**Chapter 1 – Introduction**

*Control and Coordination*

The vertebrate Central Nervous System (CNS), consisting primarily of the central ganglia (brain) and the spinal cord, samples and receives information from the external world offering top-down control over the activity of all parts of the body. Functions like exploration, food acquisition, and danger aversion, all involve complex coordination between,

* the Sensory Systems (that integrate information from the environment),
* the Memory Systems (that integrate sensory information with prior experience), and
* the Motor Systems (that integrate motor plans and execute movement).

*Projects and overall goals of the thesis*

The overall focus of the work and experiments described in this Thesis was to study Memory Systems, specifically, in terms of,

Project I: How do sensory representations transform with learning?

Project II: How does the timing of cellular activity adjust to behavioural task variables?

Project III: What is the best way to detect and score time-tuned cellular activity?

Narrowing down, we as a lab were interested in the Mammalian Hippocampus, a brain structure which is important for consolidating information (from Sensory and other Memory Systems) to enable certain kinds of short-term memory and the translation of short-term memory to long-term.

*Theories on the function of the Hippocampus*

Three main ideas of hippocampal function studied over the past few decades in increasing order of popularity are,

1. Response Inhibition - Studied mostly in the 1960’s, this perspective described the Hippocampus as important to the ability of animals to inhibit their impulses and natural, habitual, or dominant behavioral responses to stimuli, in order to select more appropriate responses. This perspective was justified by two observations with regard to animals with hippocampal damage - 1) these animals tended to be hyperactive, and 2) were unable to withhold previously learnt responses. British psychologist Jeffrey Alan Gray developed this perspective to link Hippocampal activity with anxiety (Gray and McNaughton, 2000).
2. Episodic Memory - Currently (at the time of writing) in vogue due to advances in cellular neurophysiology recording techniques, this perspective was popularized by the psychological studies on Patient H.M. (Henry Molaison), who had been suffering from epileptic seizures and had to undergo extensive Hippocampectomy (surgical destruction of the Hippocampi), as treatment. American neurosurgeon William Beecher Scoville and British-Canadian neuropsychologist Brenda Milner were pioneers of this study and were able to describe severe anterograde and partial retrograde amnesia in the patient post surgery (Scoville and Milner, 1957). Since the late 2000’s, the discovery and description of Time Cells (Pastalkova et al., 2008; MacDonald et al., 2011, MacDonald et al., 2013, Modi et al., 2014, Kraus et al., 2013, and Kraus et al., 2015), has reinvigorated this perspective.
3. Spatial Cognition - Originally popularized by the remarkable work of American-British neuroscientist John O’Keefe and his American psychologist Lynn Nadel, the link between Hippocampal function and spatial navigation and coding was solidified with the discovery and subsequent descriptions of Place Cells (O’Keefe and Dostrovsky, 1971; Morris et al., 1982; O’Keefe and Reece, 1993). This perspective is the most popular amongst the known and studied functions of the Hippocampus and has been the subject of a large body of work. Indeed, the Nobel Prize in Physiology or Medicine 2014 was awarded to John O’Keefe, May-Britt Moser, and Edvard I. Moser, for “The Brain’s Navigational Place and Grid Cell System”.

*The Hippocampus and Time Cells*

Damage to the hippocampal system has been shown to cause the impairment of long-term memory or amnesia, in human patients, rodents, and non-human primates. Interestingly, such damage to the Hippocampus seems to have no observable effect on the capacity for acquisition and expression of skilled performance. These two results suggest the role of the Hippocampus in certain kinds of memory, but not all.

Anatomically, the hippocampal system receives input from, and in turn, projects to the neocortical brain regions that serve as the site to process higher order categories and modalities of information. It is thus suggested that the Hippocampus holds a privileged position in the brain, receiving the outcomes of the computation of the brain’s various modules, and relating to them. A large majority of the cortical information is sent to the Hippocampus via the Entorhinal Cortex (EC). This information is processed in roughly three stereotactically and molecularly separable layers of cells in the following order: EC →Dentate Gyrus → CA3 →CA1. This pathway from the EC to the CA1 has three separate synaptic connections (across the layers) and is also known as the Trisynaptic Pathway. The output of the CA1 is then sent to other cortical areas.

The activity of neurons in the hippocampus of awake, behaving animals is modulated by significant stimuli or objects in the environment as well as relationships between temporally discontiguous but relevant, paired stimuli. With the discovery of Place cells, it was clear that the CA1 of rats navigating a spatial environment, showcased location specific firing fields. With the discovery of Time cells, it was shown that the CA1 of rats could elicit spatiotemporal sequences of activity whenever the animal required to make a link between stimuli or events, even with a stimulus-free period in between. This provided an important physiological parallel to the spatial learning as well as episode learning deficit seen with damage to the Hippocampus.

The hippocampus supports a robust form of synaptic plasticity called Long-Term Potentiation (LTP), viz., brief patterned activation of particular pathways produces a stable increase in synaptic efficacy that may last for hours to weeks. This is also an important mechanism often suggested as the answer to how the hippocampus manages short-term declarative memory.

In an experiment published in 2008, Eva Pastalkova and colleagues from Gyorgy Buzsaki’s lab had rats navigate a figure 8 maze, with the animal being rewarded with water, in between trials, if they managed to alternate between the left and right arms. There was a catch however. Just before launching into the left or right arms, the animal had to spend a fixed amount of time running a treadmill, held in place. This would allow self-motion cues, but with the absence of any other external stimuli. Impressively, single-units recorded from the Hippocampal CA1 cells revealed strong correlation with the time spent on the treadmill, despite the absence of external cues, and that different cells tuned to different time points, forming a spatiotemporal sequence of activation (Pastalkova et al., 2008). In a different experiment published in 2011, Christopher J. MacDonald and colleagues from Howard Eichenbaum’s lab had rats had to go around a maze and perform a olfactory task when they were first presented with an odour, then made to wait for a delay period in a cordoned off section of the maze, before being allowed to either dig for a reward or continue on the maze, depending on the odour presented. As trials progressed, Hippocampal CA1 cells were recorded (single-units) and found to not only be modulated by the decision to be taken, but also to the amount of time spent in the delay period. Experimentally, the delay period could be elongated or shortened, each having an effect on remapping of the tuning fields of the various CA1 cells, but to different extents (MacDonald et al., 2011). This study was often scrutinized since there was the confound of the animals moving in space, potentially invoking Place cells.

In 2013, the Eichenbaum group published their findings with head-fixed rats (no movement in space) performing a Delayed Match-To Sample (DMS) task with pairs of odours, where again time tuned activity could be observed with a sequence of Hippocampal CA1 cell activations, that depended on the identity of the first odour (MacDonald et al., 2013). In 2014, Mehrab Modi, Ashesh Dhawale, and Upinder Bhalla published their results with head-fixed mice learning and performing a Trace Eye-Blink Conditioning (TEC), wherein it was observed that Hippocampal CA1 cell activity sequences emerged in close relation to the acquisition of behavioural performance, thus cementing the idea that sub-populations of Hippocampal CA1 cells could bridge temporal gaps between relevant, paired stimuli, and that they did so with the activity of time-tuned cells.

Finally, it was important to study if these apparently time-tuned cells were tuned to the actual duration of time in a delay period, or whether it was more important for these cells to track the distance run. In an experiment published in 2013, Benjamin Kraus and colleagues from Howard Eichenbaum’s lab again had their rats navigate a figure 8 maze, but with a motorized treadmill in the central arm, to experimentally regulate the running speed. With this setup, the study was successful at delineating that both time spent running and distance run were important features, and that different cells could tune to either of the features (Kraus et al., 2013). Whenever Hippocampal CA1 cells showcased time-tuned activity (as opposed to space/location-tuned activity), such cells were dubbed “Time Cells” (Kraus et al., 2013; Eichenbaum 2017).

*“Single-cell, multi-trial” vs. “multi-cell, single-trial” approaches in Neuroscience*

A dominant, early perspective in Neurophysiology had been to record activity from a single cell, over many trials, under a variety of conditions (bath applications in slice physiology, different physiological conditions like stress and genetic background). For more than one recorded cell, the process would be repeated, till the dataset was complete.

An intermediate perspective was to record from multiple cells simultaneously, yet treat each cell independently for analysis towards correlation and mechanism studies, across many repeats of experimental conditions or trials (same as above).

An important and more modern perspective is to record from multiple cells simultaneously, and use this network or population activity to decode single-trial characteristics (position, time, stimulus presence, etc.) using very powerful numerical and mathematical algorithms involving (but not limited to) Bayesian Decoding and Information Theory. The essential idea is that the neuronal code of the brain is not defined by the activity of single neurons since they may only encode very specific fractions of the experience, but rather that the population encodes the full experience, using a number of distributed and redundant strategies.

* Bayesian Decoding: Using the activity of multiple, simultaneously recorded neurons to develop a likelihood estimate of the evidence (firing rate combinations) to the experimental parameter (spatial position, relative time, etc.) and combine this with the experimentally determined prior (probability ), to obtain estimates of the conditional or posterior probability of a parameter value, given evidence. Bayes’ Rule describes

P(A|B) = P(B|A).P(A)/P(B)

… where,

A: Parameter value (position, time, etc.)

B: Evidence (cellular firing rate)

P(A): Prior Probability (experimentally defined)

P(B): Probability of evidence (Firing Rate)

P(A|B): Posterior probability of parameter value given evidence

P(B|A): Likelihood estimate of evidence given parameter value (based on recordings)

This methodology has been used to not only successfully predict specific time points in a trial from population activity, but has also been used to observe that the population activity from a session of recording is able to predict time points in trials conducted on subsequent sessions of recording, up unto 3-4 sessions (Mau et al., 2018).

* Information Theory: Using recorded cellular activity to estimate how much information this activity carries about experimental parameters (position, time, stimuli, etc.). Three essential metrics have been used,

1. Information per activity spike (Ispike), in bits/spike
2. Information per unit time (Isec), in bits/sec
3. Mutual Information (MI) between evidence and parameter value, in bits

<Needs a good example here.. maybe Skaggs 1993 and 1996 (defining place cells)>

A major step forward with “multi-cell, single-trial” approaches is the benefit of resolving how each cell and inter-cell interactions contribute to stimulus representation, behavioural task variables, and other brain-intrinsic computation. Technological advances in large-scale neurophysiology recordings such as the increased density of tetrode drives, neuropixels, optical sectioning and microscopy, resonant scanning, etc., have enabled the discovery of well coordinated sequences of cellular activity such as Sharp Wave Ripples (SWRs), Replay, and behavioural timescale spatio-temporal sequences, *in vivo*. This is primarily due to a radical improvement to an experimenter’s ability to simultaneously record from multiple cells (Foster, 2017), going from yields of ~10 cells to even ~104 cells, per animal.

*A short note on Single-Unit Electrophysiology*

The most well characterized and studied function of the Hippocampus and surrounding tissue (Entorhinal Cortex, Parasubiculum, etc.) is the role these tissue systems played in Spatial Navigation and Coding. Single-Unit Electrophysiology was paramount to being able to isolate the activity from individual cells, and eventually was used to discover and describe properties of Place Cells (O’Keefe and Dostrovsky, 1971), Grid Cells (Fyhn et al, 2004, Hafting et al., 2005), Head-Direction Cells (Taube and Ranck, 1990), along with numerous other important physiological discoveries. However, even with advances in the density of tetrode recordings, the yield of recorded cells from any given animal was often limited to <100 cells. It was only with the invention of Neuropixels (Jun et al., 2017) that this yield could be expanded to ~1000 cells. At the time when I had started my experiments for this Thesis, the only way to get more cells per animal, was to use Imaging based techniques, such as Calcium Imaging by Two-Photon Microscopy, albeit with a significant cost to the recording frame rate, limiting interpretation to low-pass filtered, rate based coding profiles for cells.

*Calcium Imaging and Two-Photon Microscopy*

Typically, as cells become activated and elicit action potentials, there is often a large concomitant influx of Ca2+ ions through voltage gated calcium channels all around the perisomatic membrane, amongst other cellular compartments. Several organic dyes have been developed that reversibly bind Ca2+ ions in the cytosol and either become fluorescent or emit greater fluorescence (often with a red shift) when in this Ca2+-bound state (Paredes et al., 2008). Additionally tremendous advances in molecular biology has seen the deployment of Genetically Encoded Calcium Indicators (GECIs) that may be exogenously incorporated into the genome of target cells. These GECIs serve the same function as organic calcium dyes, but may easily be replenished in the cytosol given the cell’s natural machinery for transcription and translation, and whose Fluorescence properties can be engineered for brightness, responsivity, Ca2+ binding Kd, and fluorescence emission wavelengths. The number of cells that may be recorded by fluorescence is often only limited to either the spread of the organic dye or the imaging magnification settings, allowing for yields of 100-1000.

A major advancement in Fluorescence Imaging was the invention of Confocal and Multiphoton (typically two-photon) Microscopes, which allowed for unprecedented recording signal-to-noise by optical sectioning. Two-Photon Imaging itself was an important development for the neurophysiology of tissue greater than 300 µm in thickness, typical of rodent brain tissue, because it avoids wasteful excitation of imaging planes that are not in focus. The Two-Photon effect requires two photons of longer wavelength (lower energy per photon), to near instantaneously excite a fluorophore, a phenomenon that is most likely only possible at the focal point of the microscope objective lens. Additionally, longer wavelengths of excitation light can more easily penetrate deeper layers of tissue, due to comparatively lower scattering (Rayleigh effect).

The Hippocampus (specifically the Hippocampal CA1) was the main brain structure of interest for all my physiology experiments, and lies under about 1 mm of cortical tissue for mice, which is a depth that is difficult to image even with Two-Photon Microscopy. The typical methodology employed in such cases is to perform a cortical excavation just above the Hippocampus filling the crevice with optically clearer agarose or silicone elastomer. Even so, the Hippocampal CA1 cell body layer (Stratum Pyramidale) still lies about 150-300 µm below the external capsule and corpus callosum fibers (left intact for chronic imaging). Accordingly, we combined cortical excavation with two-photon microscopy, using a long working distance objective with a wide field of view, imaging cytosolic Ca2+ activity with the help of either an organic dye (OGB-1; acute imaging) or a GECI (GCaMP6f; chronic imaging).

An important perspective that has motivated the use of Imaging based physiology recordings (as opposed to Electrophysiological methods) other than potential yield, is that imaging provides anatomical confirmation of any particular recorded cell, and this in turn allows for

1. Unambiguous isolation of the same cell across multiple imaging sessions (across days and weeks). Single-Units are ultimately only algorithmically resolved and this can be done only for cells that are active and are represented in multiple spatially separated electrodes. However, very recently, Ashesh Dhawale and colleagues from Bence Olveczky’s lab have devised a solution to track the movement of electrodes in tissue over time and use this information to ensure chronic recording of the same units (Dhawale et al., 2017). This technique was not available at the time when I started my experiments for this thesis.
2. Unambiguous detection of the lack of activity in an otherwise recorded cell. Since the cell can be anatomically identified independent of activity, it is possible to observe the absence of Ca2+ activity. However, with the development of automated cell ROI detection (Francis et al., 2012; Maruyama et al., 2014; Pnevmatikakis et al., 2015; Pachitariu et al., 2017) that requires activity from target ROIs to confirm cell isolation, this advantage is now no longer as applicable as when cell ROIs are drawn out by hand, a technique that I had followed for many years before switching to automated detection.

*Automated ROI detection for large-scale Calcium Imaging datasets*

A number of automated ROI detection algorithms have been cited in literature that require minimal user intervention, perform relatively fast identification for a large number of identified sources (putative cells). Some popular algorithms include PCA/ICA (Mukamel et al., ), Suite2p (Pachitariu et al., ), and Non-Negative Matrix Factorization (NNMF; Pnevmatikakis et al., ), which all have been developed to the extent where comparable or oftentimes much better ROI detection is achieved than as compared to the more tedious hand-drawn ROIs which scales very poorly with orders of cells recorded.

We have strictly followed Suite2p (Pachitarius et al., ) for all physiological ROI (cell sources) described in this thesis.

*Imaging of deep-lying brain tissue*

The hippocampii (one in each hemisphere) of the mouse brain lie ~1 mm below the most superfical layers of cortex (just inside the cranium), a barrier typically too wide for typical 1-photon fluorescence imaging systems (Confocal, Spinning Disk, etc.). This poses a very difficult challenge for imaging preparations since there are hardware and other technical limits to how long the working distance of microscope objectives can be made. The use of 2-photon microscopy combined with combinations of cortical excavations (to aid physical access), microendoscopes, prisms to guide emitted fluorescence, have all been used to achieve deep brain imaging based recordings at cellular resolution, in rodents.

* Tank lab, Dombeck lab, Schnitzer lab, etc.
* Cortical excavations, Microendoscopes, prisms
* Hippocampal CA1 imaging
* MEC imaging

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All imaging preparation standardizations described in this thesis invoke two-photon calcium imaging of Hippocampal CA1 cells at cellular resolution (1 pix = ~1 µm), following cortical excavations just above the left hippocampus (Dombeck et al., 2010).

*A brief introduction to Associative Learning*

Prior to the early 20th century, Structuralism was a dominant perspective in Psychology, insisting on introspection - the observation and report of one’s own mind and thoughts. Experiments and discoveries by Ivan Pavlov at the Military Medical Academy in Petrograd (St. Petersburg), eventually led to a dramatic shift in perspective, with the birth of Classical Conditioning, a type of Associative Learning. Following the very same methodology advocated by Francis Bacon (early 17th century), quantitative data from carefully conducted animal experiments were recorded, with the idea to narrow down on a small number of hypotheses that could explain experimental observations.

Ivan Pavlov provided essential demonstrations of anticipation and made tremendous progress in understanding the circumstances on which anticipation depends, and this is why Classical Conditioning is also often referred to as Pavlovian Conditioning. Following Pavlov’s studies (Pavlov, 1927), it was proposed that Classical Conditioning was a prototypical example of Association. While it does have caveats such as “silent” learning when observable behaviour may be blocked (Mackintosh, 1983; Krupa et al., 1996), Associative learning is rich with a variety of animals and association tasks that have been crucial to study Memory and Learning over the past century.

Typically, animals require no prior training to elicit a behavioural or motor movement to biologically potent stimulus (appetitive or aversive), called an Unconditioned Stimulus (US). Examples include food, water, electrical shock, temperature shock, etc.. Without pairing with a US, a neutral stimulus elicits no observable response from an animal, and such a stimulus is called a Conditioned Stimulus (CS). Examples include simple auditory tones, flashes of light, etc..

Classical Conditioning is both the behavioural procedure as well as the learning process that results from the pairing of a previously neutral stimulus (CS) with a biologically potent stimulus (US). Repeated pairing allows animals to make implicit associations between the CS and US, and essentially anticipate the occurrence of the US, once the CS is observed. Animals report this forecasting feat by producing the same response that they would to a US, albeit often a milder version. Typical protocols for Classical Conditioning, follow the regime of Forward pairing, i.e. - the CS is presented before the US, and this temporal structure will be followed unanimously across all behaviour experiments described in this thesis.

The standardization of the behavioural task, physiological recording (imaging) preparation, as well as the custom analysis routines to look for various physiological features will be described in this thesis. Combining these multi-disciplinary approaches allowed us to develop a toolkit to study Time Cells in the Hippocampus, under strict behavioural contexts.

*Short Summaries of the 3 projects*

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– Bibliography