**Chapter 5 – Discussion**

Ramón *y* Cajal, one of the pioneers of neuroscience around 1900, utilized Camillo Golgi’s staining method to conclusively describe neurons in the brain as independent functional units connected to each other in intricate small-world networks with many billions of nodes. These neurons have since been described not just anatomically, but also on the basis of genetics, development, and neurophysiology.

In the subdiscipline of Learning and Memory, a very popular neuron type by way of such studies is the pyramidal neuron, an example of which is the Hippocampal CA1 pyramidal neuron. This thesis describes a toolkit of techniques ranging in a wide, multi-disciplinary scope, assembled with standardized hardware and software routines studying Animal Behaviour, network Neurophysiology, and Statistical Analysis.

The aim of the toolkit was to provide the experimental ability to study the Hippocampal CA1 neuron network, under strictly controlled behavioural contexts designed to train experimental mice on temporal or episodic memory tasks. Specifically, these tasks such as Trace Eye-Blink Conditioning (TEC) have previously been described to elicit Hippocampal CA1 sequences (Pastalkova et al., 2009; MacDonald et al., 2011; MacDonald et al., 2013; Modi et al., 2014; Kraus et al., 2015; Mau et al., 2018). This spatiotemporal network activity sequence is dynamic and built from individual Hippocampal CA1 pyramidal neurons showcasing time tuned activity through spiking. These cells are called Time Cells (Eichenbaum 2017).

*Engrams associated with Learning and Memory*

Eric Kandel’s experiments with the Aplysia sensory neurons studied gill withdrawal - an aversive but reliable, adaptive behaviour. The reliability of this learned response allowed the experiments to include crucial electrophysiological and neurochemical circuit dissections that ultimately lead to the discovery of the entire neural circuit orchestrating the task, even to the level of cellular signaling. This led to decades of research focussed on the plasticity of synapses across nervous systems in the animal kingdom.

The term "engram" (coined by Richard Semon) refers to the physical substrate of memory in the organism, used for storing and recalling memories. Donald Hebb’s theory of Hebbian Plasticity (Hebb 1949) postulated that memory formation was correlated to modulations in synaptic strength and connectivity. The theory critically emphasized that the pair of neurons connected through the synapse undergoing plasticity to strengthen efficacy, required the spiking activity of both neurons. In subsequent decades, research into the idea led to the theory of spike-timing-dependent plasticity (Caporale & Dan, 2008), a mechanism of synaptic plasticity based on the relative timing of activity of the neurons. However, it is still a matter of debate whether the biophysical manifestation of the engram is the synapse, the activity of the neurons, biophysical and chemical processes, but it is likely that the engram is distributed across several computational scales in the brain.

Research exploring causal relationships between the physical or functional integrity of various brain regions and overt behaviour has been crucial to mapping many brain regions to specific functions and motor responses. Technological advancements in molecular neuroscience led to the development of a number of fluorescent sensors, conditional tagging, activators and inhibitors that allowed cellular resolution tracing of the engram (Luo et al., 2018).

Experiments by Sheena Josselyn, Alcino Silva, and colleagues (Han et al., 2009; Zhou et al., 2009; Yiu et al., 2014; Rogerson et al., 2014) led to the discovery that the intrinsic excitability of a pyramidal neuron in any network positively biased the probability of recruitment to the engram (the tagged set of cells active when memory leant and recalled). The engram could now be described in terms of the cellular subpopulation involved but experiment could only identify the same in a window of time (~mins.), leading to only a static list of cells which may even have included False Positives (Type I error). Importantly, any dynamics in the spatiotemporal patterns of activity of the pyramidal neurons were not amenable to study at shorter timescales (~ms.). On the other hand, physiological recordings could describe these dynamics at short timescales, but were rarely translated to chronic measurements of the activity of the same cells across days and sessions, given technical limitations at the time.

*Engrams are likely dynamic*

Place Cells and their role in spatial navigation have been described in great detail through decades of research ever since they were first described by John O’Keefe (O’Keefe and Dostrovsky, 1971 ??). We did not explicitly study Place Cells in this thesis but some key discoveries in literature require mention, with the goal to build a case for a theory of CA1 ensemble sequences (Modi et al., 2014). Briefly, Place Cells are pyramidal neurons that showcased a higher than baseline probability of firing action potentials whenever animals navigating spatial environments visited specific locations, often in a sequence of place cells mapped to the real spatial trajectory of the animal (Frank et al., 2000; Wood et al., 2000; Ferbinteanu and Shapiro, 2003; Foster 2017).

As the animal enters these landmark locations in any spatial context, these Place Cells showcase Phase Precession, firing earlier in phase to cycles of Theta (Foster and Wilson 2007) a network rhythm clocked at ~4-10 Hz, as the animal’s position changes relative to the landmark. These navigation mapped place cell sequences are called Theta Sequences (ref?), typically mapped to a few active neurons at a time.

In very specific contexts, these place cells express activity sequences synchronized to Sharp Wave Ripples (ref?), a different network activity phenomenon clocked at ~10-30 Hz, often not tied to the animal’s location (Foster and Wilson 2006). These sequences have been described to play out typically in temporal order to models of place cell sequences describing known trajectories in space.

There is variability in the firing of Place Cells in any spatial context, and studies have mapped specific sequences to very specific trajectory goals (going towards or away from locations) with modulation by both egocentric and allocentric orientations cues (Wood et al., 2000; Frank et al., 2000; Ferbinteanu and Shapiro, 2000; Ferbinteanu and Shapiro, 2003; Davidson et al., 2009) and movement speed based estimates of distance (Moser papers).

Place Cell and Time Cell sequences have many similarities and differences in descriptive neurophysiology, but may emerge from the same memory organization principles (Buzsaki and Llinas, 2017). It is argued that there is significance to the exact phrasing of the CA1 sequence in any given context. Furthermore, a very interesting feature observed is Time-stamping, viz., time dependent overlap of ensemble responses to different contexts and behavioural parameters (Eichenbaum paper; Cai et al., 2016).

Trace Eye-Blink Conditioning is a behavioural context which has been shown to feature CA1 Time Cell Activity Sequences. Transient increases in CA1 excitability post acquisition of the task were described up to 4-5 days (Moyer et al., 1996) and could be important to the forging of the task specific spatiotemporal sequences during learning. Moreover, Trace Eye-Blink Conditioning in mice has been previously observed to elicit CA1 activity sequences even in a single session of training (Modi et al., 2014).

Internally driven as opposed to externally driven network models of activity sequences have been proposed as the mechanism driving Hippocampal CA1 sequences (Eichenbaum 2017; Buzsaki and Llinas, 2017). The CA1 neurons participating in any sequence may represent physiologically mappable attractors in temporally specific contexts. The standardized protocols described in the thesis are expected to aid in future experiments studying Hippocampal CA1 sequences.

*Studying Hippocampal CA1 sequences*

We standardized a multi-day Trace Eye-Blink Conditioning (“Chapter 2 - Behaviour”) training system for mice based on previous literature (Modi et al., 2014; Siegel et al., 2015) and could demonstrate several types of behavioural adaptations that experimental animals could learn under a variety of experiment conditions and modulations. Notably,

1. The animals typically learnt the tasks quickly, within 1-2 weeks of training.
2. Modulating the Inter-Stimulus Interval (ISI) between the CS and US results affected the expression of the conditioned response (CR).
3. A wide palette of stimuli may now be incorporated into existing protocols as either of the presented stimuli

Simultaneous large-scale recordings have been fundamental to the discovery of long spatiotemporal activity patterns with several participant CA1 neurons (Davidson et al., 2009; Foster 2017). Electrical recordings provide many orders of magnitude better temporal resolution, not to mention being a direct readout of action potentials. However at the time of the design of the thesis, imaging based approaches could yield more recorded neurons per experiment animal.

We standardized 2-photon fluorescence based chronic imaging of Hippocampal CA1 neurons *in vivo* to allow Calcium Imaging based recording of the spatiotemporal sequences across multiple days (”Chapter 3 - Imaging”). This allowed us the ability to

1. Record neurophysiology over a large population of neurons (~100), in conjunction with temporally relevant behavioural contexts and modulations, albeit at ~100 ms temporal resolution.
2. Chronically track cells across various behaviour sessions without ambiguity.
3. Allow for scalability in the per animal yield of recording neurons with the use of faster and modern 2-photon microscope hardware utilizing Resonant Scanning instead of Galvo Scanning, as well as multi-channel imaging.

*Engrams are likely distributed*

An important consideration is that while different brain regions have been studied and identified with various functions, each brain region is typically involved in a variety of functions and almost the whole brain in entirety, is involved in any real life task. Imaging multiple brain regions at near cellular resolution has been reported using Cortical Observation by Synchronous Multifocal Optical Sampling or COSMOS (Kauvar et al., 2022). However, we had limited our physiology experiments strictly to the dorsal Hippocampal CA1, in the left hemisphere. Multi-region imaging would require a second excitation-emission path, a technical and experimental paradigm which was not considered within the scope of this thesis.

Similarly, important engram motifs at subcellular spatial scales were beyond the scope of the experiments in this thesis. Multiscale models of network function (ref?) would be required to complement any physiology, as a possible way to address the issue of engram motifs at variable spatial scales.

For our chronic 2-photon calcium imaging recordings, we had sampled from ~100-150 Hippocampal CA1 neurons per mouse, using Galvo Scanning in our custom-built 2-Photon microscope (Modi et al., 2014) at a frame rate of ~14.5 Hz. We had to limit our scope of inquiry to randomly sampled populations of CA1 pyramidal neurons from the same CA1 microcircuit.

Future directions include the use of Resonant Scanning to achieve higher frame rates at better lateral resolution sampling up to ~100x more neurons.

*Mapping sequences to abstract variables*

The ubiquity of neural sequences in a wide variety of nervous systems has been discussed previously (Rajan et al., 2016; Bhalla 2017) and over a century of research has discovered remarkable physiological features that may be used to identify neurons that participate in these sequences. However, research is still required to carefully dissect out the contribution that each participant neuron has to behaviour, an important goal in neuroscience (Ranck 1970).

The use of user-configurable, categorically labeled synthetic calcium activity profiles allowed us to probe and compare a range of different Time Cell Detection algorithms, identifying strategies to best classify Time Cells. We were able to identify Temporal Information as a strong contender for the choice of algorithm for such classification (“Chapter 4 - Analysis”; published as <eNeuro paper>). The algorithms developed along the way were tested within the time scales of ~100 ms, that correspond to Replay Sequences or other behaviour timescale sequences. We expect the analysis routines to be useful in a variety of different experiments that could potentially help describe the neural code in more detail.

*Does the brain create or predict?*

*Code Availability*

All our code for Synthetic Data generation and Time Cell Analysis is available at <https://www.github.com/bhallalab/TimeCellAnalysis>.

All our code for conducting Trace Eye-Blink Conditioning (TEC) behaviour is available at <https://www.github.com/ananthamurthy/eyeBlinkBehaviour>.

Analysis scripts for evaluating TEC performance are available at <https://github.com/ananthamurthy/MATLAB/tree/master/TECAnalysis>.

– Bibliography