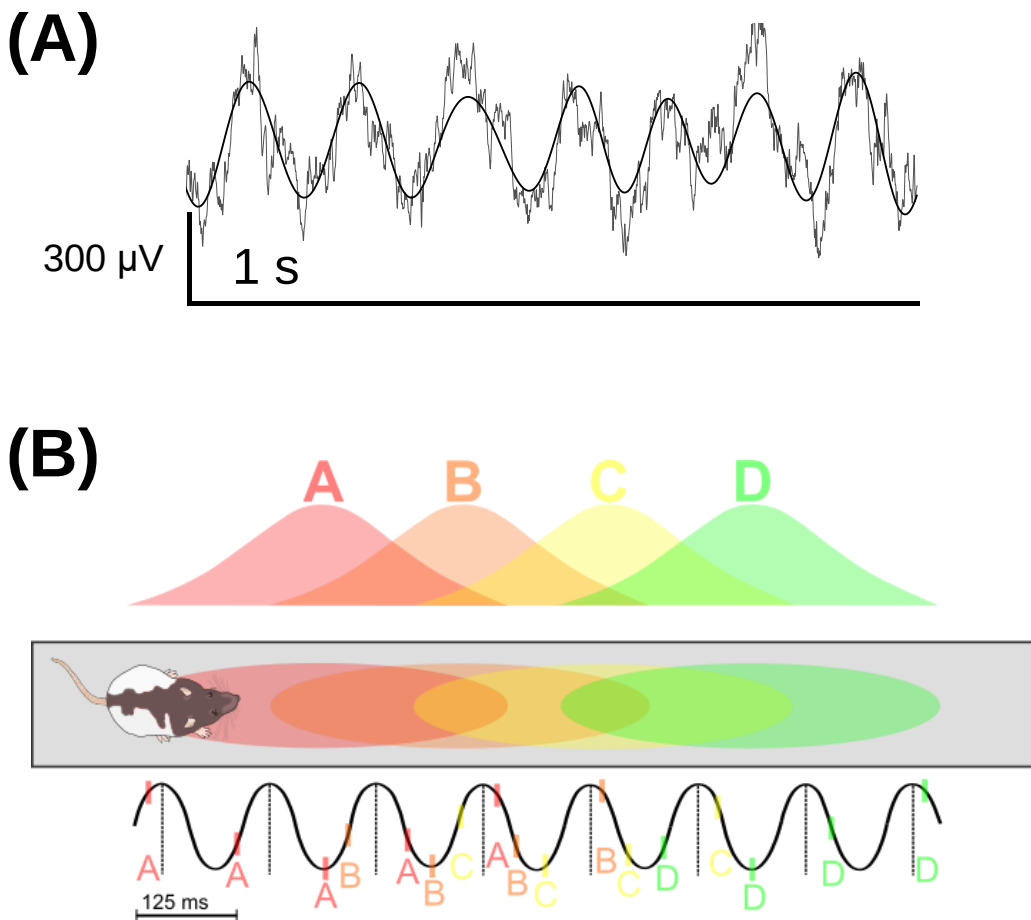


FIGURE 1.8: Theta and gamma oscillations. **(A)** A theta oscillation recorded from the LFP of dorsal CA1 while a rat was running. The raw trace is shown, overlaid with the signal filtered in the theta range, showing a 7 Hz oscillation. **(B)** Theta phase precession: A, B, C and D represent different place cells with overlapping firing fields along a linear track. The phase of the theta oscillation at which a place cell fires becomes earlier as the rat moves through its place field. Each cycle (between the dotted lines) reflects information about the rat's trajectory through the place fields (Adapted from Drieu and Zugaro, 2019).

The potentiation of synaptic connections in the hippocampus may be preferentially

expressed depending on theta phase (Pavrides et al., 1988), leading to the proposal that memory encoding and retrieval may be compartmentalised to different theta phases, allowing separation of the two processes (Hasselmo et al., 2002).

Separation of encoding and retrieval within the theta cycle has also been shown to correlate with separation of gamma oscillations within the theta cycle. Two distinct oscillation frequency bands were originally identified in the gamma range in the hippocampus – low gamma oscillations (30-50 Hz), and medium/high gamma oscillations (60-100 Hz) (Csicsvari et al., 2003; Colgin et al., 2009), although more recent evidence suggests there may be a greater number of gamma states (Lasztóczy & Klausberger, 2016; Zhang et al., 2019). Power of these oscillations is modulated by the phase of the ongoing theta oscillation (Bragin et al., 1995), as shown in Figure 1.9A. Periods of gamma oscillations may reflect periods of high responsiveness to particular inputs. In CA1, low gamma oscillations were found to be associated with CA3 inputs, facilitating memory retrieval, while high gamma oscillations were associated with sensory input from EC, facilitating memory encoding (Bragin et al., 1995; Csicsvari et al., 2003; Colgin et al., 2009).

Neuronal spiking also locks to the gamma rhythm (Buzsáki et al., 1983; Senior et al., 2008). A model of CA3 function proposed that fast cycles of high threshold states, with high inhibitory firing, may provide the conditions for pattern completion, as it causes only the patterns most strongly associated with current input activity to fire (Gardner-Medwin, 1976). A later model of theta-gamma coupling proposed that individual items may be processed on the cycles of a gamma oscillation within each theta cycle. The model used an “after-depolarisation” period, dependent on neuromodulators, which promotes excitability in the following cycle. This would mean networks of neurons that fire on a gamma cycle early in the theta oscillation on one cycle would be most excitable early in the next theta cycle, and firing of individual networks could be separated out by the gamma oscillation cycles to store multiple items in order (Lisman & Idiart, 1995; Lisman & Jensen, 2013). This predicts that, in the hippocampus, place cell ensembles would fire within the same cycle of the gamma oscillation, and there is some evidence that this may be the case (Dragoi & Buzsáki, 2006; Lisman & Jensen, 2013).

Increased theta-gamma coupling in the hippocampus has been associated with

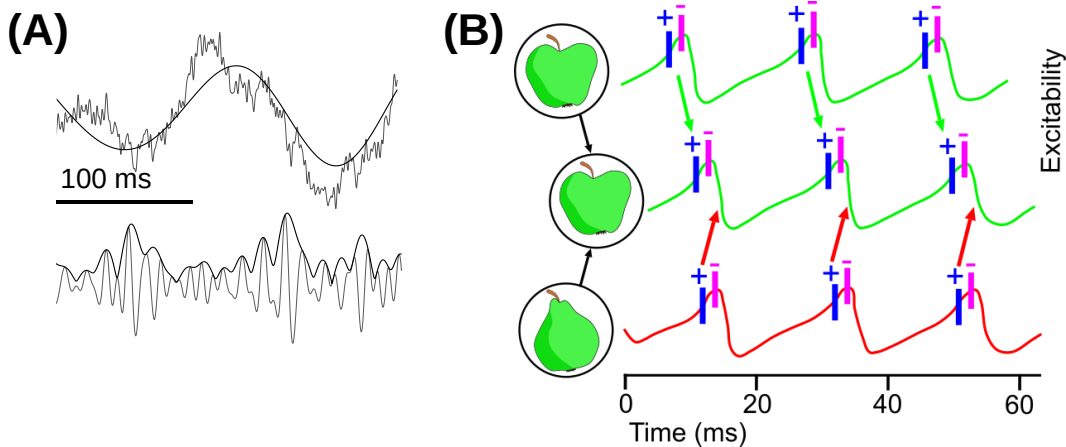


FIGURE 1.9: Modulation of gamma oscillations. **(A)** An example of theta-gamma phase-amplitude coupling. The raw signal is shown in the above trace, overlaid with the theta-filtered signal. Below, the signal is filtered in the high gamma range (60-100 Hz) – peaks of the amplitude of this oscillation occur at certain phases of the theta oscillation (above). **(B)** Communication through coherence: A stimulus entrains multiple networks into gamma rhythm within an initial region (e.g. V1), through feedback inhibition. The ensembles that fire early entrain a coherent gamma rhythm in a higher region (e.g. V4) (Adapted from Fries, 2015).

learning of item-context associations (Tort et al., 2009). As well as within the hippocampus, modulation of gamma in other regions by the hippocampal theta oscillation has been observed, suggesting the theta rhythm can be used to coordinate communication between different regions (Siapas et al., 2005; Tort et al., 2008).

How are these network oscillations generated? In the hippocampus, the theta oscillation has been shown to depend on connections with the medial septum in the forebrain that act as a pacemaker (Winson, 1978; Hangya et al., 2009). These projections consist of GABAergic cells that fire rhythmically, innervating inhibitory interneurons in the hippocampus to generate theta oscillations (Freund & Antal, 1988; King et al., 1998). Disruption of these oscillations by manipulating medial septum neurons disrupts the theta oscillation in both hippocampus and MEC, and causes spatial memory deficits (Winson, 1978; Mitchell et al., 1982; Quirk et al., 2021). Interestingly, medial septum inactivation has also been shown to reduce the periodicity of grid cell firing in the MEC, while leaving head direction selectivity intact. Spatial firing in the hippocampus remains during medial septum inactivation, suggesting theta rhythmicity is specifically necessary for periodic firing of grid cells, but not for spatially-modulated firing generally (Koenig et al., 2011; Brandon et al., 2011). Further, the theta rhythm in the hippocampus may depend at least partially on the MEC, as it is reduced following MEC inactivation (Schlesiger et al.,

2015; Zutshi et al., 2022). Hippocampal place cells also show reduced theta phase precession following MEC lesions (Schlesiger et al., 2015).

Gamma oscillations are likely the result of rhythmic inhibition onto excitatory neurons. Buzsáki et al. (1983) noted that inhibitory interneurons were consistently phase locked to the gamma oscillation, suggesting their rhythmic firing may have more to do with generating gamma oscillations, compared to pyramidal cells, which were less consistently phase-locked (Buzsáki et al., 1983; Csicsvari et al., 2003). Intracellular recordings during anaesthesia supported this, showing that inhibitory postsynaptic currents in pyramidal cells were synchronised with the gamma oscillation (Soltesz & Deschênes, 1993). Furthermore, during gamma activity in the hippocampus, firing of pyramidal cells generally precedes firing of interneurons by a few milliseconds, suggesting feedback inhibition plays a role in generating the rhythms following excitation of the pyramidal network (Csicsvari et al., 2003). Stimulation of populations of inhibitory interneurons is sufficient for generation of oscillations in the gamma frequency range (Whittington et al., 1995; Cardin et al., 2009) and optogenetic silencing of interneurons can eliminate gamma oscillations (Sohal et al., 2009). Therefore, inhibitory networks, possibly synchronised through gap junction connections (Hormuzdi et al., 2001), play a key role in generating gamma oscillations.

A number of studies have proposed that PV interneurons specifically are important for gamma generation (Fuchs et al., 2007; Cardin et al., 2009; Sohal et al., 2009; Chen et al., 2017). As described above for the mPFC, PV interneurons form perisomatic synapses with large numbers of pyramidal cells, so may have strong influence over excitability of the network and would be well-placed to produce synchronous inhibitory postsynaptic activity (Packer & Yuste, 2011; Cardin, 2016; Ferguson & Gao, 2018). However, some studies have demonstrated that SST interneurons may also be important for generating gamma oscillations (Veit et al., 2017; Antonoudiou et al., 2020).

#### **1.4.2 Coherence: theta and gamma**

Just as network oscillations can orchestrate neuronal activity and separate computational processes in one region, coherence between oscillations in different

regions can synchronise the neuronal activity in those different regions. Coherence is a measure of the degree to which two signals are related. Inter-region coherence ensures that inputs from one region arrive at another at times of high postsynaptic excitability (Fries, 2005). The updated “communication through coherence” theory suggests that networks that are preferentially excited entrain the gamma rhythm through feedback inhibition, which then entrains downstream regions with a phase delay, leading to interregional coherence that facilitates pairing of the two networks (Fries, 2015). This could increase the selectivity of transmitted responses, as synaptic inputs that arrive following this entrainment would be essentially cancelled by the inhibition resulting from the previous inputs (Figure 1.9B). This was demonstrated by an experiment in macaques, where two visual stimuli were presented simultaneously, but only one was relevant to perform a task. It was found that both stimuli entrained separate gamma rhythms in the lower visual area, V1, but only the gamma oscillation of the attended stimulus entrained gamma in the higher visual area, V4 (Bosman et al., 2012). Through this mechanism, neurons that receive projections from multiple upstream regions, which may oscillate at different gamma frequencies to one another, can synchronise with specific regions if their excitability is modulated at the appropriate frequency and phase delay. Gamma frequency is continuously modulated by balance of excitatory signals with inhibition to accommodate this (Atallah & Scanziani, 2009; Buzsáki & Schomburg, 2015).

This entrainment of gamma oscillations by upstream regions is evident in the hippocampus. Analysis of currents across the layers of the hippocampus suggest that distinct gamma rhythms in the CA1 region are generated through entrainment by CA3, or by the DG and the EC upstream of it (Bragin et al., 1995; Csicsvari et al., 2003). These may occur at different frequencies, and may reflect preferential input from CA3 for memory retrieval, or EC for memory encoding (Colgin et al., 2009). Different gamma frequencies may also be used for communication between the MEC or LEC and DG. Coherence between MEC and DG at a high frequency range was observed during a spatial memory task, while LEC was coherent with the DG at low gamma frequencies in a rewarded object discrimination task, suggesting preferential entrainment for certain functions (Fernández-Ruiz et al., 2021).

While gamma coherence may reflect transmission of synaptic input from one region to another, theta coherence may occur as a result of active synchronisation of brain regions in order to facilitate coupling. As theta is a slower oscillation than gamma,

providing around 100-200 ms periods for conduction, it can facilitate longer conduction delays associated with longer range interregional connections (Colgin & Moser, 2010). The hippocampal theta rhythm synchronises with both the EC and mPFC, through different mechanisms. As mentioned previously, interactions have been shown between mPFC and hippocampus during spatial memory tasks; such interactions are facilitated by coherence of theta oscillations between the two regions, which is correlated with correct task performance (Jones & Wilson, 2005; Siapas et al., 2005; Sigurdsson et al., 2010). This coherence is dependent on the NR (Hallock et al., 2016), and may provide optimal lags for transmission of signals in either direction, depending on the required cognitive process (Place et al., 2016).

Consequently, mPFC cells show phase-locking to the hippocampal theta oscillation, and phase precession (Siapas et al., 2005), and current position can be simultaneously decoded from both mPFC and hippocampus population firing within theta cycles (Zielinski et al., 2019). This organisation leads to increased correlations between cells firing in the two regions, which increases with acquisition of a task (Benchenane et al., 2010). This indicates that the synchronised theta rhythm between the hippocampus and mPFC, which is dependent on the NR, is important for orchestration of interactions between the networks for formation of memory ensembles.

### **1.4.3 Consolidation: ripples, delta waves and spindles**

Memory consolidation refers to the process of gradual stabilisation of memories after learning, in order to store them long-term. The idea was first suggested by Müller and Pilzecker (1900), to explain the fact that more recently-learned memories are vulnerable to interference, known as retroactive interference. This was supported by the evidence from amnesic patients showing that more remote memories were less likely to be lost than more recent ones (Scoville & Milner, 1957; Penfield & Milner, 1958). The process of consolidation in the brain involves a dual mechanism of shorter time scale synaptic changes within hippocampal networks, known as synaptic consolidation, and longer time scale active reorganisation of hippocampal and cortical networks, known as systems consolidation, which involves network oscillations (Dudai, 2004).

Synaptic consolidation refers to the molecular and structural changes that occur

at the synapse within minutes to hours after learning, leading to strengthening of its connection. Synaptic consolidation is needed in order for connections between neurons to endure long-term. In hippocampal neurons, glutamatergic activation of excitatory post-synapses through interaction with alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors) initiates sodium ion influx into the cell. This depolarisation leads to removal of a magnesium block from another glutamate receptor type, NMDA receptors, which allow calcium ions into the cell (Collingridge et al., 1983; Nowak et al., 1984). This produces a further depolarisation, as well as initiating signalling pathways that lead to synaptogenesis and remodelling, and trafficking of AMPA receptors and NMDA receptors into the synaptic membrane. This results in the post-synaptic neuron more likely to fire an action potential on further stimulation by the pre-synaptic neuron, hence consolidating the memory trace of which it is a part. This activity-dependent synaptic strengthening is known as long-term potentiation (LTP), with the opposite being long-term depression, in which certain patterns of activity lead to weakening of the synaptic connection (Bliss & Collingridge, 1993).

Synaptic memories in the hippocampus are thought to be temporary, existing for a short time window, and are prone to disruption through interference. Longer-term storage may then rely on systems consolidation, which involves the redistribution of hippocampal memories to the neocortex. Amnesic patients with hippocampal damage, such as H.M., while not being able to form new memories or remember recent events, can often recall more remote memories. And the older the memory, the more likely such patients are to be able to recall them. This suggests that memories undergo a process of transferal to cortical regions, gradually becoming independent of the hippocampus over long periods of time. This idea became the basis of the standard model of systems consolidation (Marr, 1971; Squire & Alvarez, 1995).

A later theory, the multiple-trace theory of systems consolidation, was developed to explain the fact that the hippocampus may be continually required for the retrieval of contextual episodic memories, and the extent of damage to the hippocampus may determine the extent of remote retrograde amnesia (Nadel & Moscovitch, 1997). It stated that consolidation of episodic memories involves reorganisation of memory traces both in the hippocampus and cortex, with memories gradually becoming more distributed throughout hippocampal as well as cortical networks

over time (Nadel & Moscovitch, 1997; Frankland & Bontempi, 2005). A further development to this theory suggested that prior knowledge can influence the rate of systems consolidation, with new information being integrated into existing frameworks, or “schemas”. Tse et al. (2007) used a hippocampus-dependent spatial paired-association task to show that novel paired-associations could be consolidated within 24 hours, after one training session, if rats had undergone extensive training on the task over weeks. This was emphasised by the finding that memory for novel associations could become independent of the hippocampus within 48 hours (Tse et al., 2007). This was associated with rapid upregulation of immediate early genes in the mPFC, and inactivation of the mPFC prevented consolidation of new learning (Tse et al., 2011). This suggests that cortical networks may encode memory in parallel to the hippocampus, through updating existing general schemas.

Further evidence for this parallel encoding has come from an experiment in which neurons in the mPFC, MEC and hippocampus were manipulated. It was found that contextual fear conditioning led to activation of neurons in the ACC of the mPFC, an effect which was not seen when MEC-ACC projections were inhibited. Subsequent stimulation of the activated ACC cells, known as “engram” cells, on the following day induced freezing behaviour in an unconditioned context. To test whether the mPFC engram cells were necessary for recall, they were inhibited on the following days in the conditioned context – they were not required for recall 24 hours after conditioning, but were required after day 12, coincident with observed dendritic spine maturation. On the other hand, DG engram cells were preferentially activated in the conditioned context on day 2, but were not on day 13; however, their stimulation could elicit freezing in an unconditioned context at both time points. And finally, inactivation of the DG cells led to a lack of reactivation of the mPFC engram cells on day 12, and a lack of their spine maturation (Kitamura et al., 2017). These experiments demonstrate the early encoding of a contextual memory in the mPFC, as well as a gradual maturation of the mPFC memory for remote recall, which required the ongoing activity of the hippocampal network. The memory appeared to still be stored in the hippocampus at the remote time point, but was less active.

It is clear then that over time memories become more distributed to cortical networks, although the continued dependence on hippocampus for retrieval is debated. This distribution relies on coordinated interactions between the hippocampus and cortex, which may occur during “offline” states, such as quiet wakefulness

and, importantly, slow-wave sleep (SWS).

### **Slow-wave sleep**

It is well-known that sleep in the hours following learning prevents forgetting of new information, suggesting its importance in memory (Rasch & Born, 2013). During sleep, the brain cycles through multiple different stages of characteristic activity, including rapid eye movement (REM) sleep, and non-REM (NREM) sleep. In humans, NREM makes up the initial phase of sleep, first with the lighter stages 1 and 2, then moving onto stages 3 and 4, which are termed slow-wave sleep (SWS) for the low frequency oscillations that occur in the cortical field potential. Following SWS, the brain moves to the deepest stage of sleep, REM. While it is not clear whether REM sleep is important for memory consolidation, SWS has a well-established role in declarative memory consolidation. SWS typically comes before rapid eye movement (REM) sleep in the sleep cycle, and is characterised by cortical slow wave (delta, 0-4 Hz) oscillations, thalamo-cortical spindles (10-16 Hz), and hippocampal sharp-wave ripples (100-250 Hz) in the EEG/LFP signals. These oscillatory events temporally associate with one another to coordinate networks and facilitate memory consolidation (Diekelmann & Born, 2010).

### **Ripples and reactivation**

Sharp-wave ripples (ripples) are another feature of the hippocampal LFP, identified by Buzsáki et al. (1983) as “large-amplitude irregular activity”. Ripples are irregularly occurring sharp waves in the LFP which last between 40 and 100 ms, and occur when animals are at rest or in SWS, and not during the theta oscillation. An example ripple is shown in Figure 1.10A. They are accompanied by bursts of spiking in the pyramidal layer. These neuronal spiking bursts underlie temporally-compressed replaying of events that occurred during earlier behavioural episodes (Wilson & McNaughton, 1994). Therefore, sequences of place cells are activated during ripples, in the order in which their place fields were traversed during wake (Nádasy et al., 1999; Lee & Wilson, 2002). The importance of this process for memory consolidation has been demonstrated through the disruption of ripples during SWS following learning, which causes poorer performance on subsequent

tests of memory recall (Girardeau et al., 2009; Ego-Stengel & Wilson, 2010). In addition, activation of memory ensembles during ripples has been shown to induce synaptic potentiation (Sadowski et al., 2016). Consolidation of novel experiences are upregulated, as sequences of place cells that were associated with spatial novelty are reactivated more strongly in subsequent sleep than those associated with more familiar places (O'Neill et al., 2008). Rewarded experiences are similarly upregulated (Singer & Frank, 2009).

Ripples are initiated by activity in CA3, leading to simultaneous activation of both excitatory and inhibitory neurons in CA1 (Buzsáki, 1986; Ylinen et al., 1995). Ripples are intrinsic to the hippocampus, as they persist with lesions to external regions such as EC or medial septum (Buzsáki et al., 1983; Suzuki & Smith, 1988; Bragin et al., 1995). The strong recurrent connections within CA3 are thought to be important for the initial generation of ripples, possibly occurring when excitatory and inhibitory potentials build up within populations, until a threshold is reached and synchronous burst firing can occur (de la Prida et al., 2006). Groups of interneurons show different patterns of activity during ripples, some increasing their firing, others decreasing, and some increasing only at the beginning and end of ripple events (Csicsvari et al., 1999). Stimulation of PV interneurons can trigger ripples in vitro, which has been modelled as a disinhibition of another class of interneurons, which usually act to suppress ripple generation (Schlingloff et al., 2014; Evangelista et al., 2020). A recent experiment showed that the specific timing of CA1 pyramidal cell spiking within ripple events was determined by the level of transient inhibition before the ripple event, with greater membrane hyperpolarisation before the ripple resulting in later discharge (Noguchi et al., 2022). This evidence emphasises the importance of GABAergic interneurons in organising network activity and possibly mediating the specificity of firing during hippocampal ripple events.

Reactivation of memory ensembles during SWS also occurs in the cortex. Qin et al. (1997) recorded from the hippocampus and parietal cortex before and after a maze task, and found the correlations between cells within each area, as well as between areas, were more similar to the awake maze behaviour after the task than before. This has since been shown between the hippocampus and other cortical areas, including visual cortex and mPFC (Ji & Wilson, 2007; Euston et al., 2007). Recording SWS after learning of strategy-switching rewarded task found coordinated reactivation of hippocampal and mPFC sequences that were associated with the learning of rules (Peyrache et al., 2009). Furthermore, simultaneous

reactivation of ensembles in the hippocampus and mPFC during SWS was found to correlate with those that had been associated with one another during theta coherence while learning the task (Benchenane et al., 2010). This suggests that coherence during wake supports formation of cell associations between regions, which enhances their coordinated reactivation during subsequent SWS.

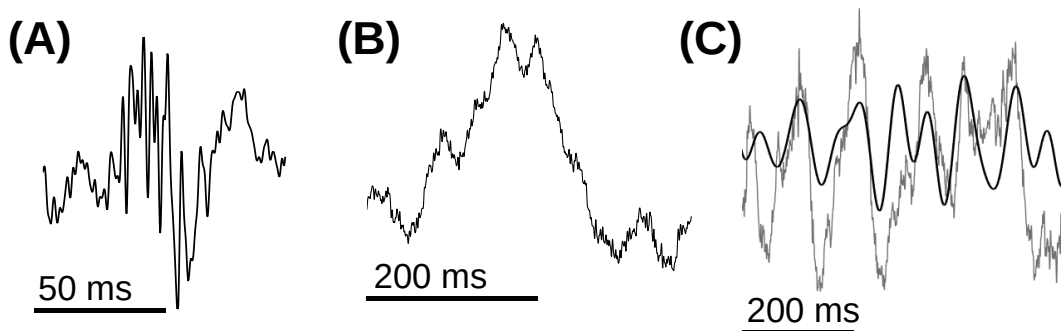


FIGURE 1.10: The LFP oscillations of slow-wave sleep. **(A)** An example of a sharp-wave ripple (100-250 Hz) recorded in dorsal CA1 of the hippocampus of a rat. **(B)** An example delta oscillation (0-4 Hz) peak recorded in the mPFC of a rat during SWS. **(C)** An example spindle oscillation (10-20 Hz) recorded in the mPFC of a rat during SWS. The bold line represents the signal filtered in the spindle range.

### Delta oscillations

This coordination of ensemble reactivation is facilitated by the network oscillations of SWS. The most striking feature of the cortical EEG/LFP during SWS is the large amplitude, slow (0-4 Hz) oscillation, known as delta. An example of a delta oscillation cycle is shown in Figure 1.10B. Cortical delta oscillations reflect UP states and DOWN states, consisting of large cortical neuronal populations transitioning between excitable states and hyperpolarised states, respectively (Steriade, Nuñez, et al., 1993b). This oscillation is regulated by the brainstem, and occurs throughout the cortex, as well as engaging subcortical and thalamic regions, and the cerebellum (Mahon et al., 2006; Ros et al., 2009; Sheroziya & Timofeev, 2014). However, delta oscillations are intrinsic to cortical circuits, and they can be generated without thalamic or callosal input, and in cortical slice preparations (Steriade, Contreras, et al., 1993; Sanchez-Vives & McCormick, 2000). UP states are generated from high densities of excitatory and inhibitory postsynaptic potentials, while a distinct lack of synaptic activity is observed during DOWN states (Steriade, Nuñez, et al., 1993b).

Cortical delta oscillations also influence the hippocampus, with excitatory and inhibitory cells in DG and CA1 shown to exhibit fluctuating membrane potentials in time with the delta oscillation (Isomura et al., 2006; Hahn et al., 2007), although these are not as clear as the UP and DOWN states in the cortex. Ripple occurrence is correlated with the cortical delta oscillation, with a tendency to occur at the onset of UP states in the cortex (Battaglia et al., 2004a; Isomura et al., 2006; Mölle et al., 2006). Therefore, cortical delta oscillations may serve to synchronise ripples with high-excitability states in the cortex to allow synchronous replay of memory sequences (Peyrache et al., 2009; Peyrache et al., 2011).

### Spindles

Modulation of ripples has also been shown by spindle oscillations in the cortex. Spindles are waxing and waning periods of 10-20 Hz oscillations that last from 500 ms to 3 seconds – an example is shown in Figure 1.10C. They are generated through rhythmic activity of thalamic reticular nucleus (TRN) GABAergic projections onto thalamo-cortical projecting cells. During the hyperpolarised state of SWS, excitatory thalamo-cortical projections produce rebound spikes following the rhythmic inhibitory postsynaptic potentials from the TRN, generating rhythmic excitatory input to the cortex (Steriade et al., 1987; Halassa et al., 2011). Synchronous spindle activity across cortex and thalamus as is seen in vivo is dependent on cortico-thalamic input, which comes from spiking during the UP state of the delta oscillation (Contreras et al., 1996). Hence, spindles occur as a result of cortical spiking at the transition from DOWN state to UP state (Steriade, Nuñez, et al., 1993a; Sirota et al., 2003; Battaglia et al., 2004a; Mölle et al., 2006), and co-occur with ripples in the hippocampus (Siapas & Wilson, 1998; Staresina et al., 2015).

In summary, delta oscillations in the cortex may influence the timing of hippocampal ripples, and hence memory ensemble reactivation in the hippocampus, leading to reactivation of coupled cortical ensembles during UP states, when synaptic plasticity can be induced to facilitate long-term storage (Sirota & Buzsáki, 2005; Diekelmann & Born, 2010).

The functional importance of the temporal coordination of ripples, delta oscillations and spindles was demonstrated by Maingret et al. (2016). In this study, an object-location (OL) task was employed, in which rats were allowed to sleep for 1

hour, then either given a 3-minute or 20-minute sample phase, followed by another hour of sleep, and a test phase 23 hours later. In the period of sleep following the sample phase, coupling between hippocampal ripples and mPFC delta and spindle oscillations was increased relative to the sleep period before the sample phase, only in the group that were given the 20-minute sample phase. This was correlated with performance in the test phase, as rats that had the 3-minute encoding phase showed no memory for the object location, but the group that had the 20-minute sample phase did. The influence of the ripple-delta-spindle coupling was then demonstrated by stimulation of the mPFC following detection of ripples, inducing delta-spindle events, with a particular temporal delay, during the period of sleep following the 3-minute sample phase. This was found to improve memory in the test phase (inferred from preferential exploration of the displaced vs stationary object), and caused reorganisation of mPFC cell activity, leading to increased cell firing associated with the displaced object. Importantly, increasing the delay between hippocampal ripples and mPFC stimulation had no effect on memory, emphasising the importance of precise hippocampal-cortical temporal coordination for consolidation (Maingret et al., 2016).

## 1.5 Disordered learning and memory – Fragile X Syndrome

As well as injury or resection, memory can also be affected by disease. Understanding how the brain processes memories in health can help us understand the impairments associated with disorders, and how they can potentially be treated or accommodated. One class of conditions in which learning and memory, as well as other cognitive functions, are often affected, are neurodevelopmental disorders.

An example of such a disorder is Fragile X Syndrome (FXS). FXS is a genetic disorder, caused by a mutation of the *FMR1* gene on the X chromosome. It is one of the most common inherited causes of intellectual disability and autism spectrum disorder (ASD), affecting around 1 in 7000 males, and 1 in 11000 females (Coffee et al., 2009; Hunter et al., 2014). The gene mutation usually occurs on a region which in most individuals contains 6-44 repeats of CGG, whereas in FXS patients it is expanded to more than 200 repeats, which causes hyper-methylation and silencing of the gene and loss of its protein product, FMRP (Verkerk et al., 1991;

Colak et al., 2014). Mutations with numbers of repeats in between these ranges (45-200) are defined as premutations, which may not cause any pathology, but can cause Fragile X-associated Tremor/Ataxia Syndrome (FXTAS), which presents with Parkinsonism later in life. Premutations can develop into full mutations if passed on another generation.

The symptoms of FXS are very variable between individuals. Intellectual disability can vary from mild learning impairment to severe cognitive disability. Other commonly observed symptoms include ASD-related behaviours, seizures, sleep problems, sensory hypersensitivity, and physical characteristics (Lozano et al., 2014). As well as genetics, environmental factors appear to have an effect on some of these symptoms (Smith et al., 2016; Dahlhaus, 2018).

The level of cognitive impairment is correlated with the level of silencing of the gene, with lower levels of FMRP causing more severe deficits. Particular cognitive deficits that have been identified in FXS include impaired executive function, short-term/working memory, visual-spatial reasoning, and visual-motor coordination (Van der Molen et al., 2010; Huddleston et al., 2014). Executive function is defined as goal-directed and future-oriented behaviours, such as inhibition, set shifting, and cognitive flexibility. Studies of whether these skills are affected is often challenging in severely cognitively impaired individuals, but evidence suggests deficits in multiple aspects of executive function in FXS. These have included poor performance in working memory tasks, as well as the Wisconsin Card Sorting task and Contingency Naming test, which assess inhibition and set-shifting behaviours (Bennetto et al., 2001; Hooper et al., 2008; Baker et al., 2011; Huddleston et al., 2014).

In non-FXS individuals, FMRP is very widely expressed, with the highest levels in the brain and testes (Devys et al., 1993). In the brain, it is expressed in neuronal and glial cells throughout the cortex, and in the hippocampus and cerebellum (Devys et al., 1993; Hinds et al., 1993; Gholizadeh et al., 2015). Animal models of FXS have been valuable to further characterise protein expression and investigate effects on behaviour and brain networks.

As FXS is monogenic in its origin, rodent models have been produced through knockout (KO) of the homologous gene, *Fmr1*. This was originally done in mice (Bakker et al., 1994), and more recently in rats, to validate findings across species and increase translatability (Till et al., 2015; Tian et al., 2017). A difference between these rodent models and the human condition is in how the gene is altered;

in FXS humans, hyper-methylation and silencing of the gene occurs during gestation, where in *Fmr1* KO (*Fmr1*<sup>-/-</sup>) mice, the protein is never present. Therefore, other models aiming to mimic this have also been studied (Bontekoe et al., 2001), as well as a conditional KO model to control the level of FMRP at different developmental stages (Mientjes et al., 2006), though these are less well-studied.

Through study of FMRP in animal models, it has been shown to have a wide variety of functions. The primary function of FMRP is its role as a protein transcription regulator, interacting with a diverse range of mRNAs in the brain (Brown et al., 2001; Darnell et al., 2011). In addition, FMRP directly binds ion channels to alter neuronal excitability (Zhang et al., 2012; Deng et al., 2013).

The role of FMRP in the signalling process of metabotropic glutamate receptor (mGluR)-dependent LTD (Huber et al., 2002) led to the mGluR theory of FXS (Bear et al., 2004). The theory was that FMRP was upregulated by activation of mGluR receptors to repress the translation of proteins involved in LTD. Thus, loss of FMRP results in uncontrolled LTD-related protein expression and internalisation of AMPARs from the membrane, leading to the characteristic immature, elongated dendritic spines seen in FXS patient and *Fmr1*<sup>-/-</sup> mouse brains (Bear et al., 2004). Support for this theory came from attenuation of some symptoms of the disorder through pharmacological blockade of mGluRs.

However, due to the widespread function of FMRP, the cause of the disorder may not be as simple as disruption of a single signalling pathway. Loss of FMRP may have different effects on cellular signalling between different cell-types and brain regions. For example, in *Fmr1*<sup>-/-</sup> mouse hippocampal neurons, most studies have found normal or subtly altered induction of LTP (Godfraind et al., 1996), while in cortical neurons, LTP is reliably shown to be deficient (Li et al., 2002; Zhao et al., 2005; Contractor et al., 2015). Changes in other neuronal properties also differ between different regions; many neuronal populations in *Fmr1*<sup>-/-</sup> rodents are hyper-excitable, but mechanisms behind changes may be brain region-specific (Deng & Klyachko, 2016; Routh et al., 2017; Deng et al., 2019).

An overall theory of FXS, and ASD in general, that has developed relates the symptoms to an imbalance of excitation and inhibition within circuits (Hussman, 2001; Contractor et al., 2015; Contractor et al., 2021). For example, increased neuronal excitability could explain the reduced audiogenic seizure threshold seen in *Fmr1*<sup>-/-</sup> mice and seizures seen in many FXS patients, as well as hypersensitivity

to sensory stimuli seen in mice and FXS patients (Contractor et al., 2015). The causal relationship with cognitive deficits is not as directly obvious, but general imbalances of excitation and inhibition within circuits are likely to cause dysregulated output that affects cognitive processes throughout the brain.

Therefore, how loss of FMRP relates to the cognitive deficits of FXS is complex, and mediated through alteration of a vast range of processes both during development and through into adulthood. Furthermore, studies in the rodent models have provided mixed conclusions on cognitive dysfunction. Initial study of the *Fmr1*<sup>-/-</sup> mouse found normal spatial learning in the Morris Water Maze, but an impairment on reversal, when the location of the platform was changed (Kooy et al., 1996; D’Hooge et al., 1997). Other studies have found similar reversal or extinction learning deficits in the *Fmr1*<sup>-/-</sup> mouse (Bhattacharya et al., 2012; Radwan et al., 2016), suggesting cognitive inflexibility which may mirror findings in FXS patients. However, this finding was not replicated in the rat model, which showed performance on Morris Water Maze reversal that was similar to wild-type (WT) littermates (Till et al., 2015). However, *Fmr1*<sup>-/-</sup> rats did show a deficit in associative recognition memory when required to bind multiple associations in an OPC task (Till et al., 2015; Asiminas et al., 2019). These differences could reflect differences in cognitive strategies or requirements between mice and rats in these tasks.

The deficit in OPC in *Fmr1*<sup>-/-</sup> rats could reflect impaired cortical-hippocampal connectivity, as hippocampal connections with perirhinal cortex, LEC, and mPFC are required for performance on this task (Chao et al., 2016; Barker & Warburton, 2020). Furthermore, place cell recordings in the hippocampus of *Fmr1*<sup>-/-</sup> mice and rats reveal characteristics largely similar to WT (Talbot et al., 2018; Asiminas et al., 2021, preprint). However, differences in stability or spatial precision are found in response to changes in external cues (Arbab, Pennartz, et al., 2018) or task conditions (Dvorak et al., 2018), and across days (Asiminas et al., 2021, preprint). This also suggests that memory impairments may be related to differences in cortical inputs to hippocampus, rather than hippocampal network deficits alone. Therefore, study of the mechanisms underlying these deficits in the rodent models may aid in understanding of cognitive impairments in human FXS patients.

## 1.6 Objectives of this thesis

This thesis aims to explore some of the remaining questions around the function of cortical-hippocampal circuitry in memory processing, through a series of experiments using rodents.

The first experimental chapter (Chapter 2) tests the hypothesis that the MEC is necessary for the anchoring of CA1 place cells to distal visual landmarks, but not proximal object cues. Multiple lines of evidence suggest the MEC may be important in formation of internal spatial representations based on landmarks, but not for representation of objects within the environment (Knierim et al., 2013). Chapter 2 details an experiment in which place cells were recorded in CA1 while mice explored an open field with either distal visual landmarks or proximal object cues. In each case, the cues were rotated and anchoring of place cells to each cue type was compared in control mice and mice with MEC lesions. The prediction was that place cells in MEC-lesioned mice would not rotate with the distal landmarks, but would with the objects.

Chapter 3 aimed to test the hypothesis that MEC stellate cells are required for hippocampus-dependent associative memory. Evidence suggests that stellate cells in layer 2 of the MEC may be required for hippocampal contextual processing (Kitamura et al., 2015). In this experiment, MEC layer 2 stellate cells were inactivated in a group of mice, and their performance on object recognition and object-context, object-place and object-place-context association recognition tasks was compared to control mice. It was predicted that the loss of MEC stellate cell input to the hippocampus would disrupt object-place-context memory.

Chapters 4 and 5 investigate the function of mPFC-hippocampal connectivity in a rat model of FXS. In Chapter 4, the hypothesis that mPFC-hippocampus connectivity in *Fmr1*<sup>-y</sup> rats may be disrupted during active exploration of novelty, was tested. Deficits in executive function in FXS patients, as well as mPFC and hippocampus-dependent behaviours in *Fmr1*<sup>-y</sup> rats suggest that PFC-hippocampal connectivity may be impaired (Huddleston et al., 2014; Asiminas et al., 2019). WT and *Fmr1*<sup>-y</sup> rats were implanted with tetrodes in the mPFC prelimbic area and dorsal CA1 of the hippocampus, and allowed to explore a familiar environment followed by a novel environment. LFPs were recorded in each region, and the power and coherence of oscillations were compared between WTs and *Fmr1*<sup>-y</sup> rats. The prediction was

that changes in hippocampus-mPFC connectivity may be observed in the novel environment compared to the familiar, and that *Fmr1*<sup>-/-</sup> rats may show altered connectivity between the two regions.

Chapter 5 then tested the hypothesis that mPFC-hippocampus interactions related to memory consolidation during sleep are disrupted in the FXS model. A lack of experience-dependent change in CA1 place cells across days observed previously in *Fmr1*<sup>-/-</sup> rats led to the hypothesis that consolidation mechanisms may be disrupted (Asiminas et al., 2021, preprint). Following each day of the novel environment protocol, rats were left to sleep in a bucket. LFPs from mPFC and hippocampus were recorded during SWS to compare memory consolidation-related patterns between the genotypes.



## **Chapter 2**

# **The role of the MEC in use of cues in the CA1 spatial representation**

## **2.1 Introduction**

Formation of spatial and episodic memories requires sensory information to be processed and integrated into an internal representation of the surrounding environment. In the hippocampus, an internal map is formed from networks of place cells, which fire when an animal is within a particular region of an environment (O'Keefe & Nadel, 1978). The location where place cells fire is influenced by associations with sensory information in the environment, anchoring to salient landmarks. This can be demonstrated by recording place cells in CA1 while a rodent explores an arena with one or more salient visual cues, such as a large polarising card on the wall, then rotating the cues around the arena between sessions; the locations where the place cells fire will rotate to follow, and fire in the same position relative to the cues (Muller & Kubie, 1987).

Which neural pathways carry this landmark information into CA1 of the hippocampus is an interesting question, as well as what types of cues are used as anchoring landmarks. The largest cortical input to the hippocampus is the entorhinal cortex (EC). The entorhinal cortex is divided into separate regions, the medial entorhinal cortex (MEC) and lateral entorhinal cortex (LEC), by anatomical and functional distinctions (Insausti et al., 1997; Kerr et al., 2007). The MEC is thought to carry information about global spatial location, whereas the LEC appears to carry more local sensory information. Electrophysiological recordings in the MEC have revealed cells which are modulated by different aspects of the animal's position in space,

including grid cells, head direction cells, border cells and speed cells (Fyhn et al., 2004; Hafting et al., 2005; Sargolini et al., 2006; Solstad et al., 2008; Kropff et al., 2015). Whereas LEC cell firing shows little spatial modulation in an open field, but seems to encode locations of objects placed within the environment (Hargreaves et al., 2005; Deshmukh & Knierim, 2011; Tsao et al., 2013).

This may suggest that the MEC provides the spatial information necessary for hippocampal place cells to form their internal map. Computational models have provided evidence for how input from networks of grid cells could summate to guide the activity of place cells (Solstad et al., 2006; Savelli & Knierim, 2010; Giocomo & representation, 2011). However, experimental evidence shows that the EC is not necessary for the spatial firing of CA1 place cells – a number of studies have shown spatial firing of place cells with lesions of the entire EC or just MEC, albeit with decreased spatial specificity and stability (Miller & Best, 1980; Van Cauter et al., 2008; Hales et al., 2014; Jacob et al., 2020). Furthermore, spatial firing in the hippocampus appears to develop before grid cell coding in the MEC (Langston et al., 2010). It appears then that the MEC contributes spatial information to place cell firing, but is not necessarily required for spatial coding in the hippocampus. Exactly what this contribution consists of is yet to be fully determined.

Another finding from a study in which the entire EC was lesioned was that hippocampal place cells were less anchored to distal visual landmarks, instead representing information about the physical properties of the maze (Miller & Best, 1980). As the entire EC was removed, it is unclear whether this was caused by removal of MEC input, or input from the EC as a whole. Recording of cells in the MEC and LEC while rats traversed a circular track with differently textured flooring, and extra-maze distal cues, showed that MEC cell firing was associated with the distal cues, while LEC firing was more associated with the local track features, when both were rotated in conflict to one another (Neunuebel et al., 2013). Scaplen et al. (2014) have since investigated this local/global distinction in terms of influence on hippocampal place cell firing, using visual cues projected onto the walls or floor of an environment. They found that large salient “landmark” cues were more likely to be used for orientation of the spatial map, than smaller “object” cues (Scaplen et al., 2014). Deactivation of the LEC then caused a greater proportion of cells to follow the landmark cues, possibly reflecting a lesser influence of local cues, and a greater influence of landmark cues, which could be provided by inputs from the MEC (Scaplen et al., 2017).

There is also some behavioural evidence to support the idea that the MEC processes distal landmarks, while the LEC processes local objects and cues (Knierim et al., 2013). Two studies have shown that rats with MEC lesions exhibit deficits in navigation in a Morris Water Maze where distal cue information is the most salient. Hales et al. (2014) showed slower acquisition in MEC-lesioned rats compared to controls. When they changed the distal cues around the maze, the control rats were able to learn the new platform location, whereas MEC-lesioned rats perseverated strongly with the old platform location. This suggests they were not using distal cue information to learn the platform location, perhaps instead using the limited cue information within the maze, and may explain their slower initial acquisition. A more recent study also showed that MEC lesions in rats slowed learning of the platform location, and reduced accuracy of navigation to the platform area when it had been removed in a probe trial (Poitreau et al., 2021).

In contrast, rats with LEC lesions showed impairment on a rewarded maze navigation task that required use of local object cue information (Kuruvilla & Ainge, 2017). However, in a local cue version of the Morris Water Maze, in which objects were placed in the water, while there may have been some impairment compared to controls, both MEC- and LEC-lesioned rats were able to perform the task (Poitreau et al., 2021). This may be due to ambiguity about whether the objects were classified as landmarks for navigation or objects for associative learning, and either strategy being sufficient for navigation to the platform.

The aim of the experiment presented in this chapter is to test the hypothesis that the MEC provides the hippocampus with distal visual landmark information, but is not required for providing the hippocampus with proximal object information. Place cells were recorded in dorsal CA1 of mice with excitotoxic lesions of the MEC, and compared to those in mice that had undergone sham surgeries. Cells were first recorded across sessions where distal visual landmarks were rotated around the arena, then across sessions where positions of objects were rotated within the arena. The place cells in control mice anchored to both distal visual landmarks as well as proximal object cues; whereas, in addition to having reduced spatial selectivity, place cells in MEC-lesioned mice were less anchored to distal visual landmarks, but anchoring to object cues was unaffected. This shows that the MEC is required for anchoring of CA1 place cells to distal visual landmarks, but not to object cues.

## 2.2 Methods

Experimental work for this chapter was performed by Dr Elizabeth Allison and Julia Thomas. All data analysis was performed by the author, partly using MATLAB scripts written by Dr Daisy Arkell, and R scripts written by Dr Owen Dando for GLMMs.

### 2.2.1 Animals

Experimentation was carried out under a UK Home Office project licence, approved by the Animal Welfare and Ethical Review Board (AWERB) of the University of Edinburgh College of Medicine and Veterinary Medicine, and conformed with the UK Animals (Scientific Procedures) Act 1986.

The subjects were 13 male wild-type C57/BL6 mice, aged around 8 weeks at the beginning of the experiment. Mice were housed in groups of 2-5 from weaning, then housed individually following surgery, on a 12-hour diurnal light/dark cycle (lights on at 07:00). The mice had *ad libitum* access to food and water in their home cages. The mice were assigned to experimental groups pseudorandomly, to receive either bilateral MEC lesions ( $n = 7$ ), or sham lesions ( $n = 6$ ). All mice were implanted with a tetrode microdrive targeted to dorsal CA1 of the hippocampus.

### 2.2.2 Tetrode implants

Implants were constructed to carry a bundle of two tetrodes, movable using a microdrive mechanism (Axona Ltd). Tetrodes were made from 17  $\mu\text{m}$  HML-coated platinum (90%)-iridium (10%) wire (California Fine Wire). Four lengths of wire were twisted together and heat-annealed using a heat gun to form each tetrode. Two tetrodes were then loaded into a metal cannula attached to the drive mechanism and connector.

The tetrodes were cut to the appropriate length just before surgery using ceramic scissors (Fine Science Tools), then plated with gold solution (Neuralynx) to an impedance of 150-200  $k\Omega$ . A wider metal cannula was placed around the drive cannula to protect the tetrodes and held in place using sterile Vaseline.

### 2.2.3 Surgery

Mice were anaesthetised using Isoflurane with oxygen in an induction chamber, then transferred to a stereotaxic frame (Kopf), on a thermostatic heat blanket. Analgesia (Rimadyl) and saline/glucose solution were administered by subcutaneous injections. The eyes were covered throughout surgery with hydrating eye gel (Viscotears). The scalp was disinfected using iodine around the incision site. An incision was then made and the skull exposed. Craniotomies were drilled at 3.5 mm lateral to Bregma (left and right), posterior to the lambdoid suture and anterior to the transverse sinus. For the lesion mice, a glass micropipette was filled with ibotenic acid (Tocris) (10 mg/ml, pH 7.4, dissolved in phosphate-buffered saline (PBS)). The pipette was lowered into the craniotomies at an angle of  $-10^\circ$  in the anterior-posterior plane, and four injections of either 20 or 40 nl were made at depths of 2.8 mm, 2.3 mm, 1.85 mm and 1.4 mm below the dura (Figure 2.1A). Following each injection, the pipette was left in place for 5 minutes before retraction to the next site. For the sham group, the dura was pierced but no injections were made. Sterile gelatin sponge (Spongostan Special, Ferrosan A/S) soaked in saline was used to cover the brain surface following injections/dura piercing.

The microdrive was then implanted. Self-tapping stainless-steel 120 TPI screws (Antrin Miniature Specialties Inc) were embedded in the skull and held in place with dental cement (Simplex rapid acrylic denture polymer, Associated Dental Products Ltd). One of these screws had a wire attached to it before surgery, for grounding the drive. The craniotomy for the tetrode implant was drilled 2 mm lateral to Bregma on the right side, and -2 mm posterior to Bregma. The dura was removed and the tetrodes lowered 0.9 mm below dura (around 0.15 mm above the dorsal CA1 pyramidal layer). The outer cannula was lowered to the skull surface to protect the tetrodes and sealed using sterile Vaseline. The microdrive ground wire was soldered to the skull screw wire. Dental cement was used to cover the injection sites and skull screws, and to fix the microdrive in place. Mice were then removed from the stereotaxic frame and placed in their home cage on a heat pad at 30 °C and recovery was monitored. They were given 10 days to recover fully and return to their pre-surgery weight, before screening began.

#### **2.2.4 Electrophysiological recording**

Mice were connected to a 32-channel recording system (Axona Ltd) via a headstage amplifier and pre-amplifier. The signal was amplified, filtered with a band-pass filter at 600-6000 Hz and single units were identified using the oscilloscope in the DACQ software (Axona Ltd). The gain and spike trigger were set at appropriate levels in order to collect spikes from putative neurons while minimising collection of noise spikes. Local field potential was recorded from one wire – the signal was duplicated and filtered with a low-pass bandpass filter at 0-300 Hz and a notch filter at 50 Hz, and recorded with a sampling frequency of 250 Hz. An infra-red LED on the headstage amplifier was tracked at a frequency of 50 Hz, by a camera placed above the environment, to record the mouse's position.

Mice were screened in a cylindrical environment until putative CA1 pyramidal cells were observed. This was assessed by width of waveform and theta-modulation in the auto-correlograms.

#### **2.2.5 Cue rotation apparatus**

The recording arena was a rust-coloured plastic flowerpot saucer 50 cm in diameter, with a 2 cm-wide and 3 cm-high rim. This was affixed to the top of an 80 cm tall stool placed in the centre of a circular black curtained environment, 2 m in diameter. The environment was lit from above so that light was evenly distributed around the curtained enclosure, and the ceiling was covered with a white sheet, to remove any visual directional cues. White noise was played through a speaker above to mask any auditory directional cues. In the distal cue sessions, a white sheet, reaching from the ceiling to floor and 1.4 m wide, and a 1 m diameter hoop covered in shiny paper to make a circle, were hung from the curtains 130° apart from one another. In the proximal cue sessions, the curtains were left bare, and three objects were attached to the inside of the arena, spaced 90° apart at the edge of the inner surface of the saucer, just next to the rim (Figure 2.1C). These were each different in shape, colour and texture, and sizes ranged from 6-11 cm tall.

### 2.2.6 Cue rotation protocol

Following recovery from surgery, mice were screened daily until multiple place cells were identified. They were then recorded for three days in the environment with distal cues, followed by three days in the environment with proximal cues, depending on whether cells could still be detected.

Each day the mice would follow the session sequence as shown in Figure 2.1B - standard 1, standard 2, 90° rotation, standard 3. Each session lasted 15 minutes, with an interval of around 5 minutes between sessions. The mouse was carried into the curtained enclosure in its home cage, which was covered in a blanket. The home cage was carried, still covered, for between one half and two turns around the arena, to mildly disorient the mouse. The cage was then uncovered and the mouse was removed and connected to the recording system. Crumbs of Wotsits (Walkers) were scattered around the arena for the mouse to forage for 15 minutes while single unit and local field potential activity were recorded. Once the session had ended, the mouse was unplugged and placed back into its home cage, which was covered again with a blanket. The arena was wiped with absolute alcohol, and the necessary cue rotations were performed. The cage was again picked up and moved around the arena a random amount between one half and two turns, and the mouse was placed on the arena and connected to the recording system again for the next session.

### 2.2.7 Data analysis

For single unit data, session files from each day were combined using a custom Matlab script and the spikes were clustered using a clustering algorithm (KlustaKwik2, developed by Kadir et al. (2014)). Identified clusters were visualised using Klusters (Hazan et al., 2006), and manually curated – noise clusters and spikes were removed where possible, and clusters with very similar waveforms were merged. Custom Matlab scripts were then used on the outputs to generate firing rate maps with 2.5 cm square bins, for each session, and to calculate mean and peak firing rates, spatial information, and sparsity for each cluster, as well as correlations of the firing rate maps between sessions. To calculate the in-field mean and peak firing rates, the pixel of the firing rate map with the highest firing rate was found, and place fields were defined as 9 or more contiguous pixels with

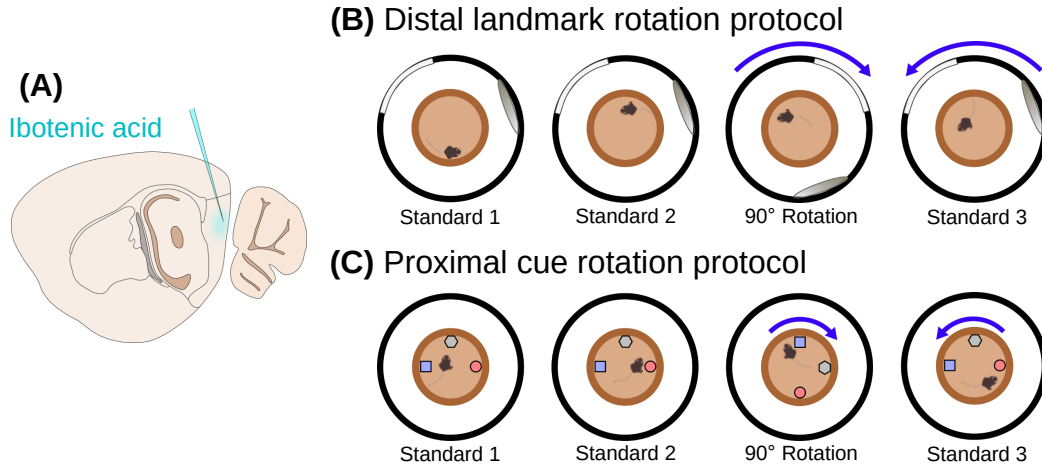


FIGURE 2.1: Schematic of injections and cue rotation protocols **(A)** Schematic of sagittal section of a mouse brain, showing injection of ibotenic acid into the MEC. **(B)** Distal cue rotation protocol: visual cues were hanging from black curtains surrounding the arena, and mice were given two sessions to explore the central platform with the cues in their standard position, followed by a clockwise rotation of 90°, then a final session with the cues returned to the standard position. All sessions were 15 minutes, with around 5 minutes between each. **(C)** Proximal cue rotation: a similar protocol was used, but with a rotation of objects within the arena (the arena itself was not moved), and no cues on the surrounding curtains.

firing rates above 20% of this value. The in-field mean and peak firing rates were calculated from only those pixels (Park et al., 2011). The speed of the mouse was calculated for every 500 ms bin, and a threshold of 3 cm/s was applied so that only spikes recorded while the mouse was travelling above this speed were included in the analysis. Movement during these bins was summed to calculate total path length for each session.

Cells were identified as putative pyramidal cells if they had a waveform width of above 250  $\mu$ s and a mean firing rate across all sessions of between 0.1-5 Hz. Only the clusters that met these criteria were taken forward in the analysis. Spatial information (SI) measures how much information about the animal's location is carried by one spike (Skaggs et al., 1993), and was calculated using the following equation:

$$SI(bits/spike) = \sum_i P_i \left( \frac{R_i}{R} \right) \ln \left( \frac{R_i}{R} \right)$$

Where  $i$  represents the bin number in the firing rate map,  $P_i$  is the probability that

bin  $i$  is occupied,  $R_i$  is the mean firing rate in bin  $i$ , and  $R$  is the overall mean firing rate. Sparsity measures the proportion of the area the mouse explored in which spikes from a cell/cluster occurred. This was calculated using the formula:

$$Sparsity = \sum \frac{(P_i R_i^2)}{R^2}$$

Where  $i$  represents the bin number in the firing rate map,  $P_i$  is the probability that bin  $i$  is occupied,  $R_i$  is the mean firing rate in bin  $i$ , and  $R$  is the overall mean firing rate.

Overall mean firing rates, in-field mean and peak firing rates, spatial information and sparsity across sessions were then fitted with generalised linear mixed-models (GLMMs) (lme4 package in RStudio)(Bates et al., 2015), in order to avoid pseudoreplication by treating cells from the same mouse as independent datapoints, as well as accounting for the variability in number of cells recorded from each mouse. Mouse and cell identity was included as a random effect, and experimental group (Control, MEC lesion), session (standard 1, standard 2, rotation and standard 3), and group x session interaction were included as fixed effects. The data were first modelled with only the interaction term and random effect included, and fit was compared to a model without that term included – the p values reported represent likelihood ratio tests between these models. Post-hoc tests were then used to compare between estimates of fixed effects for the individual sessions and groups; p values from these were Bonferroni-corrected to account for multiple comparisons.

### 2.2.8 Cue rotation data analysis

To analyse cue rotations and place field stability between sessions, firing rate maps for each session were compared in pairs: standard 1 vs standard 2, standard 2 vs rotation, rotation vs standard 3, and standard 2 vs standard 3. To compare two sessions, a Pearson's correlation was calculated between the two firing rate maps, first at their "real world" orientation, then one was rotated relative to the other at increments of 5° and a correlation was calculated at each angle. The maximum and minimum correlation values, as well as the angle at which the maximum correlation occurred, were taken for each cluster. A V-test was used on the distribution of maximum correlation angles from all cells from each mouse, and for each group, to

test whether values were uniformly distributed, or if their mean was centred around the expected angle of rotation. Expected angles for each session comparison were: 0° for standard 1 vs standard 2; 270° for standard 2 vs rotation; 90° for rotation vs standard 3; 0° for standard 2 vs standard 3. A Watson-Williams F-test was then used to assess differences in mean angle of maximum correlation between the groups. A cell was only included in this analysis if its firing rate in both of the two sessions being compared was greater than 0.1 Hz – cells that did not meet this criterium were categorised separately.

The cells were then categorised for each session pair comparison based on their maximum and minimum correlation values, angle of maximum correlation and firing rates in each session, into the following groups: stable, target rotation, off-target rotation, remap, inactive and ambiguous. Criteria were as follows:

- *Stable*: mean firing rate > 0.1 Hz in both sessions, maximum correlation > 0.5, minimum correlation < 0, angle of maximum correlation in the range of 330° to 30°.
- *Target rotation*: mean firing rate > 0.1 Hz in both sessions, maximum correlation > 0.5, minimum correlation < 0, angle of maximum correlation in the range of 240° to 300° for standard 2 vs rotation (clockwise), 60° to 120° for rotation vs standard 3 (anticlockwise).
- *Off-target rotation*: mean firing rate > 0.1 Hz in both sessions, maximum correlation > 0.5, minimum correlation < 0, angle of maximum correlation not in the range of the target rotation.
- *Remap*: mean firing rate > 0.1 Hz in both sessions, maximum correlation < 0.5.
- *Inactive*: mean firing rate < 0.1 Hz in either of the two sessions.
- *Ambiguous*: mean firing rate > 0.1 Hz in both sessions, maximum correlation > 0.5, minimum correlation > 0.5 Hz (often because of the field being close to the centre of the arena, so was rotationally symmetrical, so therefore whether it was rotating with the cues cannot be determined).

Overall proportions of categorised cells from each group were then compared using Chi-square tests, and the proportions of cells from each mouse in the two groups in

the 'stable' and 'target rotation' categories were compared across sessions using a two-way ANOVA.

Correlations at 0° were compared using a GLMM to assess differences in stability between groups.

### 2.2.9 Histology

Once all recording had been completed, mice were anaesthetised with Isoflurane and a lethal dose of sodium pentobarbital (Euthatal) was administered via intraperitoneal injection. They were transcardially perfused with phosphate buffered saline and 4% paraformaldehyde (PFA) to fix the tissues. The brains were removed and kept overnight in PFA before being transferred to 30% sucrose for cryoprotection. 32-40  $\mu\text{m}$  sagittal sections were taken using a cryostat, and half of the sections were mounted on polysine slides (Thermo Scientific). They were stained with 0.1% Cresyl-violet and coverslipped in DPX (Sigma-Aldrich), then imaged at 10x magnification using Image-Pro Plus (Media Cybernetics). Fiji (Fiji Is Just Image-J) was used to draw around the regions of MEC, presubiculum, parasubiculum, and ventral and dorsal hippocampus, as defined by Paxinos et al. (2001). The average area of these regions was taken from the control mice, then the spared tissue area in lesion mice was found and calculated as a percentage of this.

## 2.3 Results

### 2.3.1 MEC lesion histology

The area of MEC affected by the lesion varied between the 7 mice in the lesion group. Figure 2.2A-C shows representative sagittal sections from one control mouse, one mouse with a medium-sized MEC lesion, and one mouse with a large MEC lesion with clear damage to surrounding areas. Remaining MEC tissue was measured and averaged across multiple sagittal sections, and expressed as a percentage of the area of the MEC of control mice which had sham surgeries (Figure 2.2D). Average size of MEC lesion across the two hemispheres varied from 30% to 94%. Among these there were variable levels of damage to surrounding areas,

such as presubiculum, subiculum, parasubiculum and ventral hippocampus. In two of the mice, damage to presubiculum, and parasubiculum was minimal, with no damage to subiculum or hippocampus (L5 and L6). In two mice there was a large amount of MEC damage, but with significant damage to presubiculum, parasubiculum, as well as some damage to subiculum and hippocampus (L3 and L4). In the mouse with the largest MEC lesion, although the MEC was almost totally eliminated, this also included the presubiculum and parasubiculum, as well as large parts of the subiculum and ventral hippocampus (L7). For most of the following analyses, all lesion mice are grouped together; however, the effect of lesion size is considered at the end of this section.

Figure 2.2E shows two representative examples of electrode tracks, from one control mouse, and one MEC lesion mouse. Similar positions in the pyramidal cell layer of dorsal CA1 were observed between mice, as can be seen in Appendix A, Figure A.1.

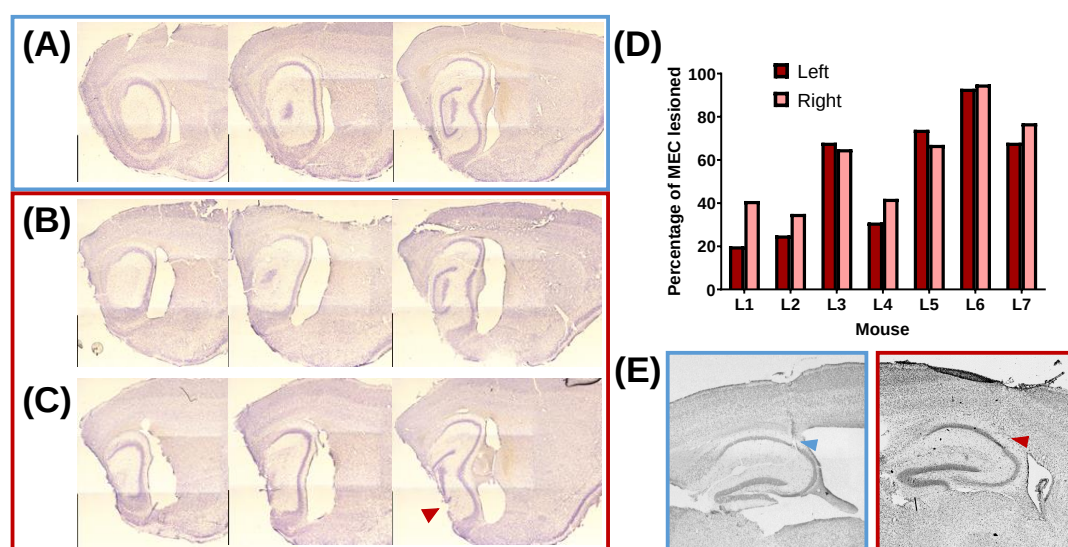


FIGURE 2.2: Lesion histology **(A)** Example sagittal sections from a control (sham lesion) mouse brain, showing an intact MEC from lateral (left) to medial (right). **(B)** Similar sections from a lesion mouse with around 70% of the MEC lesioned, with good specificity. **(C)** Example sections from a lesion mouse with a larger, less specific lesion, with almost 100% MEC lesioned, but some damage to other areas, such as ventral hippocampus, as indicated by the red arrow. **(D)** Percentage of the MEC lesioned in the left and right hemispheres of each mouse in the lesion group ( $n = 7$ ). **(E)** Example sagittal sections showing electrode tracks for one control mouse (blue box, left) and one MEC lesion mouse (red box, right).

### 2.3.2 Place cells of MEC-lesioned mice have lower spatial information and sparser fields than place cells of control mice

Mice in the lesion group appeared to show slightly longer path lengths than those in the control group, although this difference was not quite significant (group main effect: distal:  $F(1,11) = 4.73$ ,  $p = 0.0522$ ; proximal:  $F(1,11) = 1.98$ ,  $p = 0.187$ ) (Figure 2.3A). There was a main effect of session in the proximal condition ( $F(2.8,31) = 10.98$ ,  $p < 0.0001$ ), as mice tended to travel shorter distances in the later sessions (Figure 2.4A), but this pattern was not observed in the distal condition ( $F(1.5,16.3) = 0.068$ ,  $p = 0.884$ ). There were no significant group  $\times$  session interactions for either condition (distal:  $F(3,33) = 0.14$ ,  $p = 0.934$ ; proximal:  $F(3,33) = 1.5$ ,  $p = 0.245$ ). As faster speeds (which would result in longer path lengths) are typically associated with higher firing rates, it is important to take potential differences into account when interpreting firing rate data below.

In the distal cue rotation sessions, 376 putative pyramidal cells were recorded from the 6 control mice, and 212 from the 7 lesion mice. In the proximal sessions, 228 pyramidal cells were recorded from the control group, while 178 were recorded from the lesion group. Generalised linear mixed-modelling (GLMM) of mean firing rate across the distal cue sessions, including mice and cells as random factors, found no group  $\times$  session interaction (likelihood ratio test = 3.33,  $p = 0.344$ ) and no main effect of group (LRT = 0.409,  $p = 0.523$ ), but did find a main effect of session (LRT = 43.1,  $p < 0.0001$ ), with firing rate increasing across the four sessions (Figure 2.3B). Comparison of within place field mean firing rate found a significant group  $\times$  session interaction (LRT = 12.8,  $p = 0.0051$ ), with lesion mice showing generally lower in-field mean firing rates. However, Bonferroni-corrected multiple comparisons between groups for each session found no significant differences (Std1:  $p = 0.203$ ; Std2:  $p = 0.823$ ; Rot:  $p = 0.834$ ; Std3:  $p > 0.999$ ). Comparison of in-field peak firing rate produced similar results, with a significant interaction (LRT = 11.9,  $p = 0.0076$ ), but no differences between groups in individual sessions (Std1:  $p = 0.506$ ; Std2:  $p > 0.999$ ; Rot:  $p = 0.931$ ; Std3:  $p > 0.999$ ) (Figure 2.3C). Thus, firing rates did not differ significantly between genotypes in the distal landmark sessions, despite the non-significant trend for lesioned animals to travel further.

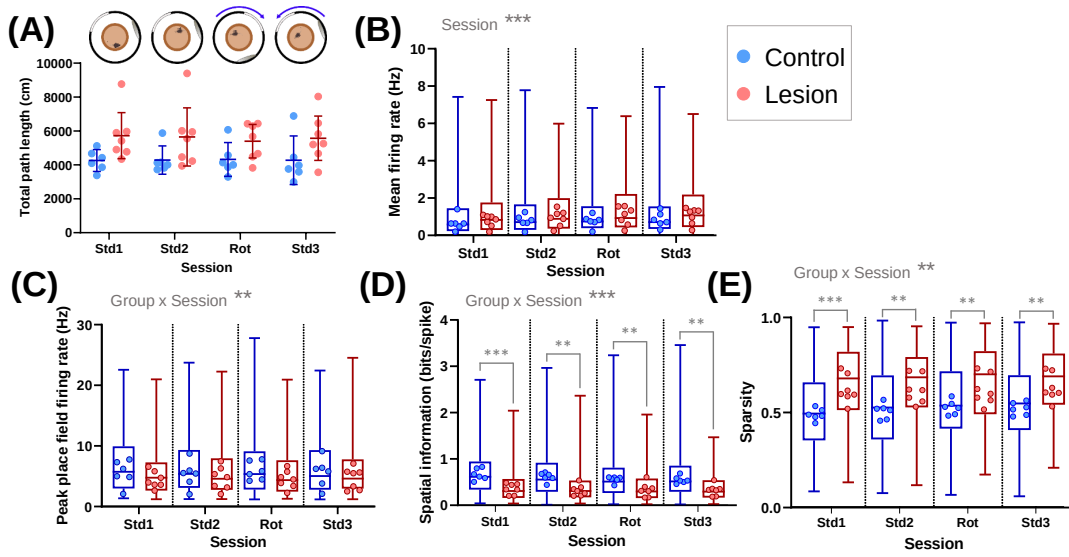


FIGURE 2.3: MEC lesion mice place cells have lower spatial selectivity in the distal landmark sessions. **(A)** Total path length of each mouse on day 1 of the distal cue sessions, with means and 95% confidence intervals (control,  $n = 6$ , lesion,  $n = 7$ ). **(B)** Mean firing rates for all cells from each group are represented by the box and whisker plots, showing median, interquartile range and range. The mean values for cells recorded from each mouse are shown as dots (control,  $n = 6$ , lesion,  $n = 7$ ). **(C)** Peak place field firing rates for all cells from each group are represented by the box and whisker plots, showing median, interquartile range and range. The mean values for cells recorded from each mouse are shown as dots (control,  $n = 6$ , lesion,  $n = 7$ ). **(D)** Spatial information values of all active ( $> 0.1$  Hz mean firing rate) cells from each group are represented by the box and whisker plots. The median values for cells recorded from each mouse are shown as dots (control,  $n = 6$ , lesion,  $n = 7$ ). **(E)** Sparsity of all active cells from each group are represented by the box and whisker plots (control,  $n = 6$ , lesion,  $n = 7$ ). The mean values for cells recorded from each mouse are shown as dots. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

In contrast, spatial properties of the cells were affected by MEC lesion. Spatial information was significantly lower in the lesion group, where the median of cells from all mice together was 0.31 bits/spike (interquartile range [0.16,0.57]), compared to controls, with an overall median of 0.62 bits/spike (IQ range [0.34,0.95]) (Figure 2.3D). A GLMM of spatial information across the four sessions showed a significant group  $\times$  session interaction (LRT = 17.5,  $p = 0.0006$ ), with Bonferroni-corrected post-hoc tests revealing significant differences between the groups in every session (Std1:  $p < 0.0001$ ; Std2:  $p = 0.0017$ ; Rot:  $p = 0.0051$ ; Std3:  $p = 0.0015$ ). The same pattern was seen in the analysis of sparsity, which is another measure of spatial firing; cells from the lesion group fired in a greater proportion of the environment, showing increased sparsity compared to the control group (Figure 2.3E).

### 2.3. Results

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A GLMM across sessions showed a significant session x group interaction (LRT = 12.2,  $p = 0.0067$ ) and revealed significant differences in all four distal landmark sessions (Std1:  $p = 0.0003$ ; Std2:  $p = 0.0016$ ; Rot:  $p = 0.0051$ ; Std3:  $p = 0.0024$ ).

The same measures were analysed for the proximal cue sessions. For overall mean firing rate in each session, there was no group x session interaction (LRT = 1.71,  $p = 0.634$ ), and no main effect of group (LRT = 1.29,  $p = 0.256$ ), but there was a main effect of session (LRT = 48.0,  $p = 0.0001$ ) (Figure 2.4A). However, for in-field mean firing rate, there was a significant group x session interaction (LRT = 23.8,  $p < 0.0001$ ). In-field mean firing rate was generally lower in the lesion group than the control group, but the lesion group increased in later sessions, so post-hoc tests showed significant differences in the first two sessions (Std1:  $p = 0.020$ ; Std2:  $p = 0.012$ ), but no differences in the final two sessions (Rot:  $p = 0.222$ ; Std3:  $p > 0.999$ ). In-field peak firing rate showed a similar pattern, with a significant group x session interaction (LRT = 27.5,  $p < 0.0001$ ), and significant differences between groups in the first two sessions only (Std1:  $p = 0.033$ ; Std2:  $p = 0.018$ ; Rot:  $p = 0.380$ ; Std3:  $p > 0.999$ ) (Figure 2.4C).

Analysis of spatial properties in the proximal cue sessions also found lower spatial precision. For spatial information, there was no significant group x session interaction (LRT = 5.61,  $p = 0.132$ ), but there were significant main effects of both group (LRT = 5.79,  $p = 0.016$ ) and session (LRT = 48.0,  $p < 0.0001$ ) individually (Figure 2.4D). Sparsity showed the same effects, with no group x session interaction (LRT = 5.04,  $p = 0.169$ ), but main effects of both group (LRT = 5.08,  $p = 0.024$ ) and session (LRT = 40.3,  $p < 0.0001$ ) (Figure 2.4E).

Therefore, spatial precision of CA1 place cells is clearly reduced in MEC-lesioned mice, shown in both the distal landmark sessions and the proximal cue sessions. Effects of MEC lesion on firing rates are less clear, but may reduce in-field firing rates slightly, although this was only significant in a subset of the proximal cue sessions.

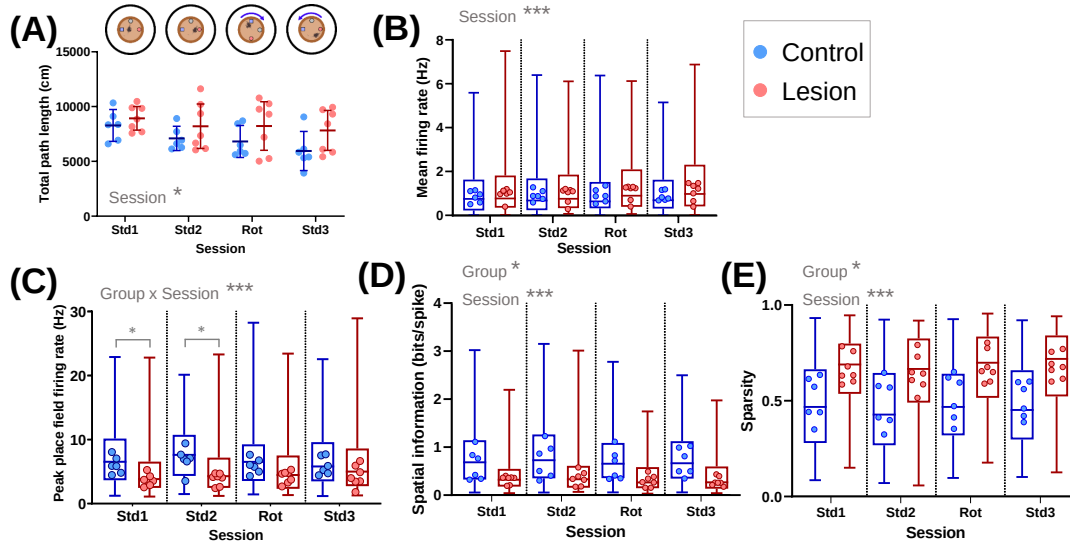


FIGURE 2.4: MEC lesion mice place cells have lower spatial selectivity in the proximal cue sessions. **(A)** Total path length of each mouse on day 1 of the proximal cue sessions, with means and 95% confidence intervals (control,  $n = 6$ , lesion,  $n = 7$ ). **(B)** Mean firing rates for all cells from each group are represented by the box and whisker plots. The mean values for cells recorded from each mouse are shown as dots (control,  $n = 6$ , lesion,  $n = 7$ ). **(C)** Peak place field firing rates for all cells from each group are represented by the box and whisker plots, showing median, interquartile range and range. The mean values for cells recorded from each mouse are shown as dots (control,  $n = 6$ , lesion,  $n = 7$ ). **(D)** Spatial information values of all active ( $> 0.1$  Hz mean firing rate) cells from each group are represented by the box and whisker plots. The median values for cells recorded from each mouse are shown as dots (control,  $n = 6$ , lesion,  $n = 7$ ). **(E)** Sparsity of all active cells from each group are represented by the box and whisker plots (control,  $n = 6$ , lesion,  $n = 7$ ). The mean values for cells recorded from each mouse are shown as dots. \*  $p < 0.05$ , \*\*\*  $p < 0.001$

### 2.3.3 MEC lesions impair influence of distal visual cues on CA1 place fields

In the distal cue rotation, cells from the control group were largely anchored to the distal visual cues, rotating accordingly, while cells from the lesion mice were much less likely to follow these cues. This can be seen in the example firing rate maps shown in Figure 2.5. The example cells in Figure 2.5A (blue box) are from control mice, with each row showing the firing rate maps of a cell across the four sessions. The first five rows show cells, each from different mice, which rotated their firing fields as predicted by the rotation of the distal cues. The bottom row shows an example of a cell that remapped between sessions. The first five rows of

### 2.3. Results

Figure 2.5B (red box) show example cells, each from different lesion mice, which remained stable across the cue rotation, and the bottom row shows a cell which remapped between sessions.

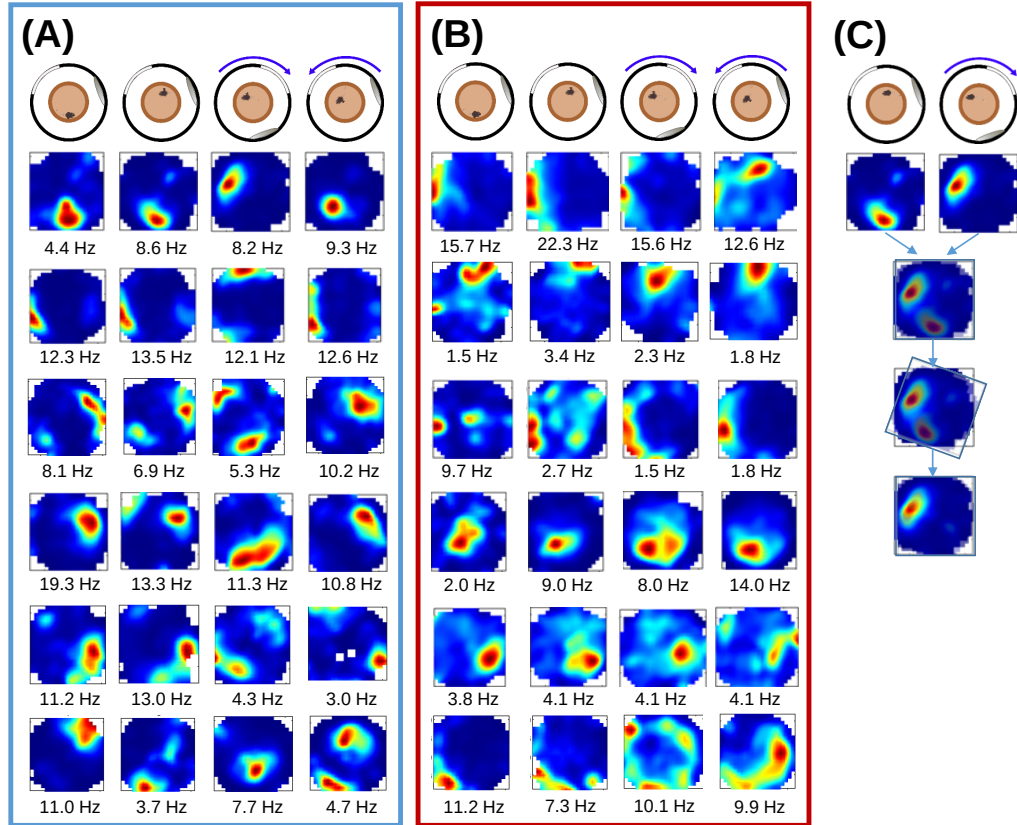


FIGURE 2.5: Example place fields across the distal cue rotation sessions **(A)** Selected example cells from control mice, showing rotation of spatial firing with the cues (top five rows; each cell is from a different mouse), with one example of a remapping cell (bottom row). Peak firing rates are indicated below. **(B)** Selected examples from the MEC-lesioned mice, showing lower spatial selectivity and stability of spatial firing as the cues were rotated (top five rows; each cell is from a different mouse). **(C)** Method for calculating rotation correlation – cell firing rate maps from one session were rotated by 5° increments relative to the firing rate maps from the session being compared, and a correlation of the two calculated. The maximum and minimum correlations, and the rotations at which they occurred were found.

To quantify the cue rotation data, correlations between firing rate maps were calculated across sessions, rotating one firing rate map relative to the other in 5° increments, as in Figure 2.5C. The rose plots in Figure 2.6A show the rotation angles at which the correlation was at its maximum value, for all cells with firing rates of above 0.1 Hz in both sessions being compared, in 10° bins. It is clear that in

the sham-lesioned mice (Figure 2.6A, blue box), cells generally remained stable across the first two standard sessions, when the cues did not move, then followed the clockwise 90° rotation of the cues between the standard 2 and 90° rotation sessions. The cells then followed the anticlockwise rotation of the cues as they were returned to their original position for the final session (standard 3). The figure also shows that cells from the MEC-lesioned mice were generally stable across the first two standard sessions, but they also remained stable both between standard 2 and the 90° rotation session (i.e. did not follow the cues), and when the distal cues were returned to their original positions (90° rotation to standard 3) (Figure 2.6A, red box). This finding, suggests that the distal visual cues had a lesser influence over the place fields in the mice with MEC lesions.

Statistical analysis of the distributions of the maximum correlation angles supported this. When comparing standard 1 and standard 2, in both the control and lesion groups, maximum correlations were clustered around 0° (control: mean vector ( $\mu$ ) of shifts = 1.2°, SE = 5.0°, 95% CI [351.5°,10.9°]; MEC lesion:  $\mu$  = 7.4°, SE = 4.8°, 95% CI [358.0°,16.8°]), suggesting they were remaining stable across the two sessions. Furthermore, the distributions of the two groups were not significantly different (Watson-Williams F-test:  $F(1,504) = 0.90$ ,  $p = 0.34$ ).

Standard 2 and the 90° rotation sessions were then compared; if cells were rotating with the cues they would show maximum correlations around 270°. In the control group, cells were clustered around 270°, ( $\mu = 269.4^\circ$ , SE = 2.5°, 95% CI [264.3°,274.3°]) but in the lesion group, there was only a small clockwise shift from 0° in the mean value ( $\mu = 341.0^\circ$  (- 19.0°), SE = 5.5°, 95% CI [330.2°,351.7°]). This meant that the two distributions were significantly different (Watson-Williams F-test:  $F(1,531) = 170.4$ ,  $p < 0.0001$ ).

Comparison of the 90° rotation and the final session, standard 3, where the cues returned to their original position, showed that cells from control mice had maximum correlations clustered around 90° ( $\mu = 90.4^\circ$ , SE = 4.6°, 95% CI [81.5°,99.4°]), representing an anticlockwise rotation to follow the cues. Cells from lesion mice again showed only a small rotation rotation ( $\mu = 14.0^\circ$ , SE = 5.4°, 95% CI [3.4°,24.5°]), and the distribution of rotations in the two groups were again significantly different ( $F(1,511) = 130.3$ ,  $p < 0.0001$ ).

As each mouse from each group contributed a different number of cells to the overall total (see Table 2.1), it is possible that data from mice from which more cells were

### 2.3. Results

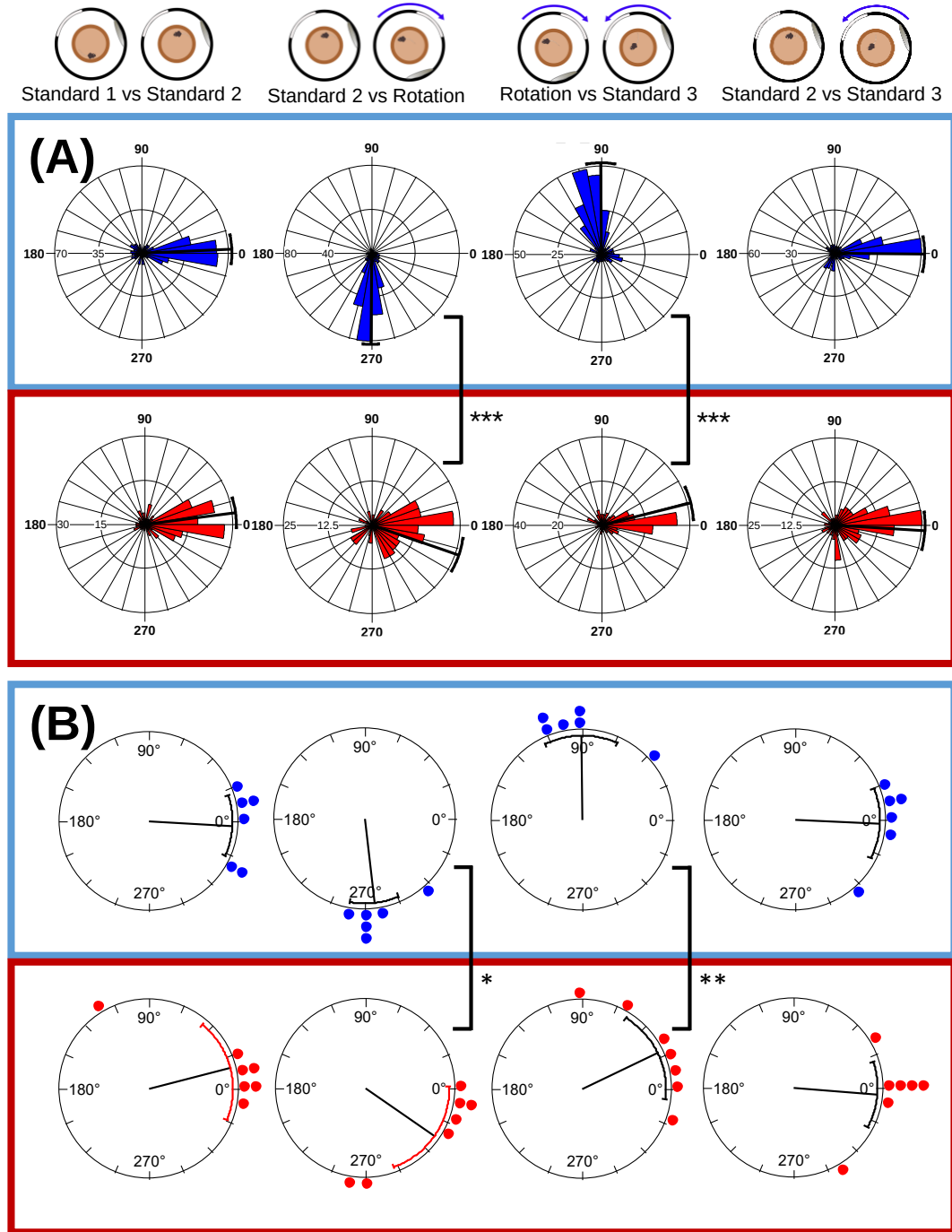


FIGURE 2.6: MEC lesion mice place cells do not rotate with distal landmarks. **(A)** Angular shift at which the maximum correlation between the session rate maps occurred, plotted for cells recorded from all mice in the control (blue) and MEC lesion (red) groups. The radial axis represents number of cells, the bold radial line denotes the circular mean of all cells, error bars show 95% confidence intervals. **(B)** Circular mean of maximum correlation angles for cells recorded from each mouse (control, blue,  $n = 6$ , lesion, red,  $n = 7$ ). Radial line represents mean of the animal means, with 95% confidence interval error bars. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

recorded might bias the data. Therefore, the mean maximum correlation values of cells from each mouse were also compared, and this is shown in Figure 2.6B. In the control group, for the first two standard sessions, each of the means from the six mice were around  $0^\circ$ , while in the lesion group, most were around  $0^\circ$  with the exception of two mice, and no difference was seen between the groups ( $F(1,11) = 0.97$ ,  $p = 0.345$ ). Comparing standard 2 with the clockwise ( $-90^\circ$ ) cue rotation showed that all of the means from the six control mice were clustered around the expected rotation of  $270^\circ$ ; in the lesion group, five of the mice appeared to have stable cells, with means close to  $0^\circ$ , while two had means around  $270^\circ$  suggesting that the cells in these two mice rotated with the distal visual cues. Despite these two mice that did show rotation with the cues, the difference between the groups was significant ( $F(1,11) = 7.50$ ,  $p = 0.019$ ). For the anticlockwise rotation back to standard 3, the mean rotations of the control mice were mainly around  $90^\circ$  as predicted, apart from one mouse showing an under-rotation; the lesion mice showed responses that were consistent with their previous average responses – five remained close to  $0^\circ$ , and the two mice that had rotated with cues between standard 2 and the rotation session showed the expected rotation of around  $90^\circ$  with the cues. This difference between the groups was also significant ( $F(1,11) = 13.89$ ,  $p = 0.003$ ). Interestingly, the two lesion mice with average responses close to the expected rotations with the cues were the two mice with the smallest lesions (see below for further analysis), suggesting the lesions may not have been sufficiently large to cause an impairment.

In order to confirm whether the repetition of the distal cue sessions across days had any effect on this result, the analyses described above were repeated using only cells recorded on the first day of recording in the distal landmark condition. The results showed a very similar pattern, even with the reduced numbers of cells (see Appendix A, Figure A.2).

As an additional measure of cell responses, maximum correlation, minimum correlation, and maximum correlation angles were all taken into account to categorise cells into types of responses. For a cell to be categorised as rotating, as well as having a maximum correlation above 0.5 for a certain angle, it must also have a minimum correlation below 0 for a different angle (see Methods for full classification). In the control group, 56% of cells followed the rotation of the cues, compared to 4% of the cells recorded in the lesion group. 32% of the cells from the lesion group remained stable when the cues were rotated, compared to just 4% in the

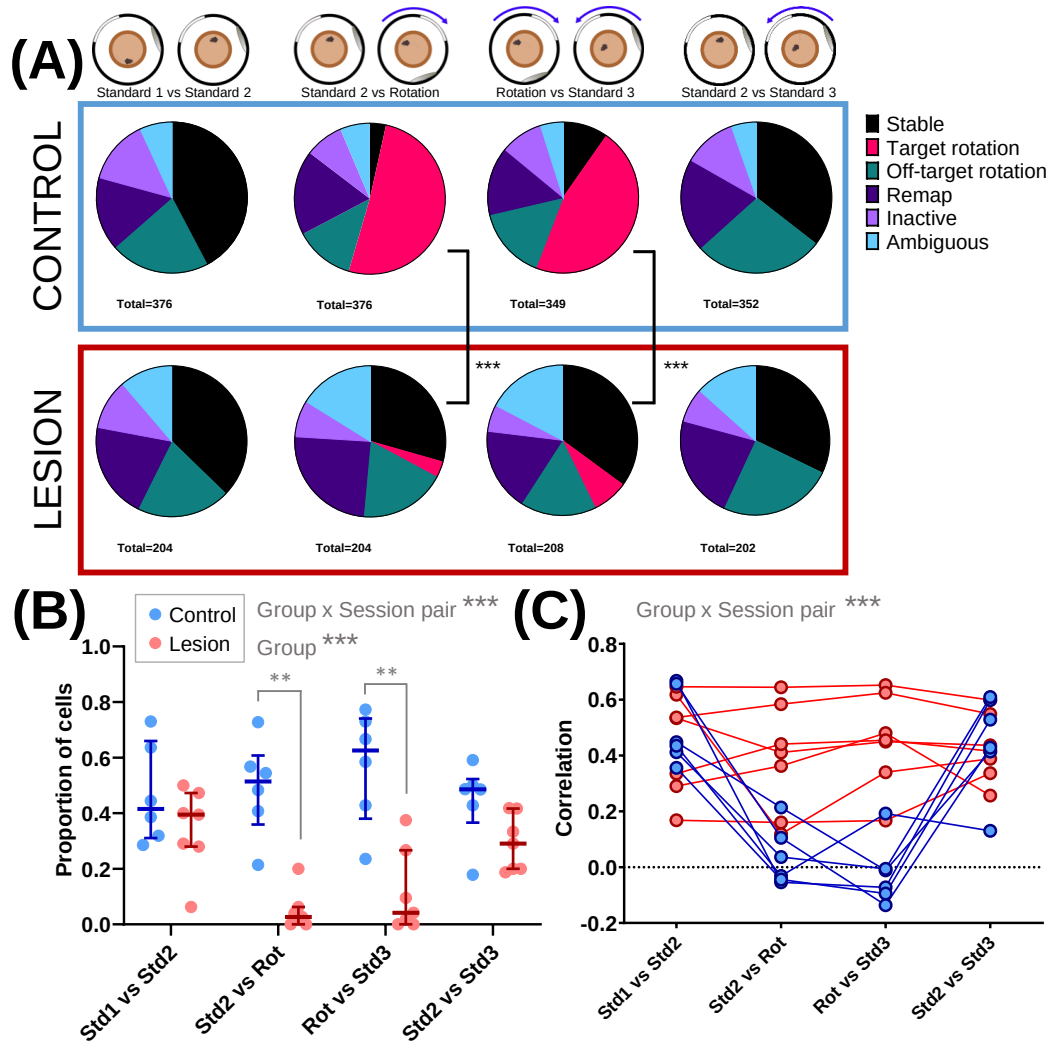


FIGURE 2.7: Lower proportions of place cells rotate with the distal cues in MEC lesion mice. **(A)** Cells recorded from control (above, blue box) and lesion (below, red box) mice, categorised according to rate map correlations between different sessions, shown as proportions of total number of cells for each session comparison. **(B)** Proportions of cells from each mouse that rotated within  $\pm 30^\circ$  of the cue rotation angle (Std1 vs Std2:  $0^\circ$ , Std2 vs Rot:  $270^\circ$ , Rot vs Std3:  $90^\circ$ , Std2 vs Std3:  $0^\circ$ ) with median  $\pm$  interquartile ranges plotted (control, blue,  $n = 6$ ; lesion, red,  $n = 8$ ). **(C)** Correlation of firing rate maps at  $0^\circ$ , showing means of all active cells recorded from each mouse (control, blue,  $n = 6$ ; lesion, red,  $n = 7$ ). Higher correlations indicate more stable firing rate maps. \*\*\*  $p < 0.001$

control group (Figure 2.7A). The proportions of cells categorised as showing each of the responses listed in Figure 2.7A were compared across groups. For the first session comparison (Standard 1 vs Standard 2), there was no difference between the groups when using a Chi-square test ( $X^2(4) = 6.672$ ,  $p = 0.154$ ), suggesting there was no difference in the number of cells that remained stable across the first two sessions. When comparing the sessions across cue rotations however, there were significant differences in the proportions for both the clockwise rotation (Standard 2 vs Rotation:  $X^2(5) = 176.9$ ,  $p < 0.0001$ ) and the anticlockwise rotation back to the standard position (Rotation vs Standard 3:  $X^2(5) = 128.2$ ,  $p < 0.0001$ ).

As the analysis described above and displayed in Figure 2.7A considered all cells from the mice of each group together, and included proportions of cells that showed off-target rotations, remapping, inactivity, or ambiguous responses, proportions of cells from each individual mouse that remained stable or rotated within the expected range were compared. Figure 2.7B compares proportions of cells recorded from each mouse that showed rotation with the cues for each session comparison (for Standard 1 vs Standard 2 and Standard 2 vs Standard 3, this is the same as proportion of cells that are stable, since the expected rotation is  $0^\circ$ ). Comparison of these values showed a significant main effect of group ( $F(1,11) = 25.74$ ,  $p = 0.0004$ ) and a significant interaction between session comparison and group ( $F(3,33) = 6.95$ ,  $p = 0.0009$ ). As expected, differences between groups were observed in proportions of cells that rotated with the cues (Sidak's multiple comparisons: Standard 2 vs Rotation:  $p = 0.0034$ ; Rotation vs Standard 3:  $p = 0.0056$ ), but there were no differences between groups in the proportions that remained stable across the sessions where the cues did not rotate (Standard 1 vs Standard 2:  $p = 0.608$ ; Standard 2 vs Standard 3:  $p = 0.200$ ).

To determine whether the lower proportion of rotating cells in the MEC lesion group could be due to lower stability between sessions, the correlation values at  $0^\circ$  of rotation were compared across the session pairs. Figure 2.7C shows mean correlation values for cells from each mouse across the session pairs; when comparing sessions across the cue rotation (Std2 vs Rot and Rot vs Std3), there is a drop in correlation in the control group, as is expected as the cells rotate with the cues, so the correlation value at  $0^\circ$  is low. In contrast, the correlations for mice in the lesion group appear to remain at similar values across all session pairs (i.e. both when the cues rotated and when they did not). To assess these differences, a GLMM was run on correlations of all cells active (mean firing rate  $> 0.1$ ) in both sessions

in each session pair, with mice and cell identity as a random factor. A significant group x session pair interaction was observed (LRT: 160.2,  $p < 0.0001$ ). Post-hoc tests showed that there was no difference between the groups when the cues remained stable, between the first two sessions (Std1 vs Std2: contrast estimate = 0.103, SE = 0.101,  $p > 0.999$ ), or between standard 2 and standard 3 (Std2 vs Std3:  $p > 0.999$ ), suggesting that between-session stability of spatial firing is unaffected by MEC lesion when the distal visual cues are stable. As expected, the control group showed a significant drop in correlation in the rotation session pairs, compared to the stable pairs (Std1 vs Std2, compared to Std2 vs Rot:  $p < 0.0001$ ). However, correlations in the lesion group were not affected by the cue rotation, and did not differ across the session pairs (Std1 vs Std2 compared to Std2 vs Rot:  $p > 0.999$ ). Hence there were significant differences between the two groups in stability across the rotation session pairs (Std2 vs Rot:  $p = 0.0003$ ; Rot vs Std3:  $p = 0.0002$ ). These analyses show that the lower proportions of cells rotating with the distal visual cues are not likely to be a consequence of reduced overall between-session spatial stability, as similar proportions of cells are stable between the stable sessions between groups, and this level of stability is maintained across the cue rotation in the MEC lesion group. It is not known what stable external stimuli the MEC lesion mice may use to maintain this stability.

#### **2.3.4 MEC lesions do not impair influence of proximal object cues on CA1 place fields**

In contrast to results from the distal cue rotation, cells recorded in the proximal cue rotations showed more similar responses between the two groups. Figure 2.8A shows example place cells across the proximal cue sessions for the control mice. The first three rows in Figure 2.8A show example place cells that showed clear rotation with the proximal objects, while the bottom three show remapping or ambiguous responses across sessions. Figure 2.8B shows similar examples from MEC lesion mice, with the first four rows clearly following the 90° rotation with the proximal cues, and the bottom two rows showing less spatially selective cells with remapping or ambiguous responses between sessions.

Maximum correlation angles for cells in each group are indicated in Figure 2.9A. In both groups, maximum correlations between the first two standard sessions were

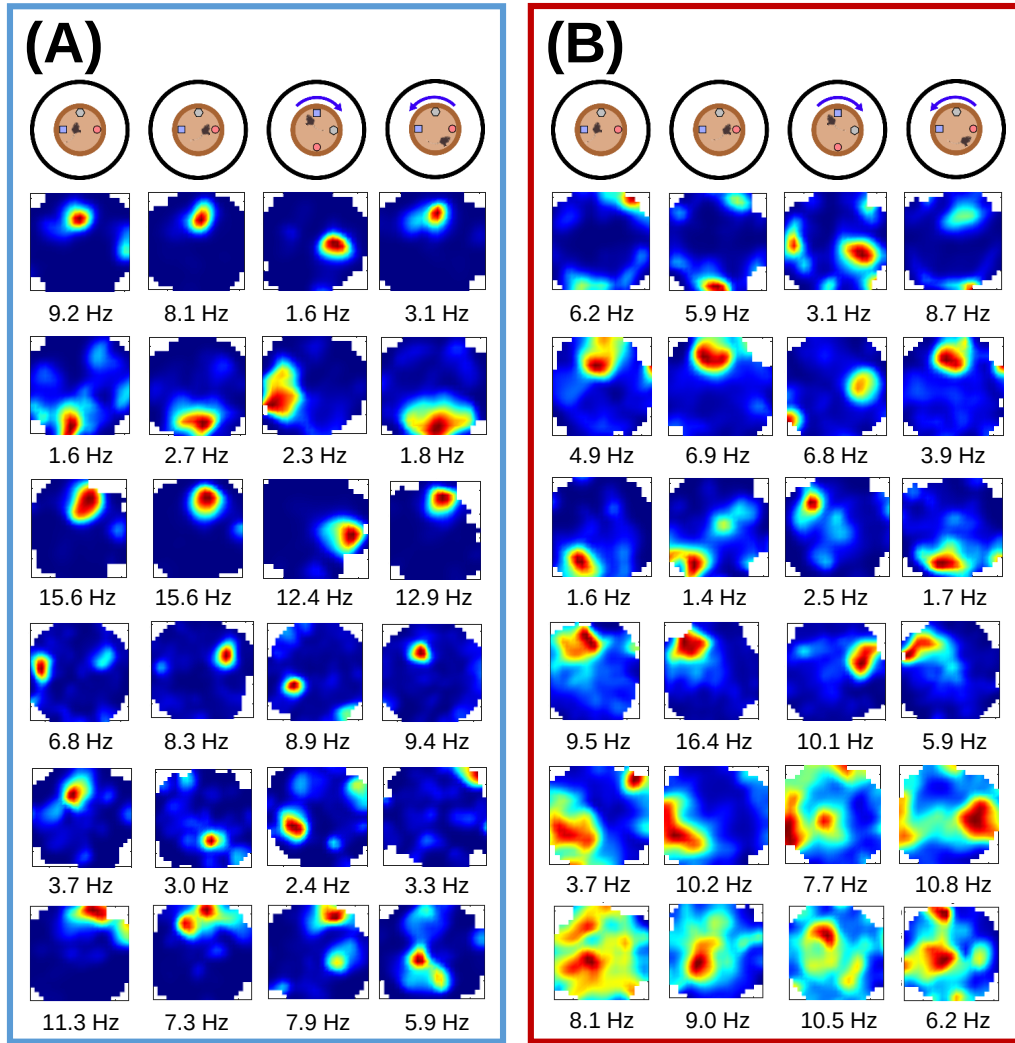


FIGURE 2.8: Example place fields across the proximal cue rotation sessions. **(A)** Selected example cells from control mice, showing cells that rotate with the cues (top three rows), and cells that show remapping across sessions (bottom three rows). Peak firing rates are shown below. **(B)** Selected examples from the MEC-lesioned mice, showing cells that rotate with the cues (top four rows), and cells with very low spatial selectivity (bottom two rows).

clustered around  $0^\circ$  (control:  $\mu = 2.9^\circ$ ,  $SE = 4.0^\circ$ , 95% CI [ $355.2^\circ, 10.7^\circ$ ]; lesion:  $\mu = 358.3^\circ$  ( $-1.7^\circ$ ),  $SE = 6.2^\circ$ , 95% CI [ $346.1^\circ, 10.4^\circ$ ]), suggesting both were remaining stable when the cues were not moved. The two distributions were not significantly different (Watson-Williams F-test:  $F(1,361) = 0.47$ ,  $p = 0.49$ ). When comparing standard 2 to the  $90^\circ$  cue rotation session, the control cells seemed to show a bimodal distribution, with some maximum correlations around the expected rotation of  $270^\circ$ , and some stable at  $0^\circ$ . The mean value was in between these

### 2.3. Results

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two values at  $\mu = 312.9^\circ$  ( $-47.1^\circ$ ) ( $SE = 7.6^\circ$ , 95% CI [ $297.9^\circ$ ;  $327.8^\circ$ ]). The cells from the lesion mice appeared to show a slightly more consistent response, generally clustering around  $270^\circ$ , with a mean of  $\mu = 296.7^\circ$  ( $-63.3^\circ$ ) ( $SE = 7.0^\circ$ , 95% CI [ $283.0^\circ$ ;  $310.4^\circ$ ]). However, the distributions of the correlations of cells in two groups were not significantly different (Watson-Williams F-test:  $F(1,367) = 3.39$ ,  $p = 0.07$ ).

Finally, comparing the rotation session to standard 3, most of the control cells were clustered around the expected rotation of  $90^\circ$ , with some remaining stable around  $0^\circ$  ( $\mu = 66.3^\circ$ ,  $SE = 10.1^\circ$ , 95% CI [ $46.6^\circ$ ;  $86.1^\circ$ ]), while the lesion cells were again mostly clustered around the expected rotation ( $\mu = 68.7^\circ$ ,  $SE = 5.7^\circ$ , 95% CI [ $57.1^\circ$ ;  $79.8^\circ$ ]). Again, there was no significant difference between the two distributions (Watson-Williams F-test:  $F(1,370) = 0.07$ ,  $p = 0.79$ ).

Looking at the individual animal means for the proximal cue sessions also suggests the lesion mice may have an increased reliance on the proximal cues compared to the controls. The means for both groups, with the exception of one control mouse, clustered around  $0^\circ$  for the first two standard sessions, and there was no difference between the groups ( $F(1,11) = 0.384$ ,  $p = 0.548$ ). The control mice means were much more variable following the  $90^\circ$  rotation of proximal cues than the lesion mice, of which four were close to the expected rotation of  $270^\circ$  (Figure 2.9B). Because of this high variability, there was again no significant difference between the groups ( $F(1,11) = 3.00$ ,  $p = 0.111$ ). In the rotation back to standard, the control mice were again very variable, although three of the means were close to  $90^\circ$ , and the lesion mice were again more reliably clustered around the expected rotation, five being close to  $90^\circ$ . Again, there was no significant difference between the groups ( $F(1,11) = 0.58$ ,  $p = 0.462$ ). The lower variability in the lesion mice compared to controls (indicated by the radial error bars in Figure 2.9B) across the rotation sessions suggests a more consistent response of the place fields in the lesion mice. This may indicate that the lack of distal cue information normally conferred by the MEC leaves the place cells more likely to rely on proximal cue information and therefore show a more coordinated rotation with the objects.

In both groups, 30% of the cells showed rotations consistent with the  $90^\circ$  cue rotation. However, a slightly higher proportion of cells from the control group (20%) remained stable across the rotation, compared to the lesion group (9%). Chi-square

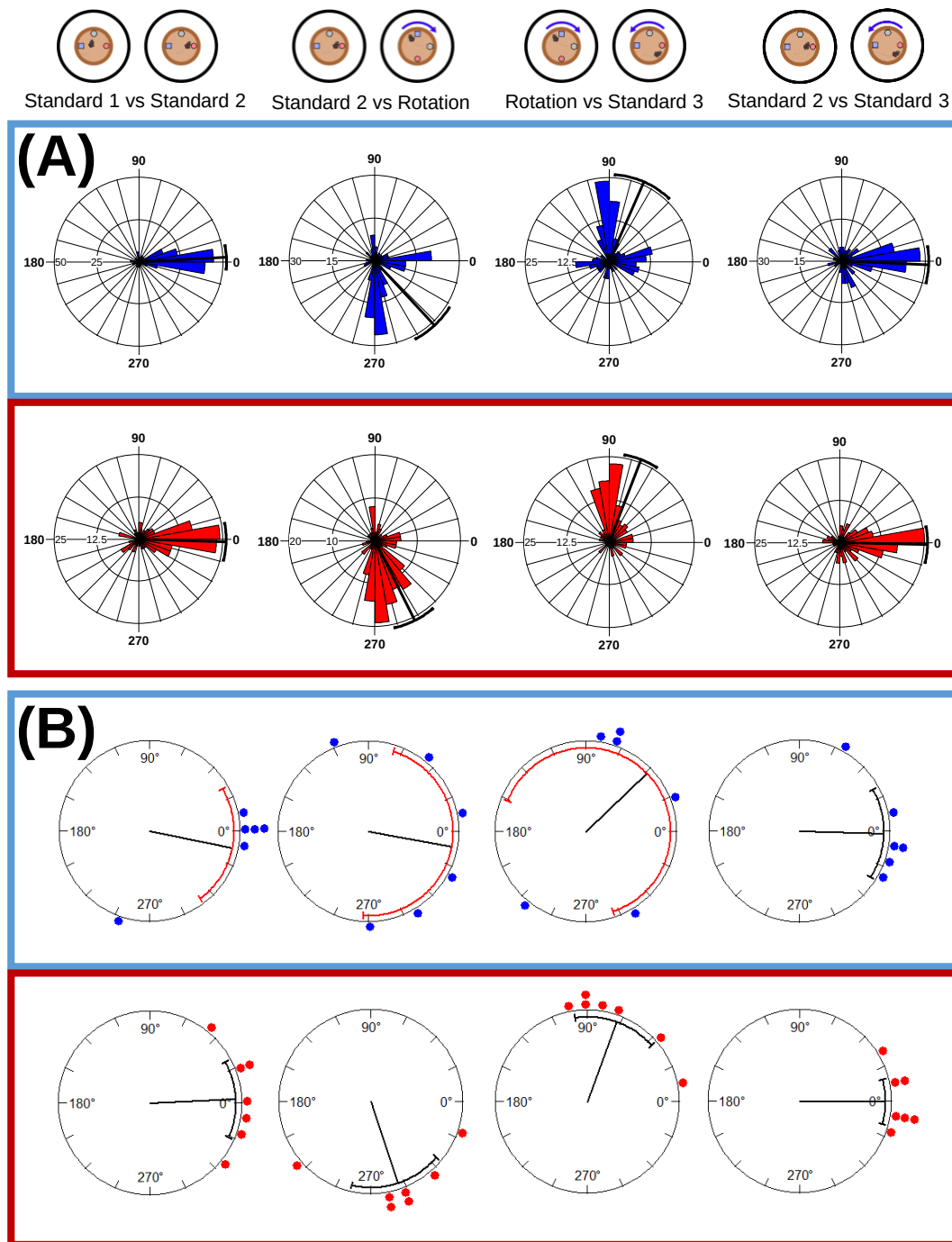


FIGURE 2.9: Place cells in both groups rotate with the proximal cues. **(A)** Angular shift at which the maximum correlation between the session rate maps occurred, plotted for cells recorded from all mice in the control (blue) and MEC lesion (red) groups. The radial axis represents number of cells, the bold radial line denotes the circular mean of all cells, with error bars showing 95% confidence intervals. **(B)** Circular mean of maximum correlation angles for cells recorded from each mouse (control, blue,  $n = 6$ , lesion, red,  $n = 7$ ). Radial line represents mean of the animal means, with 95% confidence interval error bars.

tests found significant differences between the groups in all of the session comparisons (Figure 2.10A; Standard 1 vs Standard 2:  $X^2(4) = 35.09$ ,  $p < 0.0001$ ; Standard 2 vs Rotation:  $X^2(5) = 25.00$ ,  $p = 0.0001$ ; Rotation vs Standard 3:  $X^2(5) = 28.95$ ,  $p < 0.0001$ ; Standard 2 vs Standard 3:  $X^2(4) = 12.35$ ,  $p = 0.0149$ ). Comparing proportions of cells from each mouse that showed rotations following the cues (Figure 2.10B) showed a significant main effect of session ( $F(2.61, 28.75) = 3.91$ ,  $p = 0.0226$ ), but no significant effect of group ( $F(1, 11) = 0.04$ ,  $p = 0.843$ ) and no significant interaction ( $F(3, 33) = 2.88$ ,  $p = 0.0509$ ). Multiple comparisons did not reveal any significant differences between the groups for any of the comparisons.

Figure 2.10C shows the mean correlation values at  $0^\circ$  of rotation for each mouse across the different session comparisons. From this, mice from both groups generally show a reduction in mean correlation values for the rotation comparisons compared to the stable comparisons. A GLMM was fitted to these data, which found a significant group  $\times$  session interaction (LRT = 12.1,  $p = 0.0071$ ). However, there were no differences in correlation values between groups for any of the session comparisons. There was no difference in stability between the groups when the objects remained stable (Std1 vs Std2:  $p = 0.270$ ), and there was a similar decrease in correlation at  $0^\circ$  for both groups when the objects were rotated (Std1 vs Std2 compared to Std2 vs Rot: Control:  $p < 0.0001$ ; Lesion:  $p < 0.0001$ ), and so there was no difference between the groups comparing the across the cue rotation (Std2 vs Rot:  $p > 0.999$ ).

#### **2.3.5 In control mice, higher proportions of CA1 place fields anchor to distal landmarks, compared to proximal object cues**

In Figure 2.11, proportions of cells for each mouse which rotated their firing fields to follow each set of cues was compared. In both the standard 2 vs  $90^\circ$  rotation and  $90^\circ$  rotation vs standard 3 comparisons, for the control mice (Figure 2.11A), proportions of cells rotating with distal landmarks were higher than proportions rotating with the proximal cues. Hence, a within-subjects two-way repeated measures ANOVA found a significant main effect of cue condition (distal vs proximal:  $F(1, 5) = 14.5$ ,  $p = 0.013$ ). In the MEC lesion group, there were very low proportions of cells rotating with the distal cues, and higher proportions rotating with the

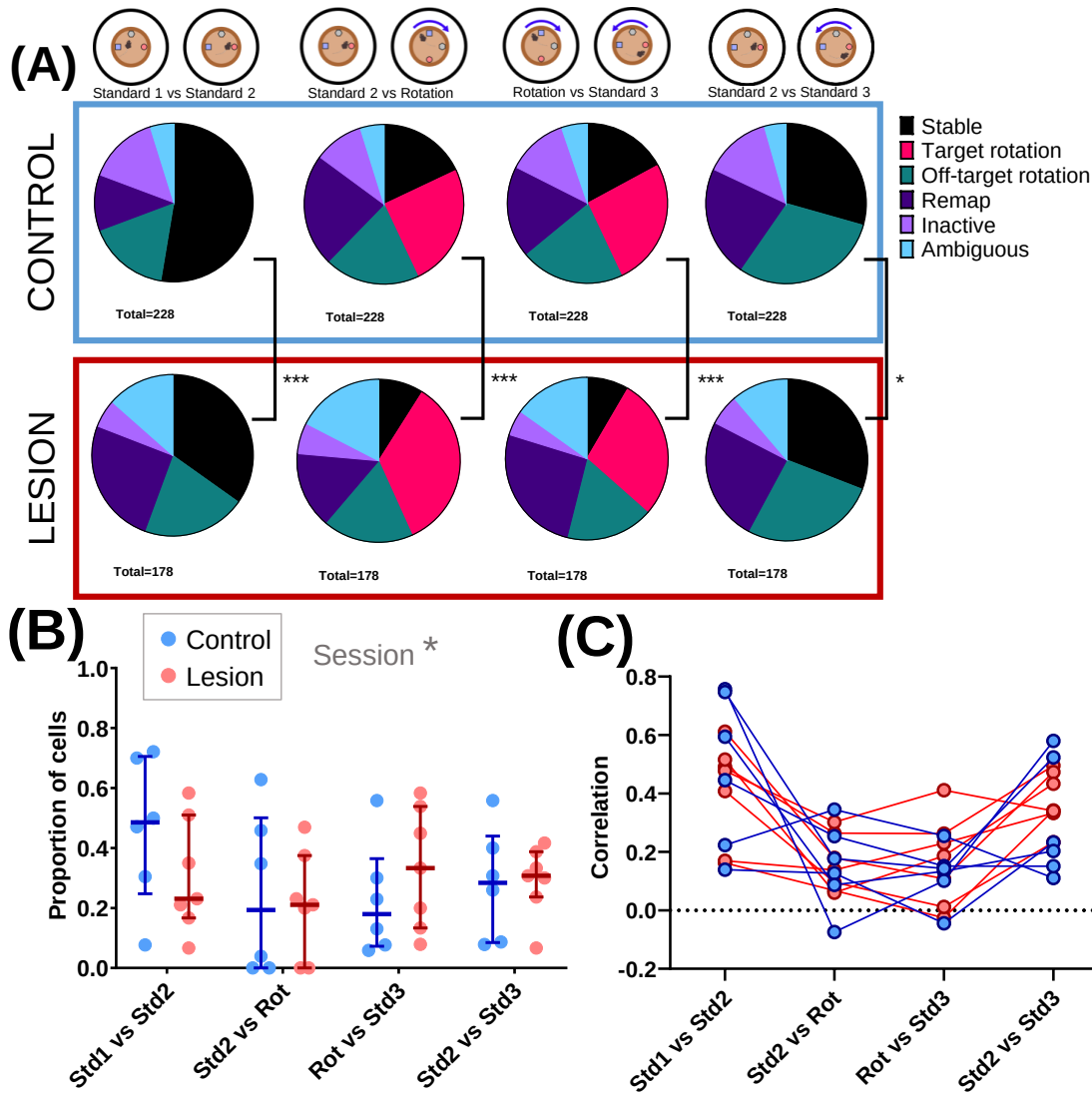


FIGURE 2.10: Similar proportions of cells rotate with the proximal cues in both groups. **(A)** Cells recorded from control (above, blue box) and lesion (below, red box) mice, categorised according to rate map correlations between different sessions, shown as proportions of total number of cells for each session comparison. **(B)** Proportions of cells from each mouse that rotated within  $\pm 30^\circ$  of the expected rotation angle, with median  $\pm$  interquartile ranges plotted (control, blue,  $n = 6$ ; lesion, red,  $n = 7$ ). \*  $p < 0.05$  **(C)** Correlation of firing rate maps at  $0^\circ$ , showing means of all active cells recorded from each mouse (control, blue,  $n = 6$ ; lesion, red,  $n = 7$ ). \*  $p < 0.05$ , \*\*\*  $p < 0.0001$

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proximal cues (Figure 2.11B). However, there was no main effect of cue condition ( $F(1,6) = 4.6$ ,  $p = 0.074$ ) in the MEC lesion mice.

This indicates that, in the control group, place cells are more likely to use distal landmarks for anchoring, compared to proximal object cues.

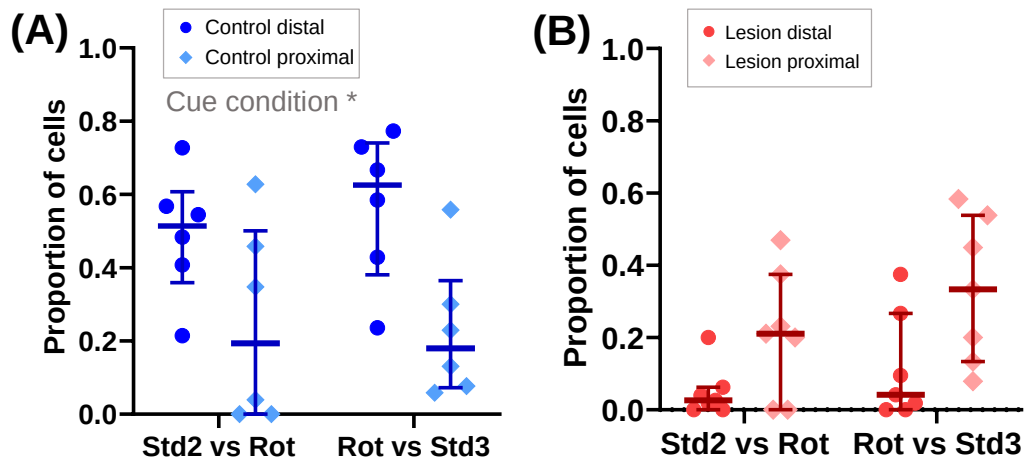


FIGURE 2.11: Higher proportions of cells in the control group anchor to distal landmarks, compared to proximal cues. **(A)** Control group data as in Figures 6 and 9. Proportions of cells from each control mouse that rotated within  $\pm 30^\circ$  of the angle of cue rotation (Std2 vs Rot:  $270^\circ$ , Rot vs Std3:  $90^\circ$ ) with median  $\pm$  interquartile ranges plotted (control, blue,  $n = 6$ ; lesion, red,  $n = 7$ ). **(B)** Lesion group data as in Figures 6 and 9. Proportions of cells from each Lesion mouse that rotated within  $\pm 30^\circ$  of the angle of cue rotation with median  $\pm$  interquartile ranges plotted (control, blue,  $n = 6$ ; lesion, red,  $n = 8$ ). \*  $p < 0.05$

One possible explanation for this may be that, as the proximal cue sessions were always recorded following the three days of distal landmark sessions, the mice were habituated to the apparatus and environment and so were no longer relying on the manipulated cues. In order to partly account for this, two of the control mice and three of the lesion mice were recorded for a further day on the distal landmark protocol, following the proximal cue days. Analysis of the cells recorded on this day showed a similar pattern to the other distal session days, with the control group largely showing rotations with the distal landmarks and the lesion mice showing a lack of rotation (see Appendix A, Figure A.3).

### **2.3.6 Larger lesions of the MEC were associated with less control of CA1 place fields by distal visual landmarks**

As mentioned in the previous sections, size of each lesion appeared to have an influence on some of the results observed. Table 2.1 gives the percentages of the MEC lesioned for each mouse, along with number of cells recorded, and mean rotation angle at which the maximum correlation occurred, for each session comparison, as represented in Figure 2.6B. The data in the table show that control mice generally show consistent rotation with the distal cues, with most showing mean rotations close to 90° in the direction of the cues. All mice with more than 36% of MEC lesioned had average rotations close to 0° when the cues were rotated. In contrast, the two mice with smaller lesions had average rotations consistent with the 90° rotation, suggesting an association between MEC lesion size and rotation with distal landmarks. Figure 2.12A shows proportions of cells that were categorised as following the initial clockwise distal cue rotation (Standard 2 vs Rotation) for each mouse, plotted against percentage of lesioned MEC. A trend can be seen, with larger lesions being associated with lower proportions of rotating cells – this correlation was found to be significant (Spearman's rank correlation:  $r(7) = -0.927$ ,  $p = 0.007$ ).

Results in Table 2.1 also showed no suggestion that damage to surrounding areas had any clear effect on rotation with the cues. The animals with lesions above 60% had variable levels of damage to other areas; mouse L5 and mouse L6 had lesions limited to the MEC, while L2 and L7 had more significant damage to other areas. These two groups of lesion mice showed comparable responses to the distal cue rotations.

### **2.3.7 Larger MEC lesions may be associated with greater proportions of CA1 place fields controlled by proximal cues**

Table 2.2 shows the same data as Table 2.1, but for the proximal cue sessions. The control mice showed very variable mean angles across the rotation sessions, with no clear indication that they were following the cues. Mice with MEC lesions tended to have mean rotation angles that followed the direction of rotation of the cues, but there was considerable variability in the extent of the rotations. There was not such

## 2.4. Discussion

| Mouse # | Lesion type | MEC lesion % | Number of cells | Mean angle    |             |             |
|---------|-------------|--------------|-----------------|---------------|-------------|-------------|
|         |             |              |                 | Std1 vs Std 2 | Std2 vs Rot | Rot vs Std3 |
| C1      | Control     | 0            | 10              | 1.2           | -47.5       | 105.6       |
| C2      | Control     | 0            | 21              | 5.9           | -89.4       | 91.5        |
| C3      | Control     | 0            | 73              | -29.2         | -90.3       | 94.5        |
| C4      | Control     | 0            | 134             | 24.4          | -93.6       | 42.3        |
| C5      | Control     | 0            | 50              | -29.6         | -95.8       | 99.4        |
| C6      | Control     | 0            | 36              | 7.0           | -82.8       | 113.0       |
| L1      | Medium      | 30           | 13              | 0.3           | -90.8       | 61.4        |
| L2      | Medium      | 30           | 11              | 115.8         | -98.7       | 92.2        |
| L3      | Medium      | 36           | 24              | 8.1           | -25.0       | 15.2        |
| L4      | Large       | 67           | 30              | -7.3          | -9.1        | 25.1        |
| L5      | Large       | 71           | 22              | 10.5          | -30.0       | 8.1         |
| L6      | Large       | 73           | 54              | 15.4          | -4.4        | 2.3         |
| L7      | Large       | 94           | 28              | -0.3          | -7.1        | -19.0       |

TABLE 2.1: Mean maximum correlation angles of each mouse in the distal cue sessions, with MEC lesion size and number of cells.

a clear relationship between mean angles and size of lesions – the four mice with angles close to  $-90^\circ$  were L2, L3, L5, and L6, which had lesions ranging from 30% to 73%. The variability among the control mice may reflect sub-populations of cells within each mouse showing a variety of responses to the cue rotation. The bimodal distribution of all control cells together shown in Figure 2.10A suggests that some cells rotate with the cues while others remain stable to the outer environment. The greater consistency in the responses of the lesion mice may indicate that their cell populations are more biased towards rotating with the proximal cues, in the absence of distal information. If this were the case, larger lesions may be associated with a higher proportion of cells rotating with the proximal cues. While correlation of proportion of cells rotating with the proximal cues with lesion size did show a positive trend (Figure 2.12B), it was not significant (Spearman's correlation:  $r(7) = 0.700$ ,  $p = 0.090$ ).

## 2.4 Discussion

This study set out to determine whether the MEC is necessary for the anchoring of the hippocampal spatial representation to distal visual landmarks. It was shown that place cells in control mice rotated their firing patterns to follow salient distal extra-maze cues, whereas those of MEC-lesioned mice did not. There was little

| Mouse # | Lesion type | MEC lesion % | Number of cells | Mean angle    |             |             |
|---------|-------------|--------------|-----------------|---------------|-------------|-------------|
|         |             |              |                 | Std1 vs Std 2 | Std2 vs Rot | Rot vs Std3 |
| C1      | Control     | 0            | 10              | -114.5        | 113.3       | -129.6      |
| C2      | Control     | 0            | 21              | -10.5         | -25.3       | 18.1        |
| C3      | Control     | 0            | 38              | 1.0           | 46.5        | 68.5        |
| C4      | Control     | 0            | 44              | 10.0          | 9.3         | -62.0       |
| C5      | Control     | 0            | 40              | 4.5           | -88.2       | 72.2        |
| C6      | Control     | 0            | 42              | 1.2           | -73.0       | 79.3        |
| L1      | Medium      | 30           | 13              | 47.8          | -140.4      | 101.9       |
| L2      | Medium      | 30           | 19              | 18.9          | -76.2       | 76.3        |
| L3      | Medium      | 36           | 6               | -43.5         | -80.7       | 42.0        |
| L4      | Large       | 67           | 23              | 24.1          | -21.0       | 91.9        |
| L5      | Large       | 71           | 23              | -4.1          | -67.7       | 94.1        |
| L6      | Large       | 73           | 48              | -7.1          | -72.2       | 71.2        |
| L7      | Large       | 94           | 36              | -16.4         | -47.7       | 13.0        |

TABLE 2.2: Mean maximum correlation angles of each mouse in the proximal cue sessions, with MEC lesion size and number of cells.

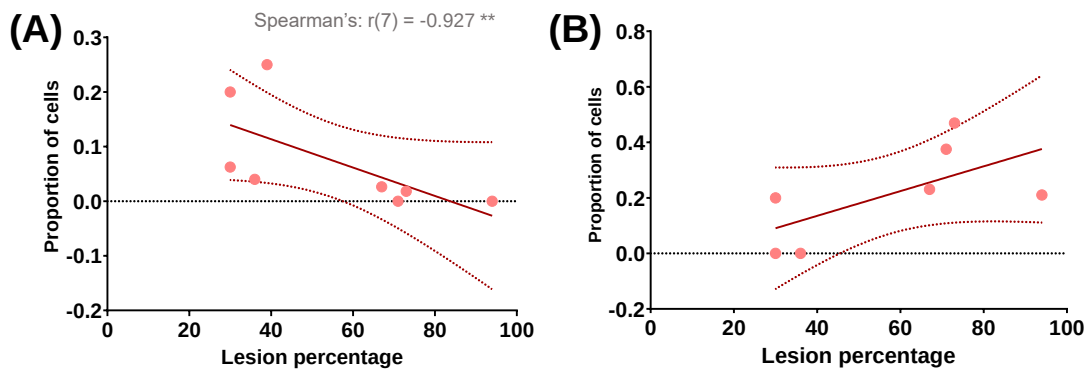


FIGURE 2.12: Larger MEC lesions are associated with lower proportions of cells rotating with distal landmarks. **(A)** Proportion of cells recorded from each lesion mouse that rotated within  $\pm 30^\circ$  of the initial clockwise rotation in the distal cue sessions, against percentage of MEC lesioned. The data were fitted with a linear regression line with dotted lines showing 95% confidence intervals. **(B)** Proportion of cells recorded from each lesion mouse that rotated within  $\pm 30^\circ$  of the initial clockwise rotation in the proximal cue sessions, against percentage of MEC lesioned. The data were fitted with a linear regression line with dotted lines showing 95% confidence intervals.  $^{**} p < 0.01$

effect, however, of MEC lesion on proportions of cells that rotate to follow proximal intra-maze object cues. This evidence therefore supports the hypothesis that the MEC is involved in processing of distal visual landmarks, and carries this information into the hippocampus, but is not required for providing information about proximal object cues to the hippocampus.

### 2.4.1 MEC lesions impair spatial coding of CA1 place cells

Firstly, this study replicates previous findings of preserved spatial firing in hippocampal place cells, but with reduced spatial information and selectivity when input from the MEC is lost (Van Cauter et al., 2008; Hales et al., 2014; Schlesiger et al., 2015; Jacob et al., 2020). Although no species difference was expected given similar cellular functioning of the MEC and CA1, these previous studies had all used rats, while the current study used mice, confirming this result to be cross-species. As some subtle differences in place field properties have been observed between the two species (Mou et al., 2018), it is useful to show similar effects in MEC-lesioned mice.

Contrary to some previous reports (Van Cauter et al., 2008; Hales et al., 2014) which showed large reductions in both mean and peak firing rates in MEC-lesioned rats, this study found only a slight reduction of place cell firing rates when measured within place fields, which was only significant in particular sessions. There are many possible reasons for such a difference, one being a species difference, as mouse place cells generally have lower peak firing rates (Mou et al., 2018). Another reason may be differences in lesion sizes; Van Cauter et al. (2008) reported damage to LEC in most rats, and Hales et al. (2014) reported close to 100% damage of superficial layers of MEC in all rats, so it is possible that a greater extent of damage to hippocampal inputs was linked to lower firing rates. It should also be noted that not every previous study has reported the same firing rate deficit – Schlesiger et al. (2015) showed no change in mean firing rates, with a small reduction in peak firing rates, while Jacob et al. (2020) showed no effect of MEC lesion on mean or peak firing rates. Furthermore, neither selective lesions of layer 3 of MEC or hyperpolarisation of layer 2 MEC inputs to the hippocampus, produced significant changes to CA1 mean firing rates (Brun et al., 2008; Kanter et al., 2017). Therefore, significantly decreased firing rate of CA1 place cells is not a consistent result of loss of MEC inputs, although there were generally at least trends towards lower peak firing rate in the above mentioned studies, as well as the current.

The stability of spatial firing between sessions also did not seem to be affected by the MEC lesions; correlations between firing rate maps for the first two sessions where the cues were stable showed no differences between groups. Hales et al. (2014) found a deficit in stability between sessions in MEC lesion rats, which worsened as the delay was increased – at the shortest delay of 2 minutes, comparable

to the delay used in this experiment, the deficit was at its lowest (similar results were shown in Schlesiger et al. (2018)). The mean correlation values reported by Hales et al. (2014) were also higher than in this study, so this effect could depend on the type of environment used, or familiarity of the environment. There is some evidence to support that this may be a species difference, as mice show lower levels of place field stability than rats when freely exploring an environment, only showing comparable levels when spatial memory is required for a task (Kentros et al., 2004; Hok et al., 2016). Therefore, the lack of task demand in the current experiment may have resulted in relatively low place field stability in both groups of mice, and any effects of MEC lesion may not have been observed for this reason. The stability of cells from the lesion group appeared to remain at similar levels of stability across the distal cue rotations, suggesting they were anchoring to other cues, possibly the limited local features of the platform. This could perhaps explain the lower place field precision, as having less reliable landmark information may decrease accuracy of location estimation.

#### **2.4.2 The MEC is required for anchoring of place fields to visual landmarks**

The data shown here show a clear difference in how place fields anchor to distal visual landmarks in mice with MEC lesions, compared to control mice. Cells from control mice were shown to rotate their spatial firing to follow a rotation of visual cues placed outside of the arena. Cells from lesion mice, however, appeared more likely to remain firing in the same position relative to the arena, which was unmoved between sessions.

Larger MEC lesions appeared to be associated with less influence of the distal cues. The two mice with the lowest percentage of MEC lesioned had the mean maximum correlation angle closest to  $-90^\circ$  (Table 1), suggesting the lesion was not sufficient to affect distal cue use. Whereas of the five mice with larger MEC lesions, four had mean angles close to  $0^\circ$ , suggesting they were remaining stable and not firing relative to the rotated cues. The fifth larger lesion mouse showed a rotation close to  $180^\circ$ , but had very few cells, so may not have a reliable mean value. There was also a significant negative correlation between lesion size and proportion of cells rotating with the cues, indicating that less intact MEC tissue leads to less

distal cue control over place cells.

The two sets of cues used in this experiment were chosen to make a clear distinction between distal visual landmark cues and proximal object cues. The environment was carefully controlled to make the visual landmarks or objects the most salient information, and to limit any other polarising cues. A previous place cell experiment placed landmarks within the environment, and found some rotational cue control in MEC-lesioned animals (Van Cauter et al., 2008). The cues were objects placed at the edges of the environment, so may not have been classed as purely landmark cues, as the animals could interact with them and move around them. This perhaps makes them more comparable to the proximal cues used here. Cues that are outside the environment cannot be moved around by the animal, and therefore will remain in a similar position relative to the overall global spatial representation. This may make them a more reliable basis for an internal map. This was demonstrated by Scaplen et al. (2014), showing that place fields were more likely to anchor to projected visual “landmark” cues that covered larger areas of the wall or floor close to the edge, compared to projected visual “object” cues that covered only a small, more central part of the floor. The current experiment lends support to the hypothesis that the MEC is the route of visual landmark information into the hippocampus.

There is some behavioural evidence in agreement with the findings of the current study. MEC disruption has shown deficits in navigational tasks where distal cue information may have been important (Hales et al., 2014; Poitreau et al., 2021; Fernández-Ruiz et al., 2021). There have been mixed results in tasks comparing distal and proximal cues in navigation with MEC or LEC lesions. Poitreau et al. (2021) used object cues within the Morris Water Maze and found successful navigation in both MEC- and LEC-lesioned rats (although somewhat impaired in MEC-lesioned rats). However, the interpretability of these results is limited by not knowing what strategy the animals are using to navigate, maybe not needing to bind object identity within the global spatial representation in order to find the platform. Lesions or disruptions of the LEC impair learning in tasks in which object identity determines the location of a reward (Kuruvilla & Ainge, 2017; Fernández-Ruiz et al., 2021). Moreover, associative object recognition tasks show that the MEC is important for object-location tasks, that may require the use of spatial information guided by visual cues (Van Cauter et al., 2013; Rodo et al., 2017; Tennant et al., 2018). The LEC is important for associative object memory, with lesion or

inactivation producing deficits in tasks where specific local objects must be associated with their positions or context (Wilson, Watanabe, et al., 2013; Vandrey et al., 2020).

In the current experiment the place fields of control mice were clearly anchored to the distal landmarks, as high proportions responded with the rotation predicted by the cues, and very few remained stable. The MEC lesion group showed levels of stability that were not different from the control group when the cues were stable, but did not rotate with the distal landmarks, and instead remained at similar levels of stability across the cue rotation. This might indicate use of some other external cues, such as local features of the platform, or odour cues left from the previous session. These would both be very limited, as the platform was circular and did not have any clear features, and was thoroughly cleaned between each session. Nonetheless, the data strongly support the hypothesis that the MEC is required to provide distal visual landmark information to CA1 hippocampal place cells.

### **2.4.3 The MEC is not required for processing of proximal object cues**

Similar to previous reports, we found lower proportions of cells that rotated their firing with the object cues compared to the distal cues in the control animals. This supports the idea that object cues are less likely to be used as a basis for the global spatial representation. In the current experiment, the object cues were placed close to the edge of the environment, but the mice could run around them by climbing on the raised rim. This distinction between the meaning of different types of cues perhaps makes sense in terms of navigation; large stationary objects outside of the immediate environment are useful for understanding where one is in terms of space, whereas it is useful to know the positions of possibly relevant objects within the immediate vicinity, but they may not be useful for locating one's self.

From the results presented here, the MEC is not required for proximal object cue information to influence hippocampal place cells. In fact, there is some evidence to suggest an increase in the influence of proximal object cues in the MEC lesion mice compared to the controls. The mean maximum correlation values for each mouse show very variable rotations in the control group, ranging from  $-88.2^{\circ}$  to  $+113.3^{\circ}$ . When all the cells are taken together, the rotations appear to show a

bimodal distribution, indicating that the networks of cells are not acting consistently, with some remaining stable relative to the arena (or room) and some firing relative to the objects. This suggests that control animals were not using the objects as landmarks on which to base their sense of space, despite them likely being the most salient information in the environment, and that they may have been using other, less salient cues within the room or features of the platform to anchor their spatial firing.

This is consistent with previous studies; in a study in which proximal and distal cues were rotated in conflict, populations of CA1 place cells were split, with some fields following the distal cues and some following the proximal (Yoganarasimha et al., 2006). While this study used textured flooring as the proximal cues, another cue conflict experiment using a similar set-up to the current experiment showed a similar split of responses (Renaudineau et al., 2007). Others have shown a lack of control of place fields by object cues (Cressant et al., 1997; Scaplen et al., 2014). Yoganarasimha et al. (2006) found that fewer cells followed the proximal cues compared to the distal landmarks, suggesting place cells are more likely to anchor to distal landmark cues. Although Renaudineau et al. (2007) reported a more equal split, this may have been due to the use of large objects placed at the very edge of the environment, which were possibly being treated as more reliable landmarks. The results presented here, while not testing a cue conflict, show some rotation of place fields with objects in the control mice, but in lower proportions than in the distal landmark condition (Figure 2.11A).

Cells in the lesion mice appeared to fire more coherently than those in the control mice in the proximal cue condition, with many following the object cues – the cell maximum correlation angles and individual mouse mean values are generally clustered around the expected rotation values, contrasting with the high variability in the control group. However, even in the MEC lesion mice, some cells did remain stable, demonstrated by the undershoot in the rotation of the cells and mean values. There did appear to be a lower proportion of cells that remained stable across the rotations than in the control group, and there was a trend in the association between lesion size and proportions of cells that rotated with the cues, but more subjects may be required to determine whether these were real effects. At any rate, we did not find any evidence that the MEC lesion had a detrimental effect on place field rotation with the proximal cues.

This result provides evidence in support of the hypothesis that the MEC is not required for the processing of objects within an environment, and representation of this within the hippocampus. This leaves open the question of where this information comes from, the LEC being the most likely candidate. While this has not been tested with cue rotation similar to the one used here, recording in the LEC has shown spatial firing related to object locations, as well as previous object locations (Deshmukh & Knierim, 2011; Tsao et al., 2013), and LEC lesions impaired performance on behavioural tasks where specific object location memory was required (Kuruvilla & Ainge, 2017; Rodo et al., 2017). A recent study also reported reduced object-associated firing in a rewarded task, with disruption of LEC activity (Fernández-Ruiz et al., 2021).

Object-associated firing has also been seen in CA1 place cells (Deshmukh & Knierim, 2013), and appears to be more prevalent in the distal part of CA1, bordering the subiculum, rather than the proximal part, bordering CA2 (Burke et al., 2011; Vandrey et al., 2020). This is consistent with the object information being provided by LEC inputs into CA1, as the LEC projects predominantly to distal CA1, while MEC projects more to proximal CA1 (Tamamaki & Nojyo, 1995). It should also be noted that Cressant et al. (1997) used coordinates that would likely have targeted proximal CA1, and found no object control of place fields, while Yoganarasimha et al. (2006) found some fields that were controlled by local cues in more distal CA1. The tetrode coordinates in the current experiment were targeted more towards proximal CA1, so were less likely to capture object-associated firing; this may explain the generally low proportions of cells following the proximal cues. Although this was variable between mice, any variation in electrode position is very unlikely to have caused this, since the same coordinates were used for all, and proximodistal positions used by Vandrey et al. (2021), where there were differences in object-associated firing, were at the two extremes of the CA1 proximodistal axis.

#### **2.4.4 The role of the MEC in hippocampal spatial processing**

Our new findings support the hypothesis that distal and proximal cue information may enter the hippocampus via different routes, with the MEC carrying information for use of distal visual landmarks to guide navigation and allocentric location within the global environment, with the LEC perhaps carrying information about

local items and their locations within the environment (Knierim et al., 2013; Wang et al., 2018; Kuruvilla et al., 2020). This fits with a previous study in which spatial firing of MEC cells followed distal landmarks, and LEC cells followed proximal cues, in a cue conflict situation (Neunuebel et al., 2013). Taken together with a recent study demonstrating the importance of the MEC for path integration information input to the hippocampus (Jacob et al., 2020), the role of the MEC may be integration of self-motion information with allothetic landmark cue information to inform an overall representation of space in the hippocampus.

Recent studies recording cells in the MEC may make this distal/proximal cue distinction less clear, showing cells which appear to encode distance and direction from an object within an environment, termed ‘object-vector cells’ (Høydal et al., 2019). A process of gradually changing the 3-dimensional objects to 2-dimensional cues on the wall of the environment, led to object-vector cells also firing relative to this cue (Andersson et al., 2021). Similarly, MEC cells fired in positions relative to visual landmarks in a virtual reality environment (Kinkhabwala et al., 2020). This evidence suggests MEC may not just process distal landmarks, but may encode spatial position relative to salient features in the environment. However, distal landmarks may be more favoured, as they are often more stable and reliable cues for anchoring. The distinction between MEC and LEC function may be more well described by the context/content division proposed by Knierim et al. (2013), with distal landmarks more often being considered a reliable framework for the overall spatial representation (context), and proximal cues being considered as features within this (content).

The MEC may also process contextual feature information, as well as spatial position within it. A study analysed cells in MEC which were not categorised as grid, border or head direction cells, but showed spatial firing patterns. The majority (around 68%) of cells recorded in this study were “non-grid spatial” cells, and were found to redistribute their firing patterns in response to environmental manipulations, such as changes in colour or shape (Diehl et al., 2017). In the same study, grid cells did not appreciably change their spatial field locations, but showed firing rate changes across the fields, similar to the hippocampal rate remapping which was seen in a subset of rats with simultaneous hippocampal recording (Diehl et al., 2017). This shows that the MEC may not be simply responsible for overall spatial position information, and encodes contextual features. The LEC may also process such information, as hippocampal rate remapping is reduced in animals with LEC

lesions (Lu et al., 2013). This suggests parallel encoding of both spatial and environmental feature information in the MEC, possibly in separate cell populations, which may inform the hippocampal spatial code.

### **2.4.5 Limitations**

Perhaps the main limitation of the experiment presented here was the use of excitotoxic lesions, and the non-specificity of such an approach. There was quite a high level of variability in terms of lesion size across the mice, with some having lost a relatively small proportion of the MEC, and some where a large amount of MEC had been lesioned, but the damage had spread to surrounding areas. This did give the opportunity to look at how greater extent of damage affected place cell responses, but it is difficult to rule out the possibility of damage to other areas having affected the responses. Three of the lesion mice had considerable damage to areas such as subiculum, presubiculum and ventral hippocampus. In three of the mice (L3, L4, L7), there was damage to the (ventral) presubiculum; the presubiculum has head direction cell activity (Taube et al., 1990), and major projections to the MEC (van Groen & Wyss, 1990; van Haeften et al., 1997), so damage could be partly responsible for the effects seen. Most studies of spatial processing have focused on the postsubiculum (also known as the dorsal presubiculum), showing head direction and grid cell activity (Boccaro et al., 2010), and requirement for CA1 distal landmark anchoring (Calton et al., 2003). The damage outside the MEC here though, was limited to more ventral areas and spared the postsubiculum. In two of those mice there was also extensive damage to the ventral hippocampus. Although the ventral hippocampus is thought to be more involved in anxiety and odour processing rather than spatial processing (Moser et al., 1993; Bannerman et al., 2003), spatial firing has been found in cells of the ventral hippocampus, and other studies have shown a role in spatial behaviour (Jung et al., 1994; Kjelstrup et al., 2008; Lee et al., 2019). The spatial firing seen is much less precise than in its dorsal counterpart (Jung et al., 1994), so it is unlikely to play a role in distal landmark processing in the dorsal hippocampus, and the damage seen here is unlikely to have contributed to the effect. While this external damage may have had some effect, there were two mice with the majority of MEC lesioned, and minimal damage to other areas (L5 and L6). These mice showed low proportions of cells

rotating with the distal landmarks and mean maximum correlations close to  $0^\circ$ , indicating lack of rotation with the distal cues, so even with the external damage in other mice, overall, the data do strongly support the conclusion that loss of MEC input is sufficient to disrupt distal cue use in CA1.

As well as a lack of specificity in the extent of the damage, the lesion approach gives a lack of temporal specificity; the damage is permanent and may lead to longer-term effects outside the MEC that are difficult to assess. A more time-limited approach such as manipulation of specific circuits using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) or optogenetics may have provided a more reliable conclusion on the effect of MEC inactivation specifically. These techniques also have the advantage of being able to compare the place cells within-subjects, when the MEC is both functional and perturbed. On the other hand, the more sudden manipulation of cells using these methods may not allow the brain to adapt to the change. Whereas with the more permanent lesion approach, we may be observing brain activity in a more physiological state, allowing assessment of the capability of hippocampal processing without MEC input.

While we can make comparisons between representation of proximal cues versus distal landmarks, and how they are processed by different hippocampal inputs, the current study only examines one input area, and examines the types of cues separately. We can compare with previous studies and speculate about the information carried by the LEC, but since the LEC was not lesioned here we cannot make solid conclusions about its role. From the current data and evidence from other studies, it can be predicted that lesions of the LEC would have the effect of reducing rotation of CA1 place fields with the object cues, while preserving rotation with distal landmarks. Some adaptation of the apparatus may be required, as some of the control mice showed low proportions of cells rotating with the objects (Figure 2.9C). This could have been due to insufficient control of other sensory information, such as odour cues on the floor of the platform, or they may have been able to anchor to the distal visual environment. It may be that CA1 place cells are not very likely to rotate with object cues in this set-up. As previously mentioned, recording in more distal CA1 may also be required, where LEC input and object firing are more prevalent (Tamamaki & Nojyo, 1995; Vandrey et al., 2021). An alternative explanation for the low proportions of cells rotating with the proximal cues could be that, as the proximal cue sessions were always after the distal landmark sessions, the increased familiarity with the environment led to more stability of the cells to

uncontrolled external cues. This is unlikely, as the proportion of cells remaining stable were not particularly high during the proximal cue rotation (Figure 2.10A), however, in future experiments it may be preferable to counterbalance the order of the cue conditions.

Following the distal and proximal cue sessions, some of the mice underwent recording in the same environment with both types of cues present, which were then rotated in conflict, similar to Renaudineau et al. (2007). However, there were not enough cells recorded in this condition to analyse the data and get a clear result. This cue conflict manipulation would have allowed us to compare the anchoring to the different types of cues directly, and the proportions of cells in the control and lesion groups that followed each type of cues. My prediction is that the control mice would show a split, with some cells following the distal landmarks, some following the objects, and others remaining stable, whereas cells in the MEC lesion mice would only follow the objects or remain stable.

#### **2.4.6 Future work**

The current study shows that lesions of the MEC produce deficits in anchoring of the hippocampal place cell representation to distal landmarks; an interesting next step would be to investigate the specific circuitry involved in this process. The MEC has multiple projections to CA1, directly via pyramidal cells, and indirectly via layer 2 stellate cells, which project to dentate gyrus and CA3. A similar recording protocol with a specific inactivation of each of these pathways would answer this question. Likewise, an LEC lesion or inactivation of specific inputs from LEC would provide evidence for the difference in cue processing between the two areas.

Directional coding in place cells has been an ongoing debate, but recent studies have shown convincing evidence for conjunctive directional modulation within place fields, which appears to be strongly biased towards visual cues (Acharya et al., 2016; Jercog et al., 2019). Unfortunately, the head direction of the mice was not recorded in this study, and efforts to estimate head direction from movement tracking were unsuccessful. However, it would be very interesting to determine whether directional coding in CA1 place cells is dependent on the MEC. This seems likely, due to the strong directional coding in the MEC, not just in head direction cells, but within grid cell fields (Sargolini et al., 2006; Gerlei et al., 2020).

Understanding the types of information carried by the entorhinal-hippocampal circuits, and their importance for memory and behaviour, is an ongoing process. Questions remain over how the loss of MEC-dependent processing of distal landmark information presented here impacts on spatial behaviour, and the specific circuits carrying this information. Further study of what sensory information becomes classed as a landmark or object within an environment, and how they are processed differently in these circuits, would be interesting to reveal more about how memories of experiences are formed in the brain. The following chapter aimed to investigate some of these questions.



## **Chapter 3**

# **The role of medial entorhinal cortex layer 2 stellate cells in associative memory**

### **3.1 Introduction**

The previous chapter reported an experiment that demonstrated the effect of complete lesion of medial entorhinal cortex (MEC) on formation of spatial memory representations. It was shown that the MEC may be crucial for the use of distal landmarks to anchor the internal spatial map in CA1 of the hippocampus. While this shows the importance of the MEC as a cortical structure in this aspect of spatial processing, the functions of specific MEC-hippocampal connections remain unclear.

Axonal projections from the MEC to the hippocampus include two main pathways; pyramidal cells in both layer 2 and 3 of the MEC synapse directly with cells in CA1 of the hippocampus, while layer 2 stellate cells (L2SCs) project to the dentate gyrus and CA3 via the perforant path, where the signals are carried via the Schaffer collaterals to CA1 (Figure 1A) (Witter et al., 2017). These projections may have different functions in terms of the information they carry into the hippocampus.

A number of studies have attempted to characterise different functional properties of the two cell types. There has been some debate about spatial firing properties of pyramidal and stellate cells in the MEC, but evidence suggests both populations show grid, head direction, speed and border firing so may not differ in spatial firing

properties (Tang et al., 2014; Rowland et al., 2018; Gu et al., 2018).

A series of papers by the Tonegawa lab found differences in functional roles of the layer 2 stellate and pyramidal cells not directly related to spatial tuning. Specific expression of calcium indicators in each of the two cell types allowed *in vivo* imaging while mice were exposed to distinct contexts – pyramidal cells showed no specific differences in firing between the two contexts, whereas a proportion of stellate cells fired in one context only. They also showed that encoding of the different contexts by different CA3 pyramidal cell populations was disrupted by inhibition of MEC L2SC input, but not MEC pyramidal cell input. Furthermore, contextual fear conditioning was found to be dependent on MEC L2SC function (Kitamura et al., 2015). MEC pyramidal cells, on the other hand, showed more speed-modulated activity, and were important for temporal association memory (Kitamura et al., 2014; Sun et al., 2015). This evidence suggests that MEC L2SCs encode contextual information, and may be required for hippocampus-dependent contextual discrimination behaviours.

Kitamura et al. (2015) used multiple different pairs of environments to test contextual discrimination of the MEC L2SCs. Higher proportions of L2SCs discriminated between context pairs that differed in multiple dimensions (i.e. floor textures as well as walls), compared to a more similar context pair that only differed in shape. This may reflect the different remapping processes that occur in the hippocampus; when two environments are very distinct, hippocampal place cells produce completely distinct spatial representations, with many place fields changing in location (Muller & Kubie, 1987; Anderson & Jeffery, 2003; Leutgeb et al., 2004). When environments only differ in certain features, such as wall colour or odour, hippocampal place fields generally remain in the same spatial location, but change their firing rates (Anderson & Jeffery, 2003). The evidence from Kitamura et al. (2015) suggested that L2SCs may be required for hippocampal global remapping between very distinct contexts.

Manipulation of MEC inputs to the hippocampus can induce global remapping of CA3 or CA1 place cells in a familiar environment, without disruption of spatial firing (Miao et al., 2015; Kanter et al., 2017), although there is some contrasting evidence regarding the type of manipulation required, as well as the extent of remapping that occurs (Zhao et al., 2016; Rueckemann et al., 2016; Kanter et al., 2017). In addition, grid cells in the MEC change their firing field locations alongside global

remapping in CA3, and other spatial cells in the MEC show changes in firing field locations in response to context changes (Fyhn et al., 2004; Marozzi et al., 2015; Diehl et al., 2017). However, there is some conflicting evidence; normal CA1 global remapping between environments differing in shape and colour placed in different rooms, was observed in MEC-lesioned rats, suggesting the MEC may not be necessary for hippocampal remapping (Schlesiger et al., 2018).

Rate remapping in the hippocampus in response to changes of features of an environment may rely on the LEC, lesions of which disrupt hippocampal rate remapping (Lu et al., 2013). This idea is also based on the fact that LEC cells appear to encode the local features of environments, rather than overall spatial location information (Neunuebel et al., 2013; Knierim et al., 2013). It could be that MEC L2SC input is required for global remapping in the hippocampus, requiring a more complete change of the spatial representation, which may have been required in response to the highly distinct contextual changes employed by Kitamura et al. (2015). Although this is disputed by the evidence from Schlesiger et al. (2018), so the role of the MEC input in hippocampal global remapping is not completely clear.

The current experiment aimed to test the requirement of MEC stellate cells for hippocampal contextual processing in associative memory. A previous experiment using targeted inactivation of the layer 2 fan cells of the LEC, cells with similar projections to the hippocampus as stellate cells of the MEC, produced a deficit in hippocampus-dependent object-place-context associative memory in mice (Vandrey et al., 2020). Here, a similar set of tasks were used to directly compare the role of MEC L2SCs in this type of associative memory.

Spontaneous object exploration tasks can be used to test recognition memory in rodents, taking advantage of their natural tendency to explore novelty for longer than familiarity. Memory for familiar objects, or object-context (OC), object-place (OP) or object-place-context (OPC) associations can be inferred if the rodent preferentially explores the novel object or object pairing. Of these associations, only OPC memory is hippocampus-dependent (Langston & Wood, 2010). Wilson et al. (2013) first demonstrated a role for the LEC in these tasks; c-fos expression was increased in the LEC of rats following OC discrimination, and LEC lesion impaired OP, OC and OPC performance, while standard object recognition remained intact (Wilson, Langston, et al., 2013; Wilson, Watanabe, et al., 2013). Recently it was shown that the LEC fan cell input to the hippocampus is required for OPC memory

in mice, but not for object or OC memory. These data suggest that inputs from LEC into the hippocampus are required for the formation of memory for associations between objects, contexts and locations within the environment. A role for the LEC in these tasks is consistent with the context-dependent object-associated firing patterns of LEC cells (Tsao et al., 2013).

Fewer studies have focused on the role of the MEC in object association tasks. The context-specific firing (Kitamura et al., 2015) and recent finding of object-vector cells in the MEC (Høydal et al., 2019) may suggest a role in these tasks. Studies in MEC-lesioned rats have shown impaired contextual discrimination and object-location association, in which one of a familiar set of objects is displaced to a novel location within the environment (Hunsaker et al., 2013; Van Cauter et al., 2013; Rodo et al., 2017). Tennant et al. (2018), using inactivation of MEC L2SCs in mice, showed an object-location discrimination impairment, suggesting MEC inputs to hippocampus are required for this type of memory.

Here the targeted viral manipulation used in Tennant et al. (2018) and Vandrey et al. (2020) was used to inactivate L2SCs of the MEC, in order to determine their function in hippocampus-dependent associative recognition memory. To allow comparison to the LEC fan cell study (Vandrey et al., 2020), a very similar task protocol was used. Due to the proposed role in contextual discrimination, it was predicted that inactivation of MEC L2SCs will lead to a deficit in tasks requiring contextual information input into the hippocampus (i.e. OPC, which is hippocampus-dependent (Langston & Wood, 2010)). The role of MEC L2SCs in spatial processing (Tennant et al., 2018) means that they may be needed for the place information needed in the OPC task as well.

## **3.2 Methods**

Some of the initial surgeries and perfusions for this experiment were performed by Dr Sarah Tennant, during training of the author. Timing of object exploration from videos was performed partly by a volunteer student, Ella Cronkshaw, and partly by the author.

#### 3.2.1 Animals

Experimentation was carried out under a UK Home Office project licence, approved by the Animal Welfare and Ethical Review Board (AWERB) of the University of Edinburgh College of Medicine and Veterinary Medicine, and conformed with the UK Animals (Scientific Procedures) Act 1986.

All mice were from a transgenic line (Sim1:Cre) made to express Cre under the control of the Single-minded homolog-1 (Sim1) promoter, in order to enable selective genetic access to the layer 2 stellate cells (L2SCs) of the MEC, as described in Sürmeli et al. (2015). The line was originally generated by GenSat and obtained from MMRRRC (strain name: Tg(Sim1cre)KJ21Gsat/ Mmucd). The line was then maintained by breeding male Sim1:Cre mice carrying the Cre transgene, with female C57/BL6jCrl wild-type mice (Charles River) to produce offspring heterozygous for the Cre transgene. Male and female mice carrying the Cre transgene aged between 8 and 12 weeks were used for experiments. All mice were housed in groups of 2 to 5 in diurnal light conditions (12-hour light/dark cycle) and had *ad libitum* access to food and water. Mice were pseudorandomly assigned to either receive a GFP virus (n = 12, 5 males, 7 females) or a TeLC virus (n = 9, 4 males, 5 females), shown previously to suppress neurotransmitter release in stellate cells when injected into MEC of Sim1:Cre mice (Sürmeli et al., 2015; Tennant et al., 2018). In total, 25 mice were run on the experiment, but 4 TeLC mice were excluded from analysis due to low virus expression found afterwards.

#### 3.2.2 Viruses

Adeno-associated viruses (AAVs) were generated in-house with a chimeric 1/2 serotype as described in McClure et al. (2011), and titered by qPCR following expression in HEK cells. The AAV1/2-FLEX-GFP virus (referred to as GFP virus) had a titer of  $5.1 \times 10^{12}$  cp/ml, and the AAV1/2-FLEX-TeLC-GFP virus (referred to as TeLC virus) had a titer of  $6.4 \times 10^{12}$  cp/ml. These viruses have been used previously in Sürmeli et al. (2015) and Tennant et al. (2018), and were shown to be selectively expressed in stellate cells within layer 2 of the MEC, and the TeLC (tetanus light chain toxin) virus was shown to suppress neurotransmitter release when expressed in these cells.

### **3.2.3 Surgery**

Surgical procedures were performed using aseptic technique. Mice were anaesthetised in an induction chamber using Isoflurane with oxygen, then transferred to a stereotaxic frame, and a thermostatic heat blanket. Analgesic (Rimadyl) was administered subcutaneously, and the area around the incision site was disinfected using Hibiscrub. An incision was made in the skin to expose the skull. The skull was levelled, and a craniotomy was drilled at 3.5 mm lateral to Bregma on the left and right, just posterior to the lambdoid suture and anterior to the transverse sinus. A glass micropipette was used to make four injections in each craniotomy, along the dorsoventral axis of the MEC. The pipette was first set at an angle of  $-16^\circ$  in the anteroposterior axis, and lowered into the craniotomy. The approximate depth of layer 2 of the MEC was determined by measuring the depth at the point where the pipette bends, signalling that it has hit the dura lining the posterior surface of the brain. It was then retracted 0.2 mm to be within layer 2 (Figure 3.1A). An injection of around 500 nL of virus was made and the pipette left in place for around 3 minutes, before being retracted. The process was repeated for angles of  $-13^\circ$ ,  $-10^\circ$  and  $-7^\circ$  to cover the MEC from its most dorsal to most ventral extent. This was then repeated for the other craniotomy. The wound was closed using absorbable sutures and Vetbond (VetTech) adhesive, and the mouse was given saline/glucose solution subcutaneously for rehydration, removed from the stereotaxic frame and placed in its cage, on a heat pad, and recovery was monitored.

### **3.2.4 Behavioural apparatus**

The box used for the object association memory tasks was a plastic crate with removable MDF walls (length 35 cm, width 30 cm, height 31 cm). The removable walls were painted with black and white stripes on one side, and the other side was covered with blue self-adhesive plastic film. The floor of the box was grey vinyl, and fitted with a removable wire mesh. The two contexts were: (A) striped walls with vinyl floor and (B) blue walls with wire mesh floor (shown in Figure 3.1C and E). Distal cues were used to provide consistent polarising cues across the two contexts: a multi-coloured pinwheel was placed above the back wall, and a yellow object hung just inside the wall to the left.

#### 3.2.5 Handling and habituation

For one week before surgery, mice were handled daily for five minutes each. Mice were introduced to being handled gradually, and the initial handling was done by picking them up when they were inside a cardboard tube, which has been shown to minimise anxiety (Gouveia & Hurst, 2013). For one week following surgery, mice were handled once in the morning and once in the afternoon for five minutes each.

After one week of post-surgery handling, habituation to the behavioural apparatus began. On the first day, mice were placed in the testing box configured as either Context A or Context B, and allowed to freely explore for 10 minutes, along with their cagemates. They were then removed and placed in a bucket lined with bedding, for 3 minutes, after which they were placed in the testing box configured as the other context for another 10 minutes, again with their cagemates. The order in which they experienced the two contexts each day was varied pseudorandomly. On the second day of habituation, each mouse was placed individually in each context for 10 minutes. This was repeated for the third day. On the fourth day, each mouse was again placed in each context alone, but with two objects that would not be used in the experiments, to habituate the mice to objects in the positions that were used in the experiment. This was repeated on the fifth and final day of habituation, before experiments began.

#### 3.2.6 Object association recognition tasks

Experiments began once mice had been habituated to the apparatus, also allowing two weeks for the viruses to express sufficiently. Testing was carried out in the morning between 8:00 and 12:00 and in the afternoon between 14:00 and 18:00.

Mice underwent testing on spontaneous object exploration protocols as shown in Figure 3.1. Each mouse performed no more than one task each day. For each phase of each trial, mice were placed into the box facing the front wall, away from the objects. They were then allowed to freely explore the arena and objects in the sample phase configuration, for three minutes. They were then removed and placed into the bucket lined with bedding, while the experimenter wiped the arena with 70% alcohol and reconfigured the context/objects as needed (this took approximately 2 minutes). The mice were then placed back into the arena in the same

way, and again allowed to explore the arena for three minutes. This was done for the one or two sample phases, depending on the specific task, and the test phase.

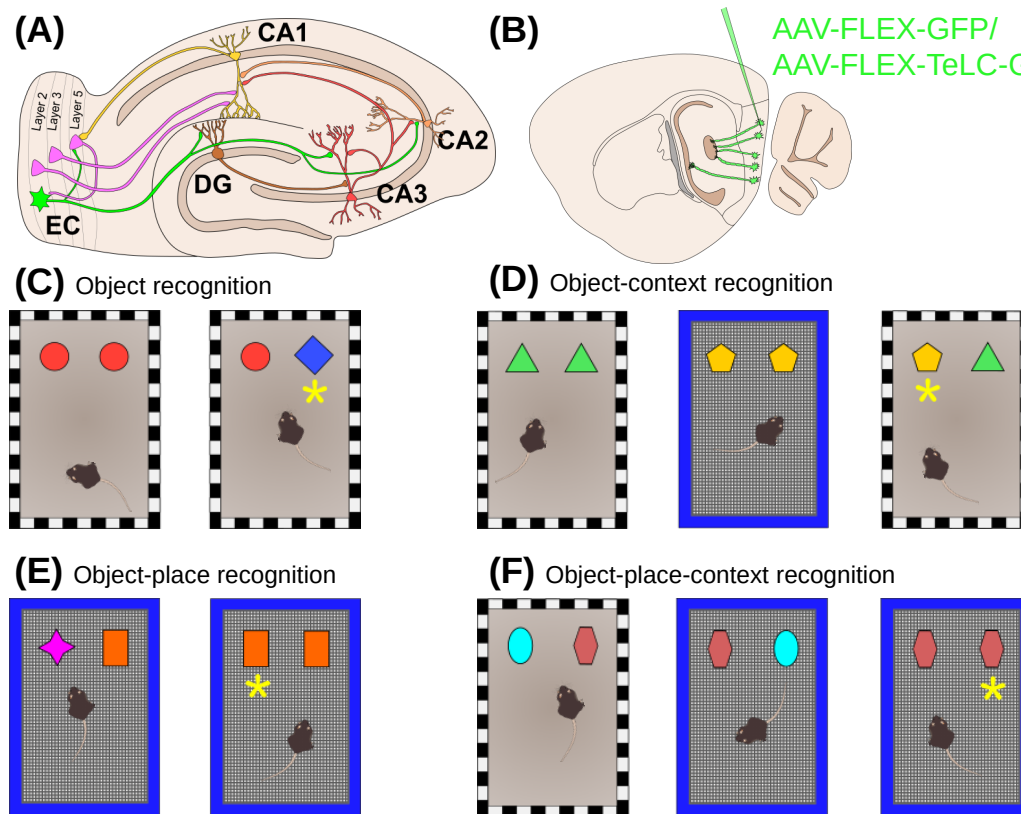


FIGURE 3.1: Virus injection and object association task protocols. **(A)** Diagram of entorhinal-hippocampal circuits. **(B)** Schematic of a sagittal section showing injection of the GFP and TeLC-GFP viruses into the MEC (bounded region). The injection was aimed at layer 2 of the MEC, with selective expression in the stellate cells, the axons of which project to dentate gyrus and CA3. **(C)** Object recognition protocol: a sample phase with a pair of identical objects, followed by a test phase with the familiar object plus a novel object (indicated by the yellow asterisk). **(D)** Object-context protocol: two sample phases in which the mice are presented with two pairs of identical objects, followed by a test phase where the object indicated with the asterisk has not been experienced in the test context before. **(E)** Object-place recognition: the sample phase features two different objects, then in the test phase the asterisk indicates an object not experienced in that position before. **(F)** Object-place-context recognition: a sample phase in each context in which the mice experience two pairs of different objects, followed by a test phase with a pair of identical objects. The object above the asterisk has not been experienced in that position within the test context previously.

The experiment was counterbalanced for which object, context and position were experienced as novel. Each mouse was tested on each of the tasks twice, experiencing the novel object/association on each side of the box for each trial, and for

OC and OPC, both contexts were experienced as the test phase context across the two trials.

Video footage of all the sessions was recorded using a camera placed above the arena. Once all experiments had been completed, an experimenter blind to mouse group, as well as which object was novel, scored the amount of time mice spent exploring each object in all sample and test phases, using a custom programme (Z-score – Oliver Hardt).

#### 3.2.7 Data analysis

Results were then analysed using a custom programme (Z-chop – Oliver Hardt). A discrimination index was calculated using the formula:

$$\frac{(\text{time spent exploring novel object (s)} - \text{time spent exploring familiar object (s)})}{(\text{time spent exploring novel object (s)} + \text{time spent exploring familiar object (s)})}$$

This was calculated for the whole test phase overall and cumulatively across time. Trials in which mice explored either sample phase object for less than 5 seconds, and/or explored test objects for a combined total of less than 10 seconds, were removed from analysis.

Discrimination indices were also calculated for the first 10 seconds of the trial, the first 20 seconds, the first 30 seconds, up to the full 180 seconds of the trial, in order to study how mice explored the objects over time. Discrimination indices were also calculated for the first 10, 20, 30, and 40 seconds of total object exploration, to remove the influence of different total exploration times from the analysis.

For each mouse, discrimination indices were averaged across the two trials of each task. If one trial was excluded due to low exploration, the other trial was used alone as the discrimination index for that mouse. No mice were removed from analysis due to low exploration. Discrimination indices of mice in each group were compared to a hypothetical value of 0, representative of chance-level exploration, using either a one-sample t-test, or a Wilcoxon signed rank test, depending on outcomes of normality tests. Differences between groups in exploration times or discrimination indices were compared using a two-sample t-test or Mann-Whitney U test, depending on normality, or a repeated measures ANOVA where multiple time points were being considered.

### **3.2.8 Histology**

Once the mice had performed all of the tasks, at no more than six weeks after surgery, mice were anaesthetised using Isoflurane and a lethal dose of sodium pentobarbital (Euthatal) was administered via intraperitoneal injection. Transcardial perfusion with phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA) was carried out to fix the tissues. Brains were excised and kept in 4% PFA overnight at 4 °C, then transferred to 30% sucrose PBS.

A cryostat was used to take 40 µm sagittal sections, and these were transferred to wells containing PBS. Every fourth section through the MEC was removed and used for staining with far-red NeuroTrace fluorescent Nissl stain (ThermoFisher) to visualise neurons. Stained sections were mounted on slides in Fluoromount-G mounting medium (ThermoFisher) and imaged using an array tomography microscope and AxioVision software (Zeiss).

Images were analysed in Fiji (Fiji Is Just Image-J). The free-hand tool was used to outline the extent of the MEC; the straight-line tool was used to connect the most dorsal point to the most ventral point, which was then bisected perpendicularly to split the mEC into dorsal and ventral sections. In each section, layer 2 was outlined and area, mean intensity, standard deviation, and maximum and minimum intensity measures were taken within these regions. Expression in other areas outside of MEC layer 2 was also assessed.

## **3.3 Results**

### **3.3.1 Histology**

Expression of the TeLC virus was assessed by calculation of the percentage of the extent of MEC where GFP fluorescence could be observed. This was averaged across sagittal sections through the MEC, and across both hemispheres of the brain. Four mice in which no GFP signal could be detected were excluded from all analyses. Figure 3.2A shows sagittal sections from an example brain which showed a clear pattern of fluorescence in layer 2 of the MEC. Figure 3.2B shows

### 3.3. Results

average percentage of the MEC layer 2 where GFP signal could be observed, from the left and right hemispheres of all mice included in analysis.

Expression of the TeLC virus observed was variable and generally low; the mouse with the highest average expression had GFP signal detected in 52% of layer 2 of the MEC in the right hemisphere, and just 19% in the left hemisphere (Mouse T1, Figure 3.2B). Three mice had average expression across the two hemispheres of below 10% (T5, T6, T9). In order to better assess the role of MEC L2SCs in associative memory, higher expression of TeLC virus, or possibly another more complete method of inactivation, may be needed. For the following analyses, all TeLC mice with detectable virus expression in at least one hemisphere (all those displayed in Figure 3.2B,  $n = 9$ ) have been included as a single group, with some measures being correlated with percentage expression in the final section.

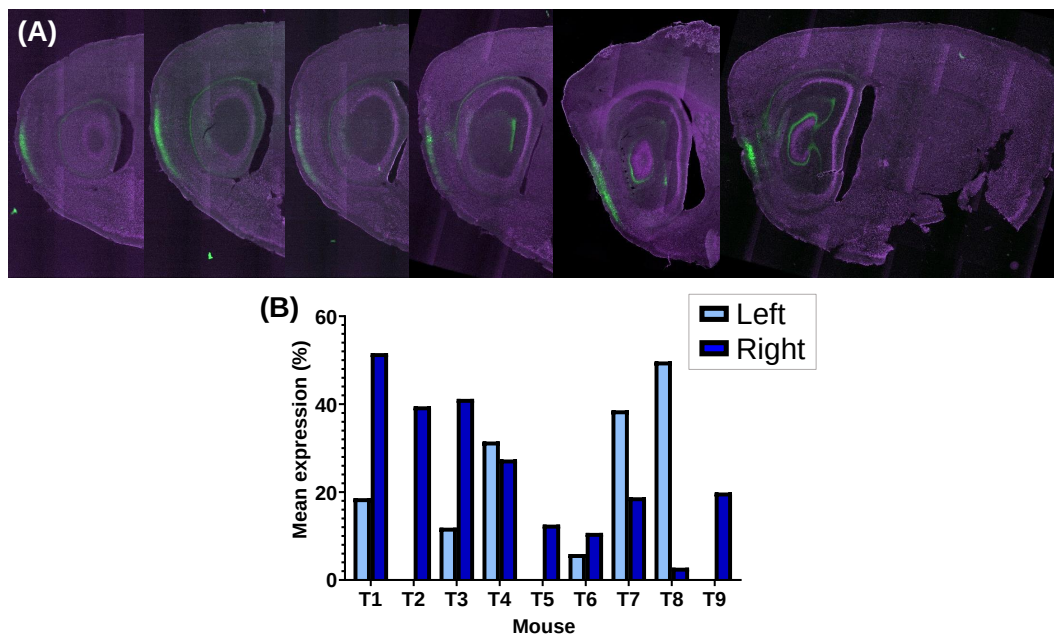


FIGURE 3.2: Expression of TeLC-GFP virus. **(A)** Example of a series of sagittal sections through the MEC, from lateral (left) to medial (right), showing neuron cell bodies in purple, with clear GFP expression in layer 2 of the MEC (green). Expression can also be seen in the dentate gyrus, where the stellate cell axons project. **(B)** Percentage of the dorso-ventral length of MEC layer 2 in which GFP expression could be detected, averaged over multiple sections, for each brain hemisphere of each TeLC mouse used for analyses ( $n = 9$ ).

### 3.3.2 Inactivation of MEC layer 2 stellate cells has no effect on object recognition memory

Mice were tested on ability to discriminate a novel from a familiar object using a spontaneous object recognition task, as shown in Figure 3.3A. Figure 3.3B shows total time spent exploring the objects in the sample and test phases, averaged across the two trials. All mice explored the objects for a total of at least 20 seconds in each phase (Sample: GFP,  $n = 12$ , mean = 39.0 s, 95% CI [34.2, 43.8], TeLC,  $n = 9$ , mean = 41.3 s, 95% CI [33.7, 50.0]; Test: GFP mean = 39.7 s, 95% CI [34.5, 44.9], TeLC mean = 44.4 s, 95% CI [37.7, 51.2]), and there were no differences between the groups in exploration time (2-way ANOVA:  $F(1,19) = 1.53$ ,  $p = 0.232$ ).

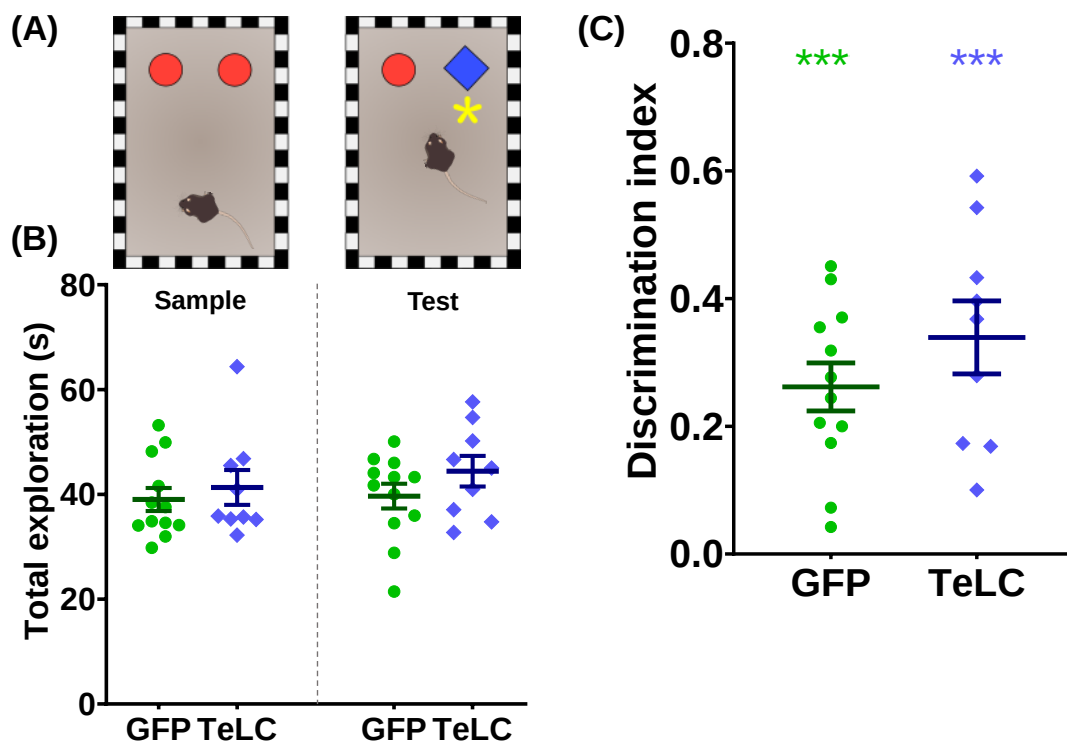


FIGURE 3.3: Both groups performed above chance level on object-recognition (OR). (A) Schematic of OR task. (B) Total time spent exploring the two objects in the sample and test phases. Symbols indicate times for each mouse, bars show group means with standard error. (C) Discrimination index of each mouse, with mean  $\pm$  SEM. (GFP, green,  $n = 12$ ; TeLC, blue,  $n = 9$ ) \*\*\*  $p < 0.001$

For the test phase, a discrimination index was calculated based on time spent exploring the novel and familiar objects (Figure 3.3C). A discrimination index of 0 indicates no preference for either object, with equal time spent exploring each

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object; a positive discrimination index indicates that the mouse spent more time exploring the novel object than the familiar, suggesting that it has a memory of the familiar object from the previous phase. In the object recognition task, all mice from both groups had a positive mean discrimination index (GFP: mean = 0.26, 95% CI [0.18, 0.34]; TeLC: mean = 0.34, 95% CI [0.21, 0.47]). A one-sample t-test was used to test each group against a null hypothesis value of 0 – this was significant for both groups (GFP:  $t(11) = 6.95$ ,  $df = 11$ ,  $p < 0.0001$ ; TeLC:  $t(8) = 5.93$ ,  $p = 0.0003$ ), suggesting a preference for exploring the novel object in the GFP and TeLC mice. Furthermore, a two-sample t-test suggested there were no differences between the two groups ( $t(19) = 1.18$ ,  $p = 0.253$ ). This evidence suggests that mice with inactivated MEC L2SCs can successfully discriminate between a familiar and a novel object.

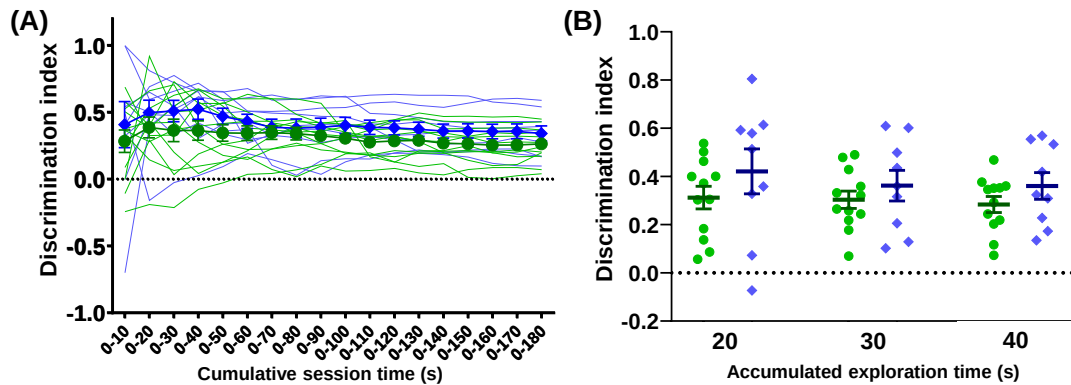


FIGURE 3.4: Cumulative discrimination indices for object recognition task. **(A)** Discrimination indices across test time, in incremental cumulative bins, from the first 10 seconds to the full 180-second session. Thin lines represent each mouse, and bold lines indicate group means  $\pm$  SEM. **(B)** Discrimination indices for the first 20, 30 and 40 seconds of total object exploration. Individual mice, with group means  $\pm$  SEM. (GFP, green,  $n = 12$ ; TeLC, blue,  $n = 9$ )

Figure 3.4A shows changes in the discrimination index across the three-minute test session, in cumulative bins, increasing in steps of 10 seconds – the discrimination index was calculated for exploration that occurred in the first 10 seconds, then the first 20 seconds, then the first 30 seconds, up to the full 180 seconds. All apart from one mouse have a discrimination index of greater than 0 from around 30 seconds, suggesting they are preferentially exploring the novel object over the familiar from early on in the trial. In order to reduce the effect of variability from different amounts of total exploration between mice, the discrimination indices were calculated only from the first 10, 20 and 30 seconds of object exploration (Figure 3.4B). Again, there was no significant effect of group when comparing these values ( $F(1,19) = 1.26$ ,  $p = 0.275$ ).

### 3.3.3 Inactivation of MEC layer 2 stellate cells may have an effect on object-context association memory

Mice were then tested on a version of the task designed to assess memory for object-context associations (Figure 3.5A). All the mice explored the objects for more than 20 seconds in all of the phases and again, there were no differences between the two groups in amount of object exploration in the two sample phases and test phase (Sample 1: GFP mean = 42.3 s, 95% CI [37.4, 47.2], TeLC mean = 45.4 s, 95% CI [34.3, 56.5]; Sample 2: GFP mean = 45.7 s, 95% CI [38.2, 53.1], TeLC mean = 48.00 s, 95% CI [39.9, 56.1]; Test: GFP mean = 46.9 s, 95% CI [37.9, 55.9], TeLC mean = 53.7 s, 95% CI [43.6, 63.8], ANOVA:  $F(1,19) = 0.77$ ,  $p = 0.390$ ) (Figure 3.5B).

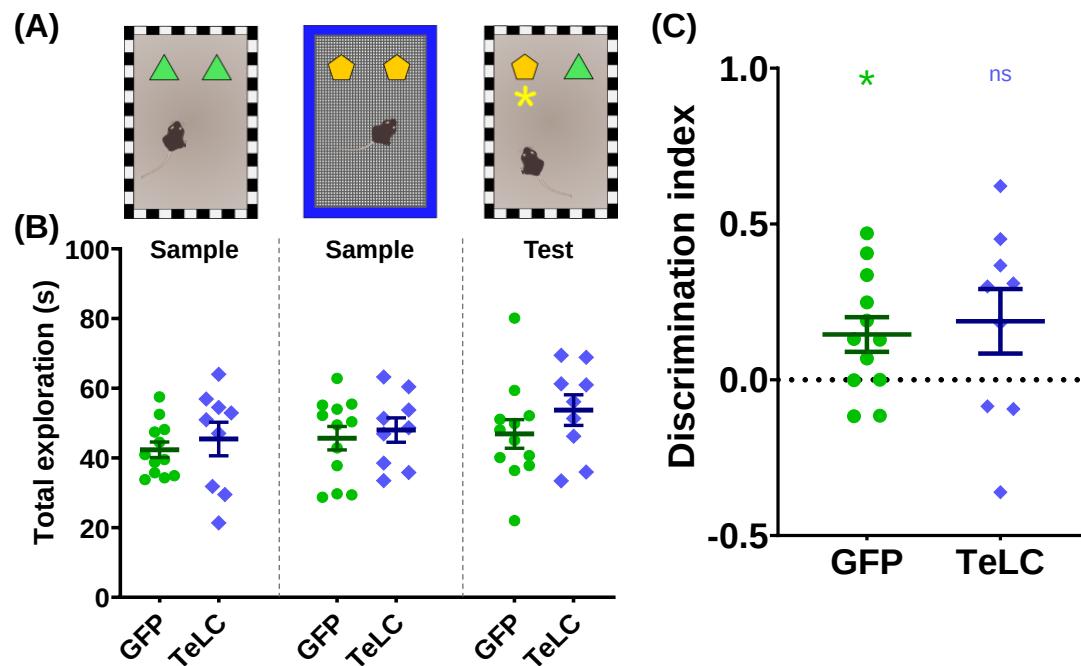


FIGURE 3.5: GFP mice performed object-context above chance level, while TeLC mice do not. **(A)** Schematic of the object-context task. **(B)** Total time spent exploring objects in the two sample phases and the test phase. Individual mouse averages, with group means  $\pm$  SEM. **(C)** Discrimination index of each mouse, and group means  $\pm$  SEM. (GFP, green,  $n = 12$ ; TeLC, blue,  $n = 9$ ) \*  $p < 0.05$ , ns  $p > 0.05$

The mice did not discriminate the novel object-context association as reliably as the novel object, with a few mice in each group showing negative discrimination indices (Figure 3.5C). The GFP group did, however, show a significant preference for the novel object-context pairing over the familiar ( $t(11) = 2.61$ ,  $p = 0.024$ ). While

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the TeLC group had a higher mean discrimination index than the GFP group (GFP: mean = 0.15, 95% CI [0.02, 0.27], TeLC: mean = 0.19, 95% CI [-0.05, 0.43]), they were not significantly above chance ( $t(8) = 1.81$ ,  $p = 0.107$ ). This is likely to be due to the higher variability in the TeLC group compared to the GFP group, and the fact that there were fewer subjects – more data would be needed to determine whether this is an effect of MEC L2SC inactivation. Furthermore, there was no significant difference between the two groups ( $t(19) = 0.387$ ,  $p = 0.703$ ).

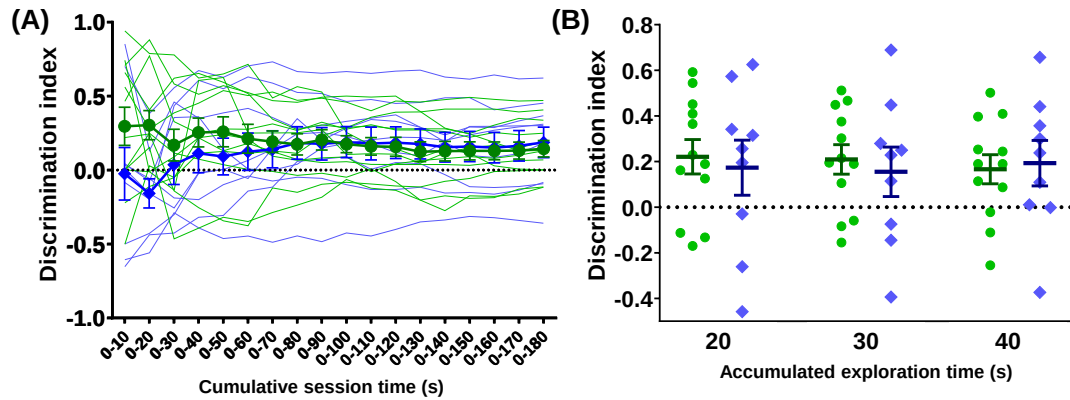


FIGURE 3.6: Cumulative discrimination indices for the object-context task. **(A)** Discrimination indices across test time. Lines shown for each mouse, and bold lines indicate group means  $\pm$  SEM. **(B)** Discrimination indices for the first 20, 30 and 40 seconds of total object exploration. Individual mice, with group means  $\pm$  SEM. (GFP, green,  $n = 12$ ; TeLC, blue,  $n = 9$ )

Looking at discrimination over time (Figure 3.6A), there were some mice which appeared to show a strong preference for the novel pairing at the beginning of the trial, then exploring the familiar to move closer to 0, while others showed a preference for the familiar pairing throughout the trial. There were no clear differences in how the two groups explored over the trial, and there is a large amount of variability throughout. This is reflected in the discrimination indices standardised by object exploration time in Figure 3.6B, with similar levels of variability in all of the measures. Similar to the results for the full trial time, the TeLC group did not differ significantly from chance at any time point (20 s:  $t(8) = 1.4$ ,  $p = 0.189$ ; 30 s:  $t(8) = 1.4$ ,  $p = 0.190$ ; 40 s:  $t(8) = 1.9$ ,  $p = 0.089$ ) whereas the GFP group showed positive discrimination at all times (20 s:  $t(11) = 2.9$ ,  $p = 0.014$ ; 30 s:  $t(11) = 3.2$ ,  $p = 0.008$ ; 40 s:  $t(11) = 2.6$ ,  $p = 0.024$ ). There were no differences between the two groups at any time point ( $F(1,19) = 0.030$ ,  $p = 0.864$ ).

### 3.3.4 Inactivation of MEC layer 2 stellate cells has no effect on object-place association memory

The object-place task assessed memory for associations of objects with their positions, as one copy of the object appeared in the same place as it occurred in the sample phase (familiar pairing), whereas the other copy of the object appeared in the location previously occupied by a different object (novel pairing) (Figure 3.7A). Again, all mice explored the objects for at least 20s in both the sample and test phases of the trial. Average total exploration ranged from around 20 to 80 seconds in the two phases (Sample: GFP mean = 46.1 s, 95% CI [38.0, 54.1], TeLC mean = 48.7 s, 95% CI [41.0, 56.5]; Test: GFP mean = 41.5 s, 95% CI [30.4, 52.5], TeLC mean = 42.8 s, 95% CI [27.7, 57.8]), and no differences were seen in total object exploration ( $F(1,19) = 0.098$ ,  $p = 0.758$ ) (Figure 3.7B).

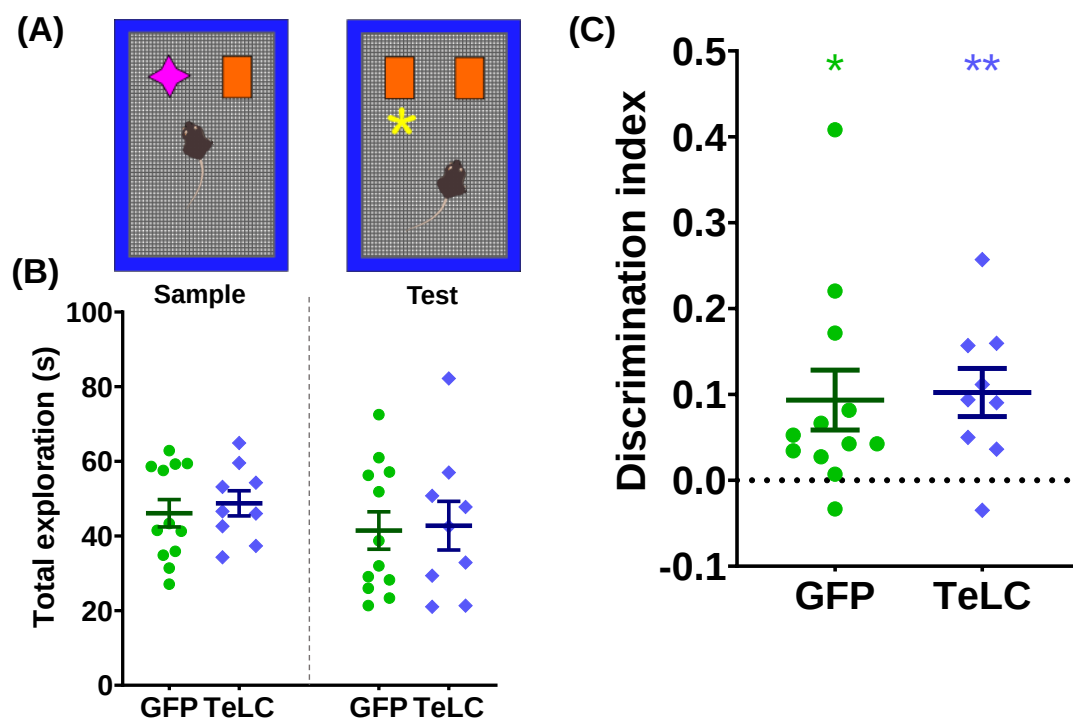


FIGURE 3.7: Both groups perform object-place (OP) discrimination above chance level. **(A)** Schematic of the OP task. **(B)** Total time spent exploring objects in the sample phase and test phase. Individual mouse averages, with group means  $\pm$  SEM. **(C)** Discrimination index of each mouse, and group means  $\pm$  SEM. (GFP, green,  $n = 12$ ; TeLC, blue,  $n = 9$ ) \*  $p < 0.05$ , \*\*  $p < 0.01$

The mice in both groups seemed to show a preference for the object in the novel position compared to the object in the familiar position, with only one mouse in

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each group showing a discrimination index of below 0. The means of both groups (GFP: mean = 0.09, 95% CI [0.02, 0.17]; TeLC: mean = 0.10, 95% CI [0.04, 0.17]) were significantly above chance (GFP:  $t(11) = 2.68$ ,  $p = 0.021$ ; TeLC:  $t(8) = 3.65$ ,  $p = 0.0065$ ). Discrimination indices did not differ between the GFP and TeLC mice ( $t(19) = 0.188$ ,  $p = 0.853$ ) (Figure 3.7C). These data suggest that inactivation of MEC layer 2 stellate cells has no effect on association of an object and its position within an environment.

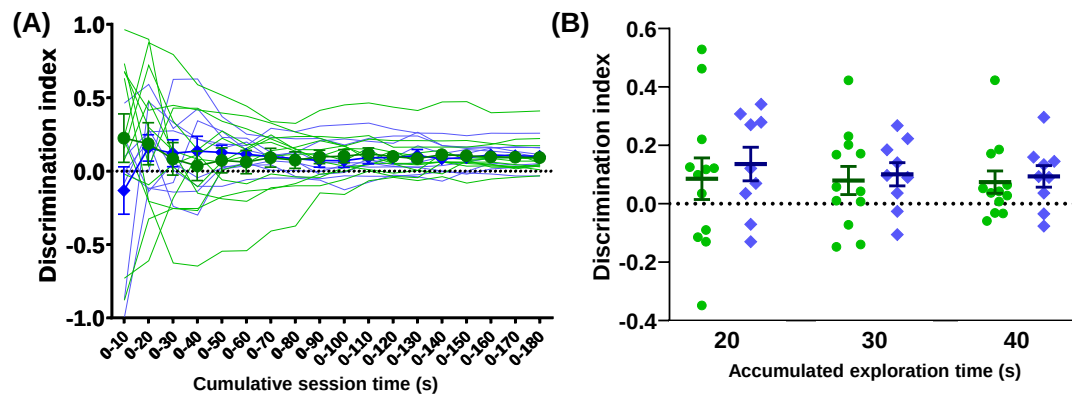


FIGURE 3.8: Cumulative discrimination indices for object-place task. **(A)** Discrimination indices across test time. Lines shown for each mouse, and bold lines indicate group means  $\pm$  SEM. **(B)** Discrimination indices for the first 20, 30 and 40 seconds of total object exploration. Individual mice, with group means  $\pm$  SEM. (GFP, green,  $n = 12$ ; TeLC, blue,  $n = 9$ )

Examination of discrimination index over cumulative time in the phase shows similar variability in each group at the beginning of the trial, which converges to just above chance level for both groups (Figure 3.8A). There was no clear difference in discrimination when standardised by amount of exploration time, and no difference was seen between the two groups in the three amounts of accumulated time ( $F(1,19) = 0.135$ ,  $p = 0.718$ ) (Figure 3.8B).

#### 3.3.5 Mice did not show memory for object-place-context associations

Finally, Figure 3.9A shows the object-place-context task, designed to assess associative memory for an object, its location, and the context. Memory for this association will be inferred if the mice show a preference for exploring an object that has not been seen in its position within the context, over one that has previously been

seen in its position in this context. Mice showed similar levels of object exploration to the previous tasks, with all exploring for more than 20 seconds on average, in all phases (Figure 3.9B) (Sample 1: GFP mean = 46.7 s, 95% CI [39.3, 54.1], TeLC = 50.7 s, 95% CI [39.9, 61.6], Sample 2: GFP mean = 46.1 s, 95% CI [41.7, 50.5], TeLC mean = 43.2 s, 95% CI [36.2, 50.3]; Test: GFP mean = 48.6 s, 95% CI [37.9, 59.4], TeLC mean = 45.1 s, 95% CI [35.0, 55.2]). Consistent with previous tasks, there were no difference in exploration between the groups ( $F(1,19) = 0.036$ ,  $p = 0.851$ ).

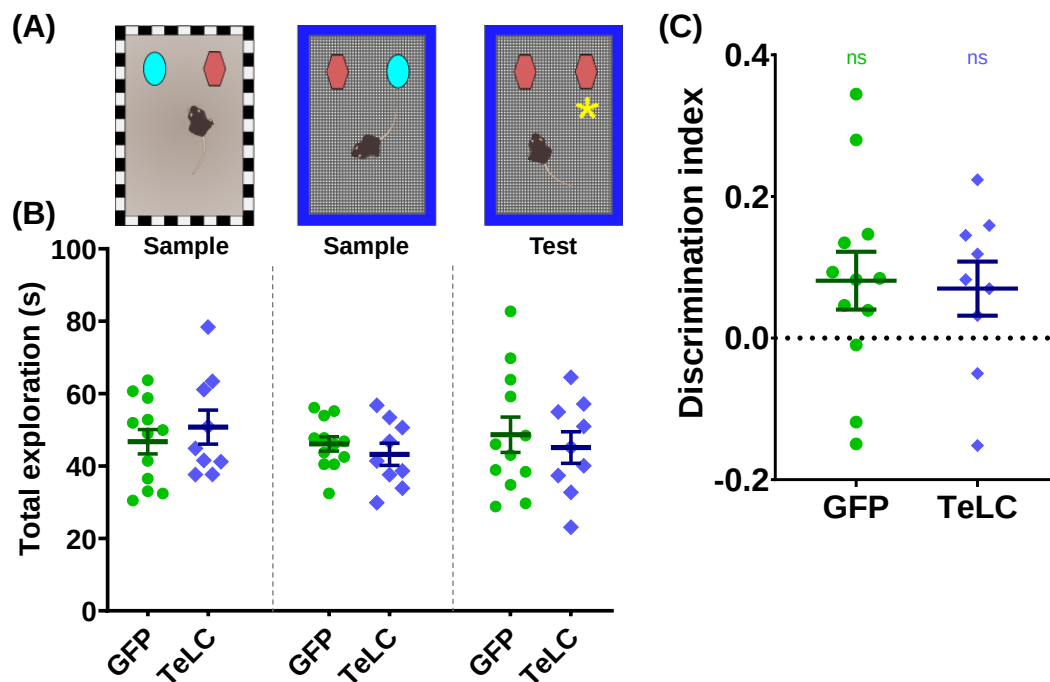


FIGURE 3.9: Neither group performed object-place-context (OPC) discrimination above chance level. **(A)** Schematic of the OPC task. **(B)** Total time spent exploring objects in the two sample phases and test phase. Individual mouse averages, with group means  $\pm$  SEM. **(C)** Discrimination index of each mouse, and group means  $\pm$  SEM. (GFP, green,  $n = 12$ ; TeLC, blue,  $n = 9$ ) ns  $p > 0.05$

Discrimination in the test phase was variable in this task, with three mice in the GFP group and two mice in the TeLC group showing preference for the familiar object-place-context association over the novel (Figure 3.9C). Although the group means are both positive (GFP: mean = 0.0811, 95% CI [-0.009, 0.171], TeLC: mean = 0.07, 95% CI [-0.02, 0.16]), neither were significantly different from chance (GFP:  $t(11) = 1.98$ ,  $p = 0.073$ ; TeLC:  $t(8) = 1.83$ ,  $p = 0.104$ ). Since neither group showed significant levels of discrimination, assessment of whether inactivation of MEC L2SCs affects this type of associative memory is not possible. The lack of significant difference between the groups ( $t(19) = 0.195$ ,  $p = 0.848$ ) gives some

indication that it may not be affected. However, more subjects would be needed to make a reliable conclusion.

Unfortunately, many of the video files for this task were corrupted, so they could not be reanalysed in order to break down exploration across time.

#### 3.3.6 Lack of effect could be due to low expression of TeLC virus

As TeLC mice did not show significant discrimination above chance in the object-context task, the discrimination indices from this task were plotted against mean expression (Figure 3.10) to determine whether any correlation could be seen. A Pearson's correlation coefficient value of  $r(9) = -0.316$  (95% CI [-0.810, 0.441]) suggested there may be a relationship, with object-context discrimination ability being reduced with greater suppression of MEC L2SC function. However, this correlation was weak and not statistically significant ( $p = 0.408$ ). As mentioned above, generally low expression may have prevented making any associations between L2SCs and function in associative memory.

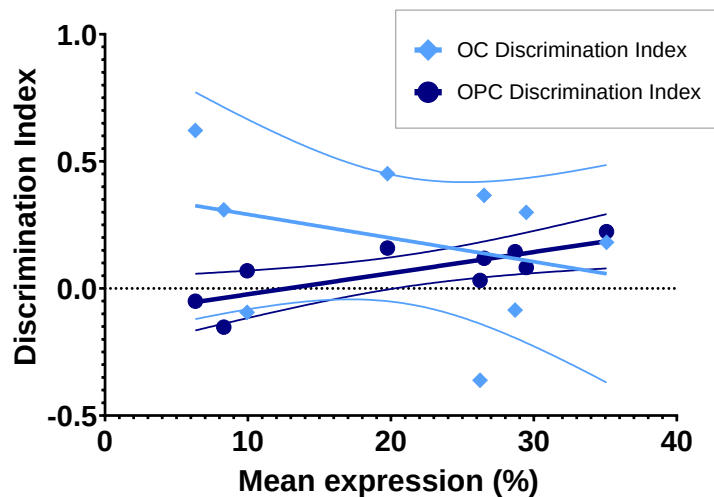


FIGURE 3.10: There was no significant correlation between expression percentage and OC or OPC discrimination. Discrimination index in the object-context task against layer 2 TeLC expression percentage (averaged across two hemispheres), fitted with linear regression line with 95% confidence interval lines (light blue). Discrimination index in the OPC task against expression percentage, fitted with linear regression line and 95% confidence interval lines (dark blue).

A correlation of OPC discrimination index against percentage of TeLC-GFP expression did not show any evidence that higher expression worsened performance. In fact, the mice showed a significant positive correlation, with greater virus expression indicating higher preferences for the novel OPC pairing ( $r(9) = 0.766$ , 95% CI [0.207, 0.948],  $p = 0.016$ ). Considering that the GFP mice did not perform this task above chance level, no conclusions about L2SC function can be made from the results of this task.

## 3.4 Discussion

The results from the current experiment are largely inconclusive. This is probably due to a combination of low virus expression and low subject numbers resulting in low statistical power. There did not seem to be any clear observable deficit caused by the inactivation of L2SCs in the MEC in any of the tasks used here. As predicted, TeLC mice were able to perform object recognition similarly to control GFP mice. Object-place memory appeared to be intact, suggesting L2SCs may not be necessary for this type of object-place association. The results for object-context and object-place-context were difficult to interpret. The TeLC mice did not perform OC discrimination above chance level, which could suggest a deficit; however, there was no difference between the two groups and it is likely that more subjects would be needed to determine whether there is any real effect of the manipulation. The OPC task was inconclusive, as neither group of mice performed the task above chance level.

### 3.4.1 L2SCs are not necessary for object recognition

Inactivation of L2SCs had no effect on memory for object identity, as the TeLC group preferentially explored the novel object over the familiar, to a similar level to the GFP group. This result was predicted, as it has been shown previously using the same manipulation (Tennant et al., 2018), and with MEC lesions (Van Cauter et al., 2013). Object identity is very likely to be processed upstream of the entorhinal cortex; the role of the perirhinal cortex in object recognition has been well-documented, with lesions producing deficits in the task (Mumby & Pinel, 1994; Ennaceur et al., 1996; Brown & Aggleton, 2001; Winters et al., 2004).

The perirhinal cortex is more strongly connected with the LEC compared to the MEC, and cells in the LEC show more firing associated with particular objects than cells in the MEC (Deshmukh & Knierim, 2011; Tsao et al., 2013; Knierim et al., 2013). Although object-vector cells have been found in the MEC, these appear to encode position relative to an object landmark, rather than firing when close to a particular object, as is seen in the LEC (Høydal et al., 2019). However, it has been shown that OR is not dependent on an intact LEC (Wilson, Watanabe, et al., 2013), and layer 2 fan cells in the LEC are not needed (Vandrey et al., 2020).

#### 3.4.2 L2SCs are not required for object-place memory

The TeLC group were able to discriminate the novel object-place pairings to a similar level as the GFP group, suggesting MEC L2SCs are not required for this type of association. This is interesting to compare to Tennant et al. (2018), where a task testing object-location association was used, and a deficit was found in MEC L2SC-inactivated mice. In this task, two identical objects are placed in a box for the sample phase, and one is displaced to a novel position within the box for the test phase, so no association must be formed between a specific object and its position, as is the case for the OP task in the current experiment. Differently to the OP task used in the current experiment, this is dependent on the hippocampus (Langston & Wood, 2010; Barker & Warburton, 2011). The lack of deficit in the current OP task may be due to the fact that the objects can only be in two positions within a square box, either the left or the right, and hence mice may not require global spatial information about the environment to determine position. While distal cues were available to the mice, they may not have been needed, as object position could be determined from an egocentric strategy. In the displaced object version, more spatial information may have been required to determine spatial information within the square box, and mice may have relied more on distal visual landmarks. This process, as outlined in the previous chapter, may be more dependent on MEC.

MEC lesion studies have also shown this. Rats with MEC lesions did not increase exploration of objects that had been displaced compared to stationary objects, as control rats did (Van Cauter et al., 2013). Another study showed a deficit in discrimination when one of four different objects was displaced; but when complexity was reduced, by using four identical objects and displacing one, MEC-lesioned rats showed similar preferences towards the displaced object as control rats (Rodo

et al., 2017). In the current study, the spatial discrimination was not very complex, as there were only two possible object positions relative to a fixed starting point, perhaps providing another reason why MEC L2SCs were not required.

It is important to note, however, that the lack of effect could have been down to the low levels of virus expression.

### **3.4.3 L2SCs are unlikely to be necessary for object-context memory**

While TeLC mice did not perform the OC task above chance level, unlike the GFP group, there is not enough evidence here to conclude that this process is dependent on MEC L2SCs. Firstly, there was no statistical difference between the discrimination indices of the two groups, the mean even being slightly higher in the TeLC group. High levels of variability are inherent to spontaneous tasks, and high subject numbers are generally needed to produce reliable results. Due to exclusion of mice with very low virus expression, there were fewer mice in the TeLC group, and this likely contributes to the lack of statistical significance. The data were skewed by one mouse that preferentially explored the familiar object pairing over the novel. This may also have been due to a preference for a particular type of object.

Looking at the group distributions, it does not appear likely that there is any difference between the two groups. Furthermore, there was no correlation between the amount of virus expression in MEC layer 2 and OC discrimination index (Figure 3.9C). From the current data, it appears unlikely that MEC L2SCs are required for OC memory. There is some evidence to suggest that MEC L2SCs may be required for contextual processing, particularly remapping of the hippocampal spatial representation. Kitamura et al. (2015) showed context-specific firing in L2SCs, and that contextual fear conditioning was affected by their deactivation; Diehl et al. (2017) also showed large changes in spatial firing in populations of MEC cells in response to context changes, along with rate changes in grid cells. Artificial depolarisation of L2SCs produced changes in CA1 spatial firing similar to contextual remapping (Kanter et al., 2017), and a number of other studies have found changes in locations of hippocampal place fields in response to manipulation of MEC cells (Miao et al., 2015; Rueckemann et al., 2016; Zhao et al., 2016; Fernández-Ruiz et al.,

2021). However, contextual information has been shown to be processed in a number of other para-hippocampal areas – Kinnavane et al. (2017) showed increased c-fos expression in areas such as LEC, perirhinal cortex and postrhinal cortex, as well as prefrontal cortex and retrosplenial cortex. Increases in c-fos have also been shown in the LEC after performing the OC task (Wilson, Langston, et al., 2013). Therefore, contextual processing for this task, requiring binding an object within a context distinct in multiple sensory dimensions, may be sufficiently processed in the LEC, not requiring the MEC pathway.

The contexts used in the current experiment, similar to Vandrey et al. (2020), were not only distinguishable visually, but also using tactile information, as a vinyl flooring was used in one context, and a wire mesh insert used in the other. These would undoubtedly have distinct odours as well, although everything was cleaned with ethanol between phases. This raises the question of whether a clear deficit would be observed if the only difference between the contexts was in visual characteristics. As described in the previous chapter, the role of the MEC in processing distal visual landmarks, but not proximal objects that can be interacted with may suggest a distinction in sensory information processing in MEC and LEC. The LEC has strong connections with the olfactory bulb and has been shown to be involved in odour learning and memory (Chapuis et al., 2013; Leitner et al., 2016). Furthermore, pharmacological blockade of NMDA receptors in the LEC disrupted olfactory-tactile associative learning (Boisselier et al., 2014), suggesting the role of the LEC may be in integrating multi-modal sensory inputs. Hence, association of objects with the different tactile characteristics of the two contexts in this experiment may have been processed in the LEC, not requiring MEC inputs to the hippocampus. Interestingly, changes in firing in MEC grid cells has been observed following manipulations of environmental odour, suggesting this information is integrated into the MEC spatial representation (Marozzi et al., 2015). It is possible that this information could come from connections between the LEC and MEC (Dolorfo & Amaral, 1998; Burwell, 2000).

#### **3.4.4 Control mice did not show evidence of object-place-context memory**

Neither group showed statistically significant discrimination above chance level in the OPC task, making it difficult to draw any conclusions from the data. There was also no statistical difference between the two groups, suggesting there was no clear difference in performance of the task caused by inactivation of MEC L2SCs. While neither group was significantly different from chance, there is a trend towards positive discrimination indices in both groups, with most mice showing preference for the novel OPC pairing. This is an indication that greater numbers of subjects may be needed to produce a statistically significant result, rather than the mice not detecting the difference at all. The fact that greater preferences for the novel pairing were seen among mice with higher levels of virus expression suggest there may be no effect of the L2SC inactivation. But due to the low discrimination values, it cannot be determined whether mice were showing any memory.

Previously OPC has been used successfully in studies using rats (Eacott & Norman, 2004; Langston & Wood, 2010; Barker & Warburton, 2011), but rarely in mice. When it has been used in mice, discrimination indices are generally low, and large group sizes are needed to see significant effects (McLean et al., 2018; Vandrey et al., 2020). It may be that this task is not as effective in mice as it is in rats, possibly due to weaker associative memory.

Of all the tasks used here, OPC was predicted as the most likely to show a deficit in mice with inactivated L2SCs, due to the requirement of a combination of spatial and contextual information. The increased complexity of this may require a combination of input from both LEC and MEC inputs into hippocampus, this also being the only task used which has been shown to be hippocampus-dependent (Langston & Wood, 2010). This is supported by the evidence that fan cells of the LEC are needed for this task (Vandrey et al., 2020), but cannot be confirmed from the current data.

#### **3.4.5 Limitations and future work**

A key limitation to all of the results presented here was the low expression of the TeLC virus within layer 2 of the MEC. In order to draw reliable conclusions from

this experiment, this would need to be improved, or a different approach would be needed. A transgenic cross to express DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) in stellate cells could produce more reliable expression (as in Kanter et al. (2017)), but may compromise on specificity. An advantage of this approach is the within-subject comparison made possible by testing with and without the receptor-activating drug. Optogenetic manipulation of the cell population is another method via which this could be achieved.

However, it may be that none of the tasks used here are affected by inactivation of MEC L2SCs. The prediction was that OPC memory may be disrupted by the L2SC manipulation, as it is hippocampus-dependent and so may require spatial and contextual input from the MEC. Previous experiments using manipulations of MEC L2SCs have shown effects on remapping in the hippocampus (Kitamura et al., 2015; Miao et al., 2015; Kanter et al., 2017), suggesting they may be required for hippocampus-dependent tasks requiring contextual discrimination, such as the OPC task. Findings presented in Chapter 2 of this thesis suggested that MEC is required for integration of distal visual landmark information into the hippocampal representation, but not proximal, multi-modal object cues. It may be that the current experiment did not require the type of information that is conveyed by the MEC; distal visual landmarks were not required for discrimination of object places, and the contexts used here differed in texture and probably in odour, as well as visual characteristics.

It would be interesting, therefore, to firstly use a different approach that could answer the question of whether MEC L2SCs are required for use of distal landmarks in spatial memory behaviour. This could be tested using a Morris Water Maze with distal cues, and rotating the cues before the probe trial. Recording place cells in CA1 in mice with inactivated L2SCs with the same cue rotation used in the previous chapter, would also help to answer the question of whether these inputs specifically are required for distal cue use. Whether MEC L2SCs are required for context discrimination is another question, and testing mice with inactivated L2SCs on a version of OPC with purely visually-discriminable contexts may provide answers as to whether the type of sensory information determines whether MEC inputs are necessary for these tasks.

However, a further limitation of this experiment was the lack of discrimination above chance level in the GFP group in the OPC task. As discussed previously, mice do

not perform this task as reliably as rats, and large numbers of subjects are often needed for sufficiently powered results. Therefore, a different approach may be needed in terms of the task used as well. Alternatively, more genetically-modified lines of rats are now being developed to allow similar access to specific cell populations as is possible in mice, so another option may be to investigate the associative memory function of MEC L2SCs in rats instead.

Another limitation may have come from how the tasks were scheduled. Previous work with the TeLC virus had shown expression was greatest from around two weeks after injection, and cell death began to occur at around six weeks. Since the experiment aimed to test the effect of stellate cell inactivation, testing had to be carried out within this time period. Tasks were run on successive days, so as more tasks were run and more objects experienced, this could have produced an interference effect that reduced the animals' ability to remember specific object associations. This would especially impair the more complex associations, such as OPC. As OPC was also run as the final task in the first run, this could explain the lack of discrimination in the GFP group. The experiment may have been improved by leaving more time between each task. Using a different technique for inactivation of the L2SCs could have allowed more time for testing.

In summary, while the current experiment provides some evidence that inactivation of MEC L2SCs does not produce deficits in object recognition, object-context, or object-place memory, a number of experimental limitations, particularly the low expression levels of the TeLC virus, prevent reliable conclusions from being made. Furthermore, control mice failed to demonstrate clear evidence of object-place-context memory, so whether MEC L2SCs are required for hippocampus-dependent object association recognition memory remains unclear.

## **Chapter 4**

# **Network oscillations of medial prefrontal cortex and hippocampus in a rat model of Fragile X Syndrome during novelty exploration**

### **4.1 Introduction**

Memory is affected in a many different neuropsychiatric disorders. Use of animal models of such disorders can help to better understand circuitry involved in memory, through how it can be affected pathologically. One example of this is the study of rodent models of Fragile X Syndrome (FXS). FXS is one of the most common inherited forms of intellectual disability and autism spectrum disorder (Coffee et al., 2009). It is monogenic, caused by a CGG trinucleotide repeat on the FMR1 gene, leading to silencing of the gene and loss of its protein product, FMRP (Pieretti et al., 1991; Verkerk et al., 1991; Colak et al., 2014).

FMRP is expressed throughout the brain, and has a wide range of functions in neurons and glia, including regulating the translation of hundreds of other proteins (Darnell et al., 2011), as well as interacting directly with ion channels to influence cell excitability (Brown et al., 2010; Deng et al., 2013). This wide-ranging and diversity of functions is reflected in the complexity of FXS as a disorder, with a range of phenotypes seen, including learning disability, autism spectrum disorder, seizures,

sensory hypersensitivity, and sleep disruption, that are expressed to varying degrees between different individuals (Lozano et al., 2014). This makes it difficult to link loss of FMRP to specific behavioural features.

In order to better understand the disorder, FXS was first modelled in mice by knock-out of the *Fmr1* gene (Bakker et al., 1994), and then in rats more recently to provide a model for cross-species validation, as well as to test more complex cognitive function (Till et al., 2015). These models have been used to study cellular and circuit level changes that could provide mechanisms for the neurological symptoms of FXS. Differences in expression of ion channels lead to changes in cellular intrinsic excitability. These changes are brain region-specific, including some cases of opposite effects seen in hippocampal and cortical cells and networks (Contractor et al., 2015). The overall effect may be hyper-excitable networks, which may help to explain some of the symptoms, such as seizures and hypersensitivity.

In terms of learning and memory, efforts to explain defects caused by loss of FMRP behaviourally have been mixed. The *Fmr1*<sup>-y</sup> mouse shows a deficits in hippocampus-dependent spatial reference memory and reversal learning in a Morris water maze (D'Hooge et al., 1997; Baker et al., 2010). However, the same task in *Fmr1*<sup>-y</sup> rats showed no difference from WT littermates (Till et al., 2015). This study did reveal an inability of *Fmr1*<sup>-y</sup> rats to discriminate novel from familiar associations between objects, places and contexts in a version of the spontaneous object exploration task. Associative memory in this task has previously been shown to rely not only on hippocampus (Langston & Wood, 2010), but on connections between the hippocampus and medial prefrontal cortex (mPFC) (Barker & Warburton, 2020). Therefore, the selective impairment in this task, but not in all hippocampus-dependent spatial memory tasks, may reflect the importance of hippocampal-mPFC connectivity in cognitive deficits caused by lack of FMRP.

There is some evidence from human FXS patients that these regions may be affected. In a working memory task, control subjects showed increased prefrontal cortex activation, measured using fMRI, in response to increased memory load; FXS patients, however, did not show modulation of prefrontal activity (Kwon et al., 2001). Additionally, decreased FMRP levels in FMR1 premutation carriers have been associated with decreased functional connectivity between mPFC and hippocampus during a working memory task (Wang et al., 2012).

Hippocampal-cortical functional connectivity in *Fmr1* rodents has not yet been directly studied *in vivo*. Currently, most *in vivo* recording studies have focused on the hippocampus. And correlates of memory have been largely normal, with some subtle changes in place cell function, gamma oscillations, and ripple activity. An experiment in *Fmr1*<sup>-/-</sup> mice used a place avoidance task to measure cognitive discrimination; mice were able to learn the location of a shock zone similarly to WT, but were slower to learn in a conflict trial, in which the shock location was changed to a new place within the arena. Furthermore, when the shock zone was turned off, WT and *Fmr1*<sup>-/-</sup> mice extinguished memory similarly, but when placed back into the arena for a second extinction session, WT avoided the previous shock zone but not to the full level, whereas *Fmr1*<sup>-/-</sup> mice initially avoided the previous shock zone fully, then transitioned to full extinction by the end of the session. These differences were shown to not be due to perseverative behaviour, as *Fmr1*<sup>-/-</sup> mice did not express continued avoidance of old shock locations (Radwan et al., 2016).

Along with this behavioural difference, Radwan et al. (2016) found accompanying differences in network oscillations in the hippocampus. WT showed task stage-dependent changes in theta-gamma phase-amplitude coupling, as well as synchrony between different layers, in the hippocampus, which were not seen in *Fmr1*<sup>-/-</sup> mice. A further study using the same behavioural protocol found that *Fmr1*<sup>-/-</sup> mice had more dominant low gamma over high gamma during conflict trials compared to WT, which suggested they may be recalling the previous shock zone location. Decoding of place cell firing during low gamma oscillations in this conflict trial found they were encoding locations closer to the old shock location as well (Dvorak et al., 2018). Another study found increased theta power and increased low gamma synchrony between tetrodes in the hippocampus of *Fmr1*<sup>-/-</sup> mice, and suggested this represented hypersynchronised neuronal activity (Arbab, Battaglia, et al., 2018). Place cell activity was also recorded during sessions where visual cues in the environment were changed; WT showed a reduction in place field stability between these sessions, reflecting the change of the environment, but *Fmr1*<sup>-/-</sup> showed no difference in stability compared to other sessions (Arbab, Pennartz, et al., 2018). This evidence suggests *Fmr1*<sup>-/-</sup> mice may have less flexible circuits that are less adaptable to conflicts compared to WT. A recent study of the *Fmr1*<sup>-/-</sup> rat found a lack of experience-related changes in CA1 place cells, as well as altered phase-locking to LFP oscillations (Asiminas et al., 2021, preprint), possibly also reflecting less flexible circuits.

The mPFC has been previously shown to be required for tasks requiring cognitive flexibility, such as strategy-switching on decision-based maze tasks (Rich & Shapiro, 2007). During such tasks, interactions between mPFC and hippocampus are observed, and appear to be important for discriminating previous irrelevant memories from currently relevant ones (Benchenane et al., 2010; Hallock et al., 2016; Guise & Shapiro, 2017). This gives further reason to examine mPFC-hippocampal interactions in *Fmr1*<sup>-y</sup> models as a potential mechanism of cognitive inflexibility.

Within the mPFC network, ex vivo methods have shown synaptic dysfunction in mPFC neurons from *Fmr1*<sup>-y</sup> mice. In addition to LTP deficits (Meredith et al., 2007; Martin et al., 2016) and intrinsic neuronal firing properties (Kalmbach et al., 2015; Routh et al., 2017), hyper-connectivity of mPFC neurons was observed during early development, from postnatal day (P) 12 to P19, then returning to WT levels by around P20 (Testa-Silva et al., 2012). This raises an interesting question of whether circuit and behavioural dysfunction in *Fmr1*<sup>-y</sup> mice are caused by the lack of FMRP during critical developmental periods, when its expression in the brain peaks (Till et al., 2012), or whether its continued absence through adulthood impacts mature circuits. There is some evidence that the latter may be the case in mPFC – Siegel et al. (2017) showed that *Fmr1*<sup>-y</sup> mice were impaired on a trace eye-blink conditioning task, which they previously found to rely on an intact mPFC. They followed on to show that conditional knockout of FMRP in the mPFC of adult mice caused an impairment in the task (Siegel et al., 2017).

While behavioural and network deficits have been observed in relation to both the mPFC and hippocampus in *Fmr1*<sup>-y</sup> rodents, functional connectivity between the two regions is yet to be investigated. The experiment described in this chapter aimed to test the hypothesis that functional connectivity between the mPFC and hippocampus is disrupted in adult *Fmr1*<sup>-y</sup> rats, while also investigating dynamics of the individual networks. Since the mPFC and hippocampus have previously been shown to interact during novelty exploration (Bygrave et al., 2019; Wang et al., 2021), LFPs were recorded in the prelimbic area of the mPFC and dorsal CA1 of the hippocampus while rats explored a very familiar environment and an initially novel environment across three consecutive days (Figure 4.1C). Characteristic oscillations in the LFPs from both regions were analysed, as well as the coherence of the two signals to assess functional interactions.

## 4.2 Methods

### 4.2.1 Animals

All experimental procedures were carried out under a UK Home Office project licence, approved by the Animal Welfare and Ethical Review Board (AWERB) of the University of Edinburgh College of Medicine and Veterinary Medicine, and conformed with the UK Animals (Scientific Procedures) Act 1986.

The subjects were male Long-Evans Hooded *Fmr1*<sup>-/-</sup> rats (n = 4) and their wild-type (WT) littermates (n = 5), aged around 8 weeks at the time of surgery. Animals were housed in groups of 2-5 per cage after weaning, until after surgery, where they were housed individually, or with one other implanted rat, separated by a perforated divider to allow some contact without risking damage to implants. Rats were given *ad libitum* access to food and water before surgery and for at least 3 days during recovery afterwards, then put on mild food restriction only after they had returned to their pre-surgery body weight, to maintain approximately 90% of free-feeding weight. Animals were kept on a diurnal 12-hour light/dark cycle (lights on at 07:00) and all experiments were performed during the light phase.

### 4.2.2 Tetrode implants

Tetrodes were made from four lengths of 12.5 µm tungsten wire (California Fine Wire) that were twisted together and heat-annealed using a heat gun. Eight tetrodes were loaded into each implant drive. Implants were made from a custom 3D-printed microdrive body from Axona Ltd., with two shafts at 7.1 mm apart to target mPFC and dCA1. Each shaft housed two cannulae (inner diameter 254 µm) adjacent to one another, each containing a pair of tetrodes which could be moved independently of one another. The structure of the drive is illustrated by Figure 1A.

To construct the drive, polyamide cannulae (Science Products GmbH) were loaded into the microdrive shafts to form a guide for narrower polyamide cannulae (inner diameter 226 µm), which were superglued to 3D-printed shuttles (the white parts in Figure 4.1A), which had screws and insect pins driven through them. This allowed vertical movement of the shuttles when the screws were turned, as they were held

in place by the pins. The tetrodes were then threaded through the inner cannulae and superglued to them, so that they could be moved vertically with the shuttles. Vaseline was applied in between the guide and inner cannulae to lubricate this movement. Two tetrodes were loaded into each inner cannula, allowing individual movement of two separate bundles of two tetrodes in each shaft. Individual tetrode wires were then threaded through the microdrive lid and gold connector pins secured them in place and stripped away their insulation. A wire was connected to the ground connector pin, and the other end of the wire was soldered onto a copper wire mesh (Dexmet) which was wrapped around the outer surface of the drive (Figure 4.1A).

The day before surgery, tetrodes were cut using ceramic scissors (Fine Science Tools) to around 4 mm, before being plated with gold solution (Neuralynx) to reduce the impedance to 150-200  $k\Omega$ . The screws were then turned to retract the tetrodes back into the drive body so that the tips were just protruding from the guide cannulae.

### **4.2.3 Surgery**

Rats were anaesthetised in an induction chamber using Isoflurane with oxygen. They were then transferred to a stereotaxic frame and analgesia (Rimadyl) was administered by subcutaneous injection. They were fixed in place with ear bars and a bite bar, and body temperature was regulated using a thermostatic heat blanket underneath the rat's body, with a rectal probe. The scalp was disinfected using chlorhexidine (Hibiscrub) and iodine before an incision was made in the skin along the midline. The skull was levelled in the anteroposterior plane, and skull screws embedded into the skull in five different places. The screw placed in the occipital bone had a wire soldered to its head for grounding the implant. Craniotomies were drilled at the two implant sites: the mPFC site was +3.2 mm anterior to Bregma, +0.5 mm to the right; the dCA1 site was -3.6 mm posterior to Bregma, and +2.5 mm to the right (Figure 4.1B shows approximate placement). The dura was pierced, and ice-cold saline was used to prevent excessive bleeding and swelling of the brain. As much of the dura from inside the craniotomy was removed as was possible without excessive bleeding, to leave a clear area for the tetrodes to advance into the brain cleanly. The implant was then attached to a stereotaxic arm and moved

downwards and adjusted so that the tips of the two sets of tetrodes were just beneath the brain surface. They were then surrounded by sterile Vaseline. Dental cement (Simplex Rapid) was applied to the surface of the skull and the shafts and base of the microdrive body to fix it in place. The ground wire attached to the skull screw was soldered onto the copper mesh on the implant and covered with dental cement. Once the drive was fixed in place, the drive screws were gradually turned to move the tetrodes further into the brain, to 0.75 mm for the hippocampal tetrodes, and 1.25 mm for the mPFC tetrodes. The skin around the implant was cleaned with saline, disinfected using bacteriostatic ointment and sutured together at the back and front as needed. Rats were then removed from the stereotaxic frame and put in a clean home cage placed on a heat pad at 30 celsius, where recovery was monitored until they regained consciousness, after which they were returned to the animal housing unit.

The rats were then left to recover for 24 hours before the screws were turned again to advance the tetrodes further. The tetrodes were advanced in small amounts (30-125  $\mu\text{m}$ ) daily for around three days, before the rats were connected to the recording system for the first time.

### 4.2.4 Electrophysiological recording

For recording, the implanted microdrive was attached to two headstage amplifiers connected to a counterbalanced cable which led to a commutator, allowing the rat to move freely around the screening environment (see Apparatus for details of the environment). The commutator was connected to a 32-channel Axona USB recording system (Axona Ltd.), via a pre-amplifier. The signal was amplified and band-pass filtered between 600 and 6000 Hz. Single units were identified and recorded in DACQ software (Axona Ltd.). The signals from one wire in each brain area were duplicated and filtered with a 0-300 Hz low-pass filter, and a notch filter at 50 Hz to remove electrical line noise, to record local field potentials with a sampling frequency of 4.8 kHz. Two infrared LEDs on one of the headstages allowed tracking of the animal's head position using a camera placed above the environment, which was recorded within the DACQ software at a frequency of 50 Hz.

The rats were plugged into the system each day and allowed to explore the screening environment. Tetrodes were advanced each day, until theta-modulated pyramidal cells could be identified in the hippocampus and the mPFC tetrodes were estimated to be at the required depth for the prelimbic area (at least 2.6 mm). This took a minimum of two weeks for each rat.

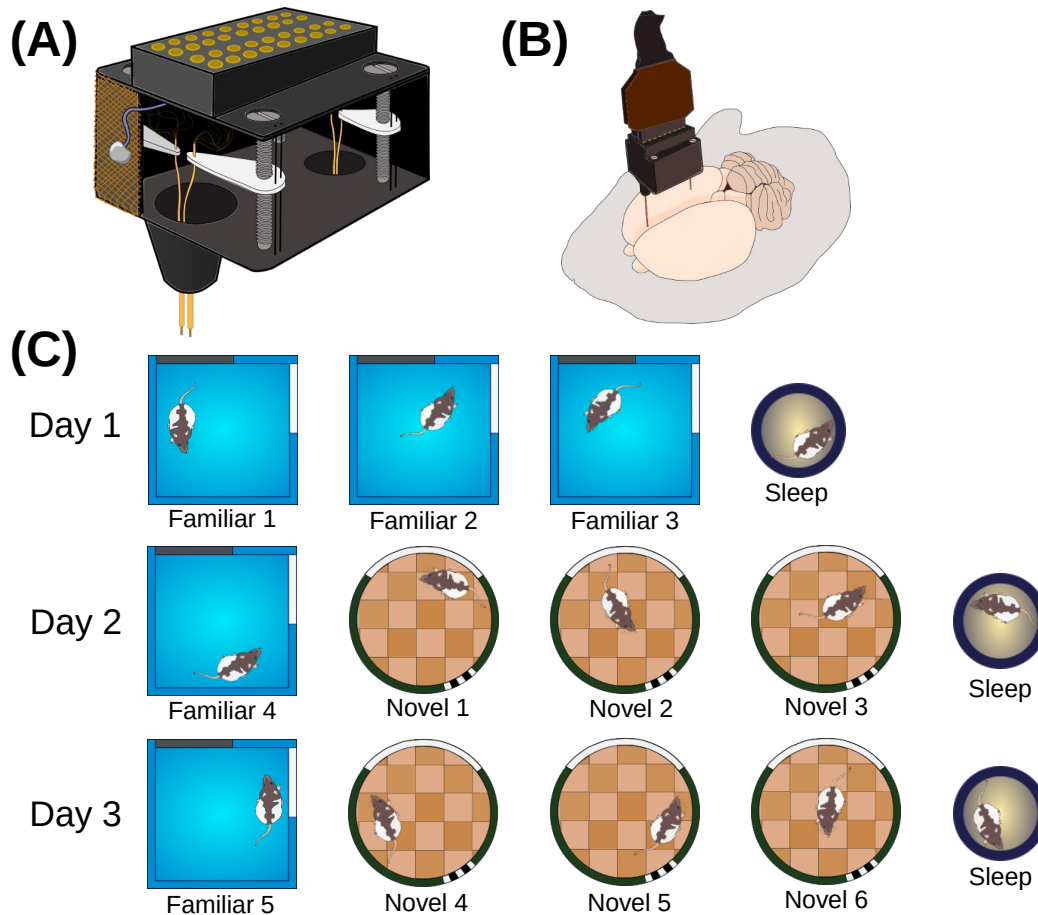


FIGURE 4.1: Electrode drive and novel environment protocol. **(A)** Design of the electrode drive used. **(B)** Approximate locations of electrodes on a rat brain. **(C)** Novel environment protocol: Each environment exploration session lasted 15 minutes, with around 3 minutes between each. Sleep sessions were 30 minutes.

#### 4.2.5 Apparatus

Screening took place in a large square wooden box, 82 cm x 82 cm with 60 cm high walls, painted blue with cues affixed to the top of three of the walls, placed in the centre of a curtained environment. The cues were one A3-sized black card

and one A3-sized white cue card, and one object – a stuffed toy sheep. The black curtain was pulled back to cover one third of the circle around the box, a white sheet was hung to cover an adjacent third, and a green bin lid and yellow bag were hung from the other side of the curtain rail to act as distal visual cues. Screening in this environment took place for at least two weeks, with two days of exposure before surgery, so this screening environment was also used as the familiar environment during the experimental protocol (Figure 4.1C). Two novel environments were used. The first novel environment was used for the first 4 WT<sub>s</sub> and 3 *Fmr1*<sup>-/-</sup> rats, and the second was used for the final one rat of each genotype. The first novel environment consisted of the same wooden box, but with a vinyl wood-effect floor insert and a circular Perspex wall placed inside, so that the walls and floor of the familiar box were not visible (diameter of the circle was 82 cm, and the walls were around 70 cm tall). The Perspex wall was spray-painted dark green, with one third around 10 cm taller and spray-painted white. A 40 cm x 30 cm black and white striped cue card (10 cm wide vertical stripes) was placed at the top, almost opposite the white section of the wall (Figure 4.1C). The black curtain was drawn fully around the box, a yellow cardboard shape (40 cm oval shape) hung from it, and a sign covered in aluminium foil (50 cm long) was placed just outside the box.

The second novel environment used was a wooden regular octagonal arena (1 m across, with 50 cm high walls) painted blue, with two adjacent walls covered with black card, one wall adjacent to this covered with striped black and white card (15 cm wide vertical stripes), and three walls adjacent to this covered with white card. The floor was covered with a textured black foam material. The arena was placed in the same position in the room as the familiar environment was, and the black curtains were drawn halfway around, with a white curtain hung around the other half. Cues included a green watering can, attached to the top of one of the walls, a yellow card star shape and large spherical white lampshade, both hung from the black curtains.

### 4.2.6 Novel environment protocol

In order to observe the effects of novelty, a protocol was designed to compare network activity in a very familiar environment with that in a totally novel one, and to test the effects of habituation to the novel environment. A three-day protocol was used, with one day of only familiar environment exploration, followed by two days

in which the rats were exposed to both the familiar environment and to the novel environment. This protocol allowed comparison between a familiar and completely novel environment, as well habituation to the novel environment both within and between days. A sleep session was recorded at the end of each day, in order to compare network activity during sleep following exposure to familiar (day 1) and novel (day 2) environments (see Chapter 5). On Day 1, rats were placed in the familiar environment for three 15 minute sessions, with Coco Pops (Kellogg's) being scattered throughout to encourage exploration. Between sessions they were removed and placed into a plastic bucket with bedding at the bottom, for two minutes while the environment was cleaned; the rat remained plugged in during this period. After the third familiar environment session, the rat was placed into the bucket which was put in the centre of the environment, for 30 minutes, so that sleep could be recorded. On each of Days 2 and 3, rats were recorded for four 15 min exploration sessions. For the first session they were placed into the same familiar environment as on Day 1, whereas for sessions 2-4 they were placed into the novel environment. Similar to Day 1, between sessions they were placed into the bucket for 2 min, and after the final exploration session each day they were recorded during a 30 min sleep session (Figure 4.1C).

#### **4.2.7 Data analysis and statistics**

The focus of the data analysis described in this thesis is on LFPs recorded in the hippocampus and mPFC. LFP recordings from the DACQ USB system, along with simultaneous animal position data, were loaded into MATLAB using custom scripts. Spectrograms were calculated using functions from the Chronux (Bokil et al., 2010) and Freely Moving Animal (FMAToolbox) MATLAB toolboxes. For each rat, the LFP signal across all 11 sessions was z-scored, and spectrograms were calculated using a window size of 10 seconds with an overlap of 5 seconds. The full spectrograms were then averaged across time windows where the rat's velocity was above 3 cm/s, assessed using position tracking data, to get the average spectrograms for each session. Estimates of power in the theta (6-10 Hz), broadband gamma (30-80 Hz), low gamma (30-47 Hz) and high gamma (60-100 Hz) bands were extracted by averaging power across the relevant frequency bands for each session.

To calculate theta-gamma phase-amplitude coupling (PAC) in each session, gamma

events were extracted from the signal using a method similar to Colgin et al. (2009) and Zheng et al. (2015). Only gamma events from times when the rat was travelling above 3cm/s were used for analysis. The signal was first bandpass-filtered in the theta band, and relevant gamma band. A Hilbert transform (using the *hilbert* function from the MATLAB Signal Processing Toolbox) was applied to each, and the imaginary component of the theta oscillation used to compute the phase at each sampling point, and the real component of the gamma oscillation used to compute its instantaneous amplitude. The standard deviation of the gamma amplitude was calculated and peaks in the gamma amplitude that were more than two standard deviations above the mean were identified. 200 ms time windows centred around these peaks were extracted and used as gamma events. If two peaks occurred within 200 ms of one another, the smaller peak was discarded. The phase of the theta oscillation at the time of each gamma event peak was found, and the circular mean of all of the peaks in each session were calculated using the circular statistics toolbox in MATLAB. The theta oscillation and gamma amplitude for each event window were then concatenated into a continuous vector in order to calculate a modulation index (MI) (Tort et al., 2008). This was done by binning the phases into  $18 \times 20^\circ$  ( $\frac{\pi}{9}$  radians) bins and finding the mean of all gamma amplitudes that occurred within each phase bin. For each bin,  $j$ , this was divided by the total of the means of all 18 bins to obtain  $p_j$ . This is used in the formula:

$$Modulationindex(MI) = \frac{\log N - \left( - \sum_{j=1}^N p_j \log p_j \right)}{\log N}$$

Where  $N$  is the number of phase bins (18).

Coherence in each session was calculated using the function *MTCoherogram* (FMAT), with time windows of 2 seconds and a 1 second overlap. This function gives the value of spectral coherence and phase coherence for each time and frequency window. Spectral coherency is calculated using the following equation:

$$C_{yx} = \frac{\Phi_{yx}(S)}{\sqrt{\Phi_{yy}(S) \Phi_{xx}(S)}}$$

Where  $\Phi_{yx}(S)$  is the cross-spectrum of the two signals, and  $\Phi_{yy}(S)$  and  $\Phi_{xx}(S)$  are the auto-spectra of signal  $y$  and signal  $x$ , respectively.

The real component of the resultant coherogram forms the spectral coherence measure used here. The imaginary component forms the phase/imaginary coherence. Measures to calculate the phase synchrony of two signals that are more independent of volume conduction were also used. The first was phase difference, where the angular difference at each sampling point was calculated and the resultant vector length of the differences for each session were found. The de-biased weighted phase lag index (dWPLI) was then calculated using the imaginary component of the cross-spectrum, before normalisation by the power as in the coherence equation. As well as being less influenced by volume conduction, this measure is less sensitive to noise and not biased by large phase differences (Vinck et al., 2011). The dWPLI was calculated using the equation:

$$dWPLI = \frac{\{\sum_{n=1}^N Im\{X\}\}^2 - \sum_{n=1}^N \{Im\{X\}^2\}}{\{\sum_{n=1}^N |Im\{X\}|\}^2 - \sum_{n=1}^N \{Im\{X\}^2\}}$$

Where  $Im\{X\}$  is the imaginary component of the cross-spectrum,  $n$  represents each time step, and  $N$  is the total number of time bins for the session.

To obtain average spectral and phase coherograms for each session, coherence was averaged over time windows where the rat was travelling at above 3 cm/s. These, as well as the average dWPLI, were then averaged within the relevant frequency bands for each session. To find an estimate of lag between the theta oscillations in the two signals, the angular difference in phase of the Hilbert-transformed bandpass-filtered signals at every sampling point was calculated, and the mean of these differences calculated.

For comparison of path length, average power, modulation indices, and coherence measures in each session, two-way repeated-measures ANOVAs were run in SPSS, with session as the within-subjects factor and genotype as the between-subjects factor. Where there was a significant effect of session, planned comparisons were used to explore the effects of novelty and familiarity (Figure 4.1C). The first tests were to determine whether there is stable activity in the familiar environment across days, so the initial familiar sessions of each day were compared (Familiar 1 vs Familiar 4, Familiar 4 vs Familiar 5). Secondly, the initial response to environmental novelty was tested, by comparing between the first novel session and the familiar session before it (Familiar 4 vs Novel 1). Habituation to novelty was then tested; habituation across days (Novel 1 vs Novel 4) and habituation

within the first novel day (Novel 1 vs Novel 3) were compared. P values were Bonferroni-corrected for the total of five comparisons. Where there were significant interactions, these comparisons were made for each group.

Where required, circular statistics were calculated using the Circular Statistics Toolbox in MATLAB (Berens, 2009). To compare mean phases across sessions and between genotypes, two-factor Harrison-Kanji tests (*circ\_hktest* function) were used. Where there were significant main effects of session or genotype, or significant genotype x session interactions, post hoc comparisons were made using Watson-Williams tests (*circ\_wwtest*), and their associated p values were Bonferroni-corrected for the number of comparisons made.

#### 4.2.8 Perfusion and histology

Once recording was complete, rats were anaesthetised with Isoflurane and given an intraperitoneal lethal dose of sodium pentobarbital (Euthatal), then transcardially perfused with phosphate buffered saline and 4% formalin to fix the tissues. The brains were removed and kept in formalin, before being transferred to 30% sucrose for cryoprotection. 40 µm sagittal sections were taken using a cryostat, and sections from around the electrode positions were mounted on polysine slides (Thermo Scientific). They were stained with 0.1% Cresyl-violet and coverslipped in DPX (Sigma-Aldrich), then imaged at 10x magnification using Image-Pro Plus (Media Cybernetics) to identify electrode tracks.

## 4.3 Results

### 4.3.1 Histology

Figure 4.2A shows example coronal sections from two WT rats (black box) and two *Fmr1*<sup>-/-</sup> rats (red box), indicating where tetrode tracks can be seen. Figure 4.2B shows adapted atlas images (Paxinos & Watson, 2006) with tetrode tip locations marked for each rat (WT, black, n = 5; *Fmr1*<sup>-/-</sup>, red, n = 4). All tetrodes were found to be located in the target areas, within the prelimbic area of the mPFC, and the pyramidal cell layer of dorsal CA1 of the hippocampus. While the same stereotaxic

coordinates were used for all rats, there was some variation in electrode placement within these regions. Tetrode locations in the mPFC ranged from around +4.0 mm to +3.0 mm anterior to Bregma, and around 0.4 mm to 0.8 mm lateral from the midline. Dorso-ventral position also varied from around 2.0 mm to 3.5 mm below the brain surface. The coordinates of the hippocampal tetrodes varied from around -3.12 mm to -3.84 mm posterior to Bregma, and between around 2.2 mm and 3.2 mm lateral from the midline. Recording took place only when theta-modulated putative excitatory units had been identified on the hippocampal tetrodes, so they can be assumed to have been within the pyramidal cell layer of dorsal CA1.

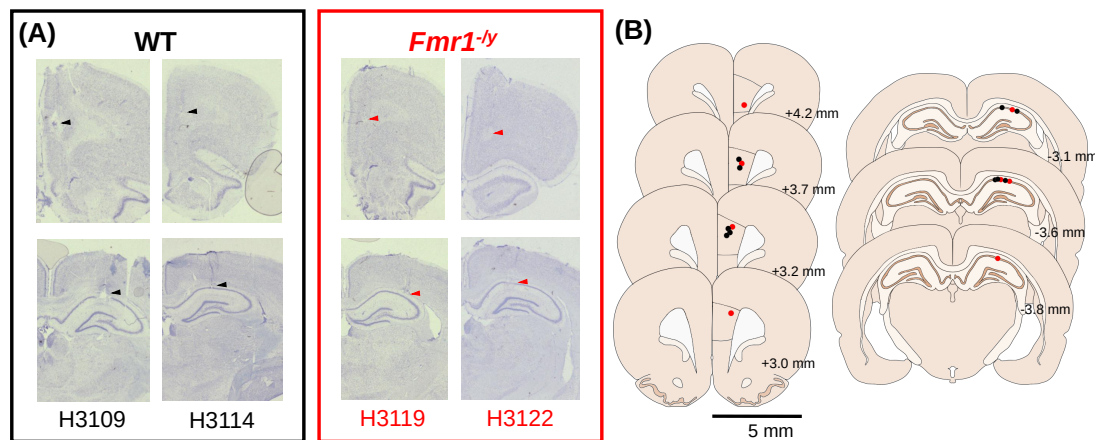


FIGURE 4.2: Example electrode tracks **(A)** Example coronal sections from two WT rats (black box) and two *Fmr1*<sup>-/-</sup> rats (red box), showing electrode tracks in the mPFC (above) and dorsal CA1 (below). Experimental numbers of rats are shown below. **(B)** All estimated electrode tip locations indicated on adapted atlas images, with antero-posterior distance from Bregma indicated (Paxinos & Watson, 2006). WT rats are represented by black dots ( $n = 5$ ) and *Fmr1*<sup>-/-</sup> rats by red dots ( $n = 4$ ). mPFC tetrodes are shown on the left, and CA1 tetrodes on the right.

#### 4.3.2 *Fmr1*<sup>-/-</sup> rats had reduced hippocampal theta power and increased hippocampal low-gamma power

As LFP oscillations can be affected by running speed, total path length in each session was first compared (this is equivalent to average velocity, as session lengths were constant) (Figure 4.3A). No differences were seen between the groups, and comparison with a two-way ANOVA found no significant main effect of genotype ( $F(1,7) = 0.12$ ,  $p = 0.738$ ), but there was a significant main effect of session ( $F(3,1,21.4) = 17.5$ ,  $p < 0.0001$ ) and a significant genotype  $\times$  session interaction

### 4.3. Results

( $F(10,70) = 2.5$ ,  $p = 0.014$ ). Path length in the familiar sessions of each day was no different for either genotype (Familiar 1 vs Familiar 4: WT:  $p > 0.999$ ,  $Fmr1^{-/-}$ :  $p > 0.999$ ; Familiar 4 vs Familiar 5: WT:  $p > 0.999$ ,  $Fmr1^{-/-}$ :  $p > 0.999$ ). There was also no significant change in path length from the familiar environment to the first novel session (Familiar 4 vs Novel 1: WT:  $p = 0.676$ ,  $Fmr1^{-/-}$ :  $p > 0.999$ ). While there were general decreases across the sessions within each day, there were no significant differences when comparing the novel sessions within day 2 (Novel 1 vs Novel 3: WT:  $p = 0.211$ ,  $Fmr1^{-/-}$ :  $p = 0.473$ ). In the WT group, there was no differences between the first two novel sessions of each day (Novel 1 vs Novel 4: WT:  $p = 0.361$ ), however, the  $Fmr1^{-/-}$  group did show a significant decrease across these two sessions (Novel 1 vs Novel 4: WT:  $p = 0.039$ ).

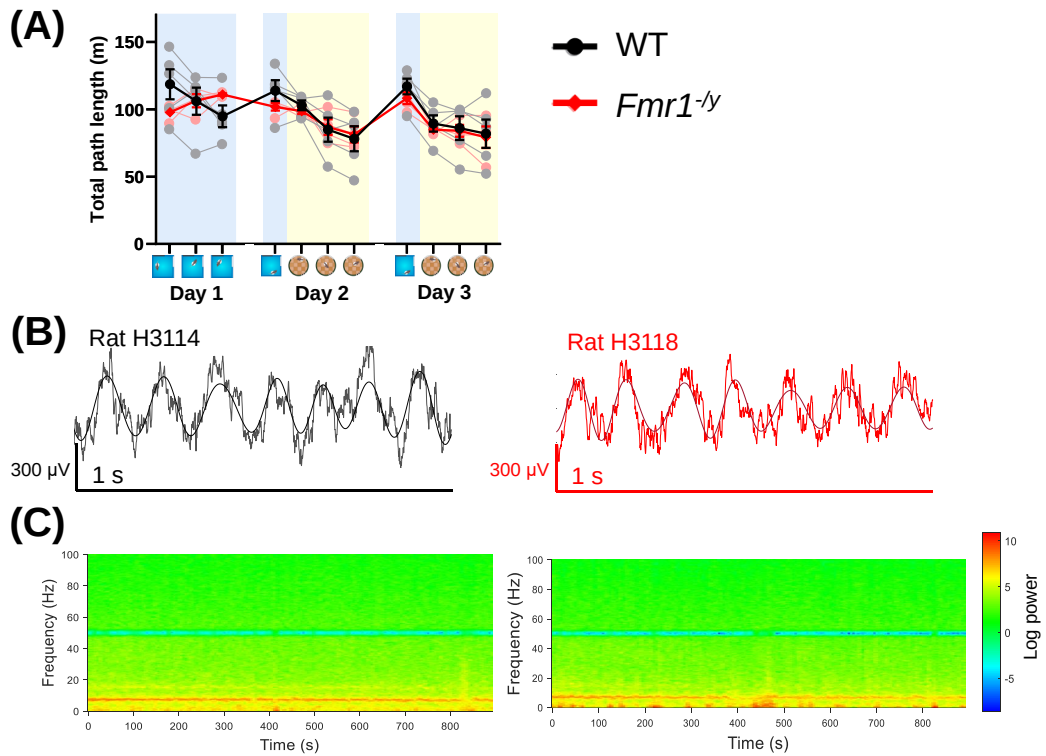


FIGURE 4.3: Path length and example spectrograms. **(A)** Path length calculated from position tracking for each session in the familiar and novel environments. Pale coloured lines show individual rat values, bold colours indicate mean  $\pm$  standard error of the mean (WT, black,  $n = 5$ ;  $Fmr1^{-/-}$ , red,  $n = 4$ ). **(B)** Example 1-second periods of LFP signal from dorsal CA1 of one WT rat (left) and one  $Fmr1^{-/-}$  rat (right), with theta bandpass (6-10 Hz) overlaid. **(C)** Example spectrograms from the same two rats as in (B). Time is represented by the  $x$ -axis, and frequency on the  $y$ -axis. Log power is represented by the colour axis.

In order to limit the effects of differences in total movement within sessions, measures of power and coherence were averaged across times where rats were moving above a speed threshold of 3 cm/s.

To assess hippocampal network activity, spectrograms were calculated from the z-transformed LFP from each session. The theta oscillation (6-10 Hz) plays an important role in the hippocampus in timing of neuronal activity, and is very prominent during active wake. Example 1-second raw traces of hippocampal LFP are shown in Figure 4.3B, and they are overlaid with the signal filtered in the theta range. Example hippocampal spectrograms are shown in Figure 4.3C, showing log power across the 1-100 Hz frequency range, over the whole 15-minute session. These were then averaged across time within each session to compare average power in each genotype (Figure 4.4A, power is plotted as the log value).

As expected, all animals showed a clear peak in the hippocampal spectrogram at around 8 Hz, indicating strong theta oscillations. From the average spectrogram, it appeared that the *Fmr1*<sup>-y</sup> rats showed a lower peak in the theta range (shown by the insert in Figure 4.4A). Power in the theta range was averaged from the spectrogram for each session and plotted across the 11 sessions of the novel environment protocol (Figure 4.4B). This showed that *Fmr1*<sup>-y</sup> rats had lower average theta power across all sessions. A two-way repeated measures ANOVA of these averages across all sessions revealed a significant effect of genotype ( $F(1,7) = 6.85$ ,  $p = 0.035$ ), and session ( $F(2.9,20.0) = 5.21$ ,  $p = 0.0088$ ), with no significant genotype x session interaction ( $F(10,70) = 0.57$ ,  $p = 0.834$ ). Post hoc tests found that theta power in the familiar environment was consistent across days (Familiar 1 vs Familiar 4:  $p = 0.138$ ; Familiar 4 vs Familiar 5:  $p > 0.999$ ). There was a clear novelty effect, with theta power increasing in the first novel session compared to previous familiar session (Familiar 4 vs Novel 1:  $p = 0.0031$ ). There was a decrease back to the baseline familiar environment level for the next novel session, suggesting the environment was no longer detected as novel (Novel 1 vs Novel 3:  $p = 0.0041$ ). There was a smaller increase in theta power for some of the rats for the first novel session of the third day, but theta power was lower than the first novel session of day 2 (Novel 1 vs Novel 4:  $p = 0.046$ ).

This novelty effect was unlikely to be due to differences in velocity during the novel session, as overall path length was no different between the sessions. Further,

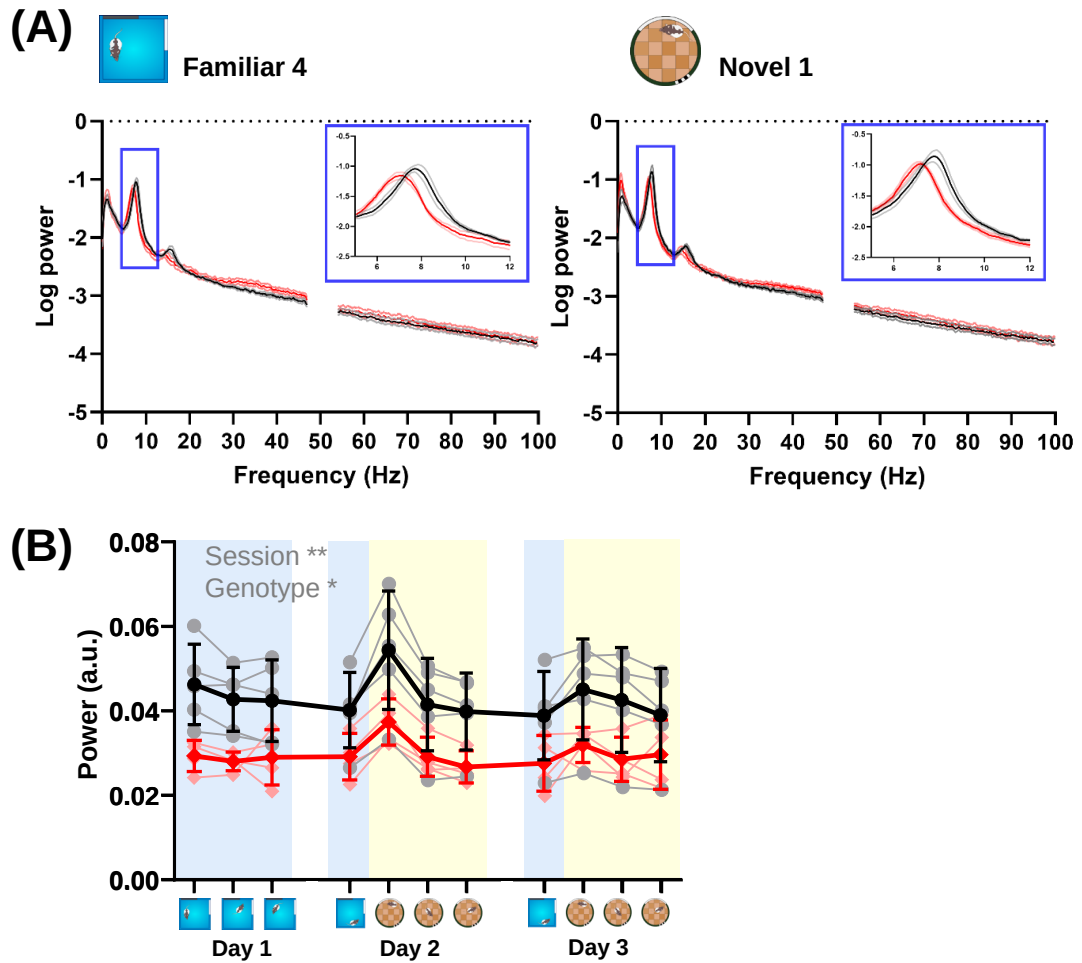


FIGURE 4.4: Hippocampal theta power is lower in *Fmr1<sup>-/-</sup>* rats, but increases with novelty in both genotypes. **(A)** Spectrograms averaged over time when rats were moving, in Familiar 4 (left) and Novel 1 (right), then averaged within each genotype. The blue box shows the average power in the theta frequency range. **(B)** Average theta power over each session in the novel environment protocol. Individual rats are shown by pale lines, and group means  $\pm$  SEM are represented by the bold lines (WT, black,  $n = 5$ ; *Fmr1<sup>-/-</sup>*, red,  $n = 4$ ). \*  $p < 0.05$ .

hippocampal theta power was averaged over times when rats were travelling at velocities within 2 cm/s bins, and theta power was clearly higher in Novel 1 compared to Familiar 4 across all velocity bins (see Appendix B, Figure B.1A).

Gamma oscillations in the hippocampus are split into multiple frequency ranges, which are associated with different inputs and functions. These include low gamma (27-47 Hz), associated with inputs from the CA3 subfield of the hippocampus, and high/medium gamma (60-100 Hz), associated with inputs from entorhinal cortex (Colgin et al., 2009). Average power of these oscillations was similarly assessed

across sessions (low gamma power shown in Figure 4.5). Figure 4.5A shows example periods of low gamma oscillation in the hippocampus – the low gamma-filtered signal is shown in bold with the raw signal behind. From the average spectrogram in Figure 4.5B, it appeared that low gamma power was slightly increased in the *Fmr1*<sup>-y</sup> rats compared to the WTs. This difference was significant, with a two-way ANOVA showing a significant main effect of genotype ( $F(1,7) = 6.26$ ,  $p = 0.041$ ), and a significant main effect of session ( $F(2.0,14.2) = 7.95$ ,  $p = 0.0048$ ), but no significant interaction ( $F(10,70) = 1.70$ ,  $p = 0.097$ ). The session effect may have come from a decrease in low gamma power from the first day to the second day, as there was a significant effect of comparison between Familiar 1 and Familiar 4 ( $p = 0.0063$ ), but not Familiar 4 and Familiar 5 ( $p > 0.999$ ). There was no significant difference in the first novel session compared to the previous familiar session (Familiar 4 vs Novel 1:  $p = 0.302$ ), or between the first novel sessions of each day (Novel 1 vs Novel 4:  $p > 0.999$ ). However, there was a significant difference between the first novel and the final novel session of day 2 (Novel 1 vs Novel 3:  $p = 0.0009$ ).

For average high gamma across sessions, there was no significant main effect of genotype ( $F(1,7) = 0.56$ ,  $p = 0.480$ ), but again there was a significant main effect of session ( $F(2.0,13.8) = 4.26$ ,  $p = 0.037$ ), with no genotype x session interaction ( $F(10,70) = 0.87$ ,  $p = 0.567$ ). High gamma power showed a similar effect to low gamma across the first two days – there was a decrease in the familiar environment from the first day to the second (Familiar 1 vs Familiar 4:  $p = 0.0024$ ), with no difference between the day 2 and day 3 familiar sessions (Familiar 4 vs Familiar 5:  $p > 0.999$ ). However, there was no effect of novelty (Familiar 4 vs Novel 1:  $p = 0.099$ ), or habituation to novelty across days (Novel 1 vs Novel 4:  $p > 0.999$ ), or within day 2 (Novel 1 vs Novel 3:  $p > 0.999$ ).

### **4.3.3 *Fmr1*<sup>-y</sup> hippocampal high gamma bursts peaked at an earlier phase of the theta oscillation**

The amplitude of gamma oscillations in the hippocampus has been shown previously to be modulated by the phase of the theta oscillation, thought to underlie separation of inputs from different regions (Colgin, 2015). To test this network coordination, bursts of gamma were identified in the hippocampal LFP, and the phase

### 4.3. Results

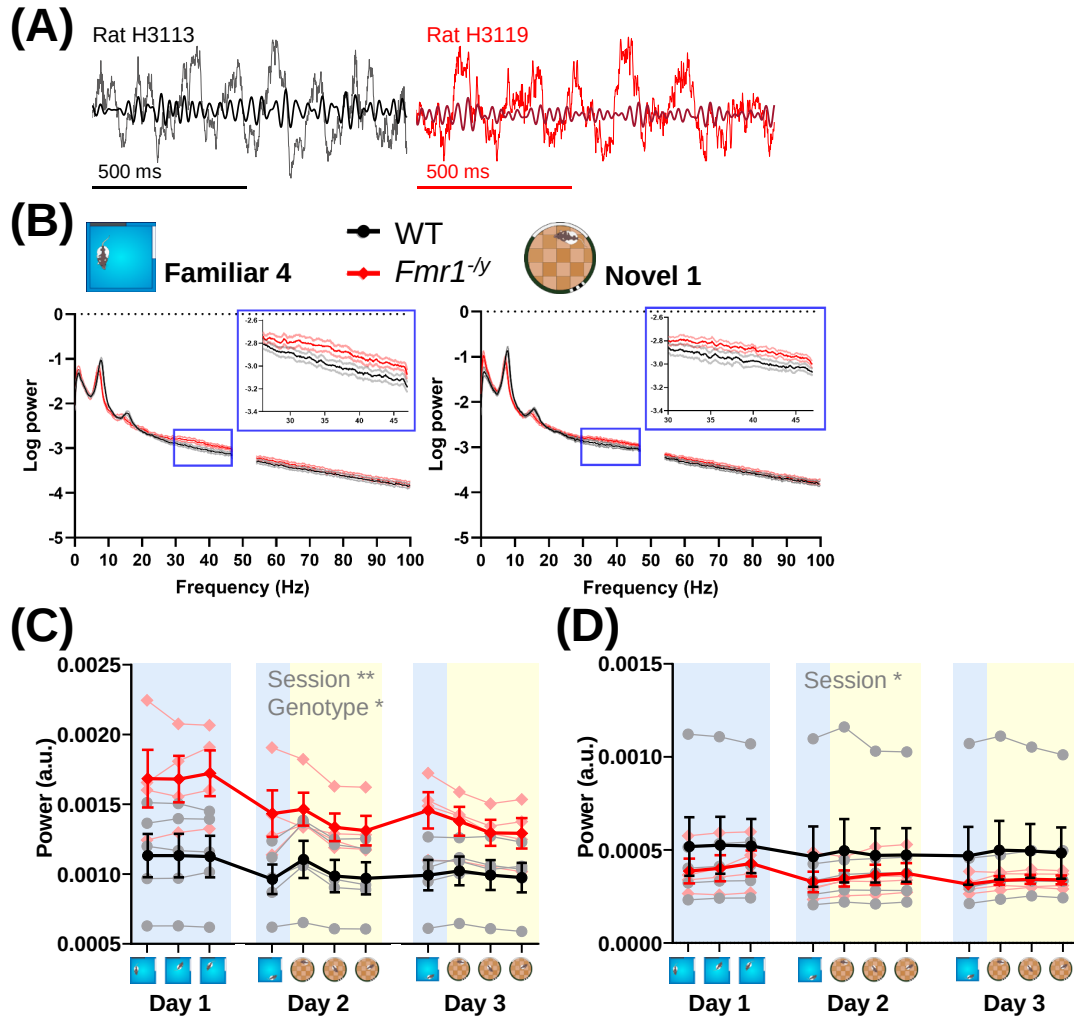


FIGURE 4.5: *Fmr1*<sup>-/-</sup> rats have increased low gamma (27-47 Hz) power in the hippocampus. **(A)** Example 1-second periods of LFP from one WT and one *Fmr1*<sup>-/-</sup>, with low gamma bandpass (27-47 Hz) of the signal overlaid. **(B)** Average spectrograms as in Figure 4.4A, but blue boxes here show the low gamma range. **(C)** Average power in the low gamma range for each session in the novel environment protocol. Individual rat values, and group means  $\pm$  SEM are shown (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(D)** Average power in the high gamma range for each session. Individual rat values, and group means  $\pm$  SEM are shown (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ .

of the theta oscillation at the time of the peak of the burst was found. Figure 4.6A shows examples of low gamma bursts where the amplitudes of their oscillation peak during the descending phase of the theta oscillation, as has been observed previously (Colgin et al., 2009). From the identified peaks, the mean theta phase of gamma bursts from each rat for each session was calculated, as well as the resultant vector length of their distribution. A modulation index (MI) was also calculated from the gamma burst time windows (Tort et al., 2008), which is a measure of the strength of the modulation. Figure 4.6B shows probability distributions across the phase of the theta cycle ( $-\pi/+ \pi$  represents the trough of the theta oscillation, with the peak at 0), binned into 18 equal bins. The bars represent the mean  $\pm$  SEM probability for all rats of each genotype. There is clear evidence that low gamma amplitude is modulated by the theta oscillation in both genotypes, with peaks in low gamma amplitude more likely to occur at the descending phase of the theta oscillation. The strength of this modulation, measured using MI, showed no significant main effect of genotype ( $F(1,7) = 1.27$ ,  $p = 0.296$ ), but there was a significant main effect of session ( $F(1.7,12.0) = 11.3$ ,  $p = 0.002$ ), with no significant interaction ( $F(10,70) = 0.70$ ,  $p = 0.721$ ) (Figure 4.7A). This session effect likely came from an apparent reduction in the MI in the novel environment compared to the familiar; however, post hoc tests found no differences. There similar levels of theta-low gamma modulation across the familiar sessions between days (Familiar 1 vs Familiar 4:  $p > 0.999$ ; Familiar 4 vs Familiar 5:  $p > 0.999$ ). The reduction seen in the first novel session was not significant (Familiar 4 vs Novel 1:  $p = 0.150$ ). There were also no differences in the novel sessions across days (Novel 1 vs Novel 4:  $p > 0.999$ ) or within day 2 (Novel 1 vs Novel 3:  $p = 0.162$ ).

Comparison of the mean phases of gamma peaks for each rat showed no clear difference between genotypes, but there was an apparent session effect in this measure as well, showing a shift to later phases of the theta oscillation in the novel environment compared to the familiar (Figure 4.7B). A Harrison-Kanji test reflected this, showing no significant main effect of genotype ( $F(1,7) = 0.19$ ,  $p = 0.666$ ), but a significant main effect of session ( $F(10,77) = 4.6$ ,  $p < 0.0001$ ), with no significant interaction ( $F(10,77) = 1.2$ ,  $p = 0.328$ ). Post hoc Watson-Williams tests found that mean phase of low gamma oscillations was similar between familiar sessions of each day (Familiar 1 vs Familiar 4:  $p > 0.999$ ; Familiar 4 vs Familiar 5:  $p > 0.999$ ). There was a novelty effect, with the mean phase of low gamma oscillation peaks shifting to a later phase of the theta oscillation in the novel session compared to the previous familiar session (Familiar 4 vs Novel 1:  $p = 0.039$ ). There was no

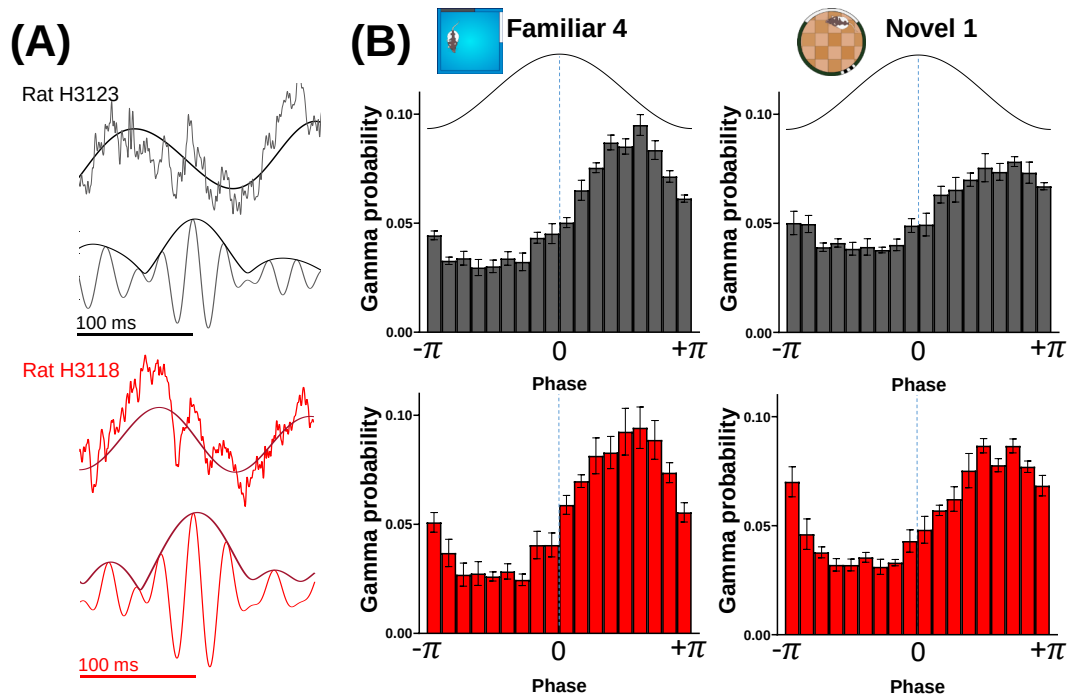


FIGURE 4.6: Both genotypes show phase-locking of low gamma events to the theta oscillation in the hippocampus. **(A)** Example low gamma events from one WT rat (black, above) and one *Fmr1*<sup>-/-</sup> rat (red, below). The top trace from each rat shows the raw signal in the paler colour, overlaid with the theta-filtered signal in bold; the trace below shows the same time period, but filtered in the low gamma band (paler colour), with its instantaneous amplitude shown by the bold line. The increase in the gamma amplitude occurs just before the trough of the theta oscillation in both examples. **(B)** Probability of low gamma event peaks occurring within bins of the theta oscillation phase, in Familiar 4 and Novel 1.  $-\pi/+ \pi$  represent the trough of the theta oscillation, and 0 represents the peak (as shown by the schematic wave cycle shown above). Bars are group means  $\pm$  SEM. (WT:  $n = 5$ , black; *Fmr1*<sup>-/-</sup>:  $n = 4$ , red).

change in mean phase in the final novel session of the day (Novel 1 vs Novel 3:  $p > 0.99$ ). Although there was less of a shift in the mean phase on day 3, there was no difference from the first novel session of day 2 (Novel 1 vs Novel 4:  $p > 0.999$ ).

High gamma bursts were also modulated by the theta phase in both genotypes. Periods of gamma oscillations with peaks around the mean phase for each genotype are shown in Figure 4.8A, and probability distributions shown in Figure 4.8B. From this, it appeared that high gamma bursts in *Fmr1*<sup>-/-</sup> rats occurred more towards the peak of the theta oscillation than in WT rats. While modulation indices of high gamma were slightly lower in *Fmr1*<sup>-/-</sup> rats compared to WT, there was no significant main effect of genotype ( $F(1,7) = 2.26$ ,  $p = 0.176$ ) (Figure 4.9A). There

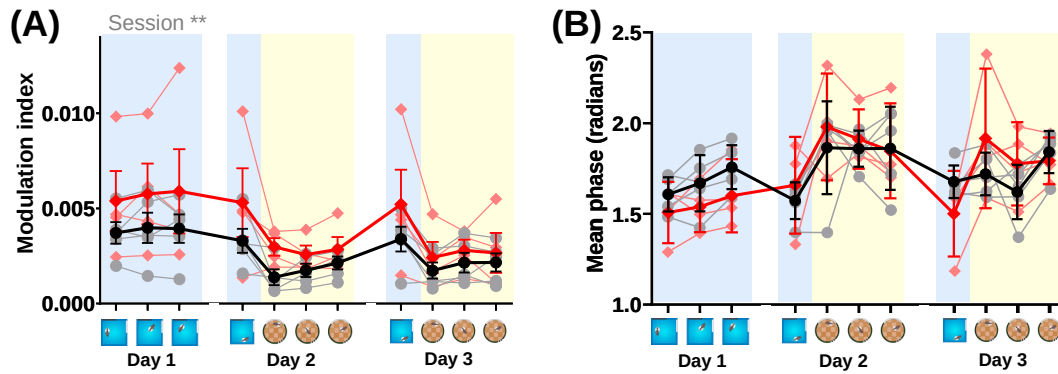


FIGURE 4.7: There was no difference in theta-low gamma phase-amplitude coupling between genotypes. **(A)** Modulation index calculated from the theta phase during high gamma event windows from each session, showing values from each rat and group means  $\pm$  SEM (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(B)** The mean phase of low gamma events in each session, from each rat, and circular mean  $\pm$  95% confidence intervals for each group (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*\*  $p < 0.01$ .

was, however, a significant main effect of session ( $F(3.0,21.3) = 5.5$ ,  $p = 0.0055$ ), as well as a significant genotype  $\times$  session interaction ( $F(10,70) = 2.0$ ,  $p = 0.046$ ). To assess the effects of the novelty, the familiar sessions were first compared – a consistent level of theta-high gamma modulation was observed in the familiar sessions across days for both genotypes (Familiar 1 vs Familiar 4: WT:  $p > 0.999$ , *Fmr1*<sup>-/-</sup>:  $p > 0.999$ ; Familiar 4 vs Familiar 5: WT:  $p > 0.999$ , *Fmr1*<sup>-/-</sup>:  $p > 0.999$ ). There was an effect of novelty in the WT group, with the strength of modulation significantly decreased in the novel environment (Familiar 4 vs Novel 1: WT:  $p = 0.0009$ ). However, this difference was not significant for the *Fmr1*<sup>-/-</sup> group (Familiar 4 vs Novel 1: WT:  $p > 0.999$ ). The WT group did not show any within-day habituation to novelty in this effect, with modulation remaining low for the novel sessions of day 2 (Novel 1 vs Novel 3: WT:  $p > 0.999$ ). There was less of a reduction in theta-high gamma MI on day 3, however, the difference between the two initial novel sessions was not significant (Novel 1 vs Novel 4: WT:  $p = 0.336$ ). In the *Fmr1*<sup>-/-</sup> group, none of the comparisons were significant (Familiar 4 vs Novel 1: *Fmr1*<sup>-/-</sup>:  $p > 0.999$ ; Novel 1 vs Novel 3: *Fmr1*<sup>-/-</sup>:  $p > 0.999$ ; Novel 1 vs Novel 4: *Fmr1*<sup>-/-</sup>:  $p = 0.336$ ).

The main difference between the genotypes appeared to be in the theta phase where high gamma bursts occurred (Figure 4.9B). In Familiar 4, the WT mean phase, calculated by finding the mean of the mean phases of high gamma events for each rat, was 1.65 radians (95% CI [1.30,2.01]), while the *Fmr1*<sup>-/-</sup> mean was 0.82 radians (95% CI [-0.21,1.85]). A Harrison-Kanji test was used to compare

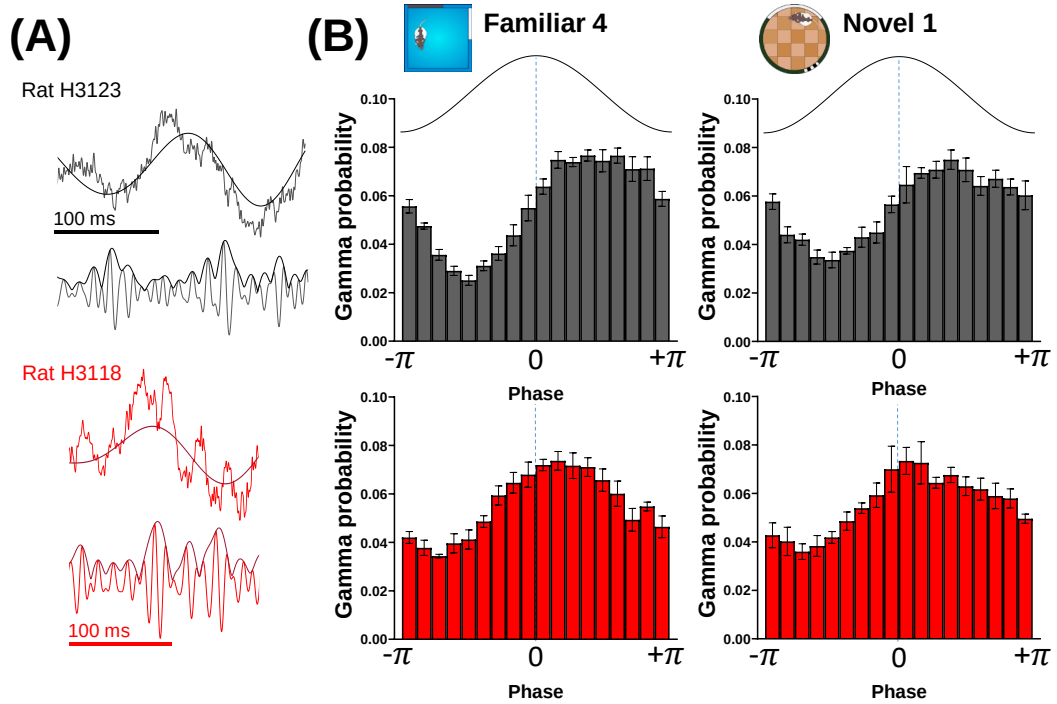


FIGURE 4.8: Both genotypes show evidence of theta-high gamma phase-amplitude coupling. **(A)** Example traces from one WT and one *Fmr1*<sup>-/-</sup> rat, showing raw signal with theta bandpass in bold, and high gamma (60-100 Hz) band-pass, with its amplitude in bold. **(B)** Average probability of high gamma event peaks occurring within bins of the theta oscillation. Bars represent means  $\pm$  SEM (WT:  $n = 5$ , black; *Fmr1*<sup>-/-</sup>:  $n = 4$ , red).

mean phases across sessions, and there was a significant main effect of genotype ( $F(1,7) = 40.0$ ,  $p < 0.0001$ ), with no significant effect of session ( $F(10,77) = 0.76$ ,  $p = 0.668$ ) or interaction ( $F(10,77) = 0.24$ ,  $p = 0.992$ ).

#### 4.3.4 *Fmr1*<sup>-/-</sup> rats had higher broad gamma power in the mPFC

Analysis of the power and timing of mPFC LFP oscillations was then performed using the same methods, to determine whether there were differences in mPFC network dynamics between groups and/or across the sessions. Example mPFC LFP traces from two rats are shown in Figure 4.10A, where theta oscillations can also be observed during active behaviour, although with lower power than in the hippocampus. This is discernible in the example spectrograms in Figure 4.10B at around 8 Hz, at certain points of the session. Analysis of theta power from the averaged spectrograms (Figure 4.11A) across sessions (Figure 4.11B) indicated

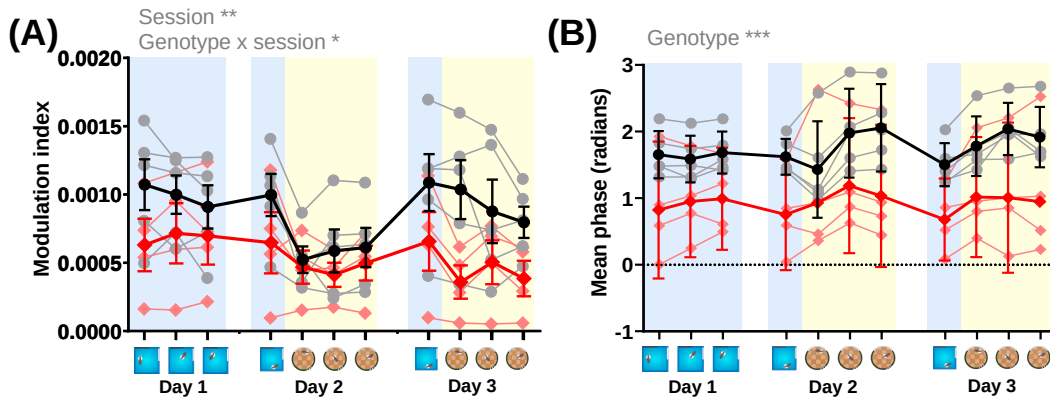


FIGURE 4.9: High gamma events are locked to an earlier phase of the theta oscillation in *Fmr1*<sup>-/-</sup> rats. **(A)** Modulation index calculated from theta phase during high gamma event windows from each session, showing values from each rat and group means  $\pm$  SEM (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(B)** Mean phase of high gamma event peaks from each rat and circular means  $\pm$  95% confidence intervals (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

that there was no significant main effect of genotype ( $F(1,7) = 0.62$ ,  $p = 0.467$ ), or session ( $F(2.7,18.6) = 1.10$ ,  $p = 0.37$ ) and the session  $\times$  genotype interaction was also not significant ( $F(10,70) = 0.88$ ,  $p = 0.555$ ).

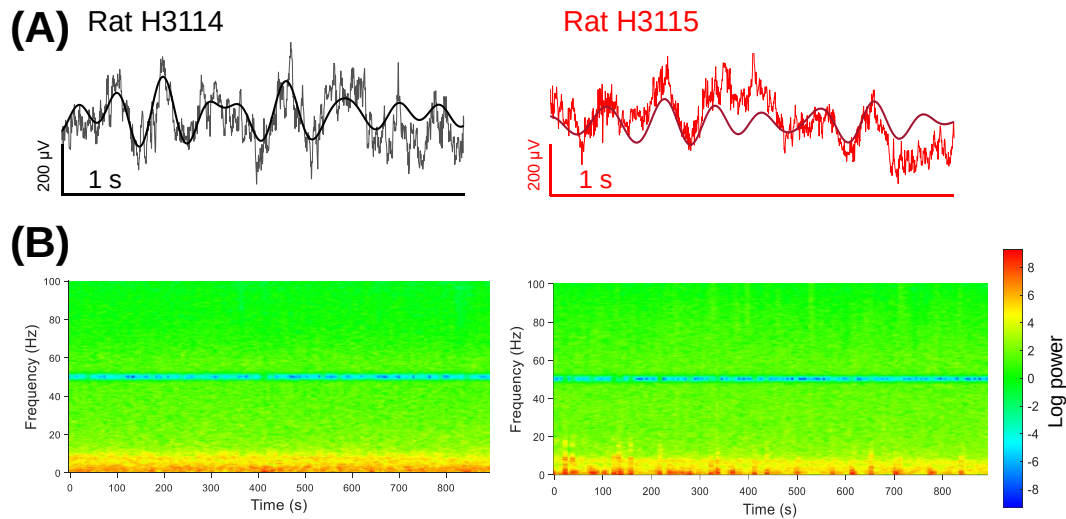


FIGURE 4.10: Example traces and spectrograms from mPFC LFP. **(A)** Example LFP traces from the mPFC of one WT rat (black, left) and one *Fmr1*<sup>-/-</sup> rat (red, right), with the theta-filtered signal overlaid. **(B)** Example mPFC spectrograms from the same two rats.

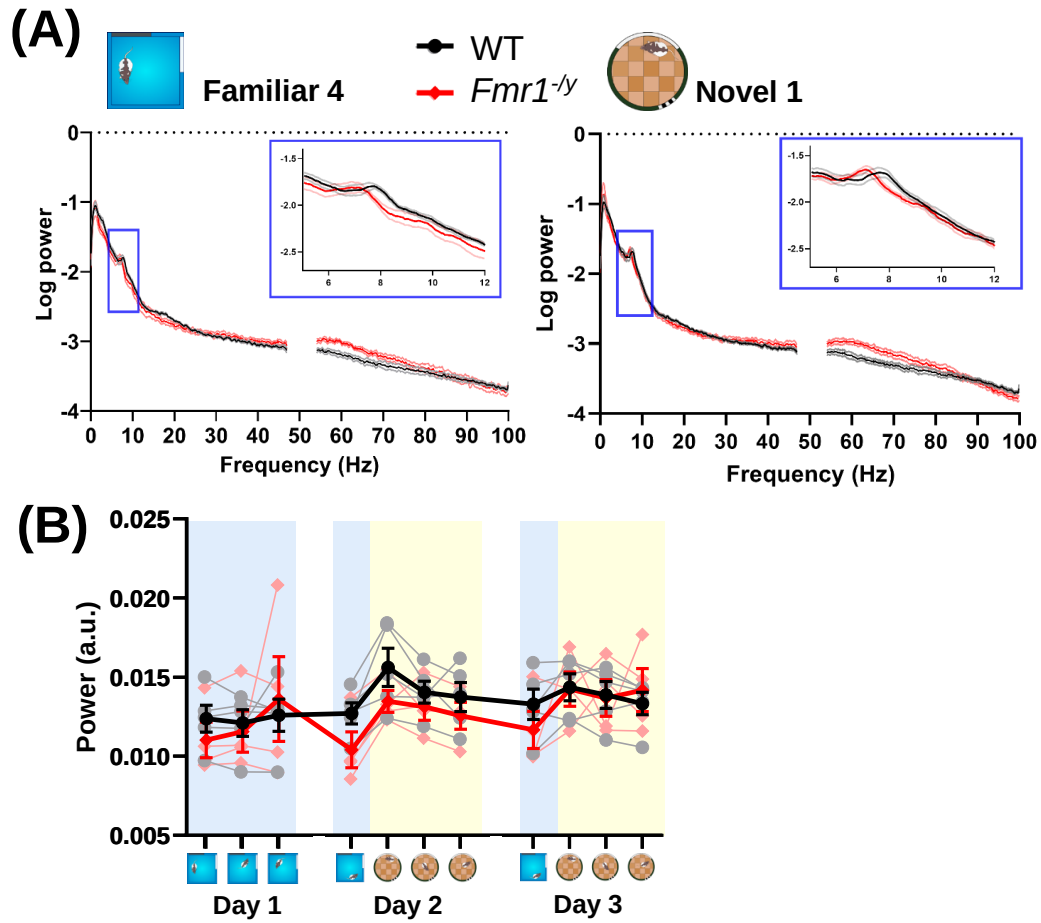


FIGURE 4.11: mPFC theta power did not differ between genotypes. **(A)** Average spectrograms from Familiar 4 (left) and Novel 1 (right), showing the theta range in the blue box. **(B)** Average theta power in each session for each rat (pale colours) and group means  $\pm$  SEM (bold lines) (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ).

Gamma oscillations are detected in the mPFC and have been linked to selective attention and cognitive flexibility (Cho et al., 2015; Kim et al., 2016). Example 1-second mPFC LFP traces are shown in Figure 4.12A, overlaid with the signal filtered in the broad gamma range (30-80 Hz). From the average spectrograms, it is clear that *Fmr1*<sup>-/-</sup> rats exhibit higher power in the 30-80 Hz cortical gamma range (Figure 4.12B). Averages for each session were compared and there was a significant main effect of genotype ( $F(1,7) = 32.3$ ,  $p = 0.0007$ ), but no significant effect of session ( $F(1.7,11.7) = 2.4$ ,  $p = 0.135$ ) or genotype  $\times$  session interaction ( $F(10,70) = 0.51$ ,  $p = 0.880$ ).

Averaging mPFC theta and gamma power over running velocity bins showed that the effects seen were unlikely to be due to any differences in movement between

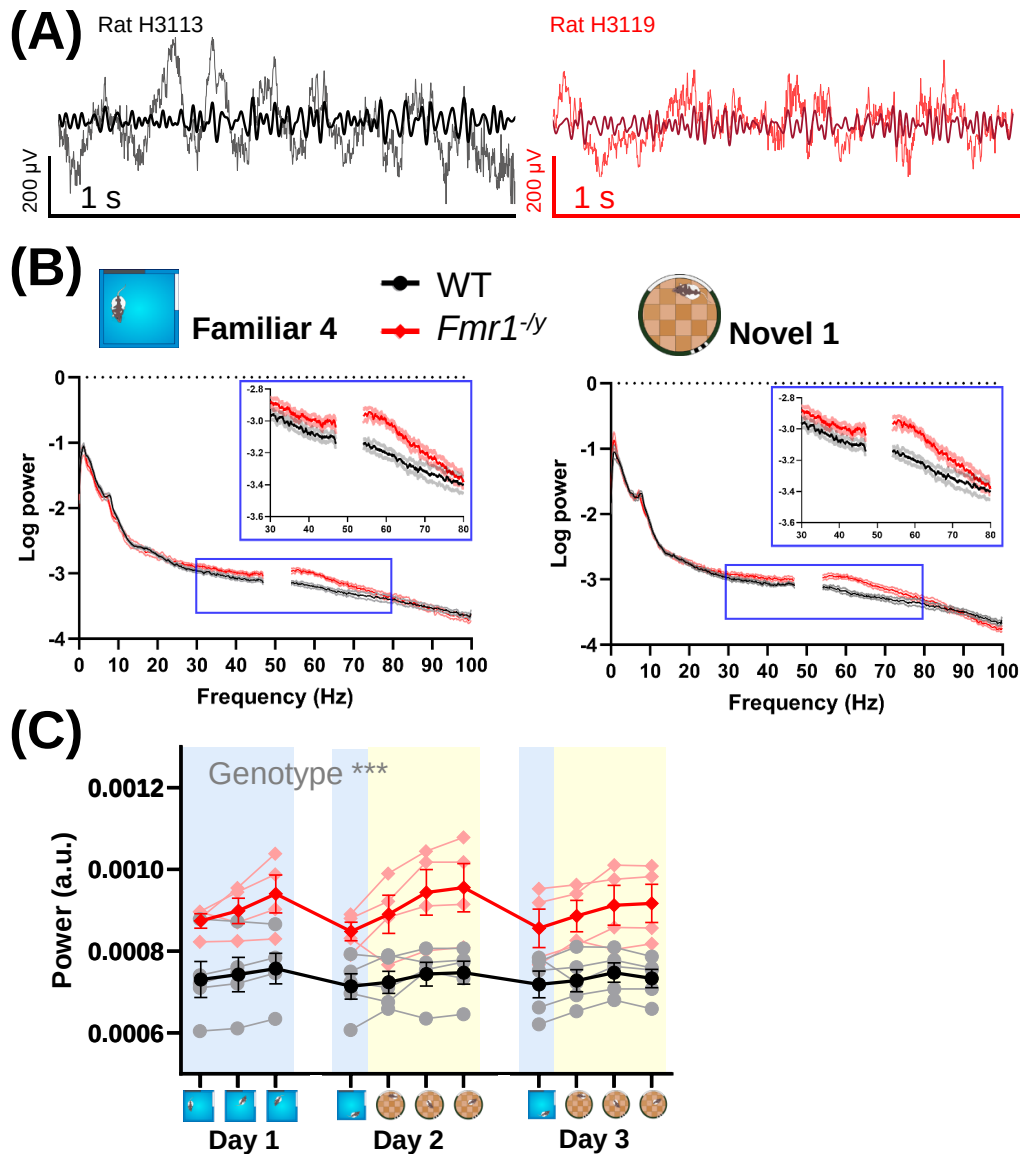


FIGURE 4.12: mPFC gamma power was higher in *Fmr1*<sup>-/-</sup> rats. **(A)** Example mPFC LFP traces from one WT rat (black, left) and one *Fmr1*<sup>-/-</sup> rat (red, right) overlaid with the broad gamma (30-80 Hz) bandpass-filtered signal. **(B)** Average mPFC spectrograms as in Figure 4.11A, but here the blue box shows the broad gamma range. **(C)** Average broad gamma power from each session for each rat, and group means  $\pm$  SEM (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*\*\*  $p < 0.001$ .

the genotypes, as theta power was similar between genotypes across all velocities, and gamma power was consistently higher in *Fmr1*<sup>-/-</sup> rats (Appendix B, Figure B.1B-C).

Phase amplitude coupling is also observed in the mPFC. Bursts in the broad gamma range (30-80 Hz) were found to be more likely to occur in the descending phase of the mPFC theta oscillation, although this was not a very strong modulation. Looking at bursts in the high gamma range, however, coupling was much stronger, with bursts occurring nearer the peak of the theta oscillation, as illustrated by the examples in Figure 4.13A, and by the probability distributions in Figure 4.13B. No significant main effect of genotype was found on theta-high gamma MI ( $F(1,7) = 0.035$ ,  $p = 0.856$ ), or session ( $F(1,7) = 2.89$ ,  $p = 0.053$ ), or for the genotype x session interaction ( $F(10,70) = 0.71$ ,  $p = 0.715$ ) (Figure 4.14C). A Harrison-Kanji test comparing the mean theta phase of the gamma oscillation peaks across sessions did find a significant main effect of genotype ( $F(1,7) = 6.53$ ,  $p = 0.016$ ), and a significant genotype x session interaction ( $F(10,70) = 2.72$ ,  $p = 0.006$ ), with no main effect of session ( $F(10,77) = 1.4$ ,  $p = 0.213$ ) (Figure 4.14D). However, for most of the sessions mean phases were very similar between genotypes, and the only post hoc between-genotype tests that were close to significance were for the final two sessions, where two of the *Fmr1*<sup>-/-</sup> rats showed large changes in mean phase (Uncorrected  $p$  values for the day 3 sessions were: Familiar 5:  $p = 0.456$ , Novel 4:  $p = 0.339$ , Novel 5:  $p = 0.035$ , Novel 6:  $p = 0.034$ ). These rats showed low modulation indices for these sessions, likely making estimation of mean phase unreliable, therefore, it is unlikely that there is a real difference between the groups in theta-high gamma PAC in the mPFC.

#### **4.3.5 mPFC-hippocampus gamma synchrony was increased in *Fmr1*<sup>-/-</sup> rats, and theta synchrony was decreased**

In order to assess functional connectivity between the mPFC and hippocampus, coherence of the oscillations recorded in the two brain areas was analysed – greater coherence is associated with enhanced connectivity between the regions. The first measure analysed was spectral coherence, a measure of correlation between signals at specific oscillation frequencies, with 0 indicating low correlation of the two signals, and 1 indicating high correlation. Spectral coherence was

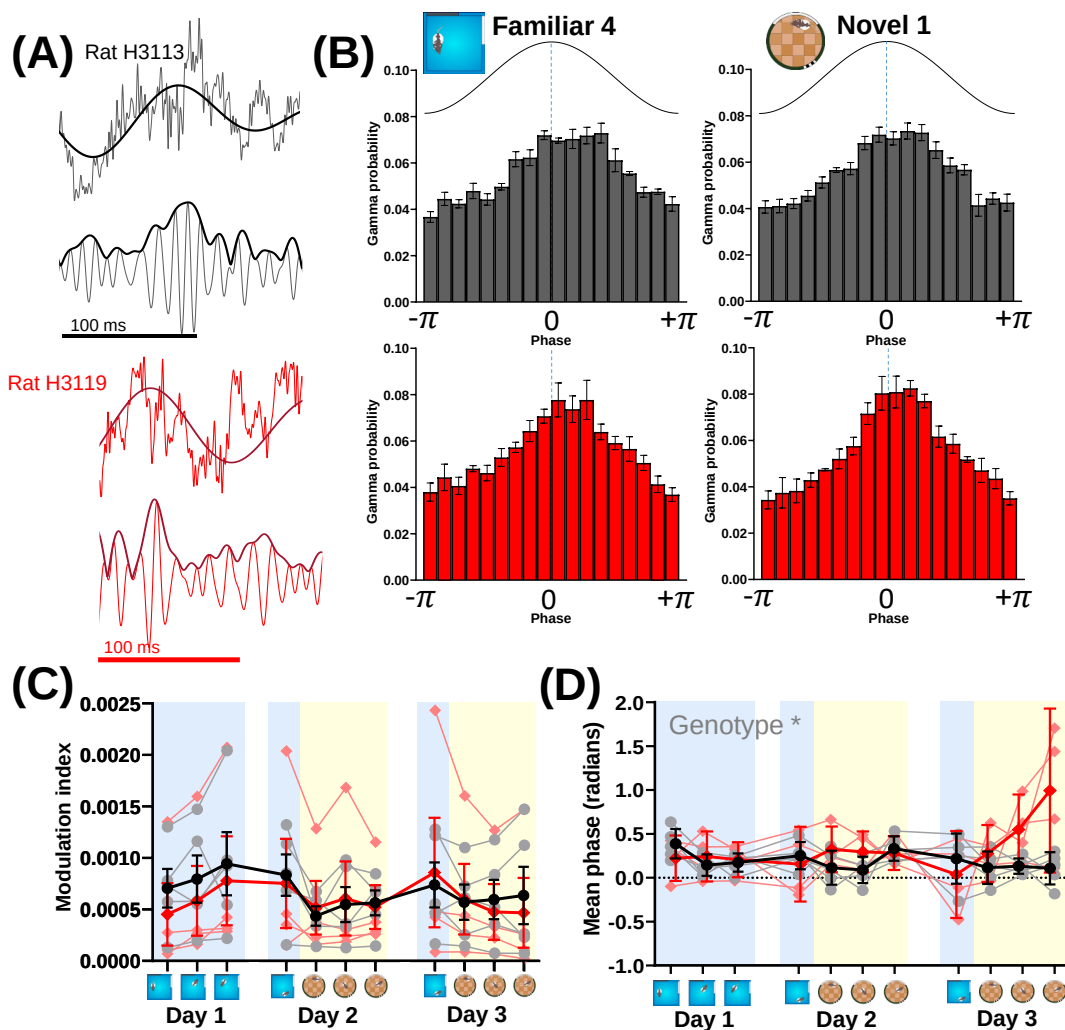


FIGURE 4.13: Both genotypes show theta-high gamma phase-amplitude coupling. **(A)** Example high gamma events in the mPFC, showing the raw LFP trace with the theta bandpass filter overlaid in bold, above the high gamma-filtered signal with its amplitude in bold. **(B)** Probability of high gamma event peaks occurring in bins of the theta oscillation. Bars show group means  $\pm$  SEM. **(C)** Theta-high gamma modulation index calculated from high gamma event windows from each session, for each rat and group means  $\pm$  SEM in bold (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(D)** Mean theta phases of high gamma event peaks for each rat in each session, and group circular means  $\pm$  95% confidence intervals (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*  $p < 0.05$ .

calculated using a sliding 2-second time window across each 15-minute session (see methods). There were peaks in the theta range in the average spectral coherograms (Figure 4.14A). There was no significant main effect of genotype when comparing average theta spectral coherence across sessions ( $F(1,7) = 1.73$ ,  $p = 0.229$ ), but there was a significant main effect of session ( $F(3.1,21.5) = 5.8$ ,  $p = 0.0043$ ), with no significant interaction ( $F(10,70) = 0.47$ ,  $p = 0.902$ ) (Figure 4.14B).

### 4.3. Results

Increases in theta coherence between the mPFC and hippocampus have previously been associated with novelty. However, the planned comparison post hoc tests did not reveal a significant difference between Familiar 4 and Novel 1 ( $p = 0.479$ ), or any of the other tests (Familiar 1 vs Familiar 4:  $p = 0.111$ ; Familiar 4 vs Familiar 5:  $p > 0.999$ ; Novel 1 vs Novel 3:  $p > 0.999$ ; Novel 1 vs Novel 4:  $p > 0.999$ ), suggesting there was no clear effect of novelty on theta coherence.

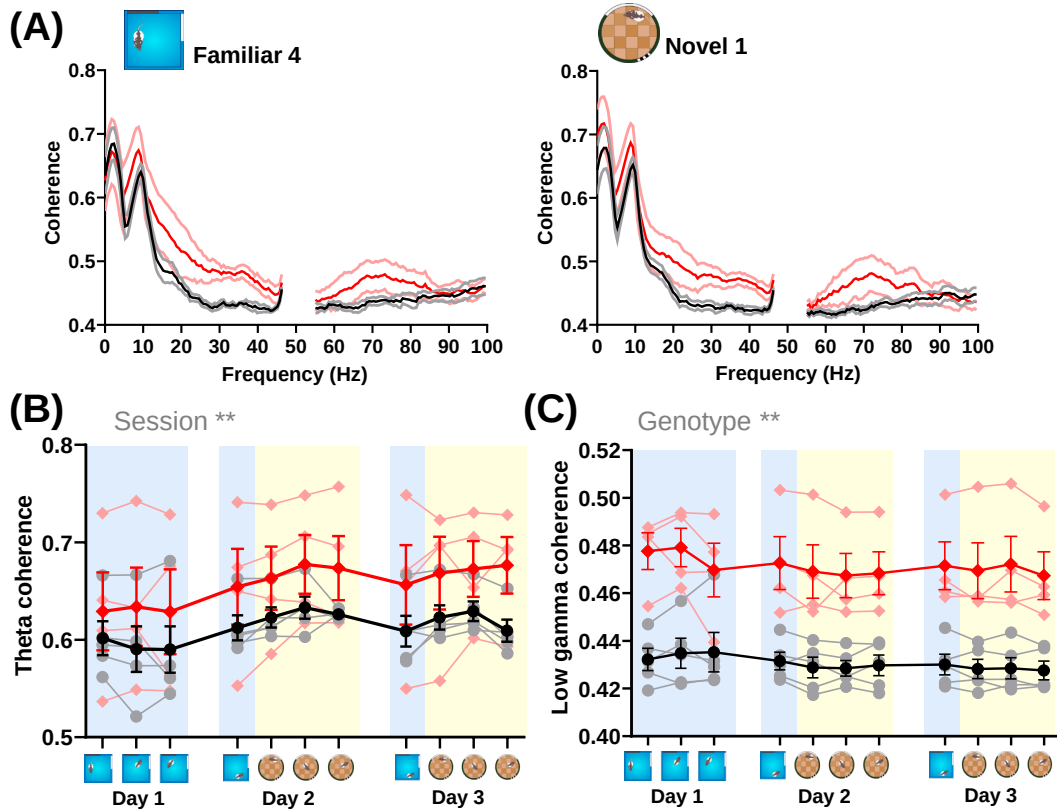


FIGURE 4.14: *Fmr1*<sup>-/-</sup> rats showed increased mPFC-dCA1 low gamma spectral coherence compared to WT. **(A)** Spectral coherograms averaged over times when the rats were moving, and within each genotype. Bold lines show genotype means, pale lines indicate  $\pm$  SEM. **(B)** Average theta spectral coherence in each session, showing each rat and genotype means  $\pm$  SEM (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(C)** Average low gamma (30-47 Hz) spectral coherence for each session, for each rat, and genotype means  $\pm$  SEM (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*\*  $p < 0.01$ .

The most obvious difference between the two genotypes in the spectral coherograms in Figure 4.14A was an increase in the gamma range in the *Fmr1*<sup>-/-</sup> rats. This was consistent across the sessions, with no obvious changes between sessions. Comparison of broad gamma spectral coherence found no significant main effect of genotype ( $F(1,7) = 5.1$ ,  $p = 0.058$ ), or session ( $F(2.6,18.4) = 2.0$ ,  $p =$

0.158), and no significant genotype x session interaction ( $F(10,70) = 0.37$ ,  $p = 0.954$ ). However, there was a significant main effect of genotype when only comparing the lower gamma frequency range of 30-47 Hz ( $F(1,7) = 18.2$ ,  $p = 0.0037$ ), with no significant main effect of session ( $F(2.5,17.4) = 1.7$ ,  $p = 0.213$ ) and no significant interaction ( $F(10,70) = 0.43$ ,  $p = 0.925$ ) (Figure 4.14C).

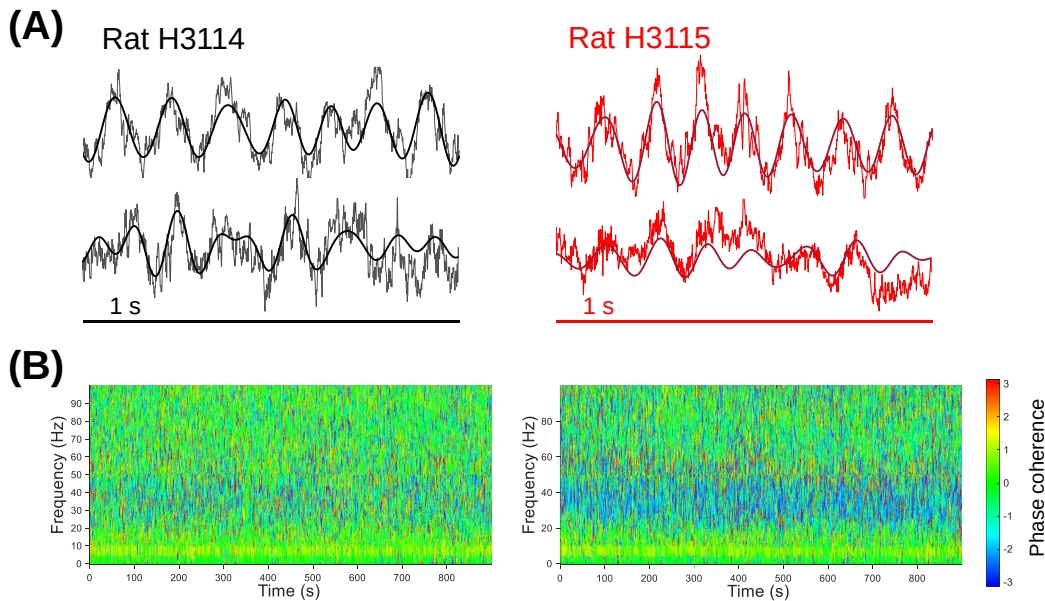


FIGURE 4.15: Example mPFC-dCA1 phase coherograms. **(A)** Example simultaneous LFP traces from one WT rat (black, left), and one *Fmr1*<sup>-/-</sup> rat (red, right), showing a period of high phase coherence in the theta oscillation (overlaid in bold). The dCA1 signal is above and the mPFC signal is below. **(B)** Example phase coherograms from the same two rats.

Spectral coherence between two regions can be affected by volume conduction of signals from a common source, as well as being affected by changes in power at a common reference, so measures of connectivity that are less affected by this confound were calculated (Nolte et al., 2004). One of these is phase/imaginary coherence, calculated using the imaginary component of the cross spectrum of the two signals. The example traces in Figure 4.15A are selected periods of high theta phase coherence – in these periods, the hippocampal signal (above) leads the mPFC signal (below) at relatively consistent delays. Example phase coherograms are shown in Figure 4.15B; the absolute value of the phase coherence indicates the strength of the phase relationship, and its sign indicates which signal is leading, based on the phase differences. Figure 4.16A shows phase coherograms averaged across times when rats were moving, for Familiar 4 and Novel 1. Here

### 4.3. Results

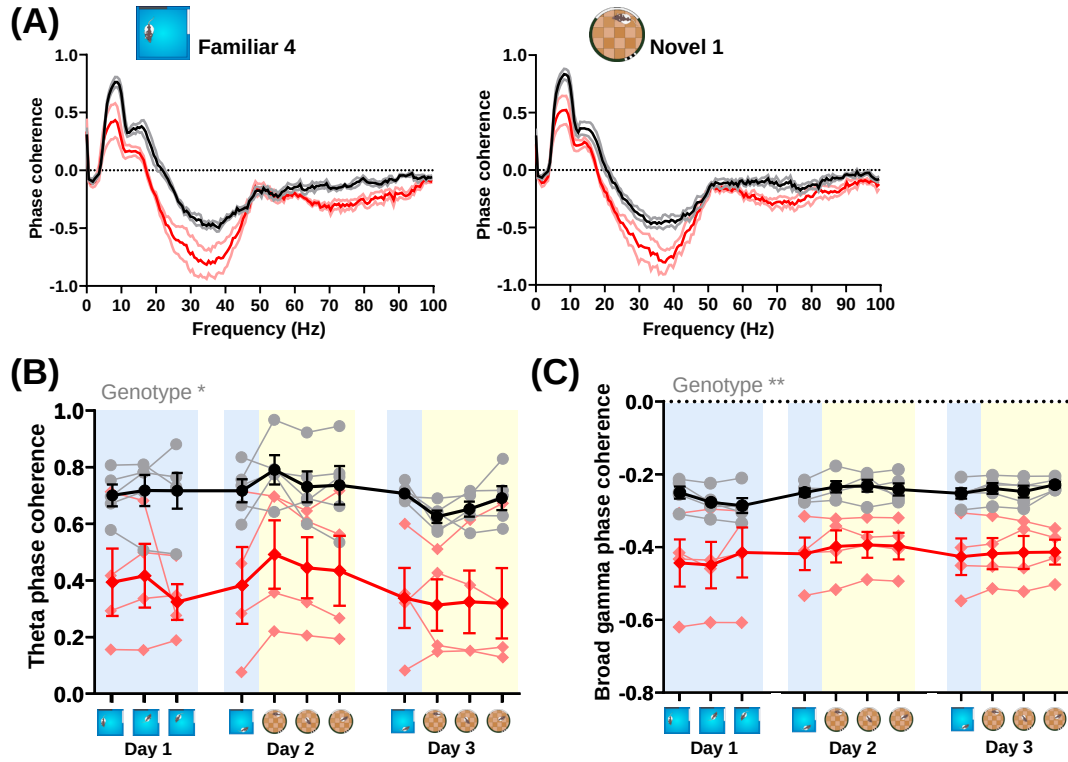


FIGURE 4.16: Theta phase coherence is lower in *Fmr1*<sup>-/-</sup> rats, and low gamma phase coherence is higher. **(A)** Average phase coherence from Familiar 4 and Novel 1. **(B)** Average theta phase coherence in each session, shown for each rat, and genotype means  $\pm$  SEM. **(C)** Average gamma phase coherence in each session, for each rat and genotype means  $\pm$  SEM (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ .

theta phase coherence appears lower in the *Fmr1*<sup>-/-</sup> rats. Averages across sessions, shown in Figure 4.16B, showed that *Fmr1*<sup>-/-</sup> rats were more variable than WT, but overall had lower theta phase coherence, and there was a significant main effect of genotype ( $F(1,7) = 6.8$ ,  $0.035$ ). There was no significant main effect of session in theta phase coherence ( $F(2.1,14.5) = 2.64$ ,  $p = 0.104$ ) and the session  $\times$  genotype interaction was not significant ( $F(10,70) = 0.39$ ,  $p = 0.945$ ). Theta phase coherence values were positive in both genotypes, indicating that the hippocampal signal was generally leading the mPFC signal. In contrast, average gamma phase coherence was negative in both genotypes, suggesting an overall lead by the mPFC signal (Figure 4.16B). Consistent with spectral coherence, the strength of the gamma phase coherence was greater in the *Fmr1*<sup>-/-</sup> rats compared to WT, with a significant main effect of genotype ( $F(1,7) = 14.5$ ,  $p = 0.0067$ ), no significant main effect of session ( $F(2.3,15.9) = 3.5$ ,  $p = 0.051$ ), and no significant genotype  $\times$  session interaction ( $F(10,70) = 1.0$ ,  $p = 0.456$ ).

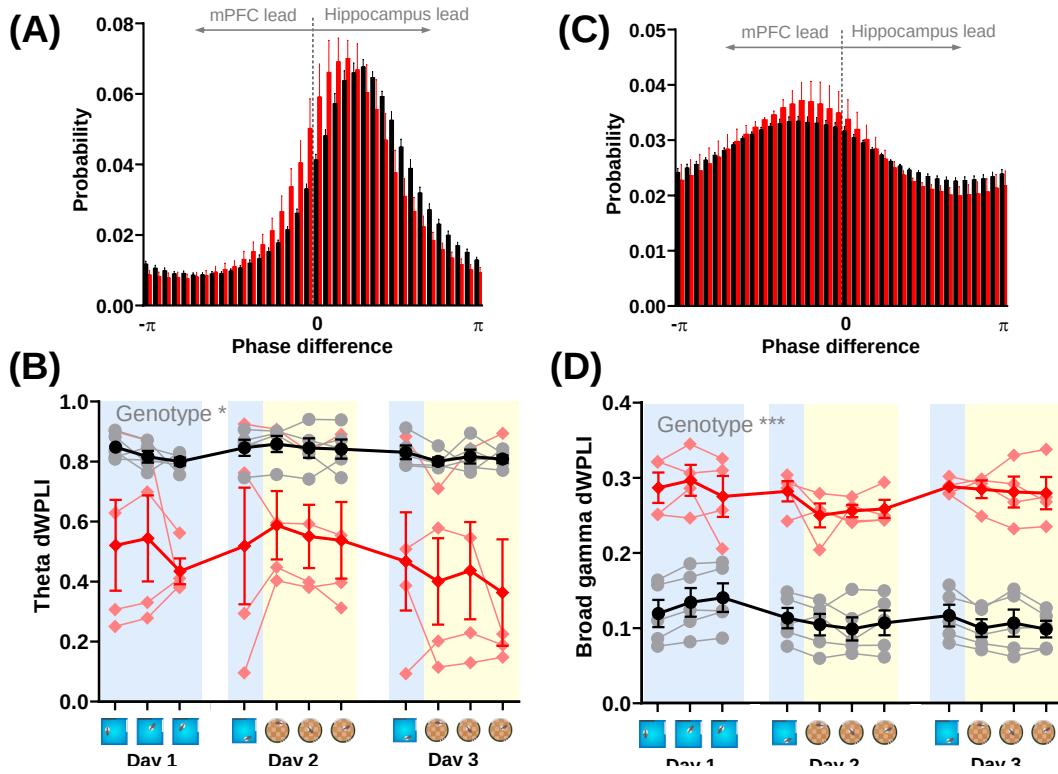


FIGURE 4.17: Gamma coherence is increased in *Fmr1*<sup>-/-y</sup> measured using debiased weighted phase lag index. **(A)** Probability distribution of phase differences between the mPFC and dCA1 theta oscillations in Novel 1. Values greater than 0 indicate that the dCA1 signal is leading the mPFC signal. Bars indicate genotype means  $\pm$  SEM. **(B)** Average dCA1-mPFC theta debiased weighted phase lag index (dWPLI) for each session, showing values from each rat and genotype means  $\pm$  SEM (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-y</sup>, red,  $n = 4$ ). **(C)** Probability distribution of phase differences between the mPFC and dCA1 broad gamma oscillations in Novel 1. Values less than 0 indicate that the mPFC signal is leading the dCA1 signal. **(D)** Broad gamma dWPLI for each session, for each rat, and showing means  $\pm$  SEM for each genotype (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-y</sup>, red,  $n = 4$ ).

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As the phase coherence is still normalised by amplitude of the signals, phase synchrony measures independent of signal power were calculated (Vinck et al., 2011). Firstly, to measure directionality and lag of the synchrony, phase difference of the Hilbert-transformed signals was found at each sampling point in each session. It should be noted that, due to the symmetrical nature of oscillations, these measures of signal directionality are only estimates, and neuronal spiking would be a more reliable determinate. Figure 4.17A shows the binned probability distribution of theta phase differences in Novel 1. Both groups show an overall lead by the hippocampal signal (positive values), with the mPFC lagging by a mean of 0.96 radians (95% CI [0.88,1.05]) in the WT in Novel 1, corresponding to a time lag of around 38.2 ms for an 8 Hz theta oscillation. This delay is similar to values observed previously for theta coherence between these regions (Siapas et al., 2005; Place et al., 2016; Wang et al., 2021). The *Fmr1*<sup>-/-</sup> showed more variable phase differences, likely due to the reduced theta coherence, but still showed an overall lead of the hippocampus over the mPFC. Secondly, the de-biased weighted phase lag index (dWPLI), a quantification of how consistent phase delays were between the two regions, was calculated for each frequency window in the cross spectrum, and averaged over time when the rats were moving above the speed threshold. This measure shows that theta phase synchrony was higher in the WT group than in the *Fmr1*<sup>-/-</sup> rats, which also appeared much more variable (Figure 4.17B). Comparing between the two genotypes across sessions, there was a significant main effect of genotype ( $F(1,7) = 8.74$ ,  $p = 0.021$ ) but the main effect of session ( $F(2.9, 20.4) = 2.1$ ,  $p = 0.133$ ) and the session x genotype interaction ( $F(10,70) = 0.81$ ,  $p = 0.621$ ) were not significant.

Using these same measures, gamma phase synchrony was assessed. Phase difference distributions showed that the mPFC gamma frequency (30-80 Hz) phase generally led the hippocampus (Figure 4.17C). Gamma phase synchrony measured using dWPLI was consistently higher in the *Fmr1*<sup>-/-</sup> group across sessions, showing a significant main effect of genotype ( $F(1,7) = 79.3$ ,  $p < 0.0001$ ), and no significant main effect of session ( $F(1.7,12.2) = 2.6$ ,  $p = 0.120$ ) and no significant interaction ( $F(10,70) = 0.986$ ,  $p = 0.464$ ).

## 4.4 Discussion

This experiment aimed to determine whether network activity within and functional connectivity between mPFC and hippocampus is affected in *Fmr1*<sup>-y</sup> rats. The main findings of the data were: (1) consistently elevated mPFC gamma power in *Fmr1*<sup>-y</sup> rats compared to WTs; (2) increased mPFC-hippocampus coherence in the gamma range in *Fmr1*<sup>-y</sup> rats, indicating hyper-synchronisation of the two networks; (3) possible alterations in the effect of novelty and novelty habituation on theta-high gamma PAC in the hippocampus. Previous studies have found increased gamma power and synchrony in other cortical regions of *Fmr1*<sup>-y</sup> mice, rats, and FXS patients. The current experiment extends this to the mPFC, and finds that connectivity with the hippocampus may be altered as well. These changes may underlie deficits in memory and cognitive flexibility.

### 4.4.1 Gamma band network abnormalities as a cross-species biomarker for FXS

The strongest and most consistent difference between the genotypes in this experiment was increased power in the gamma band in the mPFC. This adds to findings of raised gamma power in auditory and frontal cortex EEG in both *Fmr1*<sup>-y</sup> mice and rats (Sinclair et al., 2017; Lovelace et al., 2018; Kozono et al., 2020; Wong et al., 2020). Importantly, this phenotype has also been seen across multiple studies of FXS patients (Ethridge et al., 2016; Wang et al., 2017; Ethridge et al., 2019; Proteau-Lemieux et al., 2021). Ethridge et al. (2016) also went on to show a lack of synchronisation of the auditory cortex gamma oscillation to auditory stimuli, indicating how dysregulation of gamma may impact on computational processes (Ethridge et al., 2016; Ethridge et al., 2019). This same deficit was then reproduced in the *Fmr1*<sup>-y</sup> mouse model (Lovelace et al., 2018). Increased cortical gamma power therefore represents a reliable cross-species biomarker that could potentially be used to assess treatments in the animal models.

It is not clear what causes increased power of cortical gamma oscillations in FXS and its rodent models, but it may be a reflection of an imbalance of excitatory and inhibitory drive. Hyper-excitability of cortical circuits is a well-established concept in *Fmr1*<sup>-y</sup> models (Gibson et al., 2008; Zhang et al., 2014; Contractor et al., 2015).

Hyper-excitability of excitatory pyramidal cells in both layer 2/3 and layer 5 of mPFC has been demonstrated, linked to changes in expression of certain ion channels (Kalmbach et al., 2015; Routh et al., 2017). However, inhibitory networks likely play a more significant role in generating synchronous network activity such as gamma oscillations (Whittington et al., 1995; Hasenstaub et al., 2005). Recent studies have demonstrated the generation of gamma oscillations through optogenetic stimulation of parvalbumin-positive (PV) interneurons (Cardin et al., 2009; Chen et al., 2017). There have been multiple studies reporting alterations in the PV interneuron network of *Fmr1*<sup>-y</sup> mice, including changes in density, morphology and laminar distribution in sensory cortices (Selby et al., 2007; Goel et al., 2018; Wen et al., 2018) and cingulate cortex (Lee et al., 2019). Furthermore, altered GABA receptor subunit expression and reduced inhibitory drive onto layer 5 pyramidal neurons of the mPFC of adolescent *Fmr1*<sup>-y</sup> mice have been observed *in vitro* (Kramvis et al., 2020).

Although PV interneurons may be involved in generating gamma, mutant mice with reduced PV interneuron activity display increased baseline cortical gamma oscillations (Carlén et al., 2012), and gamma oscillations can be increased using optogenetic inhibition of PV interneurons (Abbas et al., 2018). A mechanism has been demonstrated using mice with a lack of functional PV interneurons, whereby apparent increases in the gamma frequency range are actually a result of asynchronous neuronal activity and higher firing rates (Guyon et al., 2021). Given the higher excitability and reduced inhibition in the mPFC of *Fmr1*<sup>-y</sup> mice, a similar mechanism could underlie the elevated gamma rhythms observed in the *Fmr1*<sup>-y</sup> rats (Routh et al., 2017; Kramvis et al., 2020). Analysis of single unit activity would provide further detail on the cause of this increase in gamma power.

Some correlational evidence for fast-spiking interneuron development playing a role in aberrant gamma oscillations in *Fmr1*<sup>-y</sup> mice comes from an experiment where *Fmr1* was selectively deactivated in excitatory neurons during development. Even though this manipulation affected only excitatory cells, a decrease in PV interneurons was observed, and cortical gamma oscillations were increased similarly to *Fmr1*<sup>-y</sup> mice (Rais et al., 2022). Furthermore, a study of development of mPFC gamma oscillations over development from P5 to P40 showed increasing frequency and power over time, that followed a similar trajectory to maturation of PV interneurons (Bitzenhofer et al., 2020). While Rais et al. (2022) did show elevated cortical gamma from P21, study of this full developmental trajectory of gamma oscillations

from early ages in *Fmr1*<sup>-y</sup> rodents may provide insight into a mechanism of aberrant cortical development in FXS.

It is difficult to determine the impact of increased gamma power on behaviour. While Ethridge et al. (2017) correlated elevated gamma levels with levels of parent-reported sensory hypersensitivity and social impairment in FXS patients, this does not necessarily indicate a causal relationship. Increased gamma power has been implicated in other neuropsychiatric disorders, including schizophrenia (Uhlhaas & Singer, 2010) and autism spectrum disorder generally (Rojas & Wilson, 2014; Filice et al., 2020), so its function has been the subject of numerous studies. While the current study cannot relate the observed gamma increase to any particular behaviour or task-related factor, it is the first finding of elevated gamma power in the local mPFC circuit of *Fmr1*<sup>-y</sup> rodents. This could have implications for the precision of mPFC circuit output, and therefore may impact mPFC-related cognitive processes that have been associated with regulation of gamma oscillations, including attention (Kim et al., 2016), working memory (Sigurdsson et al., 2010), cognitive flexibility (Cho et al., 2015), and sociability (Cao et al., 2018).

In the current experiment, hippocampal low gamma power was also significantly raised in *Fmr1*<sup>-y</sup> rats. While one study of the *Fmr1*<sup>-y</sup> mouse found increased hippocampal gamma power (Boone et al., 2018), others found no difference in baseline spectral power (Radwan et al., 2016; Dvorak et al., 2018; Arbab, Battaglia, et al., 2018). There is some evidence in these studies for dysregulated gamma oscillations though; Dvorak et al. (2018) found that *Fmr1*<sup>-y</sup> mice did not show attenuation of slow gamma power when the location of a shock zone was changed, as WT mice did. Furthermore, both Radwan et al. (2016) and Arbab, Battaglia, et al. (2018) showed increased synchronisation of gamma oscillations across neighbouring electrodes, suggesting a lack of regulation of the network oscillations. There is some evidence for asynchronous neuronal activity in the hippocampus of *Fmr1*<sup>-y</sup> rodents which could underlie the increase in power seen – hippocampal neurons show reduced spike phase-locking to the gamma oscillation in *Fmr1*<sup>-y</sup> mice and rats (Talbot et al., 2018; Asiminas et al., 2021, preprint).

Another finding of Radwan et al. (2016) were task stage-dependent differences in theta-gamma PAC. The current study did not find any differences in theta-low gamma PAC. There was no difference in the strength of theta-high gamma PAC overall, however, high gamma oscillations in *Fmr1*<sup>-y</sup> rats peaked at an earlier phase

of the theta oscillation, closer to the peak, than in WT rats. There may have also been differences in the changes in theta-high gamma PAC in the different sessions, which will be discussed further in the later section on novelty responses. The hippocampal high gamma oscillation and its modulation by the theta phase has been linked to inputs from entorhinal cortex (Colgin et al., 2009), so it is possible that the change in mean theta phase of gamma oscillations reflects mistiming of entorhinal connectivity. Entorhinal cortex neurons in *Fmr1*<sup>-y</sup> mice are hyper-excitabile, and their input to CA1 pyramidal cells via the temporo-ammonic pathway is reduced (Deng & Klyachko, 2016; Booker et al., 2020). Disrupted entorhinal input could potentially explain deficits in the lateral entorhinal cortex-dependent object-place-context task (Till et al., 2015; Asiminas et al., 2019), as well as lower sensitivity of the CA1 spatial representation to environmental cue changes in *Fmr1*<sup>-y</sup> mice (Arbab, Pennartz, et al., 2018).

#### **4.4.2 Hyper-synchronisation of mPFC and hippocampus in the gamma frequency range in *Fmr1*<sup>-y</sup> rats**

The finding of increased gamma phase synchrony also matches EEG data from human FXS patients. Wang et al. (2017) found increases in cortical functional connectivity in the gamma band, but not in lower frequencies, showing decreases in connectivity in the alpha and beta ranges. Although the current experiment shows theta frequency connectivity decreases, there may be a similar mechanism, with over-synchronous gamma disrupting synchrony between lower frequency oscillations. Further evidence for disrupted PFC-hippocampal interaction associated with reduced FMRP in humans comes from an fMRI study where reduced PFC-hippocampal connectivity during a working memory encoding task was shown in people with the FMR1 premutation. Both mPFC and hippocampal activation were similar to controls (Wang et al., 2012). This functional connectivity deficit was correlated with reduced blood levels of FMRP in those with the mutation, though there was no difference in task performance between the groups.

Due to the variability in the theta frequency connectivity in the *Fmr1*<sup>-y</sup> group, the gamma synchrony is the more reliable of the findings from the data. Hyper-synchrony at gamma frequency could potentially mean regions are less able to modulate this

connectivity when needed for communication, and inputs are less precise. This hypothesis has been suggested for the local cortical gamma power increase - raised gamma frequency synchrony between cortical layers could mean that this baseline noise impedes the specificity of oscillations related to encoding, such as phase synchronisation to auditory stimuli in the auditory cortex (Ethridge et al., 2017; Lovelace et al., 2018; Goswami et al., 2019). Furthermore, Radwan et al. (2016) showed that, in control mice, gamma synchrony between different hippocampal layers decreased during extinction trials on a place avoidance task, possibly reflecting adaptation of the circuit to new information, while this change was absent in *Fmr1*<sup>-y</sup> mice. The current experiment provides evidence that this hyper-synchrony extends to a longer-range connection, which is likely to disrupt cognitive processes that rely on regulation of mPFC-hippocampal interactions mediated by gamma oscillations.

Although the current experiment did not find any regulation of gamma connectivity across the novelty protocol in the WT group, gamma coherence between the mPFC and hippocampus has been correlated with functions such as working memory and selective memory retrieval (Sigurdsson et al., 2010; Hallock et al., 2016; Eichenbaum, 2017). Sigurdsson et al. (2010) found increases in gamma coherence between mPFC and dorsal CA1 at the choice point of a forced alternation T-maze working memory task, which correlated with performance on the task. They reported deficits in this communication in a genetic schizophrenia mouse model which showed reduced gamma coherence, so it would be interesting to see the consequences for this task of the hyper-synchronous oscillations seen in the *Fmr1*<sup>-y</sup> rats (Sigurdsson et al., 2010). An experiment using a similar task found gamma coherence at the choice point, and theta coherence during the delay, that were directionally similar to the results presented here, with mPFC leading hippocampus at the gamma frequency, and hippocampus leading at the theta frequency (Hallock et al., 2016). It was hypothesised that the gamma coherence at the choice point may reflect the mPFC influencing the retrieval of the appropriate memory representation in the hippocampus in order to complete the task. With the hyper-synchronous connectivity seen in the *Fmr1*<sup>-y</sup> rats, it may be predicted that this interaction would show inflexibility to new information, such as a reversal of goal location or strategy, in this task, continuing to show gamma coherence and retrieval of the now inappropriate memory. A similar effect has been observed in hippocampal recordings of *Fmr1*<sup>-y</sup> mice in the place avoidance task - CA1 place cells continued to represent the previous shock zone location when it had moved to a new position (Dvorak et al., 2018).

Although there may be some evidence for direct projections between the dorsal hippocampus and mPFC (Ye et al., 2017; Malik et al., 2021, preprint), ventral hippocampus appears to project more strongly to the mPFC than dorsal (Eichenbaum, 2017). Therefore, indirect pathways may mediate their synchronisation. Hallock et al. (2016) went on to deactivate the nucleus reuniens (NR) to show that it was required for theta and gamma coherence between dorsal hippocampus and mPFC in the T-maze task. There is some evidence that thalamo-cortical pathways are altered in *Fmr1*<sup>-/-</sup> mice; altered firing of *Fmr1*<sup>-/-</sup> mouse somatosensory cortex neurons in response to thalamo-cortical input has been shown (Domanski et al., 2019), although this is within a different cortical circuit, and how these results relate to network oscillations is unclear. Nonetheless, characterisation of the mPFC-NR-hippocampus pathway in *Fmr1*<sup>-/-</sup> rodents could provide a potential mechanism for the hyper-synchronous activity observed here.

An alternative explanation for the observed differences may be that the circuit defect observed within the mPFC - elevated gamma frequency power - affects its inputs to the hippocampus. This could be through direct interactions, as a recent preprint reported projections from the mPFC to dorsal hippocampus. The projections are inhibitory and may synapse with VIP interneurons in dorsal CA1, which target other inhibitory interneurons, which then inhibit the pyramidal cell network. Activation of the mPFC-hippocampal pathway was found to increase low gamma power in both regions, as well as phase synchrony between them, similar to the results presented here. The authors also suggested this pathway may facilitate novelty exploration behaviour and increase object-responsive firing in the hippocampus, although the methods behind the task used may require clarification (Malik et al., 2021, preprint). Previously, such top-down influence of the mPFC on hippocampal memory has been attributed to an indirect connection via the EC, particularly the LEC, as well as the perirhinal cortex, a pathway which is necessary for OPC memory (Chao et al., 2016; Eichenbaum, 2017; Barker & Warburton, 2020). Whichever specific pathway is responsible for top-down control of hippocampal memory by the mPFC, the current experiment provides physiological evidence that mediation of this interaction may be disrupted in *Fmr1*<sup>-/-</sup> rats. Alongside the previous behavioural evidence that OPC memory is impaired, this provides a possible mechanism for memory and cognitive flexibility deficits in FXS (Till et al., 2015; Asiminas et al., 2019).

### **4.4.3 Hippocampal theta power and theta-high gamma phase-amplitude coupling were altered in response to novelty**

The measure that was reliably and significantly modulated by novelty was theta power in the hippocampus (Figure 4.4B). This has been seen previously during novelty exploration behaviour (Wang et al., 2021). Both genotypes clearly showed this response, and there was no difference in the magnitude of this change. No increase in coherence related to novelty was seen in the current study, although it has been reported previously (Bygrave et al., 2019; Wang et al., 2021). Average mPFC theta power did not show an overall significant effect of session, but was reliably increased in the first novel session compared to the previous familiar session (Figure 4.11B). This may have been due to low power in the current experiment, and more subjects may be needed to determine if mPFC theta power also increases with a novel environment. Although this change didn't appear to be any different between the genotypes.

In the WT rats, there was also a decrease in the strength of hippocampal theta-high gamma PAC in the novel environment compared to the familiar environment, with no significant changes in high gamma power across sessions. This change persisted through at least the three novel sessions of Day 2, differently to the theta increase, which only lasted for Novel 1, before returning to similar levels as the familiar environment for Novel 2 and Novel 3. There is a chance that the theta-high gamma modulation change could be explained by differences in running speeds between sessions, as the theta phase of gamma oscillation peaks varies with speed (Chen et al., 2011). Even though only gamma events from times when the rats were travelling above the speed threshold of 3 cm/s were used, in later sessions of the day where the rats were generally travelling shorter distances, there may have been more variation in speed during the session, causing more variability in where the peaks occurred. This lower consistency may have caused the changes in MI seen. This could also explain the changes seen across sessions in mean theta phase of low gamma (Figure 4.7B) and high gamma (Figure 4.9B), as lower running speeds lead to gamma occurring at later phases of the theta oscillation (Chen et al., 2011). Therefore, in order to understand whether there were any clear effects of novelty on theta-gamma PAC, the mean phases and modulation indices may need to be assessed taking into account the rat's velocity at the time of each gamma event.

While this is likely to at least partially explain the reduction of high gamma modulation by the theta phase in the novel environment, it is strange that there is a significant drop only from Familiar 4 to Novel 1, as path length did not show a similar change between these sessions. Another study has shown an increased influence of running speed on hippocampal low and high gamma oscillation power in a novel environment compared to a familiar one. Specifically, high gamma oscillations had higher power at higher speeds, and this effect was stronger in a novel environment compared to a familiar one (Kemere et al., 2013). There could be a similar effect on theta-gamma PAC, with the environmental novelty causing speed to have a greater varying effect on theta phase of gamma oscillations, leading to the reduction in overall average modulation seen in the current data. This increased theta phase separation of inputs with different behavioural states in a novel environment may help to facilitate the processing of current sensory information from the entorhinal inputs alongside other processes (Colgin, 2015). Again, further analysis of the speed modulation of theta-high gamma PAC is required to make more solid conclusions from the current data. In addition, this novelty effect was not significant in the *Fmr1*<sup>-y</sup> group, suggesting there may be less dynamic modulation of theta phase of high gamma events. However, this was not consistent between rats, with two showing similar reductions to WT. Nonetheless, comparison of the effect of running speed on theta-high gamma PAC in the novel environment between genotypes would also be interesting to compare.

A previous study found differences in CA1 place cell habituation to novelty across days in *Fmr1*<sup>-y</sup> rats, showing a deficit in experience-dependent changes in place cell firing that occurred across a similar protocol over two days (Asiminas et al., 2021, preprint). It was suggested that the hippocampus may respond to novelty similarly on both days in the *Fmr1*<sup>-y</sup> rats. The increase in hippocampal theta power observed with novelty on day 2 in the present study is not repeated on day 3, with most rats showing an attenuated increase in theta power from Familiar 5 to Novel 4 compared to Familiar 4 to Novel 1. This suggests that the hippocampal network does not respond to novelty similarly on both days, as demonstrated by the place cell activity (Asiminas et al., 2021, preprint). However, this effect may be somewhat apparent in the theta-high gamma PAC. The reduction in the first novel session is significant in the WT, but not in the *Fmr1*<sup>-y</sup> rats, but was observed in two of the four. Then on day 3, the WT showed generally higher modulation indices in the novel environment, although the difference between Novel 1 and Novel 4 was not significant. The *Fmr1*<sup>-y</sup> rats, on the other hand, showed a strong reduction on day

3 in response to the novel environment. However, these effects were not consistent between rats, and not statistically significant, so more subjects may be required to make reliable conclusions.

#### **4.4.4 Limitations and future directions**

Perhaps the biggest limitation in this experiment was the lack of changes in connectivity at the different stages of the novelty protocol. It was expected that increases in theta coherence would be observed during the first novel environment session, as has been seen previously during exploration of novelty (Bygrave et al., 2019; Wang et al., 2021). Although there were increases in theta power in both areas, with no difference between the genotypes in this change, there were no clear changes in theta coherence in relation to the novelty in the WT, using the various measures shown here. One possible reason for this may be the lack of temporal resolution – coherence shown here is averaged across all moving time in each session. But coherence over the course of the first novel session was also checked, and there was no apparent attenuation over time. In Wang et al. (2021), an object exploration task was used and only periods of object exploration were measured, allowing comparison of LFP oscillations during novel object exploration with those during familiar object exploration. This allowed a more specific comparison than the one shown here, and potentially could account for the lack of novelty-related connectivity changes seen.

While differences between WT and *Fmr1*<sup>-/-</sup> rats in hippocampus-mPFC functional connectivity were seen, in the gamma band, and possibly in the theta band, the interpretation of this is limited by the fact that no specific connectivity changes were shown associated with particular task-related or behavioural factors. So while the differences seen can still be interpreted as resting state changes, a more specific task would be useful to determine what, if any, effect this may have on behaviour. Another possibility to explain the lack of novelty-related connectivity changes is that mPFC-hippocampal connectivity is not required for discrimination of different environments, which may be more distinct than during discrimination of more similar objects, as used in Wang et al. (2021). Following the novel environment protocol, most of the rats included here were run on object exploration tasks, one of which was an object-in-place variation known to depend on mPFC-hippocampal connections (Barker & Warburton, 2011). It will therefore be interesting to analyse whether

specific connectivity patterns can be observed between hippocampus and mPFC during this task, and whether there are differences in the *Fmr1*<sup>-y</sup> rats which may underlie established deficits in object associative memory tasks (Till et al., 2015; Asiminas et al., 2019).

After this, some of the rats were trained on a reversal learning task on a double Y-maze (similar to Ainge et al., 2007). Decision-based maze tasks similar to this are well characterised in terms of the mPFC-hippocampus interactions seen (Jones & Wilson, 2005; Sirota et al., 2008; Benchenane et al., 2010; Sigurdsson et al., 2010), so it would be interesting to see whether the network changes shown here impact on these specific interactions, and whether performance on the task is affected concurrently. This could be important, as network deficits seen in *Fmr1*<sup>-y</sup> mice are often subtle, and only apparent during some kind of conflict (Radwan et al., 2016; Dvorak et al., 2018).

Hippocampal theta has been shown to modulate mPFC gamma oscillations during working memory and strategy switching tasks (Sirota et al., 2008; Tamura et al., 2017). No evidence for mPFC gamma modulation by the hippocampal theta frequency was found here in either genotype, so given the findings of raised mPFC gamma power and gamma hyper-synchrony, it would be interesting to study this relationship in a task in which it is observed. There was evidence of theta-gamma PAC within each region, with the mPFC gamma generally locked closer to the peak of the mPFC theta than hippocampal gamma was to the hippocampal theta oscillation. This could possibly synchronise the times of gamma bursts, as the mPFC lags the hippocampal theta signal (Sirota et al., 2008). Individual mPFC neurons also phase-lock their spiking to the hippocampal theta oscillation so analysis of the single unit data recorded here could provide further insight into network connectivity.

Since FXS is a neurodevelopmental disorder, and FMRP levels are highest in the brain during early development (Till et al., 2012; Gholizadeh et al., 2015; Razak et al., 2020), measurement of the developmental trajectory of network states would be an interesting direction for future research. As mentioned previously, characterisation of gamma oscillations in *Fmr1*<sup>-y</sup> rodents may reveal the trajectory of dysfunctional cortical development in FXS. As mPFC development is protracted relative to some other brain regions, with synaptic pruning ongoing into adolescence, it may be an interesting network to study.

Finally, the current study is limited in subject numbers. In some measures there was considerable variability between individuals, which could be caused by variations in noise in the signals, or in tetrode positions, so addition of more subjects would minimise the effect of these factors and improve the robustness of the findings. There is also the possibility that differences in oscillations reflect differences in behaviour between individuals, rather than network activity itself; this confound was limited by only including data from time when the animals were moving above a speed threshold. Further, there were no differences in path length between the genotypes, and no obvious behavioural differences from observation. The key findings of increased mPFC gamma power and mPFC-hippocampal gamma synchrony showed relatively large effect sizes, as well as reflecting differences in FXS patients, so are likely to be reliable.

In conclusion, the results provided here show clear differences between *Fmr1*<sup>-y</sup> and WT rats in baseline network activity in both hippocampus and mPFC, as well as differences in interactions between the two areas. These provide reliable biomarkers that could be used to test treatments for FXS, as well as providing evidence for underlying mechanisms of cognitive deficits. Future analysis of hippocampus-mPFC interactions during specific behavioural tasks requiring connectivity between the two regions will help to determine the impact of these network changes on cognitive function.

## **Chapter 5**

# **Memory consolidation mechanisms during slow-wave sleep in a rat model of Fragile X Syndrome**

### **5.1 Introduction**

During slow-wave sleep (SWS), interactions between the hippocampus and cortical networks take place which are thought to underlie memory consolidation (Diekelmann & Born, 2010). SWS typically comes before rapid eye movement (REM) sleep in the sleep cycle, and is characterised by cortical slow wave (delta, 0-4 Hz) oscillations, thalamo-cortical spindles (10-20 Hz), and hippocampal sharp-wave ripples (ripples, 100-250 Hz) in the EEG/LFP signals.

Ripples are associated with the reactivation of memory sequences formed in the hippocampus during wake behaviour (Wilson & McNaughton, 1994; Lee & Wilson, 2002). This reactivation is important for memory consolidation, evidenced by studies in which ripples are suppressed. Suppression of ripples in rats, via electrical stimulation of ventral hippocampus, during periods of rest and sleep following a training on a spatial memory task, leads to impaired learning on the task (Girardeau et al., 2009). Furthermore, when CA1 place cells are optogenetically silenced during ripples occurring while mice learn a spatial memory task, they show a lack of learning-dependent increases in stability and precision (Roux et al., 2017). This is consistent with the idea that reactivation during ripples contributes to consolidation of place field “memory” for the spatial environment.

Long-term memory consolidation involves redistribution of memory among hippocampal and cortical circuits (Frankland & Bontempi, 2005). For this purpose, coordination of hippocampal and cortical activity is required. Reactivation of behaviourally-relevant memory sequences also occurs in the mPFC during SWS (Euston et al., 2007; Peyrache et al., 2009). Synchronisation of this activity between hippocampus and mPFC may be facilitated by cortical delta oscillations, ensuring that replay occurs during the UP state, where cortical excitability is high and conditions for synaptic plasticity are optimal (Steriade, Nuñez, et al., 1993b; Sirota et al., 2003; Diekelmann & Born, 2010). Reactivation in the cortex may be further organised by spindles, which occur following the DOWN state of the delta oscillation (Amzica & Steriade, 1998; Peyrache et al., 2011). Ripple activity also appears to be modulated with cortical spindle oscillations (Siapas & Wilson, 1998; Sirota et al., 2003; Battaglia et al., 2004a; Mölle et al., 2006; Staresina et al., 2015), perhaps reflecting the simultaneous reactivation of cortical and hippocampal networks. The importance of this temporal coordination has been shown through stimulation of deep layers of the mPFC when ripples were detected in the hippocampus, which was shown to enhance the coupling between ripples, delta waves and spindles. Inducing this precise coupling following the sample phase of an object-location task was associated with improved discrimination in the test phase 24 hours later, as well as reorganised mPFC neuronal activity (Maingret et al., 2016). This suggests that this hippocampal-mPFC interaction is important for memory consolidation across a relatively short timescale. There is increasing evidence to suggest that spatial and episodic memories are encoded in parallel in the mPFC and hippocampus, with interactions between the regions over time leading to maturation of the mPFC memory (Tse et al., 2011; Bonnici et al., 2012; Kitamura et al., 2017).

Sleep problems are a common feature of intellectual disability and autism spectrum disorders (Robinson & Richdale, 2004; Goodlin-Jones et al., 2008), and occur with a prevalence of around 30-50% in FXS patients (Kronk et al., 2010; Kidd et al., 2014). This commonly includes deficits in REM sleep time (Musumeci et al., 1995; Miano et al., 2008). Evidence from the *Drosophila* model of FXS showed that *Fmr1* may play an important role in circadian rhythms and sleep need (Inoue et al., 2002; Bushey et al., 2009). In *Fmr1*<sup>-y</sup> mice, circadian disruption was subtler, showing very slightly shorter circadian periods than WT, measured using wheel-running activity (Zhang et al., 2008). Saré et al. (2017) also showed that *Fmr1*<sup>-y</sup> mice spent less total time asleep during the light phase than WT, which only became apparent during adulthood.

While SWS deficits are not usually observed in FXS patients or *Fmr1*-null models, memory consolidation-associated neurophysiology has not been specifically tested in FXS patients. Some changes in SWS neurophysiology have been reported in the *Fmr1*<sup>-/-</sup> mouse model. One study recorded unit activity in the hippocampi of *Fmr1*<sup>-/-</sup> mice during sleep, with the main findings being a reduction in REM time (similar to what has been reported in FXS patients), as well as ripples with longer durations and lower neuronal firing rates during SWS (Boone et al., 2018). Previous *in vitro* studies have also found that the duration of cortical UP states is longer in *Fmr1*<sup>-/-</sup> mice than in WT controls (Gibson et al., 2008). However, a different study using whole-cell recordings in barrel cortex of sleeping *Fmr1*<sup>-/-</sup> mice found no differences in UP state durations, but showed hyper-synchronised cortical cells with increased firing rates during quiet wakefulness or SWS, compared to WT mice (Gonçalves et al., 2013). These changes could possibly result in less precise reactivation of memory sequences and disrupt consolidation.

Some indirect evidence that memory consolidation may be affected in *Fmr1*<sup>-/-</sup> rodents comes from behavioural tasks testing long-term memory. A fear-conditioning experiment found that *Fmr1*<sup>-/-</sup> mice showed impaired recall of a contextual fear memory 1-3 days after training, and reported deficits in reactivation of CA1 memory engrams during recall (Li et al., 2020). *Fmr1*<sup>-/-</sup> mice were also impaired on a long-term object-location task, with a 24-hour delay, while showing no deficit in short-term object-location memory (Seese et al., 2014), suggesting that memory encoding during such tasks may be normal, while longer term retrieval is impaired, possibly due to deficient consolidation mechanisms. These impairments could be due to impaired hippocampal consolidation alone, given the relatively short timescales. However, recent evidence has suggested that contextual fear conditioning memories are encoded in parallel in the mPFC (Kitamura et al., 2017), and hippocampal-mPFC interactions were shown to be important for object-location memory consolidation over 24 hours (Maingret et al., 2016).

So far, there have been no studies examining sleep in *Fmr1*<sup>-/-</sup> rats. However, evidence from Asiminas et al., (2021, preprint) may provide some further justification to investigate function of offline memory consolidation mechanisms. Hippocampal place cells were recorded in *Fmr1*<sup>-/-</sup> rats as they explored a novel environment in six sessions across two days; in WTs, place cells showed significant increases in precision of their spatial firing across the two days, whereas *Fmr1*<sup>-/-</sup> rats failed to show this change. There were few differences in place cell properties within days

between the two genotypes, suggesting the differences seen were in refinement of the spatial representation over time, rather than firing properties during wake behaviour (Asiminas et al., 2021, preprint).

The current experiment aimed to test the hypothesis that mPFC and hippocampal activity associated with memory consolidation during SWS is disrupted in *Fmr1*<sup>-y</sup> rats. WT and *Fmr1*<sup>-y</sup> rats implanted with tetrodes in the mPFC and dorsal CA1 of the hippocampus were recorded during a 30 min sleep session on each day following exploration sessions in the novel environment protocol (as described in Chapter 4). During these sleep sessions, Delta waves and spindles were detected in the mPFC LFP, and ripples in the hippocampus, and their temporal interactions were analysed. As interactions between ripples and mPFC delta and spindle oscillations have been shown to increase after learning (Maingret et al., 2016; Binder et al., 2019), the activity in the sleep session on Day 1 (after familiar environment exploration) was compared with that on Day 2 (after novel environment exploration), to test the hypothesis that consolidation of novel experiences is impaired in *Fmr1*<sup>-y</sup> rats. Overall, *Fmr1*<sup>-y</sup> rats showed lower ripple incidence, and ripples were longer in duration than WT. Additionally, in *Fmr1*<sup>-y</sup> rats, hippocampal ripples showed alterations in temporal organisation around delta waves, and ripple activity was less coupled to the phase of mPFC spindle oscillations. This represents preliminary evidence that consolidation mechanisms are disrupted in *Fmr1*<sup>-y</sup> rats, as SWS time was variable, and the lower numbers of ripples observed in *Fmr1*<sup>-y</sup> rats may have affected analysis of interactions. Furthermore, no novelty-induced changes in memory consolidation-related activity were seen.

## 5.2 Methods

The sleep recordings that were analysed in this chapter were recorded during the experiment described in Chapter 4. Therefore, the animals, tetrode implants, surgeries, electrophysiological recording and perfusions are as described in the previous chapter (Chapter 4). Briefly, animals were adult male Long-Evans Hooded *Fmr1*<sup>-y</sup> rats (n = 4) and their wild-type (WT) littermates (n = 5), implanted with tetrodes in the mPFC PrL region, and dorsal CA1 of the hippocampus.

### 5.2.1 Sleep recordings

At the end of each day of recording (Days 1-3), rats were placed in an opaque plastic bucket (40 cm tall, 25 cm diameter) lined with bedding material, which was placed in the centre of the environment they explored in the final session of the day. On Day 1, sleep recording took place with the rat in the bucket in the centre of the familiar environment; on Days 2 and 3, sleep recording took place in the bucket placed in the centre of the novel environment (Figure 5.1). Recording was started once the rat had settled, and lasted 30 minutes. All analyses reported here are from the sleep sessions on Days 1 and 2.



FIGURE 5.1: The first two days of the novel environment protocol used in Chapter 4, with the sleep sessions shown at the end of the day. Sleep sessions lasted 30 minutes. Only the first two days are analysed here, to contrast sleep after the familiar sessions only, and after sessions in a novel environment.

### 5.2.2 Slow-wave sleep classification

Slow-wave sleep (SWS) periods were classified using an automated K-means clustering algorithm based on that described in Peyrache et al. (2011). Spectrograms were calculated from the hippocampal and mPFC LFPs using the *MTSpectrogram* function (FMAToolbox), using 8-second sliding windows with steps of 1 second. Average power in the delta (0-4 Hz), theta (5-10 Hz) and spindle (10-20 Hz) ranges were calculated for each step of the spectrogram, as well as average velocity from the position tracking. Delta power in the mPFC, theta/delta power ratio in the hippocampus, spindle power in the mPFC, and velocity in each of the 1 second time

step bins were each z-scored, and used to cluster all of the bins across the 30-minute session using the MATLAB *kmeans* function with 3 different clusters. The cluster centroid associated with highest mPFC delta power was defined as SWS. A minimum of 10 successive windows (10 seconds) identified as SWS were defined as periods of SWS. The automatic classifications for each session were checked against the spectrogram figure (as in Figure 5.2A and B) to verify. The other two clusters identified wake and REM sleep, but these were not analysed here.

### **5.2.3 Delta wave, spindle and ripple detection**

Delta waves were detected as in Mölle et al. (2006), by first bandpass-filtering the LFP during SWS periods in the delta frequency range (0-4 Hz). The filtered signal was then differentiated and z-scored. The zero crossings of the differential were identified, representing the peaks and troughs of the delta filtered signal. Delta wave positive peaks were identified as sequences of trough-peak-trough in the z-scored signal that crossed either the high peak threshold of +2 and the low peak threshold of 0, or the low peak threshold of 1 and high trough threshold of -1.5. Delta waves were discarded if shorter than 0.2 seconds, or longer than 1 second. The inverse of this process was used to find negative peaks, finding peak-trough-peak sequences that crossed the high trough threshold of -2 and the low peak threshold of 0, or the low trough threshold of -1 and high peak threshold of +1.5.

Spindles were detected as in Peyrache et al. (2011). The LFP during SWS periods was bandpass-filtered in the spindle range (10-20 Hz), and the root mean square (RMS) envelope of the filtered signal calculated using a time window of 100 ms. This was smoothed by calculating the moving average over a 100 ms time window. The standard deviation of the bandpassed signal was used as a threshold, and spindle events were defined as periods where the RMS envelope was above this threshold for longer than 500 ms. A random selection of spindles were plotted to verify detection.

Ripples were detected similarly to spindles, by filtering the hippocampal LFP signal in the ripple range (100-250 Hz) and calculating the RMS envelope using a 20 ms smoothing window. The detection threshold was defined as 3 standard deviations of the filtered signal for the peak, and ripples were defined as periods where the

RMS envelope was above 1 standard deviation around this peak for over 25 ms and less than 150 ms (Mölle et al., 2006; Peyrache et al., 2011).

### 5.2.4 Data analysis

Average power and coherence measures were calculated similarly to Chapter 4, using the Chronux and FMAT toolboxes in MATLAB. Differently to Chapter 4, the LFP signal was not z-transformed before calculating the power spectrograms, as this standardisation would have been heavily skewed by the total SWS time recorded. Spectrograms and coherograms were then averaged across time windows that were classified as SWS.

Spindle and ripple power spectral density were calculated using the *pwelch* function in the MATLAB Signal Processing Toolbox on each individual event time window, then averaging across all events for the session. This average was then corrected for the 1/f component, using coefficients from its linear regression, and the peak frequency value found from this (Schlesiger et al., 2015).

To calculate ripple power around delta peaks, periods of the hippocampal LFP signal from 1 second before to 1 second after each of the peaks of detected delta waves were filtered in the ripple frequency range (100-250 Hz). They were Hilbert-transformed to obtain instantaneous ripple power, which was then smoothed using a 20 ms window moving average. These 2-second periods of ripple power were averaged across all of the detected delta waves and z-scored. A similar process was used to find spindle power around delta peaks, filtering the mPFC signal in the spindle frequency range (10-20 Hz), and using a 100 ms moving average to smooth the instantaneous power.

Delta phase modulation of ripple events was found by first calculating the phase of the delta oscillation using a bandpass filter and Hilbert transform, then finding the phase at times of ripple or gamma peaks. Mean phase and resultant vector length were found for all events in the session.

For spindle-ripple phase-amplitude coupling (PAC), each mPFC spindle event was isolated and its instantaneous phase found using Hilbert transformation. The Hilbert transformation of the hippocampal signal filtered in the ripple band during each

spindle window was used to find ripple amplitude for each event. A modulation index of spindle phase by ripple amplitude was calculated for each of these windows using the formula (method described in more detail in Chapter 4):

$$\text{Modulation index (MI)} = \frac{\log N - \left( - \sum_{j=1}^N p_j \log p_j \right)}{\log N}$$

Where  $j$  is the phase bin,  $p_i$  is the mean ripple power probability for each spindle phase bin, and  $N$  is the total number of phase bins (18) (Tort et al., 2008).

Most measures were compared across the two days using a two-way repeated measures ANOVA, with genotype as the between-subjects factor and day as the within-subjects factor. Bonferroni-corrected multiple comparisons were used where there was a significant genotype x day interaction. Three-way repeated-measures ANOVAs were used to compare average ripple and spindle power before and after delta peaks, and across days. In these analyses, genotype was used as the between-subjects factor, and the time period around the delta peak, as well as day, were the within-subject factors. Circular statistics were computed in MATLAB, using the Circular Statistics Toolbox (Berens, 2009). For comparison across days, Harrison-Kanji tests were used, with Bonferroni-corrected Watson-Williams tests to make individual comparisons between genotypes or days, where appropriate.

## 5.3 Results

### 5.3.1 There were no differences in SWS time between genotypes

During the 30-minute sessions, all rats (WT,  $n = 5$ ;  $Fmr1^{-/y}$ ,  $n = 4$ ) displayed clear periods of SWS that could be identified in the LFP signal and from the spectrograms. Figure 5.2 shows representations of LFP activity from two rats during sleep sessions. Figure 5.2A shows an example spectrogram from one WT rat, with the hippocampal signal above, and the mPFC signal below, with the rat's velocity plotted at the bottom. Times of ripples are represented by magenta symbols on the hippocampal plot, and times of spindles are represented by black symbols on the

### 5.3. Results

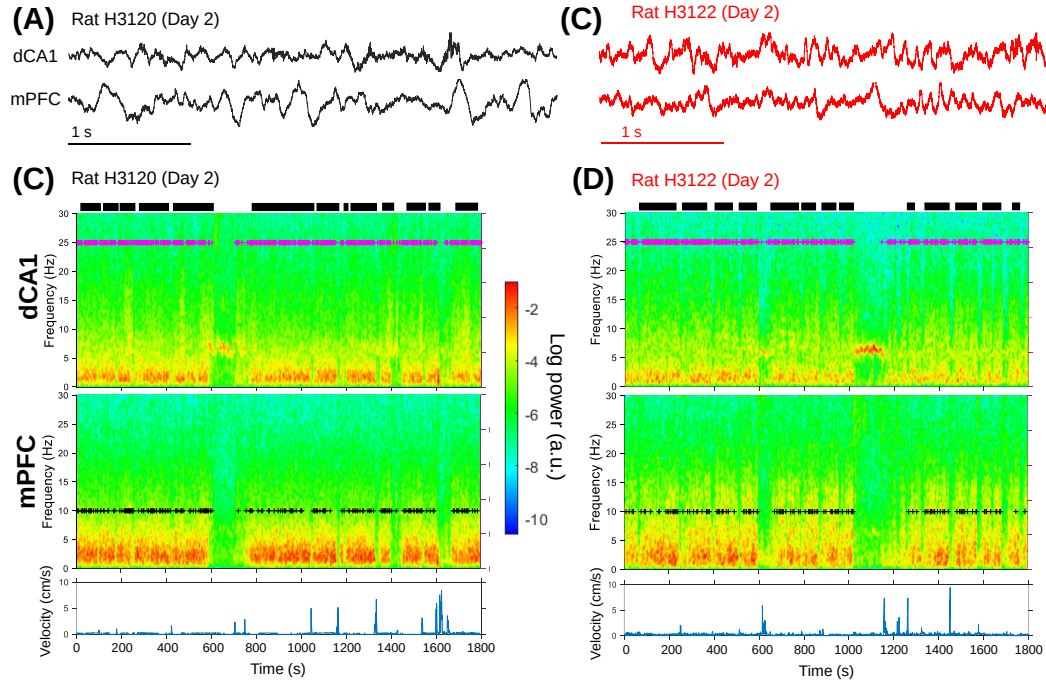


FIGURE 5.2: Example sleep spectrograms. **(A)** Example 4 seconds of raw data from one WT rat during a SWS period, showing the dCA1 signal above, and the mPFC signal below. **(B)** Example 4 seconds of raw data from one *Fmr1*<sup>-/-</sup> rat. **(C)** Spectrograms from one WT rat sleep session, showing the power of the hippocampal LFP signal from 0-30 Hz (above) and the power of the mPFC signal (below). On the above hippocampus spectrogram, times of ripples are indicated by magenta symbols. On the below mPFC spectrogram, times of spindles are plotted with black symbols. Black bars at the top indicate detected slow-wave sleep periods. Velocity of the rat is plotted in cm/s at the bottom. **(D)** Shows the same as **(C)** for an example *Fmr1*<sup>-/-</sup> rat.

mPFC plot. Figure 5.2B shows an example session from an *Fmr1*<sup>-/-</sup> rat. Cortical delta power (0-4 Hz), cortical spindle power (10-20 Hz), hippocampal theta/delta power ratio (theta power 5-10 Hz), and velocity measured by position tracking, were binned and used to classify periods of time as SWS using an automatic K-means clustering algorithm (indicated by the black boxes on the mPFC plot) (Peyrache et al., 2011).

The total time rats spent in SWS during the 30 minute session was no different between genotypes (two-way ANOVA, main effect of genotype:  $F(1,7) = 2.03$ ,  $p = 0.198$ ), but there was a significant difference across the two days ( $F(1,7) = 44.3$ ,  $p = 0.0003$ ) with no genotype x day interaction ( $F(1,7) = 0.89$ ,  $p = 0.377$ ). Both genotypes spent more time in SWS on Day 2 (novel environment day) than on Day 1 (familiar environment day) (Figure 5.3A). However, it is not clear whether this is

related to the novelty vs familiarity of the environments, or to other factors (such as the number of exploration sessions preceding sleep). To test fragmentation of SWS, the number of bouts of SWS on each day were compared (Figure 5.3B). No differences were found between genotypes or between days (Genotype:  $F(1,7) = 0.40$ ,  $p = 0.547$ ; Day:  $F(1,7) = 0.85$ ,  $p = 0.388$ ; Genotype x Day:  $F(1,7) = 0.04$ ,  $p = 0.859$ ). Similarly, there were no differences between mean SWS bout length (Figure 5.3C) (Genotype:  $F(1,7) = 3.71$ ,  $p = 0.095$ ; Day:  $F(1,7) = 1.46$ ,  $p = 0.266$ ; Genotype x Day:  $F(1,7) = 0.21$ ,  $p = 0.659$ ).

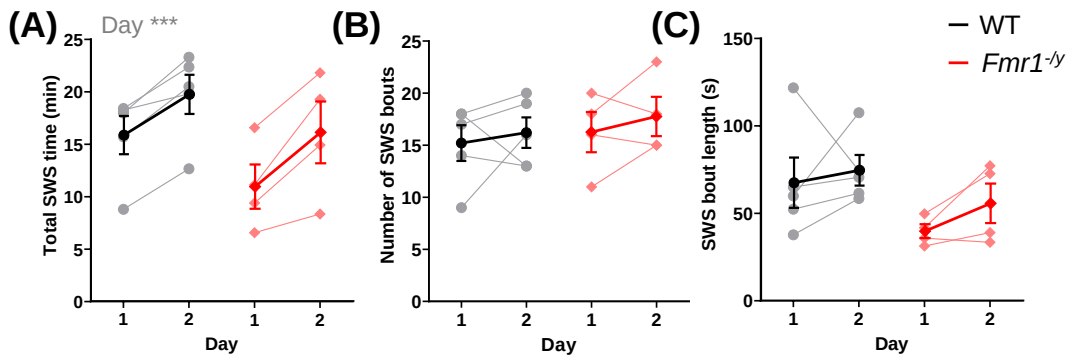


FIGURE 5.3: Slow wave sleep time was no different between genotypes, and was greater on Day 2 for both genotypes. **(A)** Proportion of the sleep session on each day that was spent in SWS. Values for each rat are shown by the pale colours, and genotype means  $\pm$  SEM are shown in bold (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(B)** Number of bouts of SWS on each day. **(C)** Mean length of SWS bouts of each day. \*\*\*  $p < 0.001$ .

### 5.3.2 *Fmr1*<sup>-/-</sup> rats have lower mPFC delta power and higher mPFC-hippocampus gamma coherence, compared to WT, during SWS

As initial analyses to compare SWS network states between the genotypes, average spectral power, coherence, and phase synchronisation in relevant frequency ranges were calculated. Delta oscillations are prominent in the cortex during SWS, reflecting cycles of synchronous excitable UP states and hyperpolarised DOWN states (Steriade, Nuñez, et al., 1993b). This is less clearly seen in the hippocampal LFP. Spindle oscillations are also recorded in the cortex during SWS, associated with reactivation of memory ensembles (Peyrache et al., 2009). Gamma oscillations are also seen during SWS, in both humans and rats, possibly underlying

interregional communication, although their role in memory consolidation is unclear (Peyrache et al., 2011; Valderrama et al., 2012; Ferraris et al., 2018). Firstly, spectrograms as shown in Figure 5.2 for each rat were averaged across all time windows that were identified as SWS by the clustering procedure, and averaged for each genotype. Figure 5.4A shows the average hippocampal SWS spectrograms for the Day 2 session. The average hippocampal spectrograms were very similar between genotypes from 0-100 Hz, and there were no significant main effects of genotype on average delta power (Figure 5.4B, Genotype:  $F(1,7) = 0.39$ ,  $p = 0.553$ ; Day:  $F(1,7) = 0.14$ ,  $p = 0.717$ ; Genotype x Day:  $F(1,7) = 0.06$ ,  $p = 0.811$ ), spindle power (Figure 5.4C, Genotype:  $F(1,7) = 0.73$ ,  $p = 0.420$ ; Day:  $F(1,7) = 1.3$ ,  $p = 0.298$ ; Genotype x Day:  $F(1,7) = 0.07$ ,  $p = 0.800$ ) or low gamma power (Figure 5.4D, Genotype:  $F(1,7) = 0.149$ ,  $p = 0.711$ ; Day:  $F(1,7) = 2.5$ ,  $p = 0.160$ ; Genotype x Day:  $F(1,7) = 0.07$ ,  $p = 0.800$ ). These results suggest that there were no differences in the power of LFP oscillations in the hippocampus between the genotypes, and no differences between the two days after the familiar and novel environment exploration.

For the mPFC, the average spectrograms for the two genotypes appeared similar, but the *Fmr1*<sup>-y</sup> rats appeared to show lower power in the low frequency ranges, as shown in the Day 2 spectrogram in Figure 5.4E. Average power in the delta range was significantly lower in the *Fmr1*<sup>-y</sup> rats compared to WT, showing a significant main effect of genotype ( $F(1,7) = 7.6$ ,  $p = 0.028$ ), with no significant main effect of day ( $F(1,7) = 4.9$ ,  $p = 0.063$ ) and no significant interaction ( $F(1,7) = 0.6$ ,  $p = 0.453$ ) (Figure 5.4F). There were no differences in power in the spindle frequency range, both between genotypes and across days (Figure 5.4G, Genotype:  $F(1,7) = 0.861$ ,  $p = 0.384$ ; Day:  $F(1,7) = 2.5$ ,  $p = 0.156$ ; Genotype x Day:  $F(1,7) = 3.9$ ,  $p = 0.088$ ). There was also no significant difference in mPFC gamma power between genotypes ( $F(1,7) = 0.001$ ,  $p = 0.973$ ) (Figure 5.4H), in contrast to the results seen during wake, described in Chapter 4. There was no significant main effect of day ( $F(1,7) = 2.3$ ,  $p = 0.175$ ) and no significant interaction ( $F(1,7) = 0.5$ ,  $p = 0.508$ ). Therefore, *Fmr1*<sup>-y</sup> rats appeared to show lower power in the delta range during SWS, perhaps reflecting less synchronised cortical networks, which may have an impact on reactivation for memory consolidation. There were no effects of day on power of any of the oscillations, suggesting the novelty before sleep on Day 2 did not change average LFP power during SWS.

To test connectivity between the hippocampus and mPFC during SWS, coherence

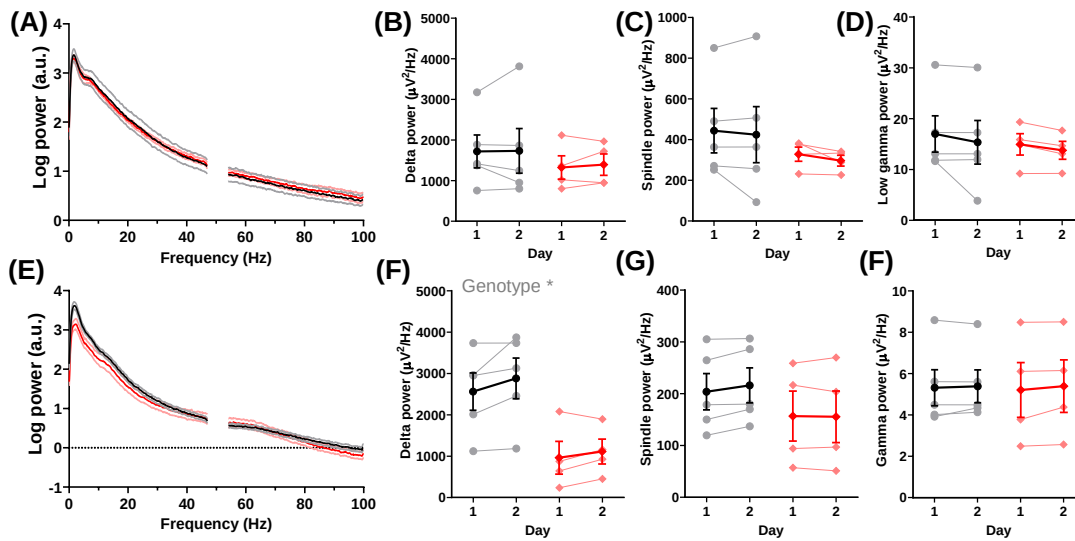


FIGURE 5.4: Average SWS mPFC delta power was lower in *Fmr1*<sup>-/-</sup> rats. **(A)** Average hippocampal SWS spectrogram from Day 2. Bold lines show the mean for rats of each genotype, pale lines show  $\pm$  SEM. **(B)** Average power in the delta range (0-4 Hz) from the hippocampal signal. Pale colours show values for each rat, bold lines indicate mean  $\pm$  SEM. **(C)** Average power in the spindle range (10-20 Hz) from the hippocampal signal. **(D)** Average power in the low gamma range (30-47 Hz) from the hippocampal signal. **(E)** Average mPFC SWS spectrogram from Day 2. Bold lines show the mean for rats of each genotype, pale lines show  $\pm$  SEM. **(F)** Average power in the delta range (0-4 Hz) from the mPFC signal. Pale colours show values for each rat, bold lines indicate mean  $\pm$  SEM. **(G)** Average power in the gamma range (30-80 Hz) from the mPFC signal (WT, n = 5; *Fmr1*<sup>-/-</sup>, n = 4). \* p < 0.05.

and phase synchrony measures were obtained. Figure 5.5A shows spectral coherence between the two signals, averaged over periods of identified SWS. Similar to recordings taken during wake described in the previous chapter, coherence was raised in the gamma range in the *Fmr1*<sup>-y</sup> rats compared to the WT. Spectral coherence was averaged across frequency bands relevant to SWS; these were delta (0-4 Hz), spindle (10-20 Hz), and gamma (30-80 Hz). Delta coherence appeared quite variable between rats, and there were no significant main effects of genotype or day and no significant interaction for spectral coherence within this band (Genotype:  $F(1,7) = 1.41$ ,  $p = 0.274$ ; Day:  $F(1,7) = 0.64$ ,  $p = 0.634$ ; Genotype x Day:  $F(1,7) = 0.59$ ,  $p = 0.468$ ) (Figure 5.5B). There were also no significant main effects of day or genotype or interaction between them on spectral coherence in the spindle band (Genotype:  $F(1,7) = 0.92$ ,  $p = 0.367$ ; Day:  $F(1,7) = 0.63$ ,  $p = 0.631$ ; Genotype x Day:  $F(1,7) = 2.5$ ,  $p = 0.155$ ) (Figure 5.5C). Gamma spectral coherence was the only frequency range where a significant main effect of genotype was seen ( $F(1,7) = 8.82$ ,  $p = 0.021$ ), with no significant main effect of day ( $F(1,7) = 2.58$ ,  $p = 0.152$ ), or interaction ( $F(1,7) = 1.4$ ,  $p = 0.263$ ) (Figure 5.5D).

The phase/imaginary coherence between hippocampus and mPFC during SWS was for each of the frequency bands. Average phase coherograms (Figure 5.6A) also showed the exaggerated synchrony in the gamma band in the *Fmr1*<sup>-y</sup> rats. Averages in the delta band were very variable, with some indicating the hippocampus signal was leading (positive values), and some indicating the mPFC was leading (negative values). The main effects of genotype and day and the interaction were all non-significant (Genotype:  $F(1,7) = 0.94$ ,  $p = 0.601$ ; Day:  $F(1,7) = 0.94$ ,  $p = 0.365$ ; Genotype x Day:  $F(1,7) = 0.17$ ,  $p = 0.690$ ) (Figure 5.6B). Values for spindle and gamma phase coherence were generally negative, indicating that the mPFC signal leads the HPC at these frequencies. Spindle phase coherence showed no difference between genotypes ( $F(1,7) = 0.50$ ,  $p = 0.500$ ) or days ( $F(1,7) = 1.50$ ,  $p = 0.260$ ), with no significant interaction ( $F(1,7) = 2.2$ ,  $p = 0.178$ ) (Figure 5.6C). Gamma synchrony was increased in *Fmr1*<sup>-y</sup> rats when measured by average phase coherence, showing a main effect of genotype ( $F(1,7) = 6.30$ ,  $p = 0.040$ ), with no significant change across days ( $F(1,7) = 0.087$ ,  $p = 0.776$ ), and no significant genotype x day interaction ( $F(1,7) = 0.084$ ,  $p = 0.781$ ) (Figure 5.6D).

De-biased weighted phase lag index (dWPLI) was also calculated, with the average across all SWS periods on Day 2 shown in Figure 5.7A. This was averaged over the gamma band, and results were consistent with the previous measures, showing a

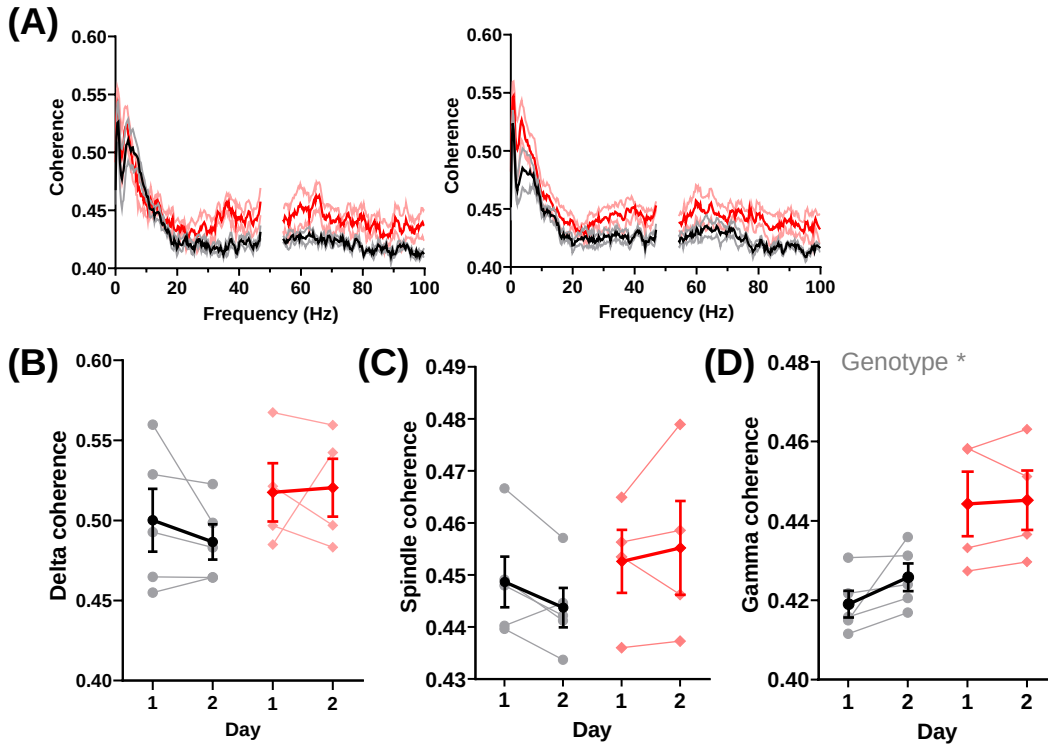


FIGURE 5.5: Gamma spectral coherence was increased during SWS in *Fmr1*<sup>-/-</sup> rats. **(A)** Coherograms for Day 1 (left) and Day 2 (right), averaged over all SWS time in the session. The WT mean is shown by the black line, with grey lines showing  $\pm$  SEM. The *Fmr1*<sup>-/-</sup> mean is shown by the red line, with pale red lines indicating  $\pm$  SEM. **(B)** Average magnitude coherence in the delta (0-4 Hz) range. **(C)** Average coherence in the spindle (10-20 Hz) range. **(D)** Average coherence in the broad gamma (30-80 Hz) range. \*  $p < 0.05$ .

main effect of genotype ( $F(1,7) = 9.0$ ,  $p = 0.020$ ), with no significant effect of day ( $F(1,7) = 2.68$ ,  $p = 0.146$ ), or interaction ( $F(1,7) = 0.05$ ,  $p = 0.826$ ) (Figure 5.7B). The findings of the analysis of power and coherence during SWS were that *Fmr1*<sup>-/-</sup> rats showed lower delta power in the mPFC, and that the mPFC and hippocampus were hyper-synchronised, similar to during wake. These were overall genotypic effects, which were unrelated to the familiar and novel environment days.

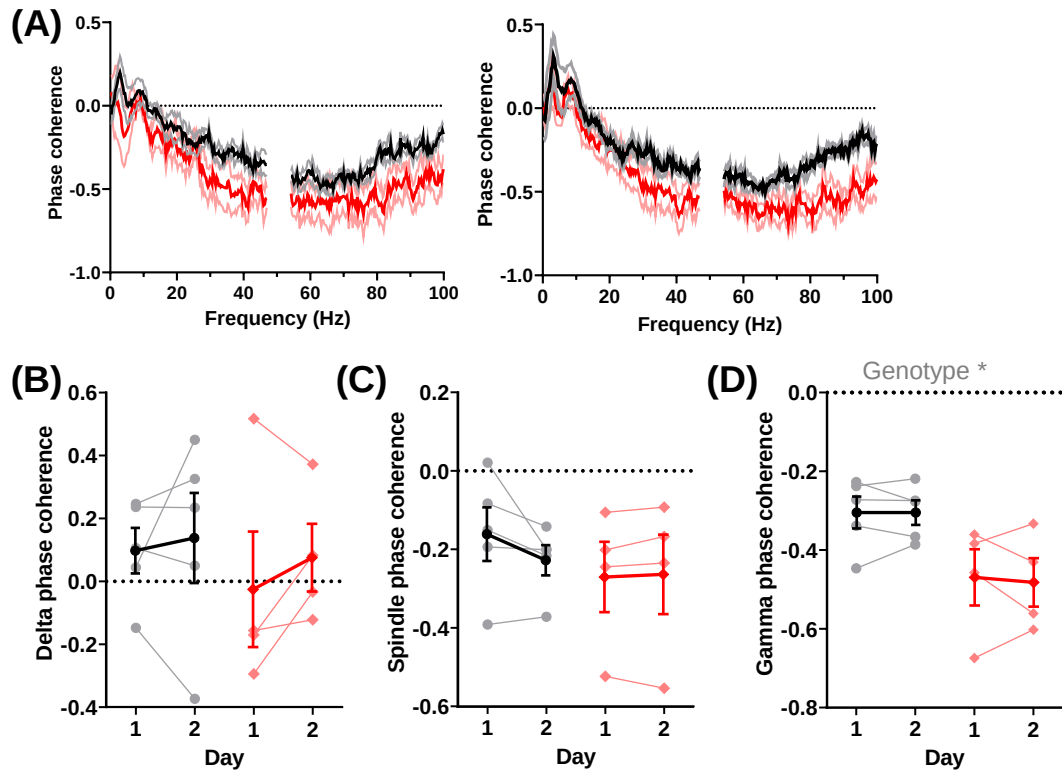


FIGURE 5.6: SWS gamma phase coherence is increased in *Fmr1* rats. **(A)** Phase coherograms for Day 1 (left) and Day 2 (right) averaged over all SWS time during the session. The WT mean is shown by the black line, with grey lines showing  $\pm$  SEM. The *Fmr1*<sup>-/-</sup> mean is shown by the red line, with pale red lines indicating  $\pm$  SEM. **(B)** Average magnitude coherence in the delta (0-4 Hz) range during SWS. **(C)** Average coherence in the broad gamma (30-80 Hz) range during SWS. **(D)** Average coherence in the spindle (10-20 Hz) range during SWS. \*  $p < 0.05$ .

### 5.3.3 *Fmr1*<sup>-/-</sup> rats showed similar numbers of mPFC delta waves and spindles to WT, but fewer and longer duration hippocampal ripples

Memory consolidation is thought to be coordinated by the temporal organisation of cortical delta oscillations and spindles, with hippocampal ripples (Diekelmann & Born, 2010). Since ripples, spindles, and delta oscillation cycles occur as discrete events, times of such events that were above a certain threshold were detected in the LFP signals (see Methods for descriptions of detection procedures). The properties of these events were then compared across genotypes, to determine whether there were any differences in generation of the oscillations. As increases in density of ripples and spindles during SWS following learning have previously

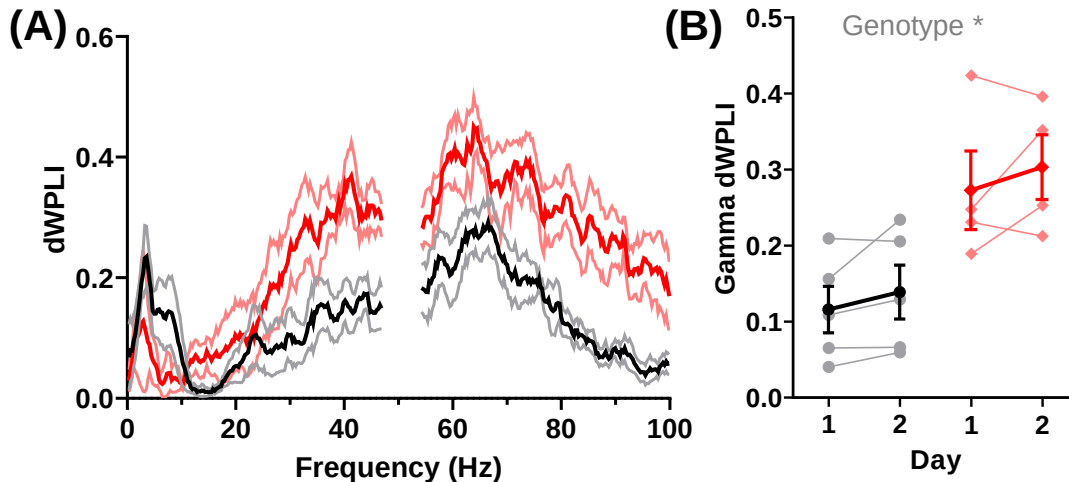


FIGURE 5.7: Gamma phase synchrony is higher in *Fmr1*<sup>-/-</sup> rats. **(A)** dWPLI averaged over SWS periods during the Day 2 sessions. Bold line represents the mean of each group, with  $\pm$  SEM in paler colours (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(B)** Average gamma dWPLI for each day. Bold lines represent means  $\pm$  SEM, with individual rats in paler colours (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*  $p < 0.05$ .

been observed (Eschenko et al., 2006; Eschenko et al., 2008), these measures were also compared across the two days, with the prediction that increases in density may be seen in Day 2 (following exposure to the novel environment), compared to Day 1 (following the familiar environment).

The total number of events detected during SWS, alongside total SWS time for each rat, are shown in Table 5.1. This shows that there was considerable variability in many of these measures. Figure 5.8A shows example raw signal mPFC delta waves from one rat of each genotype, and number of delta waves per minute of SWS is plotted in Figure 5.8B. There were no significant differences in number of above threshold delta waves during SWS between genotypes ( $F(1,7) = 2.21$ ,  $p = 0.180$ ), or across days ( $F(1,7) = 0.54$ ,  $p = 0.486$ ), with no significant genotype  $\times$  day interaction ( $F(1,7) = 1.1$ ,  $p = 0.322$ ). A previous in vitro study of *Fmr1*<sup>-/-</sup> mice found prolonged cortical UP states (Gibson et al., 2008), which can be estimated from the delta oscillation in the LFP (Saleem et al., 2010). So here, negative peak to negative peak delta cycle durations were calculated (Mölle et al., 2006), and the median duration for each rat is plotted in Figure 5.8C. There were no significant differences in delta wave duration (Genotype:  $F(1,7) = 2.64$ ,  $p = 0.149$ ; Day:  $F(1,7) = 2.0$ ,  $p = 0.201$ ; Genotype  $\times$  Day:  $F(1,7) = 1.0$ ,  $p = 0.346$ ). As this delta wave detection was centred around the positive peak, corresponding to the DOWN state,

### 5.3. Results

| Rat   | Total SWS time (min) |       | Number of ripples |       | Number of spindles |       | Number of delta waves |       |
|-------|----------------------|-------|-------------------|-------|--------------------|-------|-----------------------|-------|
|       | Day 1                | Day 2 | Day 1             | Day 2 | Day 1              | Day 2 | Day 1                 | Day 2 |
| H3109 | 18.4                 | 22.4  | 641               | 757   | 115                | 142   | 794                   | 962   |
| H3113 | 15.7                 | 20.5  | 729               | 845   | 119                | 146   | 631                   | 842   |
| H3114 | 18.3                 | 19.8  | 708               | 731   | 133                | 158   | 871                   | 976   |
| H3120 | 18.0                 | 23.3  | 723               | 879   | 156                | 143   | 826                   | 1059  |
| H3123 | 8.8                  | 12.7  | 457               | 675   | 55                 | 88    | 334                   | 494   |
| H3115 | 11.1                 | 19.3  | 435               | 729   | 74                 | 107   | 389                   | 675   |
| H3118 | 6.6                  | 8.4   | 177               | 170   | 50                 | 64    | 216                   | 309   |
| H3119 | 16.6                 | 21.8  | 393               | 464   | 102                | 130   | 723                   | 905   |
| H3122 | 9.4                  | 14.9  | 240               | 427   | 99                 | 111   | 482                   | 531   |

TABLE 5.1: Total SWS time, and total numbers of hippocampal ripples and mPFC spindles and delta waves detected, for each rat. WT rats are shaded in grey; *Fmr1*<sup>-/-</sup> are shaded in pale red.

the detection procedure was reversed to find negative troughs that may reflect UP states; no differences were found in the durations of these events either (Genotype:  $F(1,7) = 4.32$ ,  $p = 0.076$ ; Day:  $F(1,7) = 0.076$ ,  $p = 0.791$ ; Genotype x Day:  $F(1,7) = 0.31$ ,  $p = 0.593$ ). Therefore, there did not appear to be genotypic differences in the durations of synchronised cortical activity measured using the delta frequency oscillation.

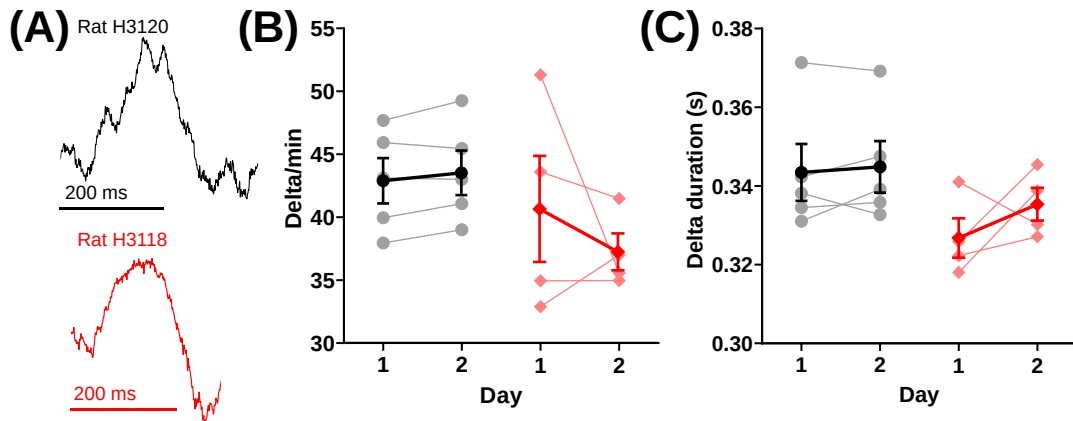


FIGURE 5.8: There were no genotypic differences in delta wave properties. **(A)** Example raw signal traces showing mPFC delta waves from one WT (black) rat and one *Fmr1*<sup>-/-</sup> (red) rat. **(B)** Number of delta waves detected per minute of SWS for each day. Bold lines represent means  $\pm$  SEM, with individual rats in paler colours (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(C)** Median durations of mPFC delta waves for each rat (paler colours), and group means  $\pm$  SEM in bold (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ).

Example mPFC spindles from one rat of each genotype are shown in Figure 5.9A. Number of mPFC spindles per minute were similar to previously observed values (Eschenko et al., 2006), and showed no significant main effect of genotype ( $F(1,7) = 0.04$ ,  $p = 0.848$ ), and no significant main effect of day ( $F(1,7) = 2.24$ ,  $p = 0.178$ ), with no significant interaction ( $F(1,7) = 0.71$ ,  $p = 0.428$ ) (Figure 5.9B). Hence there were no differences between the genotypes in spindle density, and the novel environment exploration did not appear to result in upregulated spindle density in either genotype. Spindle event duration and frequency were also calculated, to test for differences in spindle generation between the genotypes. Average spindle duration showed no main effect of genotype ( $F(1,7) = 0.07$ ,  $p = 0.801$ ), no significant main effect of day ( $F(1,7) = 0.03$ ,  $p = 0.868$ ), and no significant interaction ( $F(1,7) = 0.207$ ,  $p = 0.663$ ) (Figure 5.9C). The average peak frequency of spindles was calculated by finding the 1/f-corrected average power spectral density across all SWS spindle events for each rat, and the mean of these for each genotype are plotted in Figure 5.9D. The frequency at which power spectral density was highest was found for each rat and plotted in Figure 5.9E. The average peak frequency of spindles was similar between genotypes and across days (WT mean = 14.6 Hz; *Fmr1*<sup>-y</sup> mean = 14.5 Hz; Genotype:  $F(1,7) = 5.15$ ,  $p = 0.058$ ; Day:  $F(1,7) = 1.4$ ,  $p = 0.276$ ; Genotype x Day:  $F(1,7) = 0.31$ ,  $p = 0.593$ ).

As well as increased spindle density, hippocampal ripple density has previously been observed following learning on a task (Eschenko et al., 2008). It was predicted that the novel environment day may have a similar effect. In Figure 5.10A, example hippocampal ripples from one rat of each genotype are shown. Figure 5.10B compares number of detected ripples per minute of SWS in sessions of each day. *Fmr1*<sup>-y</sup> rats had fewer ripples per minute in SWS periods than WT; a two-way ANOVA across days found a significant main effect of genotype ( $F(1,7) = 8.26$ ,  $p = 0.024$ ). There was also a significant main effect of day ( $F(1,7) = 8.01$ ,  $p = 0.025$ ), as generally fewer ripples were detected per minute of SWS on Day 2 (after the novel sessions) than on Day 1 (after the familiar sessions), contrary to predictions of the effect of novelty, but no significant genotype x day interaction ( $F(1,7) = 0.12$ ,  $p = 0.743$ ). An increase in average duration of hippocampal ripples has previously been shown in *Fmr1*<sup>-y</sup> mice (Boone et al., 2018). As shown in the mouse model, ripples in the *Fmr1*<sup>-y</sup> rats were found to be longer in duration than ripples in the WT group. The group mean for the WTs on Day 1 was 67.0 ms (95% CI [60.6,73.5]), while the *Fmr1*<sup>-y</sup> mean was 79.2 ms (95% CI [72.0,86.4]), and there was a significant main effect of genotype ( $F(1,7) = 13.5$ ,  $p = 0.008$ ), with

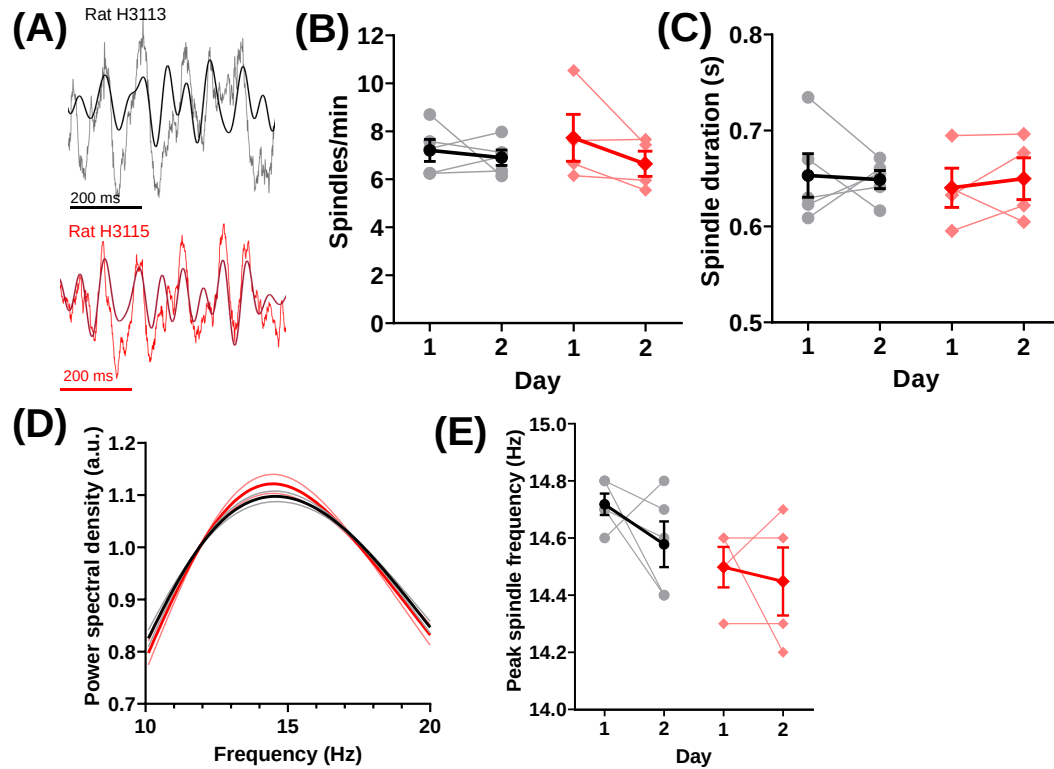


FIGURE 5.9: There were no genotypic differences in mPFC spindle properties. **(A)** Example mPFC spindles from one WT (black) rat and one *Fmr1*<sup>-/-</sup> (red) rat. **(B)** Number of spindles detected per minute of SWS for each day. Bold lines represent means  $\pm$  SEM, with individual rats in paler colours (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(C)** Median durations of mPFC spindles for each rat (paler colours), with group means  $\pm$  SEM in bold (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*  $p < 0.05$ . **(D)** Average power spectral density of mPFC spindles, showing the group mean in bold  $\pm$  SEM in paler colours (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(E)** Peak spindle power spectral density frequency for each rat (paler colours) and group means  $\pm$  SEM in bold (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ).

no significant main effect of day ( $F(1,7) = 0.61$ ,  $p = 0.459$ ) and no significant interaction ( $F(1,7) = 0.07$ ,  $p = 0.799$ ). Boone et al. (2018) also found lower frequency ripples in *Fmr1*<sup>-/-</sup> mice. Here, peak spindle and ripple frequencies were found using a similar procedure. Figure 5.10D shows average 1/f-corrected ripple power spectral density for each genotype. There was no main effect of genotype on peak ripple frequency ( $F(1,7) = 2.43$ ,  $p = 0.163$ ), however, there was a significant main effect of day ( $F(1,7) = 19.9$ ,  $p = 0.005$ ), with ripple frequency increasing for both groups from Day 1 to Day 2. There was no significant genotype  $\times$  day interaction ( $F(1,7) = 0.08$ ,  $p = 0.783$ ) (Figure 5.10E). These findings suggest that *Fmr1*<sup>-/-</sup> rats may have lower incidence of hippocampal ripples than WT rats overall, and ripples may have longer durations. A decrease in the number of ripples following the novel

environment day (Day 2) compared to the familiar environment day (Day 1) was observed in both genotypes, although an increase was predicted.

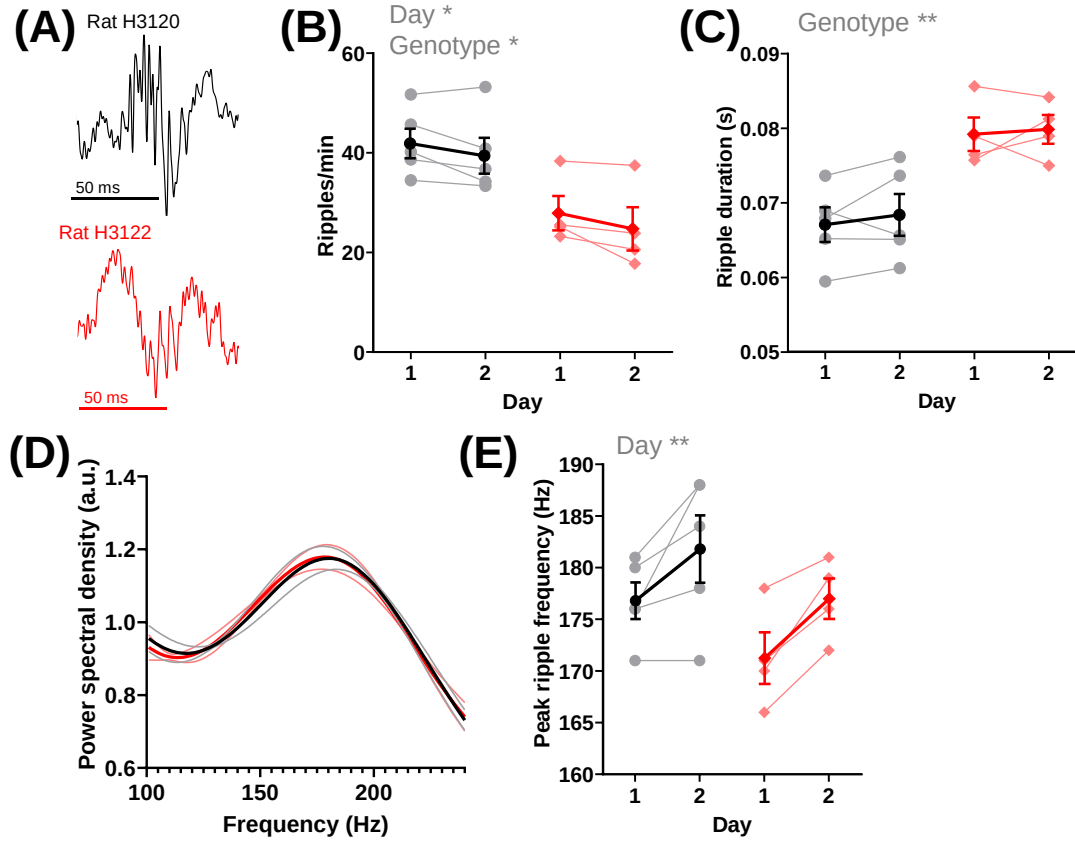


FIGURE 5.10: *Fmr1*<sup>-/-</sup> rats had fewer and longer duration hippocampal ripples. **(A)** Example hippocampal ripples from one WT (black) rat and one *Fmr1*<sup>-/-</sup> (red) rat. **(B)** Number of ripples detected per minute of SWS for each day. Bold lines represent means  $\pm$  SEM, with individual rats in paler colours (WT, black, n = 5; *Fmr1*<sup>-/-</sup>, red, n = 4). **(C)** Median durations of hippocampal ripples for each rat (paler colours), and group means  $\pm$  SEM in bold (WT, black, n = 5; *Fmr1*<sup>-/-</sup>, red, n = 4). **(D)** Power spectral density of hippocampal ripples, showing the group mean in bold  $\pm$  SEM in paler colours (WT, black, n = 5; *Fmr1*<sup>-/-</sup>, red, n = 4). **(E)** Peak ripple power spectral density frequency for each rat (paler colours) and group means  $\pm$  SEM in bold (WT, black, n = 5; *Fmr1*<sup>-/-</sup>, red, n = 4). \* p < 0.05, \*\* p < 0.01.

#### 5.3.4 Hippocampal ripples may be mistimed relative to the mPFC delta oscillation in *Fmr1*<sup>-/-</sup> rats

Ripple activity in the hippocampus is temporally organised around the delta oscillation in the mPFC, thought to underlie synchronisation of reactivation in the two networks for memory consolidation (Mölle et al., 2006). Furthermore, Maingret et al. (2016) showed that the number of positive delta peaks in the mPFC that were temporally associated with hippocampal ripples, increased after memory encoding. Therefore, it was predicted that hippocampal ripple activity may be more modulated around the delta oscillation following novelty (Day 2), compared to following familiarity (Day 1), in the current experiment. It was also predicted that there may be differences in this relationship between the genotypes. Figure 5.11A shows example raw traces where ripples were detected in the hippocampal signal around 100 ms before positive delta wave peaks in the mPFC signal (Maingret et al., 2016). Because of the variable numbers of ripples and delta waves detected in each genotype, it was difficult to assess this relationship through timing of events. Therefore, the hippocampal ripple frequency (100-250 Hz) power was averaged within 2-second periods around all detected mPFC delta peaks and z-scored. Means  $\pm$  SEM for each genotype are shown in Figure 5.11B, with Day 1 above, and Day 2 below. As previously shown in WT rats, there was a sharp decrease in hippocampal ripple power around 100 ms before the positive peak of the delta oscillation, in both genotypes (Figure 5.11B), reflecting low spiking activity in both areas (Mölle et al., 2006). This was then followed by an increase in hippocampal ripple power as the mPFC delta wave reaches the negative peak, reflecting increases in spiking in both regions (Mölle et al., 2006) – in the *Fmr1*<sup>-/-</sup> rats, hippocampal ripple power appeared to increase sooner after the positive peak than in the WTs (blue shaded region, Figure 5.11B). The modulation of ripple power around the delta wave peak was compared using a three-way repeated-measures ANOVA, in order to compare the average power before and after the mPFC positive delta peak, whether this modulation changed following the novel environment day, and whether this was different between genotypes. Time period and day were used as within-subject factors, and genotype was the between-subjects factor.

Using this analysis, there was no main effect of genotype ( $F(1,7) = 5.0$ ,  $p = 0.059$ ), as both genotypes showed low hippocampal ripple power in the 100 ms before the mPFC delta peak (green shaded region, Figure 5.11B). There was a significant

main effect of time period ( $F(1,7) = 8.5$ ,  $p = 0.022$ ), indicating that ripple power begins to rise following the positive delta peak (blue shaded region, Figure 5.11B). There was also a significant time period x genotype interaction ( $F(1,7) = 9.8$ ,  $p = 0.017$ ), indicating that *Fmr1*<sup>-y</sup> rats showed a greater increase in ripple power in the post-delta peak period than WTs (blue shaded region, Figure 5.11B). There was no significant main effect of day ( $F(1,7) = 4.0$ ,  $p = 0.085$ ), day x genotype interaction ( $F(1,7) = 1.6$ ,  $p = 0.249$ ), or day x time period ( $F(1,7) = 0.01$ ,  $p = 0.908$ ), suggesting that there was no difference in this delta-ripple relationship following the novel environment sessions compared to the familiar. However, the three-way interaction of genotype x time period x day was significant ( $F(1,7) = 7.5$ ,  $p = 0.029$ ), likely reflecting the fact that this genotype effect on timing was less pronounced on Day 2 compared to Day 1.

To explore this timing difference further, ripple power was averaged around negative delta wave peaks for each rat on each day (Figure 5.11C). In this analysis, ripple power showed increases around the negative peak (0 seconds) in both genotypes, and on both days, reflecting coordinated high activity in hippocampus and mPFC (Mölle et al., 2006). However, while in the WT rats this ripple power increase occurred just after the mPFC delta negative peak (Figure 5.11C, blue shaded region), ripple power in the *Fmr1*<sup>-y</sup> rats appeared to increase before the negative peak (green shaded region), then decrease again following the delta peak (blue shaded region). A three-way ANOVA was used to compare average ripple power in the 100 ms before and after the negative delta peak. There was a significant main effect of time period ( $F(1,7) = 9.5$ ,  $p = 0.018$ ), and a significant time period x genotype interaction ( $F(1,7) = 11.4$ ,  $p = 0.012$ ), reflecting the differences in the time period in which ripple power increased between the genotypes. There was no significant main effect of genotype, as both increased within the combined time period, and there was no main effect of day ( $F(1,7) = 0.01$ ,  $p = 0.921$ ), no significant day x genotype interaction ( $F(1,7) = 0.8$ ,  $p = 0.415$ ), and no significant time period x day interaction ( $F(1,7) = 0.7$ ,  $p = 0.430$ ). There was no significant genotype x time period x day interaction ( $F(1,7) = 3.2$ ,  $p = 0.118$ ).

Therefore, there was an apparent difference in the timing relationship between hippocampal ripples and the mPFC delta oscillation in *Fmr1*<sup>-y</sup> rats. However, as this analysis may have been limited by variability in both total number of detected delta waves (Table 5.1) and measured ripple power, in order to quantify this delta-ripple timing relationship via another method, the phase of the mPFC delta oscillation

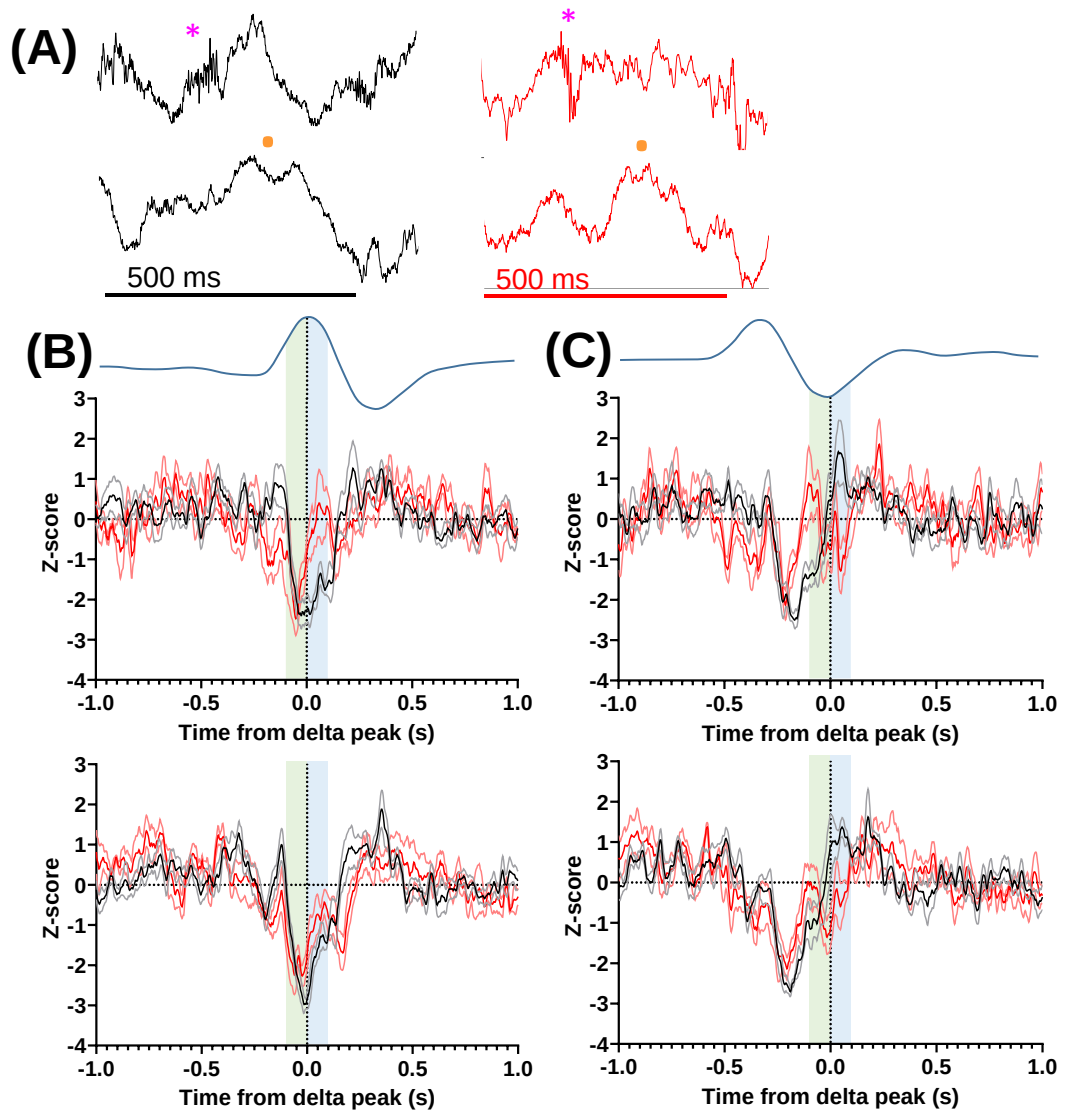


FIGURE 5.11: Ripple power increased earlier following delta peaks in *Fmr1*<sup>-/-</sup> rats. **(A)** Example ripple-delta events from one WT rat (black) and one *Fmr1*<sup>-/-</sup> rat (red). Magenta asterisks show the time of ripples in the hippocampal signals (above), and orange dots indicate the peak of delta waves in the mPFC signal (below). **(B)** Average hippocampal ripple power around the positive peaks of delta waves, on Day 1 (above) and Day 2 (below) (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). Bold lines represent genotype means, paler lines represent  $\pm$  SEM. An example average delta wave is shown above. **(C)** Average hippocampal ripple power around negative delta peaks on Day 1 (above) and Day 2 (below) (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). Bold lines represent genotype means, paler lines represent  $\pm$  SEM.

was found at the times of detected hippocampal ripple event peaks. Figure 5.12A shows the average probability of ripples occurring in each  $18^\circ$  ( $\frac{\pi}{9}$  radian) phase bin, for WT (grey) and *Fmr1*<sup>-/-</sup> rats (red) on Day 1 (above) and Day 2 (below). From this, it appeared that ripples from *Fmr1*<sup>-/-</sup> rats were timed to an earlier phase of the delta oscillation, occurring in higher probabilities slightly closer to the trough ( $-/+ \pi$ ) than ripples of WT rats. The populations of all ripples for the two genotypes were tested against one another using Watson-Williams tests, and there was a significant difference between genotypes on both days (Day 1:  $F(1,6836) = 123.1$ ,  $p < 0.0001$ ; Day 2:  $F(1,7411) = 25.0$ ,  $p < 0.0001$ ), although this was limited by low resultant vector lengths. The mean phase of ripples for each rat was then calculated, and the two groups compared (Figure 5.12B). A Harrison-Kanji test across the two days found a significant main effect of genotype ( $F(1,7) = 10.2$ ,  $p = 0.0065$ ), with no significant main effect of day ( $F(1,7) = 0.39$ ,  $p = 0.542$ ) and no significant interaction ( $F(1,7) = 1.6$ ,  $p = 0.233$ ). To test whether this possible difference was due to a difference in strength of the phase-locking relationship, resultant vector length was calculated for each rat, and there was no significant main effect of genotype ( $F(1,7) = 1.01$ ,  $p = 0.350$ ), or day ( $F(1,7) = 4.6$ ,  $p = 0.070$ ), and no significant interaction of genotype and day ( $F(1,7) = 0.12$ ,  $p = 0.740$ ).

This shows that there may be differences in coordination of hippocampal ripples and the mPFC delta oscillation. As a precise temporal relationship between these two oscillations is important for memory consolidation, differences could underlie memory consolidation deficits in *Fmr1*<sup>-/-</sup> rats.

### 5.3.5 Delta-spindle sequences are observed in both genotypes

In the cortex, spindle oscillations have been shown to follow delta waves, with spindles perhaps underlying reactivation of cortical networks during the UP state for consolidation of memories (Mölle et al., 2006; Peyrache et al., 2009; Maingret et al., 2016). Examples of mPFC spindles following delta waves are shown in Figure 5.13A. Average power in the spindle frequency range (10-20 Hz) was found around all detected delta wave positive peaks and an average was found for each rat – these were then averaged and plotted in Figure 5.13B. Although there is some variability, both genotypes show a gradual increase in mPFC spindle power following delta wave peaks, as has been observed previously (Mölle et al., 2006; Peyrache et al., 2009). To quantify the stages of this, average power was calculated

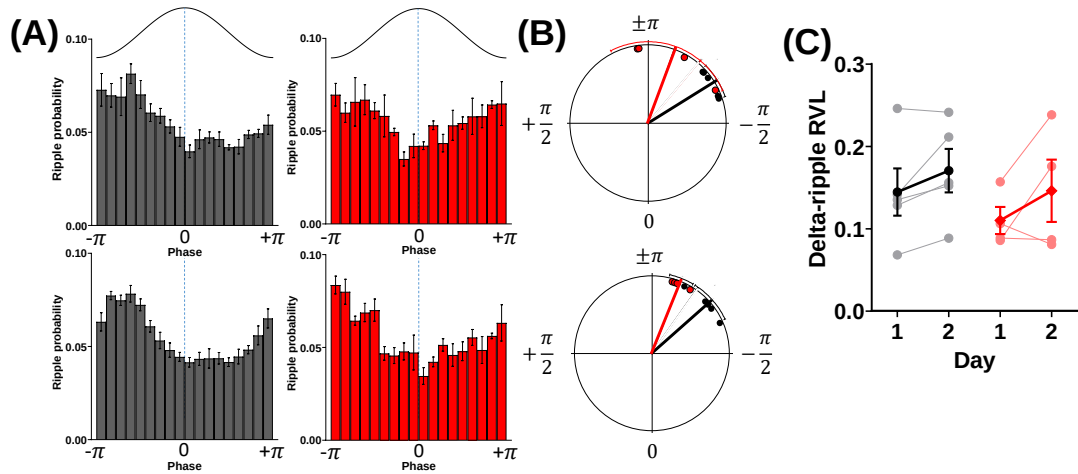


FIGURE 5.12: *Fmr1*<sup>-/-</sup> ripples may be phase locked to an earlier phase of the delta oscillation. **(A)** Probability of hippocampal ripple occurrence over delta oscillation phase bins for Day 1 (above) and Day 2 (below). Bars represent means  $\pm$  SEM (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ).  $\pm\pi$  represents the delta trough, and 0 represents the peak. **(B)** Circular plots showing the mean delta phase of hippocampal ripples from each rat for Day 1 (above) and Day 2 (below). Dots represent each rat, and bold lines represent group means (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). **(C)** Resultant vector length of mPFC delta phases of hippocampal ripples for each rat (paler colours), with genotype means  $\pm$  SEM in bold (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ).

within 250 ms before the delta peak, 250 ms after, and 250-500 ms after, within the shaded regions of Figure 5.13B, as this reflects a similar timescale to that used by Maingret et al. (2016) to identify coupled delta-spindle events. These were then compared with a three-way ANOVA.

There was a main effect of time period ( $F(1,7) = 70.6$ ,  $p < 0.0001$ ), as the spindle power increased across the three periods in both genotypes. There was no significant main effect of genotype ( $F(1,7) = 2.9$ ,  $p = 0.133$ ), and no significant interaction between genotype and time period ( $F(1,7) = 1.6$ ,  $p = 0.232$ ). Maingret et al. (2016) reported greater numbers of delta-spindle events following encoding, so it was predicted that exposure to the novel environment on Day 2 before sleep may cause a greater increase in spindle power following delta peaks compared to the familiar day (Day 1). However, there was no significant main effect of day ( $F(1,7) = 4.5$ ,  $p = 0.071$ ), and no significant interactions of day and time period ( $F(1,7) = 1.2$ ,  $p = 0.325$ ) or day and genotype ( $F(1,7) = 3.9$ ,  $p = 0.090$ ). There was also no significant genotype  $\times$  time period  $\times$  day interaction ( $F(1,7) = 0.02$ ,  $p = 0.974$ ). This suggests that spindles are coupled with delta waves similarly in WT and *Fmr1*<sup>-/-</sup> rats.

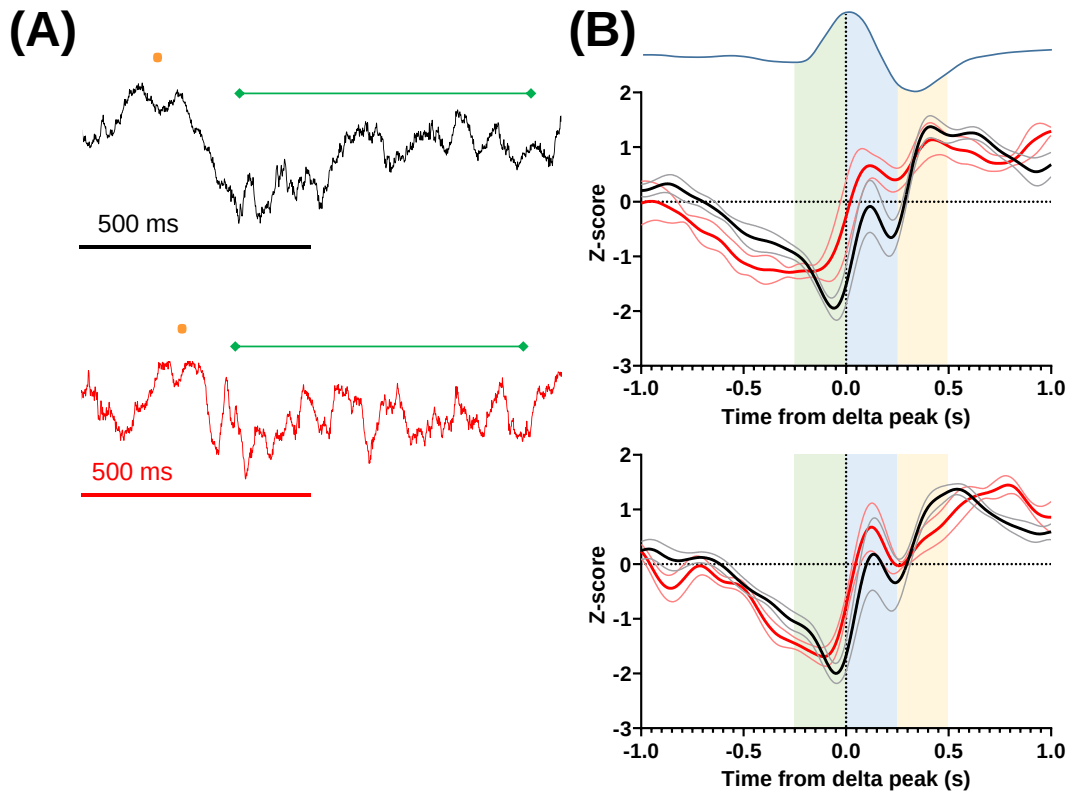


FIGURE 5.13: Spindles follow delta waves in both genotypes. **(A)** Example raw traces showing delta-spindle events in the mPFC LFP, from one WT rat (black) and one *Fmr1*<sup>-/-</sup> rat (red). Orange dots represent delta peaks, the green lines represent spindle events. **(B)** Average mPFC spindle power around delta wave peaks (0 seconds), showing increasing power following the delta wave peak. Bold lines represent genotype means, and paler coloured lines indicate  $\pm$  SEM (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ).

### 5.3.6 Ripples were less modulated with spindle phase in *Fmr1*<sup>-y</sup> rats

Coordinated modulation of hippocampal ripples and the phase of the cortical spindle oscillation has been shown previously, possibly reflecting the synchronised re-activation of hippocampal and cortical ensembles (Sirota et al., 2003; Peyrache et al., 2009; Phillips et al., 2012). Figure 5.14A shows the mean of the spindle frequency range (10-20 Hz) filtered signal from all WT (above, black) and *Fmr1*<sup>-y</sup> (below, red) mPFC spindle events detected on Day 2, centred around the trough closest to the amplitude peak of the spindle events. The lines in the paler colours represent power in the ripple frequency band (100-250 Hz) (smoothed using a 20 ms moving average) in the hippocampal signal during these spindle events. Peaks in the WT ripple power can be seen occurring at a similar frequency to the spindle oscillation, suggesting ripple power is modulated with the spindle phase. In the *Fmr1*<sup>-y</sup> signal, this appears less clear. This relationship was quantified by calculating modulation indices of mPFC spindle phase over hippocampal ripple band amplitude during spindle events, and finding the mean modulation index across all spindle events for each rat on each day (Figure 5.14B). There was a significant main effect of genotype ( $F(1,7) = 21.3$ ,  $p = 0.018$ ), indicating that hippocampal ripple power was less modulated by spindle phase in *Fmr1*<sup>-y</sup> rats than WTs. There was no main effect of day ( $F(1,7) = 1.2$ ,  $p = 0.301$ ), and no significant interaction ( $F(1,7) = 0.34$ ,  $p = 0.581$ ). This suggests that, in *Fmr1*<sup>-y</sup> rats, reactivation of hippocampal and mPFC ensembles may be less synchronised, which could impair memory consolidation.

## 5.4 Discussion

The aim of this experiment was to determine whether mPFC and hippocampal network activity associated with memory consolidation during SWS are disrupted in *Fmr1*<sup>-y</sup> rats. Significantly lower hippocampal ripple incidence was observed in *Fmr1*<sup>-y</sup> rats compared to WTs, along with greater ripple durations. Differences in modulation of ripples by cortical activity were also observed between genotypes; the data showed that hippocampal ripples were mistimed relative to the mPFC delta oscillation. Hippocampal ripple activity was also less modulated with the

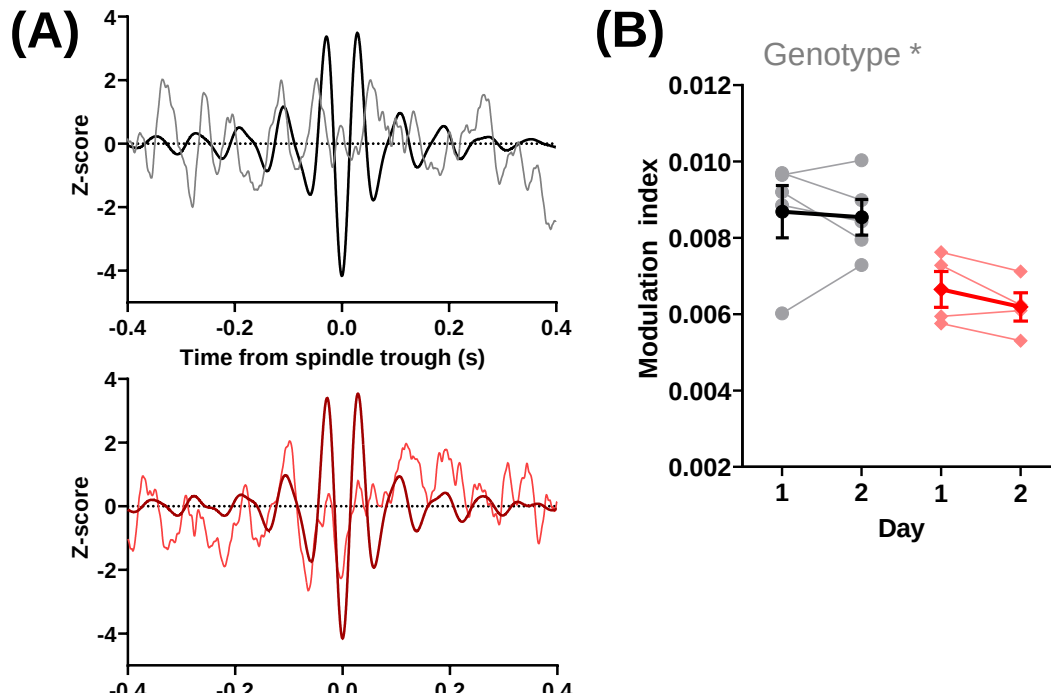


FIGURE 5.14: Hippocampal ripple power is less modulated by mPFC spindles in *Fmr1*<sup>-/-</sup> rats than WT. **(A)** Average ripple power during spindles. Bold lines show average spindle-filtered (10-20 Hz) mPFC signal for all spindles from each genotype, centred around the negative peaks. Pale lines show average hippocampal ripple (100-250 Hz) power during all mPFC spindles. **(B)** Spindle-ripple phase-amplitude coupling modulation index averaged over every spindle event for each rat (paler colours) and genotype means  $\pm$  SEM in bold, across each day (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ). \*  $p < 0.05$ .

phase of mPFC spindle oscillations. However, these represent preliminary findings which should be verified with longer recordings and more subjects. Changes in hippocampal ripple activity as well as cortical-hippocampal interactions may contribute to dysfunctional memory consolidation leading to cognitive deficits in *Fmr1*<sup>-/-</sup> rats and FXS patients.

#### 5.4.1 mPFC-hippocampus gamma coherence was increased in sleep as well as wake in *Fmr1*<sup>-/-</sup> rats

LFP power was largely similar between the genotypes, apart from lower power in the delta range seen in the mPFC of the *Fmr1*<sup>-/-</sup> rats (Figure 5.4). As this slow oscillation during SWS represents a highly synchronised cortical network state, cycling between high activity UP states and hyperpolarised DOWN states, lower

power in this range suggests the network is less synchronised. This would be consistent with a previous finding in *Fmr1*<sup>-y</sup> mice, in which firing rates are higher in barrel cortex during UP and DOWN states, indicating overall less coordinated firing patterns (Gonçalves et al., 2013). However, this analysis is limited by the lack of standardisation of the signals, and could be a result of differences in electrode impedances, or position within the cell layers of the mPFC. There was no difference in mPFC gamma power between the genotypes during SWS. This is different to the data obtained from the familiar and novel environment exploration discussed in Chapter 4, where mPFC gamma power was consistently higher in *Fmr1*<sup>-y</sup> rats. Interestingly, when the LFP was z-transformed before calculating SWS spectrograms, gamma power was significantly higher in *Fmr1*<sup>-y</sup> rats – this is likely due to the lower recorded delta power, as this would be the most prominent oscillation in the sleep session, so would be used to standardise the rest of the signal. Therefore, the results of LFP power analysis in the sleep sessions are difficult to interpret, but these results suggest that *Fmr1*<sup>-y</sup> rats show a reduced low frequency to high frequency power ratio. This could reflect an asynchronous state, and is similar to activity observed in mice with a lack of PV interneuron activity (Guyon et al., 2021). However, since the signals were not standardised, single unit activity would be needed to assess whether the activity observed here represents a similar phenomenon.

This reduced synchronisation into delta oscillations in the mPFC of *Fmr1*<sup>-y</sup> rats, paradoxically, could also explain the elevated gamma coherence between mPFC and hippocampus observed during SWS. Increased spiking during SWS that is less organised by the delta oscillation may have caused higher levels of non-specific synaptic input between the mPFC to hippocampus, possibly via other regions. There is some evidence that gamma oscillations coordinate communication between the mPFC and hippocampus during SWS, although whether there is a function for memory consolidation is not known (Ferraris et al., 2018). Therefore, this was not the main focus of this study, but it is nonetheless interesting that raised gamma coherence is also seen during SWS in *Fmr1*<sup>-y</sup> rats compared to WT.

### 5.4.2 *Fmr1*<sup>-/-</sup> rats show fewer and longer duration sharp wave-ripples

The finding of longer duration ripples agrees with a previous study of hippocampal activity in *Fmr1*<sup>-/-</sup> mice (Boone et al., 2018), suggesting this may be another cross-species effect of a lack of Fmr1. Although Boone et al. (2018) did not report any differences in ripple incidence, reduced occurrence of ripples was noted in *Fmr1*<sup>-/-</sup> mouse hippocampal slices in a recent preprint (Pollali et al., 2021, preprint), so further study of this may be required. Alternatively, decreased ripple incidence could be a potential species difference in the *Fmr1*<sup>-/-</sup> rodent models. Lower peak ripple oscillation frequencies were observed in *Fmr1*<sup>-/-</sup> mice (Boone et al., 2018), however, there was no difference between the genotypes in peak ripple oscillation frequency found in the current experiment (Figure 5.10E). Here, there was a trend towards this, with *Fmr1*<sup>-/-</sup> rats generally showing lower frequency ripples, but this was not statistically significant, so more subjects may be required to determine whether there are real differences in ripple frequency. Alternatively, this may be a species difference, as ripple oscillations have higher frequencies in WT rats when compared to WT mice (Mou et al., 2018). In agreement with this, the mean ripple frequencies are higher in both the WT and *Fmr1*<sup>-/-</sup> rats in the current study compared to those previously reported in the mice (Boone et al., 2018).

The finding of lower frequency ripples in *Fmr1*<sup>-/-</sup> mice was suggested to represent a change in interneuron firing during the oscillation (Boone et al., 2018). In the *Fmr1*<sup>-/-</sup> rats, ripples had longer durations than in WTs without a significant difference in the frequency of the oscillation, although there was a trend towards lower frequencies in *Fmr1*<sup>-/-</sup> rats. Longer duration ripples could suggest a recurrence of excitatory activity within CA3 and CA1 networks. Ripples are thought to be intrinsic to the hippocampus, generated spontaneously in CA3 (Buzsáki & Schomburg, 2015). The theory is that the fast oscillation of the ripple is generated by recurrent excitatory connections among CA3 pyramidal cells, activating both excitatory pyramidal and inhibitory interneurons in CA1 (Buzsáki et al., 1983; Csicsvari et al., 1999; Buzsáki & Schomburg, 2015). CA3 pyramidal cells are hyper-excitable in *Fmr1*<sup>-/-</sup> mice, showing lower action potential thresholds, broader action potentials and increased firing rates *in vitro* (Deng et al., 2013; Deng et al., 2019). Since the effects of this *in vivo* are not known, it is difficult to predict how this may affect ripple generation in the current study, however, it is possible that hyper-excitability

among CA3 pyramidal cells could lead to recruitment of more pyramidal cells and extension of the duration of bursts during ripples.

Another potential mechanism could be differences in GABAergic interneuron activity between the genotypes. Different groups of CA1 interneurons show different firing patterns during ripples, indicating there may be different populations involved in the initiation, oscillation, and termination of ripples (Csicsvari et al., 1999; Klausberger et al., 2003). The observed reduction in ripple incidence indicates that initiation of ripples may be impaired, with the prolonged duration perhaps due to reduction of termination. PV interneurons show increased firing during ripples, and their stimulation can induce ripples *in vitro* (Klausberger et al., 2003; Stark et al., 2014; Schlingloff et al., 2014), so could be affected in *Fmr1*<sup>-y</sup> rats, where ripple incidence is reduced. A recent study showed that the timing of individual pyramidal cells spiking during ripples is determined by the level of inhibition they receive prior to the ripple onset, suggesting specific sequences of pyramidal cell spikes are orchestrated by inhibitory networks (Noguchi et al., 2022). As individual interneuron populations have not been fully characterised in *Fmr1*<sup>-y</sup> rodents, it is difficult to provide a clear mechanism for the observed changes in ripple duration and incidence. However, inhibitory drive onto pyramidal cells is reduced in the hippocampus in *Fmr1*<sup>-y</sup> mice, including reduced tonic inhibition (Sabanov et al., 2017). The combination of this with the hyper-excitability of CA1 pyramidal cells in *Fmr1*<sup>-y</sup> rodents (Booker et al., 2020; Asiminas et al., 2021, preprint) may lead to discoordination of the precise inhibitory-excitatory interaction that occurs to orchestrate population bursts during ripples. This provides motivation for future research to investigate reactivation of ensembles during hippocampal ripples, as well as firing patterns of hippocampal interneurons, in *Fmr1*<sup>-y</sup> models, in order to understand how their discoordination may interfere with memory consolidation.

The reduced ripple incidence could reflect a reduction in reactivation of hippocampal memory sequences in *Fmr1*<sup>-y</sup> rats. As suppression of ripples results in impaired memory (Girardeau et al., 2009; Ego-Stengel & Wilson, 2010), this could contribute to deficits in memory. Asiminas et al. (2021, preprint) found that *Fmr1*<sup>-y</sup> rats showed a lack of CA1 place field refinement across days in a novel environment, using a similar protocol as described in the previous chapter. Reduced synaptic plasticity induced by ripples between the two days of the task may have resulted in the differences seen (Sadowski et al., 2016; Asiminas et al., 2021, preprint).

Silencing of place cell activity during ripples in mice had similar effects to those observed by Asiminas et al. (2021), with place cells showing less spatially-selective place fields following silencing during ripples (Roux et al., 2017). Therefore, the reduction of ripple activity observed here could provide an explanation for differences observed in the place cells of *Fmr1*<sup>-y</sup> rats (Asiminas et al., 2021, preprint).

An alternative explanation for the difference in ripple density between genotypes could be that ripples were less likely to be detected in *Fmr1*<sup>-y</sup> rats compared to WT. As the detection threshold is based on the standard deviation of the hippocampal signal filtered in the ripple band, higher baseline power in this range outside of ripple events could result in fewer ripples being detected, as they would be smaller in amplitude relative to the baseline ripple frequency. Although, comparison of spectral power in the ripple frequency range during SWS did not find any clear differences between genotypes. The hyper-excitability of pyramidal cells in CA1 of *Fmr1*<sup>-y</sup> rats (Asiminas et al., 2021, preprint) could result in higher firing rates generally during SWS, as was observed in the *Fmr1*<sup>-y</sup> mice (Boone et al., 2018), which could be detectable in the 100-250 Hz range used for ripple detection. However, analysis of single unit activity would be required to confirm this speculation.

There was a change in ripple frequency across the two days, with higher ripple frequency during sleep following the novel environment sessions (Day 2), compared to sleep following the familiar environment sessions (Day 1). This could reflect an increase in pyramidal cell firing rates during ripples. Higher CA1 pyramidal cell firing rates are seen during exploration of a novel environment compared to a familiar one (Nitz & McNaughton, 2004), and cell firing rates during wake are correlated with their firing during subsequent SWS ripples (Hirase et al., 2001). However, this effect did not differ between genotypes, consistent with the fact that the hippocampal network shows similar responses to novelty, such as theta power increase, in *Fmr1*<sup>-y</sup> rats (see Chapter 4).

### **5.4.3 Coordination of hippocampal activity with the cortical oscillations may be disrupted in *Fmr1*<sup>-y</sup> rats**

Models of memory consolidation involve a hippocampal-cortical interaction that mediates redistribution of hippocampal memory traces to the cortex (Frankland

& Bontempi, 2005). The mechanisms of this interaction are not fully understood, but ripples in the hippocampus are correlated with increases in cortical firing rates and reactivation patterns (Battaglia et al., 2004a; Peyrache et al., 2009). This may be a bidirectional relationship, with ripples initiating reactivation, and the timing of ripples being influenced by cortical activity (Mölle et al., 2006; Sullivan et al., 2011; Rothschild et al., 2017; Helfrich et al., 2019; Abadchi et al., 2020). This results in an organisation of ripple activity around cortical delta and spindle oscillations, which is important for memory consolidation (Sirota et al., 2003; Isomura et al., 2006; Maingret et al., 2016; Latchoumane et al., 2017). These relationships were explored in the current data, and compared between WT and *Fmr1*<sup>-/-</sup> rats.

Differences were identified in these timing relationships between the genotypes. Firstly, ripples appeared to be timed to an earlier phase of the mPFC delta oscillation in *Fmr1*<sup>-/-</sup> rats compared to WT. This was shown by an increase in ripple power sooner following the positive delta peak (Figure 5.11B-C), and a difference in the mean delta phase of ripple events (Figure 5.12A-B). This could reflect a discoordination of hippocampal and mPFC activity, which could cause deficits in the memory consolidation process. A limitation of these measurements is that variable numbers of both delta waves and ripples were detected in the different rats (Table 5.1), with significantly fewer ripple events, and a trend towards fewer delta waves, in *Fmr1*<sup>-/-</sup> rats. However, the fact that both measures showed that ripples were mistimed relative to the mPFC delta oscillation in *Fmr1*<sup>-/-</sup> rats, it can be concluded that this may be a real effect.

This difference in timing could be explained by hippocampal neurons responding differently to depolarisation by the cortical delta oscillation. Isomura et al. (2006) demonstrated that the membrane potentials of hippocampal neurons are influenced by the cortical delta oscillation under urethane anaesthesia, in which the brain undergoes a state resembling SWS. They also observed the modulation of ripple occurrence by delta phase during natural SWS, as reported in the current experiment (Isomura et al., 2006). The earlier delta phase of ripples in *Fmr1*<sup>-/-</sup> rats therefore could reflect hyper-excitability of CA1 neurons, possibly initiating ripple population bursts earlier in response to membrane depolarisation. This could result in mistiming of reactivation between hippocampus and cortical populations, which is coordinated by the delta oscillation. As mentioned previously, interneuron networks may be important for the generation of synchronous ripple bursts, and hippocampal interneuron membrane potentials are also modulated by the cortical

oscillation (Isomura et al., 2006; Hahn et al., 2007). The earlier delta phase of ripples could therefore, also reflect differences in interneuron discharge in *Fmr1*<sup>-y</sup> rats.

Possible discoordination of mPFC and hippocampal activity was also reflected in the decrease in modulation of ripple-band power by mPFC spindle phase in *Fmr1*<sup>-y</sup> rats (Figure 5.14). This has been observed in previous studies as a possible mechanism of coordinated reactivation of hippocampal and cortical ensembles for memory consolidation (Sirota et al., 2003; Peyrache et al., 2009; Clemens et al., 2011; Staresina et al., 2015), and discoordination of this relationship has been seen previously in a rat model of schizophrenia (Phillips et al., 2012). The mechanism for this relationship is not known, although it is possible that firing of direct projections from the hippocampus to the mPFC drives reactivation of mPFC ensembles, leading to the generation of spindles through feedback projections with the thalamus. This is evidenced by a study showing that mPFC spiking follows hippocampal ripples with a delay, and the subsequent spindle power is correlated with the magnitude of the initial hippocampus burst (Wierzynski et al., 2009). In addition, the timing relationship between hippocampal ripples and mPFC spindles is disrupted by the optogenetic inhibition of direct ventral and intermediate CA1 projections to mPFC (Binder et al., 2019). Therefore, the reduced spindle-ripple modulation observed in the *Fmr1*<sup>-y</sup> rats could potentially be a result of reduced hippocampal synaptic input to mPFC. Analysis of single unit activity would be needed to verify this.

These data provide some preliminary evidence that cortical-hippocampal coordination during SWS may be disrupted in *Fmr1*<sup>-y</sup> rats. This discoordination could have consequences for memory consolidation, as improved temporal association of hippocampal ripples with delta and spindle activity in the cortex has been linked to improved memory (Maingret et al., 2016; Latchoumane et al., 2017). The mistiming of ripples in relation to mPFC delta oscillations observed in *Fmr1*<sup>-y</sup> rats could cause mistiming of the coordinated reactivation of memories in each area (Peyrache et al., 2009). Long-term memory deficits, which may rely on consolidation of memories from hippocampus to mPFC, have been shown in *Fmr1*<sup>-y</sup> mice, and this could provide a potential mechanism for such impairment. Changes in coupling between the mPFC and hippocampal activity may also provide further evidence that connectivity between the two regions is impaired generally in *Fmr1*<sup>-y</sup> rats.

### 5.4.4 Limitations and future work

The main limitation of this dataset may be the relatively short recording sessions, which limited the amount of SWS time recorded for each rat (see Table 5.1). This led to variation in many of the measures, as some rats slept for almost the entire session, while others were more restless and a relatively high proportion of the session was spent awake. While most of the timing relationships could still be observed, these may have been strengthened by longer recordings. The sessions were limited to 30 minutes because the initial focus of the experiment was on the hippocampal and mPFC activity recorded during exploration of the familiar and novel environments reported in Chapter 4. However, the preliminary results of this chapter may provide justification to record longer periods of mPFC-hippocampal activity during sleep in *Fmr1*<sup>-y</sup> rats. Comparison between the genotypes would also be strengthened by only comparing similar amounts of SWS time between the different rats. Since the subject numbers are also currently low, this would have limited the dataset significantly in its current form, so instead measures have just been standardised by SWS time. However, it is possible that the overall amount of SWS could have had an impact, for example on quantification of temporal interactions between ripple, delta and spindle events. This could also explain some of the changes across days, so future analyses may need to down-sample all data to the shortest epochs of SWS. As all event occurrence measurements were standardised by total SWS time, this variation should not have had too great an effect, so that results can reasonably be interpreted.

The current recordings did not yield sufficient periods of REM sleep to analyse, but longer recording times would mean that this could be another network state to investigate in *Fmr1*<sup>-y</sup> rats. This could be especially valuable as studies have identified a deficit of REM sleep among FXS patients (Musumeci et al., 1995; Miano et al., 2008). However, the focus of this analysis was on memory consolidation processes during SWS so analysis of REM was not prioritised.

It would be interesting to compare whether there are any differences in upregulation of memory consolidation after learning between the genotypes. Upregulation of ripple density, spindle density and temporal associations of ripples with delta waves and spindles have been shown after learning in previous studies (Gais et al., 2002; Eschenko et al., 2006; Eschenko et al., 2008; Maingret et al., 2016). It was predicted that there might be changes in the cortical-hippocampal interactions

during SWS across the two days related to the familiarity/novelty of the environment in the preceding exploration sessions. However, there were no clear effects of exploring a novel environment before sleep on Day 2 compared to exploring a familiar environment before sleep on Day 1, apart from an increase in ripple oscillatory frequency during sleep following the novel environment exploration in both groups. This could possibly have been due to the differences in amount of SWS time recorded on each day. It could also have been due to the sessions being across two days, where previous studies have generally made comparisons between sleep sessions on the same day before and after a task (Girardeau et al., 2014; Maingret et al., 2016). Although, this could also be a strength of the current study, as the sleep recordings were carried out at around the same time on each day. It would be interesting to compare sleep following training on a rewarded task, where motivation is increased and it would be expected for upregulation of consolidation to be most required. The novelty used in the current experiment may not have represented a novel enough experience to significantly affect consolidation mechanisms measured from the LFP, as the experience of exploring an environment over multiple sessions is common to both days (Duszkiewicz et al., 2019). Therefore, in order to see upregulation of spindles and ripples, as well as their interactions, a comparison may need to be made between sleep following a very familiar experience and sleep following either learning of a task, or a more novel experience than the one used here.

Comparison of the organisation of cellular activity between genotypes would provide further characterisation of memory consolidation processes. Hippocampal and mPFC ensembles have been shown to reactivate during sleep after a learning task (Peyrache et al., 2009). Given the longer duration ripple activity in *Fmr1*<sup>-y</sup> rats, it is likely that the dynamics of ensemble reactivation are different, perhaps disrupting the timing with mPFC activity. The possible mistiming of ripples relative to the cortical delta oscillation and reduced spindle phase modulation found here may reflect the disruption of hippocampal-mPFC coordinated reactivation, but cannot be fully confirmed without recording neuronal spiking. Examination of single unit activity would also help to identify UP and DOWN states during SWS, which can be more accurately estimated from population firing than the LFP alone (Saleem et al., 2010). This would help to identify whether findings of altered cortical UP state activity *in vitro* in *Fmr1*<sup>-y</sup> mice are matched by intrinsic *in vivo* activity in rats (Gibson et al., 2008; Gonçalves et al., 2013).

#### 5.4. Discussion

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The current experiment finds a number of differences in memory consolidation-associated activity in the mPFC and hippocampus of *Fmr1*<sup>-/-</sup> rats compared to WT. Differences in ripple properties could reflect dysfunctional reactivation of memory ensembles in the hippocampus which could underlie lack of place field refinement across days (Asiminas et al., 2021, preprint). Alterations in coordination of ripples with mPFC activity could further disrupt memory consolidation in *Fmr1*<sup>-/-</sup> rats and could underlie learning and memory deficits in FXS.



## Chapter 6

# Conclusions

The experiments presented in this thesis aimed to investigate the function, and dysfunction, of cortical-hippocampal connections in spatial and episodic memory. The first two experiments investigated the impact of loss of input from the MEC on hippocampal memory processes. The subsequent experiments then investigated the connection between the hippocampus and mPFC, and how interactions may be affected by intellectual disability.

### 6.1 The role of the EC in hippocampal memory formation

Chapters 2 and 3 assessed the role of the MEC in forming a spatial context representation for hippocampal memory. The effect of lesion of the entire MEC on use of distal landmarks by place cells in CA1 of the hippocampus was first tested, then the effect of L2SC inactivation on hippocampus-dependent associative memory was investigated. The following will summarise the main results of these two sections, and discuss the implications for episodic memory formation in the entorhinal-hippocampal system.

Through recording CA1 place cells in mice with MEC lesions, the experiment in Chapter 2 demonstrates a key function of the MEC input to the hippocampus: to provide information about distal visual landmarks on which to anchor the spatial representation. Specifically, hippocampal place cells did not rotate with distal visual landmarks in lesioned animals. However, they did show anchoring to proximal

cues within the environment (objects), indicating that proximal cue information for objects must access the hippocampus via a different route. CA1 place cells in MEC-lesioned mice also showed a reduction in spatial selectivity, possibly due, at least in part, to the loss of reliable distal cue information. However, the spatial information of place cells in the lesion mice was also lower than controls in the proximal cue condition, suggesting another source of location information is impaired. This may be a result of loss of path integration mechanisms, shown to be reliant on the MEC (Jacob et al., 2020).

These findings build on previous research showing that the MEC is required for spatial navigation, and provide clear evidence in support of the theory that proximal and distal cue information in the hippocampus are provided by separate input streams (Knierim et al., 2013). It was predicted based on previous research that the MEC would provide distal cue information to the hippocampus, as spatial cell types in the MEC also orient to distal landmarks, and loss of MEC input impairs hippocampus-dependent spatial tasks that are likely to rely on distal cues (Fyhn et al., 2007; Hales et al., 2014; Tennant et al., 2018; Poitreau et al., 2021). However, the data shown in Chapter 2 demonstrate the direct consequence of loss of MEC input on distal landmark use in the CA1 spatial representation. Proximal information such as objects, on the other hand, are likely to be processed by the LEC, as cells in the LEC show object-associated firing (Deshmukh & Knierim, 2011; Tsao et al., 2013), and loss of LEC input to the hippocampus causes impairments in hippocampus-dependent object associative memory tasks, including OP, OC and OPC (Wilson, Watanabe, et al., 2013; Vandrey et al., 2020). Furthermore, our data are consistent with a recent experiment showing that disruption of LEC inputs reduced object-associated firing in the DG of the hippocampus, while disruption of MEC inputs had no effect on this (Fernández-Ruiz et al., 2021).

Another finding of the experiment in Chapter 2 was that, in control mice, rotation of proximal cues caused a lower proportion of place cells to rotate than rotation of the distal landmarks. This is consistent with experiments where landmark- or object-sized cues were projected onto the floor of an environment, showing that more place cells rotated with cues that covered a larger proportion of the floor (Scaplen et al., 2014). These results demonstrate differences in how types of cues are treated by the hippocampus – distal landmarks are more reliable for anchoring of the spatial representation than proximal objects. This may reflect the different

functions of the two types of information, with distal cues being used for determination of current location in relation to the outside environment, and proximal cues being represented as items within the environment. It would be interesting to determine the effect of LEC lesions in the cue rotation protocol used in Chapter 2, in order to further test the separation of inputs for these cue types in the place cell representation.

In a further experiment using the same apparatus, Scaplen et al. (2017) reported an increase of CA1 place cell rotation with “landmark” visual cues when the LEC was inactivated. However, there was no effect of LEC inactivation on rotation with the visual “object” cues, though there was an increase in spatial information compared to when the LEC was not inactivated. Very low proportions of cells rotated with the “object” cues in the control conditions in both studies (Scaplen et al., 2014; Scaplen et al., 2017), so it would be interesting to study the effects of LEC inactivation in conditions with multimodal objects, as used in the experiment described in Chapter 2, where there appeared to be more representation of the object cues. Further, the researchers did not inactivate MEC, so the role of the MEC in use of the projected floor “landmark” cues was not determined. An interesting further investigation may be to examine the key differences in cues that determine their use as a landmark or object, and the dependence of the MEC and LEC for representation in the hippocampus. Scaplen et al. (2017) used purely visual cues in their experiment, which were always on the floor of the environment, so it may be interesting to test the differences in representation of different sensory modalities in the hippocampus, and their dependence on MEC and LEC. The rotation experiment used in Chapter 2 could perhaps be expanded to include a version with pure odour cues, for example. It may be predicted that representation of odour information in the hippocampus would be dependent on LEC inputs, as the LEC receives strong projections from the olfactory system, and LEC activity shows modulation during odour discrimination and associative learning (Igarashi et al., 2014; Leitner et al., 2016; Nilssen et al., 2019). However, different odours have been shown to cause shifts in MEC grid cell firing, suggesting odour information is also represented in the MEC (Marozzi et al., 2015). Therefore, it may be possible that the MEC is required for rotation with odour cues if they can be used as landmarks to define the spatial representation.

Chapter 3 aimed to determine the function of a particular MEC-hippocampus pathway in contextual associative memory. Multiple lines of research have shown that

MEC L2SCs may be required for defining context in the hippocampus (Latuske et al., 2018). One study showed context-specific firing of MEC L2SCs (Kitamura et al., 2015), a number of studies have shown changes in MEC cell spatial firing between different contexts (Fyhn et al., 2007; Marozzi et al., 2015; Diehl et al., 2017), and manipulations of L2SCs can artificially induce changes in hippocampal place cell firing that are similar to remapping between contexts (Kanter et al., 2017). Hence, it was hypothesised that MEC L2SCs provide the hippocampus with information about spatial context. Therefore, it was predicted that mice with inactivated MEC L2SCs would show impaired performance on a hippocampus-dependent object-place-context association task. Results from this experiment were largely inconclusive. L2SCs were inactivated through injection of a virus that causes the selective expression of tetanus light chain toxin in the stellate cell population. However, this yielded low levels of expression so it is likely that the population of cells affected by the manipulation was not large enough to make robust conclusions about function. Additionally, control mice failed to show discrimination between the novel and familiar OPC pairing, so assessment of L2SC-inactivated mice on this task, which was predicted to be impaired, was not possible.

Therefore, questions still remain over the role of L2SCs of the MEC in contextual processing for associative memory. The role of the MEC in hippocampal contextual processing is unclear, as results from experiments have been variable. Multiple studies have shown global remapping-type responses of hippocampal place cells in response to manipulations of the MEC, including L2SCs specifically. Moreover, inactivation of L2SCs results in impaired contextual discrimination by CA3 place cells, and impaired contextual fear conditioning (Kitamura et al., 2015; Kanter et al., 2017; Latuske et al., 2018); however, global remapping in CA1 place cells has been shown in rats with complete MEC lesions (Schlesiger et al., 2018). Whether the hippocampal place cells remap to contextual changes in the absence of MEC inputs may depend on a myriad of external factors, including the sensory modality and extent of context changes. For example, it may be that very large changes, such as changing the room in which the different context is experienced, as well as the sensory features of the environment, is enough of a different experience for inputs other than the MEC to support global remapping in the hippocampus (Schlesiger et al., 2018). While contexts that are (presumably) in the same room, although differing in multiple sensory dimensions, cannot elicit distinct population encoding in CA3 without sufficient input from MEC L2SCs (Kitamura et al., 2015).

Global contextual remapping therefore may result from a summation of the differing features – if enough inputs to the hippocampus are modulated by external changes, completely distinct maps will be generated. Consequently, it may be possible for the hippocampus to show remapping in the absence of L2SCs, and even the entire MEC, if the remaining inputs from other regions are modified significantly enough by the context change for the hippocampus to represent the environments with different spatial maps. Other factors may include the animal's attention and perception of distinctions between different environments, which may be more difficult to control, but are nonetheless important for place cell stability (Kentros et al., 2004; Colgin et al., 2010).

Hence, future study of the role of MEC L2SCs in contextual processing will require these factors to be considered where possible. One way in which this could be achieved is by inactivating MEC L2SCs and testing place cell remapping in response to varying degrees of carefully controlled context changes. This could include changes only in visual characteristics, shape, odour, or texture of the context, as well as combinations of these, and the external surroundings, possibly changing the location of the context or polarising external cues. Demonstrating which of these factors are not represented by changes in place cell firing in animals with inactivated MEC L2SCs may help to determine specifically which types of contextual information these inputs convey. As results in Chapter 2 determined that MEC inputs are required for use of distal cues in the hippocampal representation, it could be that contextual changes based on distal information, such as changing the external cues around the environment, do not induce place cell remapping in MEC L2SC-inactivated animals. Place cell rate remapping in response to changes of only visual characteristics of the environment may be processed by the LEC, as lesions have been shown to reduce this response (Lu et al., 2013).

Studying how place cells respond to and represent cues, objects and contextual features have provided important insight into the functional mechanisms of hippocampal memory and the supportive role of the EC. As the results of Chapter 2 and other studies show, there is some distinction in the roles of MEC and LEC. However, the fact that hippocampal spatial firing can be preserved in the absence of MEC, along with remapping in distinct environments, suggest that other regions provide some level of spatial and contextual input. There also remains some level

of inconsistency to the context/content theory of MEC/LEC function. Studies assessing the effects of lesions of each region on behaviour have shown results suggesting levels of task demand may dictate the requirement of LEC and MEC. For example, Rodo et al. (2017) showed that the MEC was required for detecting a novel object-location, but only when all of the objects were different, not when they were identical, and the LEC was required for detection of a novel object when four different objects were used. Yoo and Lee (2017) investigated the effects of MEC and LEC inactivation as rats were trained to perform a spatial (T-maze turn) or non-spatial (interaction with an object) response to particular visual scenes. MEC inactivation impaired only the spatial response condition, while LEC inactivation impaired only the non-spatial response condition. This result suggested that the MEC is not required for visual scene discrimination, as may have been predicted from the context/content hypothesis. However, this does not necessarily test the use of spatial context (Yoo & Lee, 2017). In another comparison between the effects of MEC and LEC lesions, it was shown that the LEC (but not the MEC) is required for a navigation task based on local object cues. However, counter to their predictions, the MEC was not required for a task that required use of distal landmarks (Kuruville & Ainge, 2017). In contrast, the MEC has been shown to be required in a Morris water maze task based on distal cues, while the LEC did not appear to be required for a proximal cue version of the task (Poitreau et al., 2021) (although, as previously discussed, it is possible these cues were processed as landmarks). Therefore, it is challenging to distinguish the roles of MEC and LEC with behavioural tasks, as compensatory strategies may be used in the absence of inputs. However, there is evidence to suggest that the functional distinction of MEC and LEC may not be as clear-cut as previously thought.

A full understanding of this may require further study of other regions in the medial temporal system and how inputs mediate the constituent parts of EC activity (Figure 6.1). For example, investigation of the firing of cells in the postrhinal cortex (POR in Figure 6.1) found modulation by visual cue changes (Burwell & Haffeman, 2003), object-location selectivity (Furtak et al., 2012), as well as modulation by egocentric head direction (LaChance et al., 2019). A recent study also showed that postrhinal cells can also respond to multiple visual landmarks (LaChance et al., 2022). The postrhinal cortex was previously thought to project only to the MEC, but more recent evidence suggests that it may project to LEC and MEC equally (Doan et al., 2019; Nilssen et al., 2019). Therefore, the postrhinal cortex may provide a major source of spatial input to both the MEC and LEC, possibly for anchoring to

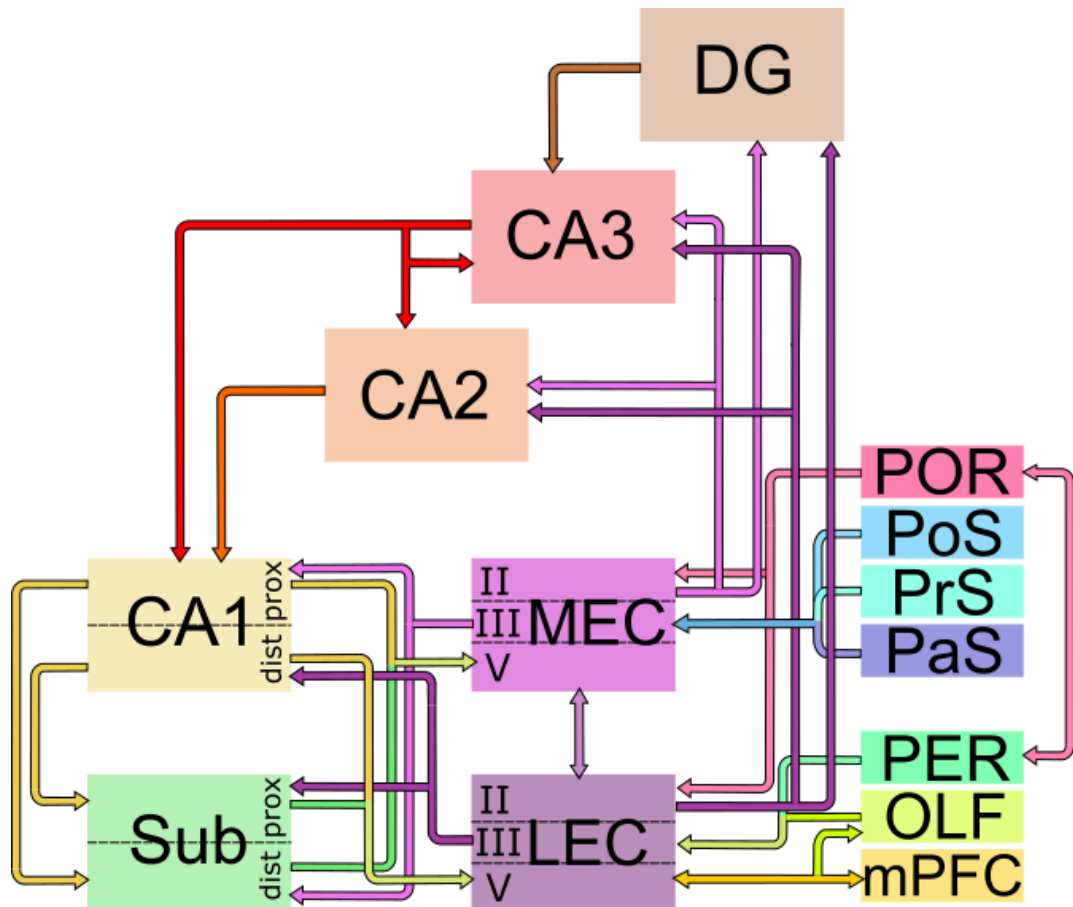


FIGURE 6.1: Some of the main excitatory connections of the hippocampus and entorhinal cortex. Sub = subiculum; POR = postrhinal cortex; PoS = postsubiculum; PrS = presubiculum; PaS = parasubiculum; PER = perirhinal cortex; OLF = olfactory system (Adapted from Nilssen et al., 2019).

visual cues, and conveying egocentric spatial information particularly to the LEC (Wang et al., 2018). The perirhinal cortex, on the other hand, is thought to project mainly to the LEC, and only weakly to the MEC (Burwell & Amaral, 1998), which is consistent with its role in object processing (Brown & Aggleton, 2001). Both perirhinal and postrhinal lesions cause object-context and contextual fear impairment, and their disconnection from one another causes the same effects (Norman & Eacott, 2005; Heimer-McGinn et al., 2017). This suggests that combination of object and context information may occur upstream of the EC. In order to understand this system, an interesting avenue of future research may be further studying the functions of connections between regions upstream of the hippocampus. This may include studying the effects of inactivation of projections from postrhinal cortex to MEC on anchoring of MEC cells to visual cues.

Studying the connections between the MEC and the LEC may provide interesting insights into the information carried by each, and allow them to be viewed more as carrying integrated information about the outside environment for memory formation. The fact that context is represented by the firing within both regions (Tsao et al., 2013; Diehl et al., 2017), could implicate the projections between the two in providing a unified context for processing of both spatial position and external items within it. Anatomical data suggest that superficial pyramidal cells of the MEC project to the LEC, while superficial LEC pyramidal cells project more within the LEC (Nilssen et al., 2019). There is also evidence to show that activity of L2SCs in the MEC is necessary for the sequential embryonic development of the hippocampus, followed by the LEC (Donato et al., 2017). This may point to the MEC as being the origin of overall spatial context, on which episodic experience is built within other regions such as the LEC. The role of the LEC may then be in binding object information from upstream regions such as perirhinal cortex, with the contextual information provided by the MEC, to form the basis of episodic memory. A representation of time may be more important to the LEC (Tsao et al., 2018), as items within the spatial context are likely to develop over an episode. An interesting experiment may be to disrupt the projections from the MEC and test the effects on LEC spatial and contextual firing.

Clearly there is an important role for the MEC in forming spatial context, which is further evidenced by the results of Chapter 2, showing that the representation of space in the hippocampus based on distal visual landmarks is disrupted by MEC lesions. Chapter 3 was inconclusive, although it would still be interesting to determine whether MEC L2SCs are required for associative memory based on contextual discrimination. Although different tasks may be needed to test the requirement of MEC for spatial context, rather than sensory context.

## **6.2 Disrupted memory network activity in Fragile X Syndrome**

In the final two experimental chapters, a different hippocampal connection important for episodic memory was studied in the context of a neurodevelopmental disorder. Hippocampal and mPFC network activity were analysed in a rat model of FXS, an inherited intellectual disability. These experiments aimed to characterise

any genotypic differences in network function which may underlie cognitive deficits seen in FXS and its rodent models.

In Chapter 4, differences in local field potentials were observed between *Fmr1*<sup>-y</sup> rats and WT rats as they explored a familiar and a novel environment; *Fmr1*<sup>-y</sup> rats showed increased gamma power in the mPFC, and alterations of functional connectivity between mPFC and hippocampus. No specific differences were observed in hippocampus-mPFC connectivity in relation to the novelty in the task, so results cannot be interpreted in relation to this specific function of the circuit. Therefore, a short-term objective of future research would be to assess the functional connectivity between these two regions in *Fmr1*<sup>-y</sup> rats in a task of cognitive flexibility or associative memory, in order to more directly demonstrate how the differences observed here are affected when the circuit is challenged in a task that relies on hippocampal-mPFC interactions. It could be predicted that the hyper-synchronisation of mPFC and hippocampus observed in Chapter 4 may cause the system to be inflexible when interactions are needed. As deficits have been observed in *Fmr1*<sup>-y</sup> mice during tasks with conflicts, such as when changing the shock location in a place avoidance task (Radwan et al., 2016), a test of cognitive flexibility may be most interesting, for example a spatial/response strategy-switching task (Rich & Shapiro, 2009). It would also be interesting to examine mPFC single unit activity in such a task, with the prediction being that neurons of *Fmr1*<sup>-y</sup> rats would show impaired extinction of previously-learned rule representations. This would provide more direct evidence for the functional consequences of the physiological changes described in Chapter 4.

Since one of the major justifications for studying hippocampus-mPFC connectivity in the *Fmr1*<sup>-y</sup> rats was the deficit in OPC memory (Till et al., 2015), investigation of the whole mPFC-EC-hippocampal circuitry would also be interesting, as this task is dependent not only on the hippocampus and mPFC, but also on the entorhinal cortex (Chao et al., 2016; Barker & Warburton, 2020). Evidence that the hippocampus receives reduced input from the EC in *Fmr1*<sup>-y</sup> mice and rats provides further reason to examine this pathway as a possible source of memory impairments (Booker et al., 2020; Asiminas et al., 2021, preprint). The longer range pathways between hippocampus and mPFC, and mPFC and EC are yet to be characterised in *Fmr1*<sup>-y</sup> rodents, so this thesis is the first evidence that connectivity between mPFC and hippocampus is disrupted.

The results of Chapter 5 also found differences in network activity, including altered functional connectivity between the hippocampus and mPFC in *Fmr1*<sup>-y</sup> rats during slow wave sleep. Although, similarly to Chapter 4, no differences in connectivity were observed related to whether the sleep was recorded after exploration of the familiar (Day 1) or novel (Day 2) environments in the familiarity/novelty protocol, interactions between the two areas that have been previously linked to memory consolidation were observed on both days, in both genotypes, but with some alterations in *Fmr1*<sup>-y</sup> rats. In both genotypes, hippocampal ripples showed evidence of temporal organisation around the mPFC delta oscillation, as ripple activity was decreased during the hyperpolarised DOWN states in the mPFC signal (Mölle et al., 2006). However, in the *Fmr1*<sup>-y</sup> rats, ripple incidence was decreased, and average duration of ripple events was longer than in WT. Differences were also observed in timing of ripples in relation to the delta oscillation, as the *Fmr1*<sup>-y</sup> rats showed earlier ripple activity increases as the mPFC delta oscillation transitioned to the depolarised UP state. Further, in the WTs, there was evidence of modulation of ripple power with the phase of mPFC spindle oscillations, and this was significantly lower in *Fmr1*<sup>-y</sup> rats.

Activity during SWS can be considered as a reflection of circuit function without the confound of current behavioural differences, so as well as telling us something about the process of memory consolidation in *Fmr1*<sup>-y</sup> rats, these data may provide an indication of the general state of the circuit. The findings therefore suggest general deficits in hippocampal-cortical connectivity in *Fmr1*<sup>-y</sup> rats. The mistiming of ripples with delta oscillations may be a result of hyper-excitable cells within the hippocampus, possibly with the combination of reduced inhibitory drive. There is some evidence to suggest that the influence of ripple power on spindle oscillations results from a direct interaction mediated by hippocampal-mPFC projections (Wierzynski et al., 2009; Binder et al., 2019); therefore, the results may indicate that this functional connectivity is impaired in *Fmr1*<sup>-y</sup> rats.

Chapter 5 represents preliminary data, as there was considerable variability in length of SWS time between rats, so longer recordings would be needed to increase reliability of the findings. Investigation of single unit activity during sleep following a spatial memory task would provide more solid conclusions related to memory consolidation mechanisms, as coordinated reactivation of task-related memory ensembles in mPFC and hippocampus could be investigated (Peyrache et al., 2009). It would be expected that, due to the differences in ripple duration,

there may be differences in the reactivation of hippocampal ensembles, possibly in the specificity of cells firing. Furthermore, as binding of networks during coherent oscillations appears to play an important role in hippocampal-mPFC coordinated replay, the specificity of this may be disrupted by the hyper-synchronicity during wake. Disruption of this process could have significant effects for episodic memory, as consolidation during sleep is an important process.

Both Chapter 4 and 5 describe network-level differences in the hippocampus and mPFC of *Fmr1*<sup>-/-</sup> rats compared to WT. Likely one of the most reliable of these findings was the elevated gamma power in the mPFC of *Fmr1*<sup>-/-</sup> rats. Increased gamma power appears to be a feature of many cortical circuits in *Fmr1*<sup>-/-</sup> rodents and FXS patients (Ethridge et al., 2016; Lovelace et al., 2018), representing a cross-species biomarker of the disorder. This may be a reflection of imbalanced excitatory and inhibitory circuitry, as a result of hyper-excitability of principle cells, in combination with reduced inhibition. Gamma oscillations and interneuron function are associated with regulation of network activity, and increasing the specificity of output signals. Gamma oscillations are thought to provide the function of compartmentalising the firing of different ensembles into separate oscillation cycles, to decrease the interference between different encoded items (Lisman & Jensen, 2013; Fries, 2015). In sensory cortices, alterations of gamma synchrony may cause hypersensitivity to sensory stimuli, a common characteristic of autism spectrum disorder, which has been correlated with elevated gamma power in FXS (Ethridge et al., 2017). Extension of this observation to the mPFC is possibly important in understanding the cognitive profile of FXS. In the mPFC, gamma oscillations have been associated with interneuron firing underlying selective attention, and stimulation of interneurons in the mPFC has also been shown to accelerate extinction of previously learned responses (Sparta et al., 2014; Kim et al., 2016). This suggests mPFC gamma oscillations reflect a network organisation process that ensures flexible selection of relevant behavioural outputs. Thus, the dysregulation of this organisational process could result in the working memory and cognitive inflexibility deficits seen in *Fmr1*<sup>-/-</sup> rodents and FXS patients.

Over-synchronous interactions between the hippocampus and mPFC, and possibly other medial temporal regions that are connected with the mPFC, may cause effects specifically related to episodic memory. As a key function of the mPFC in declarative and episodic memory is thought to be the reduction of interference between similar patterns, inflexible connections may severely disrupt this process,

limiting the ability to disambiguate hippocampal representations (Eichenbaum, 2017; Guise & Shapiro, 2017). A similar lack of circuit flexibility was seen within the hippocampus in *Fmr1*<sup>-y</sup> mice during a place avoidance task, in which *Fmr1*<sup>-y</sup> mice did not show attenuation of gamma coherence between cell layers, as WT mice did (Radwan et al., 2016). Determining whether the consistently elevated coherence between mPFC and hippocampus shows similar inflexibility in memory tasks will help to better understand the deficits caused by this.

FMRP has a very wide range of functions, including regulating the translation of a large number of proteins, and interacting directly with ion channels, affecting cell excitability (Darnell et al., 2011; Deng et al., 2013). Additionally, variable effects of loss of FMRP have been observed in neurons of different brain regions. However, it appears that gamma power is elevated in multiple cortical regions of mice, rats and humans with a lack of FMRP, suggesting a common network state which could explain multiple different symptoms. There is increasing evidence suggesting that a deficit of inhibitory interneuron activity plays a key role in many of the effects of FXS, and other causes of autism spectrum disorder (Contractor et al., 2021). Interneuron activity has not been extensively studied *in vivo* in *Fmr1*<sup>-y</sup> rodent models, but where it has been reported on, desynchronization of interneuron activity, with reduced phase-locking to the gamma oscillation, has been observed in the hippocampus (Talbot et al., 2018). Another study of *Fmr1*<sup>-y</sup> mice (and female *Fmr1*<sup>-/-</sup> mice) found impaired visual discrimination ability, which was linked to deficits in excitatory cell tuning in the visual cortex, along with decreased interneuron firing (Goel et al., 2018). Similar study of interneuron activity in both the mPFC and hippocampus of *Fmr1*<sup>-y</sup> rats in relation to memory tasks would be interesting to determine mechanisms of memory impairments in FXS.

Altered gamma oscillations have also been reported in individuals with autism spectrum disorder from causes other than FXS, as well as some other rodent models of autism spectrum disorder (Rojas & Wilson, 2014; Contractor et al., 2021). This suggests that this may be a reflection of a common mechanism of autism spectrum disorder conditions generally. An imbalance of circuit excitatory and inhibitory drive has been proposed as a general mechanism across a wide range of disorders. Recent evidence has shown that some differences from WT mice result not only from the direct effects of loss of FMRP, but also as a result of homeostatic mechanisms compensating for imbalances of excitation and inhibition in *Fmr1*<sup>-y</sup>

rodents (Antoine et al., 2019; Booker et al., 2020; Liu et al., 2022). This adds another level of complexity to understanding the mechanisms of FXS, and presents a significant challenge for developing therapies. For example, if decreased inhibitory activity is the result of a homeostatic mechanism, rather than a direct effect of lack of FMRP, attempting to treat the disorder through rescue of interneuron function during development could have detrimental effects (Contractor et al., 2021). As mentioned previously, it is possible that different mechanisms act within different brain regions, so study of circuit composition and network activity across the whole brain is required.

Equally, study of changes across development are crucial. Initiating production of FMRP within the mPFC of *Fmr1*<sup>-y</sup> mice during adulthood can rescue a behavioural deficit on a task relying on mPFC function, suggesting at least some cognitive impairments may be due to continued absence of FMRP in the mPFC (Siegel et al., 2017). However, this study used a relatively simple behaviour, and other deficits of function of the mPFC may be caused by aberrant development. Asiminas et al. (2019) found, in addition to different developmental trajectories in object association abilities in *Fmr1*<sup>-y</sup> rats, that deficits in LTP in the mPFC, and LTD in the hippocampus, are observed only at particular developmental time points. This suggests that the function of FMRP may change in both regions over this time. The development of mPFC is protracted relative to other brain regions, with synaptic pruning ongoing into adolescence, which has been described as a critical window for functional development (Chini & Hanganu-Opatz, 2021). Projections from the hippocampus may have an important role in early development of the mPFC. In rodents, through neonatal development, mPFC oscillations increase in frequency to reach adult gamma frequencies by around P25, which is within a range thought to be analogous to the adolescent period in humans (Bitzenhofer et al., 2020; Chini & Hanganu-Opatz, 2021). Before P12, bursts of lower frequency oscillations are seen, which occur in response to activation of hippocampal projections to the mPFC (Ahlbeck et al., 2018). Therefore, it is possible the increased coherence between the hippocampus and mPFC observed in the data presented in this thesis could have implications for development, possibly causing aberrant maturation of the mPFC. Recording the developmental trajectory of this pathway could generate data that might explain many of the cognitive symptoms observed in FXS. During this time and continuing through until around P60, there is also a gradual increase of inhibitory activity in the mPFC (Caballero & Tseng, 2016). Fully characterising all of these changes in circuitry in *Fmr1*<sup>-y</sup> rodents could provide a clear mechanism

for cognitive deficits in FXS, as well as identifying particular periods and targets for treatments.

To add yet another level of complexity, as mPFC development goes on into adolescence, the effects of experience may have a longer critical window of impact (Kolk & Rakic, 2022). Loss of FMRP may have a greater effect on how the brain reacts to environmental stressors, impacting development more greatly. Effects of environmental factors could be a source of variability in network activity and cognitive symptoms between different individuals. In people with FXS, environmental factors, such as familial warmth, have been correlated with reduced severity of many symptoms of the disorder (Smith et al., 2016; Dahlhaus, 2018), and there is some evidence to suggest that early social enrichment can have beneficial effects for hippocampal neuronal development in *Fmr1*<sup>-y</sup> mice (Oddi et al., 2015). Effects of such interventions on network activity and development, especially in the mPFC, would also be an interesting area for future research.

In summary, this thesis reports the first analysis of long-range connectivity in *Fmr1*<sup>-y</sup> rats, showing alterations in the oscillatory connectivity between the mPFC and hippocampus, a key circuit for episodic memory encoding and retrieval. Hyper-synchrony between the two regions could reflect inflexibility in the circuit, which could disrupt normal function. There may also be impairments in the interactions between the regions for memory consolidation during sleep.

### **6.3 Function and dysfunction of cortical connections with the hippocampus**

This thesis reports on the function of two different cortical connections with the hippocampus, with important roles in memory processes. As well as demonstrating deficits in hippocampal processes following lesion of a major cortical input, the MEC, or as a result of loss of FMRP linked to a neurodevelopmental disorder, many processes remain – the hippocampus can still generate spatial firing and many behavioural abilities remain intact. This is testament to the complexity and plasticity of the brain, and its resilience to disruptions.

## Appendix A

### Chapter 2 Supplementary Figures

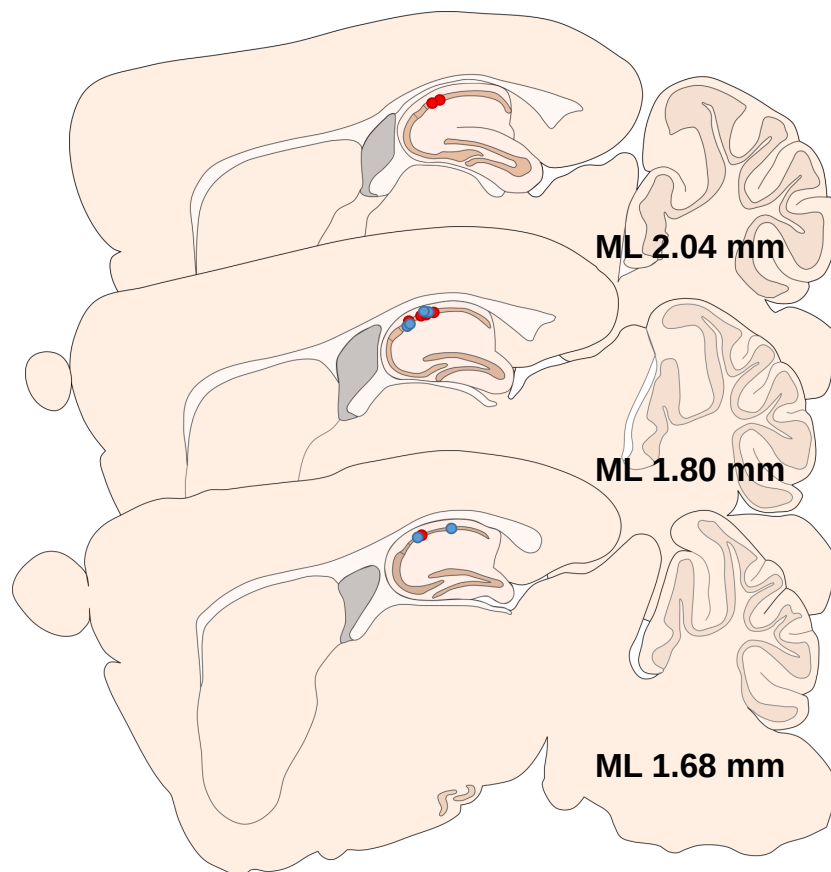


FIGURE A.1: Estimated tetrode tip locations of all mice from histology. Blue dots represent control mice (n = 6), red dots represent MEC lesion mice (n = 7). Approximate ML measurements were determined from sagittal mouse brain atlas images that most closely matched the section showing the tetrode tracks.

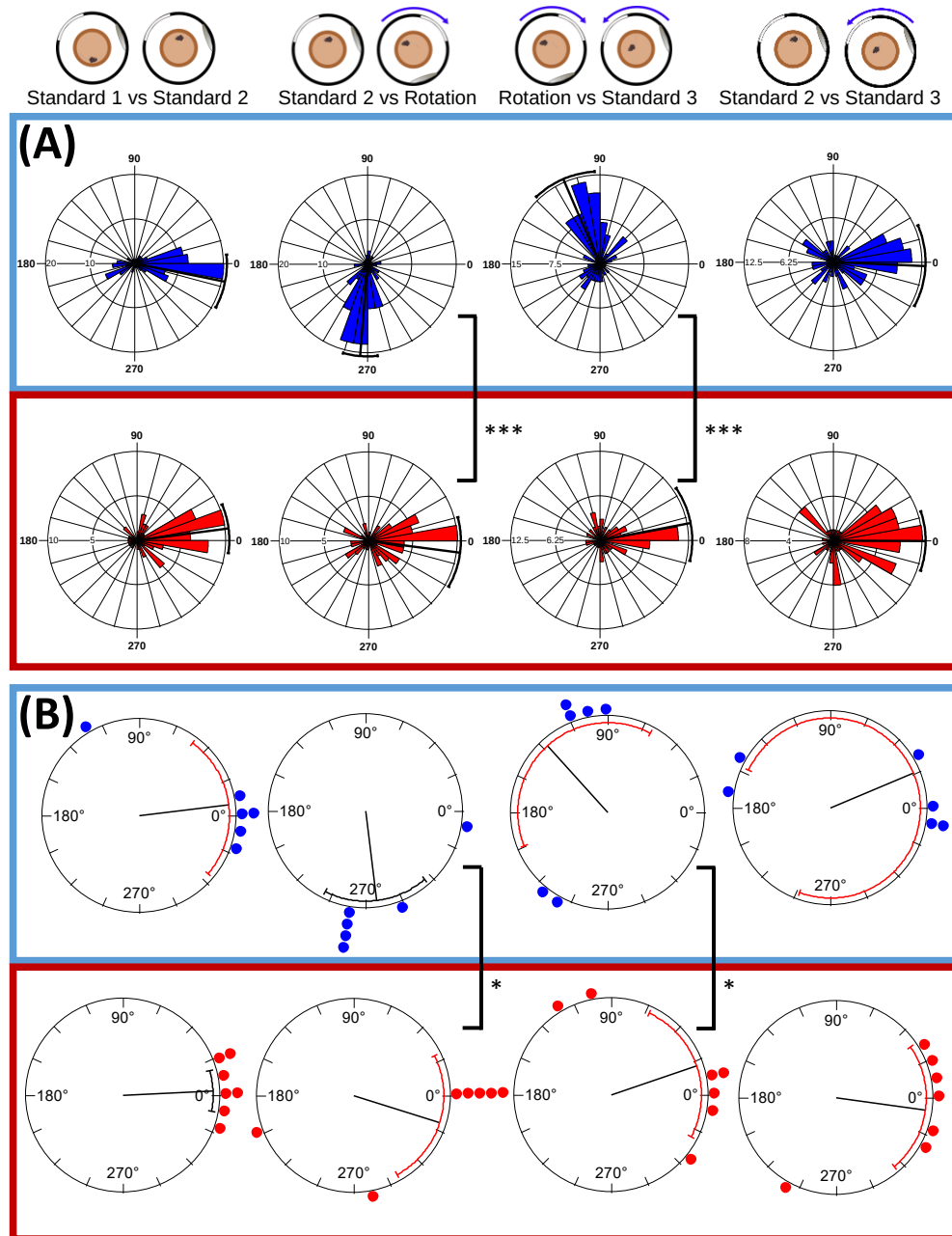


FIGURE A.2: Analysis of cell maximum rotational correlation angles (as in Figure 2.6), using only cells from first day of recording in the distal landmark condition. Consistent with data pooled from across the three recording days, cells from control mice show rotations with the distal landmarks, whereas cells from lesion mice remain stable. Asterisks denote Watson-Williams test p-values: **(A)** Std1 vs Std2:  $F(1,166) = 2.85$ ,  $p = 0.093$ ; Std2 vs Rot:  $F(1,180) = 58.6$ ,  $p < 0.0001$ ; Rot vs Std3:  $F(1,180) = 55.4$ ,  $p < 0.0001$  (may be unreliable due to low concentration); Std2 vs Std3:  $F(1,171) = 0.028$ ,  $p = 0.868$  (may be unreliable due to low concentration). **(B)** Std1 vs Std2:  $F(1,11) = 0.27$ ,  $p = 0.612$ ; Std2 vs Rot:  $F(1,11) = 5.79$ ,  $p = 0.035$ ; Rot vs Std3:  $F(1,11) = 8.26$ ,  $p = 0.015$ ; Std2 vs Std3:  $F(1,11) = 0.48$ ,  $p = 0.504$ .

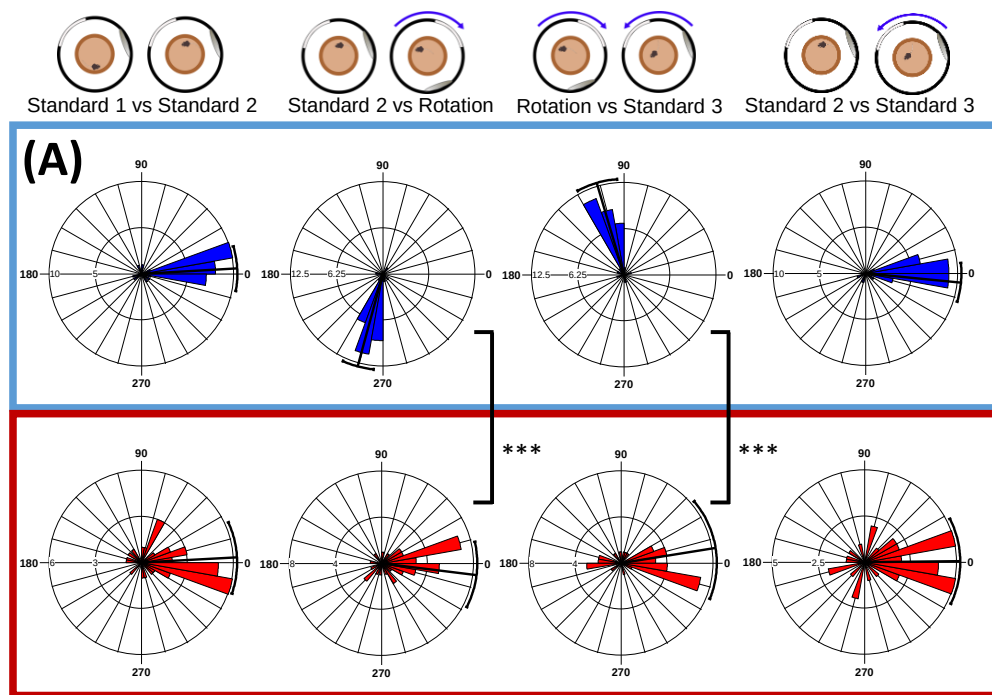


FIGURE A.3: Analysis of cell maximum rotational correlation angles (as in Figure 2.6A), using only cells from the fourth day of recording in the distal landmark condition, following the proximal cue sessions (from two control mice and 3 lesion mice). Consistent with the main data, cells from control mice show rotations with the distal landmarks, even after mice have been exposed to three days of the proximal cue protocol, whereas cells from lesion mice continue to remain stable. Asterisks denote Watson-Williams test p-values: Std1 vs Std2:  $F(1,66) = 0.0003$ ,  $p = 0.987$ ; Std2 vs Rot:  $F(1,68) = 70.7$ ,  $p < 0.0001$ ; Rot vs Std3:  $F(1,68) = 38.5$ ,  $p < 0.0001$ ; Std2 vs Std3:  $F(1,66) = 0.20$ ,  $p = 0.656$ .



## Appendix B

### Chapter 4 Supplementary Figures

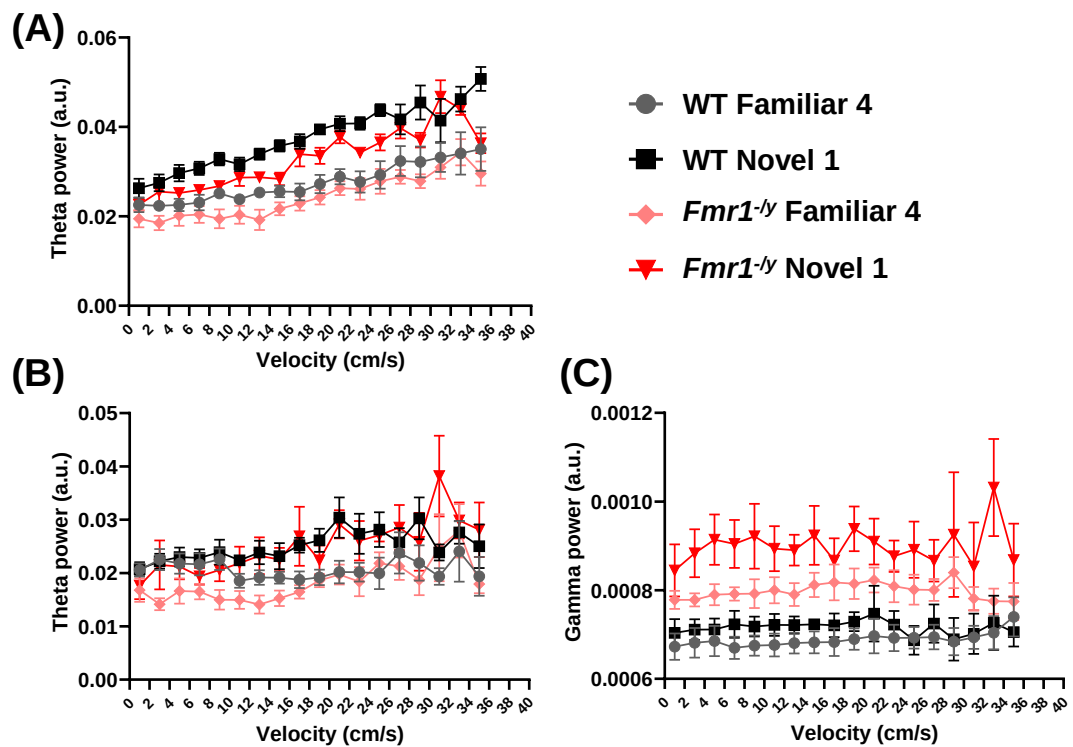


FIGURE B.1: Power of oscillations against running velocity of rat. **(A)** Hippocampal theta power, averaged over 2 cm/s velocity bins. Mean  $\pm$  SEM of each group (WT, black,  $n = 5$ ; *Fmr1*<sup>-/-</sup>, red,  $n = 4$ ) are shown, for sessions Familiar 4 (paler colours) and Novel 1 (bold colours). **(B)** Theta power in the mPFC, averaged over 2 cm/s velocity bins. **(C)** Gamma power in the mPFC, averaged over 2 cm/s velocity bins.



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