Characterizing Single Neuron Activity Patterns and Dynamics Using Multi-Scale Spontaneous Neuronal Activity Recordings of Cat and Mouse Cortex

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Neuroscience)

The University of British Columbia
(Vancouver)

November 2017

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Abstract

Throughout most of the 20th century the brain has been studied as a reflexive system with ever improving recording methods being applied within a variety of sensory and behavioural paradigms. Yet the brains of most animals (and all mammals) are spontaneously active with incoming sensory stimuli modulating rather than driving neural activity.

The aim of this thesis is to characterize spontaneous neural activity across multiple temporal and spatial scales relying on biophysical simulations, experiments and analysis of recordings from the visual cortex of cats and dorsal cortex and thalamus of mouse.

Biophysically detailed simulations yielded novel datasets for testing spike sorting algorithms which are critical for isolating single neuron activity. Sorting algorithms tested provided low error rates with operator skill being as important as sorting suite. Simulated datasets have similar characteristics to in vivo acquired data and ongoing larger-scope efforts are proposed for developing the next generation of spike sorting algorithms and extracellular probes.

Single neuron spontaneous activity was correlated to dorsal cortex neural activity in mice. Spike-triggered-maps revealed that spontaneously firing cortical neurons were co-activated with homotopic and mono-synaptically connected cortical areas, whereas thalamic neurons co-activated with more diversely connected areas. Both bursting and tonic firing modes yielded similar maps and the time courses of spike-triggered-maps revealed distinct patterns suggesting such dynamics may constitute intrinsic single neuron properties. The mapping technique extends previous work to further link spontaneous neural activity across temporal and spatial scales and suggests additional avenues of investigation.

Synchronized state cat visual and mouse sensory cortex electrophysiological recordings revealed that spontaneously occurring activity UP-state transitions fall into stereotyped classes of events that can be grouped. Single visual cortex neurons active during UP-state transitions fire in a partially preserved order extending previous findings on high firing rate neurons in rat somatosensory and auditory cortex. The firing order for many neurons changes over periods longer than 30-minutes suggesting a complex non-stationary temporal neural code may underly spontaneous and stimulus evoked neural activity.

This thesis shows that ongoing spontaneous brain activity contains substantial structure that can be used to further our understanding of brain function.
Lay Summary

The brains of all animals including mammals are spontaneously active yet much of neuroscience research has focused on studying the brain’s response to specific stimuli such as specific odors, pictures or sounds.

The work presented in this thesis is aimed at characterizing brain activity that occurs spontaneously. The focus is on recording the spontaneous activity of single neurons and relating it to other nearby neurons and to other more distant areas of the brain.

It is shown that existing single neuron detection methods are adequate for analysis. Single neurons fire simultaneously with other neurons to which they are anatomically directly connected with neurons from deeper areas of the brain firing in more unique patterns. Spontaneously active single neurons in visual areas of the brain are also shown to fire in specific orders that appear to change over time.
Preface

The author’s PhD researched has been published or is in review in 6 journals and has been presented in 9 conference posters.

The author’s work relating to biophysical modeling described in Chapter 3 has been published in one journal Hawrylycz et al., 2016, one pre-print journal (Jun et al., 2017a - currently in preparation for peer-reviewed journal submission), one article in review (Jun et al., 2017b) and was presented at multiple conferences (Mitelut et al., 2014, 2015; Gratiy et al., 2015, 2016; Vyas et al., 2016). An additional publication on the Allen Institute network model is currently in preparation (Gratiy et al., 2017).

The optical imaging work described in Chapter 4 has been published (Xiao et al., 2017) and was presented at two conferences (Mitelut et al., 2016; Xiao et al., 2016).

The work described in Chapter 5 has been presented at one conference (Mitelut et al., 2015) and is being prepared for publication.

Additional publications resulting out the author’s PhD research not discussed in this thesis include: a python-based toolbox used for analysis of widefield calcium activity recordings (Haupt et al., 2017); a publication on the use of SpikeSorter (Swindale et al., 2017); and a fourth research project on the neural correlates of spontaneous mouse behaviour initiation which was presented at two conferences (Mitelut et al., 2017a,b) and is being prepared for publication.

Experiments on cats were carried out by Nicholas V. Swindale and Martin Spacek (Swindale and Spacek, 2014). Experimental work in cats was covered by UBC Ethics Certificates A04-0098 and A11-0280. Experiments on mice were carried out by the author. Experimental work in mice was covered by UBC animal application certificates A14-0266 and A13-0336.

The author declares no conflicts of interest.
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- **Results**
- **Discussion**

**GCaMP6 mapping of spontaneous activity of auditory and visual cortex neurons**

- **Background**
- **Results**
- **Summary**
- **Discussion**

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- **Background**
- **Results**
- **Summary**
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List of Abbreviations

α-BTX  α-bungarotoxin
AP  action potential
CCH  cross correlation histogram
COM  centre of mass - referring to histogram distributions
CSD  current source density
EEG  electroencephalogram
EMD  earth mover’s distance
fMRI  functional magnetic resonance imaging
FP  fluorescent proteins (or false positive)
fps  frames per second
FWHM  full width half maximum
GECI  genetically encoded calcium indicator
GFP  green fluorescent protein
GUI  graphical user interface
IM  intra muscular
ISI  inter-spike interval
IV  intra-venous
LEC  LFP event class
LGN  lateral geniculate nucleus
LFP  local field potential
L2/3  layer 2/3 neurons
L4  layer 4 neurons
L5  layer 5 neurons
MUA multiunit activity
NSG Neuroscience Gateway, UCSD
NRT thalamic reticular nucleus
PC principal component
PCA principal component analysis
PETH peri-event time histogram
PL peak latency
PTP peak-to-peak amplitude of extracellular event
REM rapid eye movement
RF receptive field
RMS root mean square
STA spike-triggered average
STD standard deviation
STM spike-triggered-map
SVM single value metric
SWS slow wave sleep
V1 primary visual cortex
VSD voltage sensitive dye
TC thalamo-cortical neuron
WFOM wide field optical mapping
Note to Reader

In Chapter 5, the term LFP-event-class, i.e. LFP event class (LEC), is used interchangeably with UP-state transition. The basis for this equivalence is explained in detail in the chapter. Briefly, several publications have linked large amplitude multi-channel LFP deflections to single-neuron UP-state transitions (e.g. Volgushev et al., 2006; Chauvette et al., 2010). The equivalence is also made based on findings made within the chapter, e.g. LECs have large amplitudes and occur at a rate previously associated with UP-state transitions and single neurons spike substantially during LEC events. As the author did not carry out single neuron patch pipette recordings to confirm the traditional definition of UP-state against the LECs, the equivalence between LECs and UP-state transitions is provided as putative at this time. However, Chapter 5 contains an analysis of the shortcomings of a single-neuron patching approach for defining global UP-state transitions and the superiority of the LEC defined method.

In Chapter 5 the term stable neuron is used to refer to spike sorted neurons that had voltage PTP amplitudes $>75\mu$V and that fired consistently throughout the entire recording periods considered (see also Methods). These stable neurons were unlikely to have substantial spike-sorting errors and were chosen based on qualitative attributes. In the absence of ground truth data, they were determined to be the best neurons available (in those specific datasets) for analysis.
Acknowledgements

My first supervisor, Nicholas Swindale, who 4 years ago gave me an opportunity to work in neurobiology despite my limited knowledge and years of being out of academic pursuits. Nick never failed to answer my never-ending stream of questions (many of them by email) and always gave me wise advice that I came to appreciate days, months or years later. His scientific integrity and insistence on clear evidence for conclusions will guide my research for the rest of my career.

My second supervisor, Timothy Murphy, who welcomed me into his lab 2 years ago and gave me encouragement and support while learning new experimental methods. Even a few minutes of advice from Tim on research and career directions was always significant in shaping my interests and thinking.

My external supervisor, Costas Anastassiou, who hired me at the Allen Institute for Brain Science over 3 years ago based on our shared enthusiasm for modeling despite my lack of neurobiology experience. Costas provided me with research and financial support for years and I will forever be indebted to the opportunities provided by him, Christof Koch, Michael Hawrylycz and many others at the Allen Institute who have worked hard to built a unique place for doing neuroscience.

My colleague Martin Spacek whose practical advice, shared code and home made beer eased the difficulties of navigating the interdisciplinary research projects tackled in this thesis.

My many other colleagues who helped me along the way: Allen Chan, for his help, advice and support on working with mice and optical imaging. Jamie Boyd for his patience and for sharing his wisdom of both hardware and experimental approaches. Alex McGirr for our many discussions on spontaneous neural activity in healthy and diseased brains. Sergey Gratiy for his friendship, our many insightful discussions and providing me with a powerful modeling tool for carrying out my research. Lastly, my friend and colleague Bruno Herculano for listening to my complaints during expected, but still challenging, graduate school experiences.

Amitava Majumdar and the staff at the Neuroscience Gateway at the University of California, San Diego, which provided me with more than 1-million core-hours (and counting) of free cluster time and with prompt and effective support with the many challenges involved in running complex network simulations on remote servers thousands of kilometers away.

The thousands of software developers who made my research possible by providing free software
tools: Linux, Python, Geany, Git, L\TeX, Bib\TeX, GIMP.

The work presented here was supported by funding from Canadian Institutes of Health Research (CIHR), the Natural Sciences and Engineering Research Council of Canada (NSERC), the University of British Columbia neuroscience bursary program and the Allen Institute for Brain Science.
Introduction

“Traditionally studies of brain function have focused on task-evoked responses. By their very nature, such experiments tacitly encourage a reflexive view of brain function. Although such an approach has been remarkably productive, it ignores the alternative possibility that brain functions are mainly intrinsic, involving information processing for interpreting, responding to and predicting environmental demands. Here I argue that the latter view best captures the essence of brain function, a position that accords well with the allocation of the brains energy resources.”

Raichle, 2010

The brain of the Portia (genus) spider (Salticidae family) contains 600,000 neurons and can fit "comfortably on the head of a pin" (Prete, 2004). Yet the spiders are capable of complex hunting behaviours including: trial and error behaviour against novel prey or novel situations and remembering the new approaches (Wanless, 1975); solving problems and exhibiting sequences of behaviour mimicking prey-spider web signals (Harland and Jackson, 2000); and making hunting plans that can take up to one-hour and involve detouring past an incorrect route while loosing sight of their prey (Wilcox and Jackson, 2002). How can such a small animal exhibit complex behaviour requiring planning and delay of automatic hunting instincts? The answer - for the most part - is we do not know, and only very recently have electrophysiologists successfully made neurophysiology recording in the brains of such small organisms (Menda et al., 2014).

The human brain contains almost 100 billion neurons and can also support complex, unique and high-level abilities such as executive-functions, long-term memory and social behaviours. In both spiders and humans the most interesting and cherished behaviours are not related to the processing of immediate stimuli in the environment, e.g. recognizing an odor or a colour. Yet, largely due a limited understanding of nervous systems and limitations in experimental tools, most of 20th century systems neuroscience studies have focused on characterizing sensory processing, for example, how sensory systems instantiate fast circuits (i.e. on the scale of 1ms-1sec) to process stimuli (e.g. visual, olfactory or auditory inputs; Marr and Poggio, 1976). Recently, however, it is becoming more accepted that brains do much - if not most - of their work during periods of spontaneous activity and that incoming sensory stimuli largely modulate such activity (Fox and Raichle, 2007; Raichle, 2010). If what drives brain evolution is the development of systems that avoid purely reflexive behaviours with ever increasing delays between sensory experiences and behaviour, then understanding spontaneous (i.e. not stimulus evoked) neural activity is the key to understanding higher brain function. This thesis is aimed at investigating spontaneous neural activity across multiple spatial and temporal scales. It relies on cat visual cortex and mouse cortex and thalamus recordings and focuses on characterizing what single, spontaneously firing neurons can tell us about neural activity during both spontaneous and stimulus evoked periods.
Background

Making sense of the meaninglessly large (Barlow, 1961) neural activity patterns from brain recordings remains a significant - if not the most central - challenge of neuroscience. Theoretical and analytical approaches have generally referred to this problem as that of identifying the **neural code** that is implemented by nervous systems. In seeking to answer these and other foundational questions, modern experimental neuroscience methods have improved substantially and allow for the study of neural activity with unprecedented temporal and spatial scale and precision. Sensory systems (e.g. visual cortex of mouse) can now be studied with millisecond precise recordings from dozens to 100 or more single neurons using electrophysiology tools (with hundreds to thousands of neurons soon being possible Jun et al., 2017b) and with lower temporal resolution but across a few millimeters using imaging methods to capture the activity of as many as 10,000 neurons simultaneously (Pachitariu et al., 2017).

Yet, it is unclear whether new technologies alone employed in sensory areas using existing and future **reflexive experimental paradigms** will significantly advance our understanding of brain function. In fact, it has been known for many years that brains are spontaneously active consuming 10 times as much energy as expected by weight alone (Clarke and Sokoloff, 1999) with intrinsic brain activity consuming merely 5% less energy than used during intense mental effort (Sokoloff et al., 1955).

The debate about whether the primary task of nervous systems is to represent and process sensory stimuli versus being intrinsically active to predict environmental and internal demands is more than 100 years old (Sherrington, 1906; Brown, 1914). Some theoretical work suggested that even sensory systems (which make up only a part of cortical tissue) are not merely tasked with representation of stimulus or relay of their content but also at "reducing redundancy" (Barlow, 1961) in sensory information and passing only errors to internal systems that perpetually generate and evaluate models of the world. Thus, sensory (and most other neural) systems presumably make **unconscious inferences** (Clark, 2013): they compare inferences with incoming stimulus information and only errors between the expectation and the stimulus are propagated to higher brain regions. Other similar theories followed, such as **predictive coding** theories - which claims that all brain areas (not just low level sensory systems) are tasked with producing and testing hypotheses about the external world against low level sensory input (Rao and Ballard, 1999). More recently, discussions have also focused on the intrinsic properties of cortical circuits, e.g. as central pattern generators (CPG) (Yuste et al., 2005) and viewing even the psychological **ego** as a result of systems that predict behaviour and environment demands (Llinas, 2001).

Thus, focusing exclusively on reflexive neuroscience paradigms (i.e. recording neuronal activity in response to stimuli) may be insufficient for a complete understanding of brain function. The reason is that there is mounting evidence that sensory systems are driven by other non-sensory cortical areas as well as subcortical structures to be spontaneously active and are only **partially**
modulated by incoming sensory stimuli (Raichle, 2010).

However, reflexive neuroscience should not be abandoned. Many, if not most findings on cortical function have been based on studying the response - often the firing rates - of neurons during sensory stimulation (for example, Adrian, 1926; Hubel, 1959; Shadlen and Newsome, 1994). This traditional approach has lead to the **firing-rate** neural coding theory which states that neurons represent information (presumably for other, downstream neurons) through their firing rates. There is ample evidence for firing rate based coding, for example: visual cortex neurons (Hubel, 1959), LGN and V1 neurons in awake and attending monkeys (Oram et al., 1999), single patched neurons in the barrel cortex of anesthetized rats (Latham et al., 2006; London et al., 2010), modeling (Softky, 1995; Shadlen and Newsome, 1998; Oram et al., 1999) and many other studies. And decision making studies over the past two decades have shown neurons in parietal cortex (a higher-level association area in monkeys) increase their firing rates to reflect accumulation of optimal stimuli over multiple stimulus presentations spanning many seconds (Gold et al., 2007).

But over the past couple of decades evidence of precise **spike timing** relationships being present in cortex, especially low sensory cortical areas, has been slowly accruing. At the single neuron level, the evidence that precise input times are important has been available for almost two decades in the form of spike-timing dependent plasticity (STDP) (Bi and Poo, 1998). In recordings of multiple neurons there has also been evidence of spike timing is important in many studies: auditory systems (Jeffress, 1948), visual psychophysics experiments (Burr and Ross, 1979), cat visual cortex (Gray et al., 1989), chains of firing neurons ("synfire chains") in monkey and other cortical recordings (Abeles and Gerstein, 1988; Abeles, 1991), theoretical approaches (Thorpe, 1990), anesthetized cat visual cortex slices (Mainen and Sejnowski, 1995), monkey frontal cortex during behavioural tasks (Vaadia et al., 1995), extrastriate (visual) cortex of behaving monkeys (Bair and Koch, 1996), retinogeniculocortical pathway in cat (Usrey et al., 2000), somatosensory thalamocortical pathway in rat (Ahissar and Arieli, 2001), hippocampus (Harris et al., 2002, 2003), thalamocortical neurons (Salami et al., 2003), rat auditory cortex (Wehr and Zador, 2003), visual cortex and simulations (Azouz and Gray, 2003), mouse visual cortex (Ikegaya et al., 2004), human somatosensory afferents (Johansson and Birznieks, 2004), cat visual cortex (Fregnac and Zador, 2005; Havenith et al., 2011), at visual cortex (Meliza and Dan, 2006), retina (Hausseit et al., 2007; Gollisch and Meister, 2008), rat somatosensory and auditory cortex (Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013; Luczak et al., 2015), tactile perception (Mackevicius et al., 2012), gerbil inferior colliculus (auditory hub) (Garcia-Lazaro et al., 2013). Some of the evidence for spiking timing being important suggests that neurons that prefer the presented stimulus respond earlier during the presentation: theory (Thorpe, 1990; VanRullen et al., 2005), auditory cortex (Heil, 2004); somatosensory cortex (Johansson and Birznieks, 2004); retina (Gollisch and Meister, 2008).

However, combining the study of spike timing with spontaneous activity paradigms is very challenging. The reason is that there are no precise triggers as in the case of stimulus or behaviour paradigms (e.g. stimulus ON-time or behaviour onset) for triggering single neuron activity analysis.
A good starting point, however is to note that spontaneous neural activity can have non-random structure. In fact, an early hypothesis that the "machinery" underlying spontaneous cortical activity during slow-wave-sleep *K-complexes* (i.e. spontaneously occurring transitions in neurons from hyperpolarized non-spiking to active, spiking states) was used by cortex to process sensory stimuli (Amzica and Steriade, 1998a) lead to findings of preserved temporal structure in neuron firing order. In particular, several studies over the past decade have found that the firing order of high firing rate neurons is similar during spontaneous (including anesthetized) states as during processing (Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013; Luczak et al., 2015). Ironically, spontaneous activity transitions occurring during sleep (in particular, slow-wave-sleep) when sensory stimulus does not seem to propagate to higher cortical areas (e.g. executive function areas) may provide an *intrinsic* trigger which can enable the study of neural activity similar to reflexive methods which use the onset of sensory stimuli or behaviour as triggers. Other findings over the past two decades using two-photon imaging and single neuron patch clamping methods also suggest that co-activated neuronal ensembles during spontaneous activity can also be similar to patterns observed during stimulus evoked periods (Cossart et al., 2003; Ikegaya et al., 2004; Miller et al., 2014; Carrillo-Reid et al., 2015) with some suggesting a move away from the single neuron to ensembles as the basic units of cortical processing (Yuste, 2015).

While there are interesting hypotheses and theoretical concepts about cortical function, arguably most modern neuroscience discoveries continue to be driven by novel experimental paradigms with electrophysiology and more recently optical imaging, being the principal methods for studying neural systems with high temporal and spatial precision.

Electrophysiology is unarguably the most common and successful method for investigating neural activity over the past 150 years and has been the main method of recording for most of the studies discussed so far. While it has been known since the 17th century (Jan Swammerdam) that frog muscles respond to electrical stimulation, recordings from single nerve fibers were only made in 1928 (Adrian and Bronk, 1928) and were followed by glass micro-electrode recordings of single neurons in cat hippocampus in 1940 (Renshaw et al., 1940). By that point it had been known for some time that single neurons have periodic large "negative" electrical deflections (now called somatic "action potentials", APs) following early observations by Emli du Bois Reymond in 1943 (see Schuetze, 1983 for a review). In addition to direct single neuron recordings, electrical activity in the brain has also been measured by inserting a wire or glass electrode into neuronal tissue and recording the total "extracellular" electrical potential (relative to a reference) from all (nearby) sources. The earliest extracellular recordings were made by Steve Kuffler beginning in the 1940s in muscle (Kuffler, 1946) and cat retina (Kuffler, 1953). Tungsteen microelectrodes were developed (Hubel, 1957; Green, 1958; Hubel, 1959) and lead to significant findings in cat visual cortex (Hubel and Wiesel, 1962). Many other innovations followed, including tetrodes that rely on groups of 4 wires bundled together (Gray et al., 1995), to silicon polytrodes that can simultaneously record from dozens or hundreds of sites (Drake et al., 1988). Extracellular recordings capture the total Local Field...
Potential (LFP) of both single neuron and collective neuron electrical activity from all nearby cells including synaptic activity, sodium and calcium spiking and even much slower glial electrical activity (Buzsáki et al., 2012). While some have found that more than 95% (i.e. of the total amplitude) of the LFP originates from current sources <250µm from the extracellular contact (Katzner et al., 2009) some studies have suggested LFP can represent neuronal electrical activity from up to ∼1cm away (Yoshinao and Charles, 2011). Such differences in estimates likely arise from differences in tissue across different areas, animals or neuronal activity states (e.g. cortical state). There have been multiple approaches for developing high-density silicon multisite electrodes (polytrodes) over the past two decades (Drake et al., 1988; Henze et al., 2000; Harris et al., 2000; Csicsvari et al., 2003; Blanche et al., 2005a; Berényi et al., 2014) with the most recent electrodes containing ≈700 (Jun et al., 2017b) and ≈1000 (Lopez et al., 2017) channels (up to 384 simultaneously accessible).

While single neuron spikes remain central to understanding neuronal function, one limitation to using high-density extracellular electrodes to detect single neuron activity is that single unit isolation - i.e. "spike sorting" - can become very challenging and computationally expensive when recording many neurons on hundreds of electrode channels. In fact, even for older electrode recordings containing only a few simultaneously acquired voltage channels, sorting algorithm development requires "ground-truth" datasets - i.e. datasets containing both extracellular voltage records for sorting and the actual spiking patterns of neurons (i.e. the rasters) in order to verify and quantify the sorting results. There are very few in vivo and in vitro ground-truth datasets. However, with modern computational modeling tools, datasets can also be generated using biophysically detailed in silico (i.e. simulated) cortical networks that can generate large numbers of ground-truth spiking rasters and arbitrary electrode layout configurations. Computational modeling of single neurons and simulations of network activity started in with single compartment models over 70 years ago (McCulloch and Pitts, 1943). Dendritic processing was incorporated into models using "cable theory" (Rall, 1964) and with the advent of modern computers, new simulation programs were developed (e.g. NEURON, Hines, 1986; Hines and Carnevale, 1997; Migliore et al., 2006). Most recently, multi-purpose, highly biophysically detailed, cortical network simulations of rat somatosensory (Markram et al., 2015) and mouse visual cortex (Hawrylycz et al., 2016; Gratiy et al., 2017) are becoming available. Building highly detailed multi-scale models of cortex will be central to integrating the very large - and growing - experimental datasets and they can also play important roles in improving experimental and analytical approaches in addition to spike sorting.

In addition to electrophysiological recordings and modeling research, over the past three decades new optical imaging methods have enabled the recording of neuronal activity as a function of intracellular calcium concentrations (i.e. calcium imaging; Mank et al., 2008; Peterka et al., 2010; Broussard et al., 2014; Lin and Schnitzer, 2016) or as a function of membrane voltage (i.e. voltage sensitive dyes - VSDs; Peterka et al., 2010). The most common calcium imaging methods rely on fluorescent proteins (FPs) that report the intracellular concentrations of calcium in neurons. Much work in the past 15 years has focused on improving a particular class of calcium reporters
that green FPs (GFP) called GCaMP, (Nakai et al., 2001), with GCaMP6f (Chen et al., 2013b), in particular, showing a 28 fold fluorescence change from 0-1M calcium concentrations providing a substantially high signal-to-noise-ratio (SNR). There are some problems such as photobleaching (fluorophore loosing its fluorescence) and phototoxicity (excitation light damaging neurons) and single action potential detection is still not possible as calcium signals are biased to reporting single neuron spiking bursts (than tonic spiking modes; Theis et al., 2016). In contrast to intracellular calcium probes, membrane voltage reporters (VSDs) are ideal for recording the excitation state of a neuron as they have fast responses and even report sub-threshold as well as supra-threshold neural activity (Cohen et al., 1974; Blunck et al., 2004; Peterka et al., 2010). Over the past few decades dyes developed for invertebrates have been quite successful in reporting single neuron activity yet mammalian dyes are still limited to providing signals of ensemble averages of post-synaptic responses (Kenet et al., 2003). Lastly, genetically encoded calcium indicators (GECIs) in mice have greatly simplified calcium imaging (e.g. Madisen et al., 2010, 2015) and many available mouse lines targeting specific neuronal populations are now available (Taniguchi et al., 2011; Madisen et al., 2015), though only a few genetically encoded voltage indicators are currently available (e.g. Siegel and Isacoff, 1997).

In sum, over one hundred years of neuroscience investigations have identified several important questions in systems neuroscience, with arguably the most central goal being to understand the neural code employed by the spiking of neurons during both stimulus processing and spontaneous activity. Many experimental tools have been developed to increase neuron yield and targeting specificity with more complex computational modeling tools becoming available for use in neuroscience. While our understanding of spontaneous activity lags behind our knowledge of stimulus and task evoked neural responses, a number of recent novel approaches are paving the way for closing this gap.

**Thesis summary**

This thesis is aimed at investigating spontaneous neural activity in electrophysiology and imaging recordings. It leverages existing (e.g. cat visual cortex recordings) and new (e.g. mouse cortex) recordings and computational tools (e.g. Allen Institute for Brain Science network models) and connects novel findings to existing studies on both spontaneous and stimulus evoked activity.

Chapter describes experiments and computational methods. For this thesis a number of animal recordings were made and several computational toolboxes were developed and employed. First, cat visual cortex electrophysiology recordings acquired previously (Swindale and Spacek, 2014) are briefly described. Mouse sensory cortex and thalamus electrophysiology and imaging recordings acquired specifically for this thesis are described next. Last, the computational methods employed in this thesis are described in detail including several unique approaches to analysis of neuronal activity.
Chapter describes in vitro datasets and in silico modeling methods employed for spike-sorting algorithm testing. The outstanding problem of testing spike-sorting algorithms is described along with a historical background of spikesorting suite development and biophysically detailed single neuron and network models. A novel in vitro dataset acquired by previous researchers (Anastassiou et al., 2015) is reviewed and several spike sorting tests results are discussed. The remainder of the chapter focuses on simulations of extracellular activity and the results of spike sorting such datasets. First, there is a brief review of the Allen Institute biophysically detailed model of mouse V1 and the Blue Brain Project (BBP) single neuron models. Next a number of sorting results based on simulations of hundreds to several thousand neurons are presented and reviewed (Mitelut et al., 2014, 2015; Jun et al., 2017a). The chapter ends on a discussion of the realism of simulated datasets for spike-sorting testing and ongoing efforts to develop largely automated spikesorting methods (e.g. Jun et al., 2017b).

Chapter describes simultaneous widefield imaging and electrophysiology recordings experiments and analysis for generating dorsal cortex neuronal activity maps from single neuron spontaneous activity. There are three main sections. The first section has been previously published (Xiao et al., 2017) and contains simultaneous electrophysiology and calcium imaging recordings and analysis obtained from transgenic GCaMP6 mouse recordings in barrel cortex and sensory thalamus. The aim was to characterize how the spontaneous activity of cortical and subcortical neurons (recorded extracellularly) correlates with widefield bilateral dorsal cortex activity (recorded via calcium imaging). The findings are that barrel cortex neurons are co-activated (during awake and anesthetized states) with expected functional networks involving bi-hemispheric barrel and motor cortex areas (i.e. "consensus" maps). In contrast, thalamic neurons are shown to have greater diversity of co-activation and also reveal more complex temporal dynamics in relation to cortex (for example, some thalamic neurons prefer to fire during large scale cortical depression phases). The remaining two sections of the chapter briefly examine additional datasets of recordings using GCaMP6s mice (section 2) and VSD recordings (section 3) in visual and auditory cortex. The findings of these last two sections largely confirm the approach of the first section, but show that both auditory and visual cortex neurons may have more diverse maps and spatio-temporal dynamics than barrel cortex neurons.

Chapter describes a novel method for clustering spontaneously occurring UP-state transitions during synchronized cortical states and investigates single neuron firing order during such transitions. This chapter relies on previously acquired cat visual cortex electrophysiology recordings coupled with newly acquired mouse sensory cortex electrophysiology and imaging datasets. A novel method is developed that is similar to spike sorting which can identify and classify UP-state transitions in cat V1 and mouse sensory cortex using local field potential (LFP) from extracellular recordings. The LFP events are clustered into distinct classes (i.e. LECs) based on their waveform similarity. It is also shown that LECs can be similar within and across animals and that they can be localized in time with high temporal precision (≈5-15ms). Additionally, it is shown that >90% of all
neurons exhibit spiking latency peaks during LEC-defined UP-state transitions even in visual cortex - similar to previous findings in rat (non-visual) cortex. Perhaps most importantly, it is shown using multiple methods that many neurons can change their latency peaks or distributions during synchronized states (i.e. relative to UP-state transitions) and can also change their firing order outside of UP-states and during desynchronized cortical states. The firing order changes occur over periods of many minutes (e.g. 30-120 minutes) and rates of change vary across neurons suggesting that any underlying spike timing dependent neural code must employ a transient coding/decoding scheme.

In sum, the work in this thesis is aimed at improving single unit isolation (via spike sorting algorithm testing) and characterizing spontaneous neural activity in cat visual cortex and mouse sensory cortex across multiple spatial and temporal scales. The findings of unique cortical maps and drifting firing order are novel and suggest further avenues for research into spontaneous neural activity.
Experimental and Analytical Methods

Electrophysiology

Cat acute electrophysiological recordings

Experimental procedures were carried out in accordance with guidelines established by the Canadian Council on Animal Care and institutional protocols approved by the Animal Care Committee of the University of British Columbia. The experiments were carried out by Nicholas V. Swindale and Martin Spacek and the methods have been described in full elsewhere (Swindale and Spacek, 2014). Five cats were used in total: three were normal domestic cats, while two were heterozygous lipoprotein lipase deficient, left over from an unrelated study (Table 1). Cat ID’s (used throughout this thesis) are: C1, C2, C3, C4 and C5. Initial stages of each animal experiment were performed with the supervision of a veterinarian. For 3 of the 5 cats (C3, C4, C5), initial sedation was by intra muscular (IM) injection of dexmedetomidine (25 µg/kg) and initial analgesia by IM injection of butorphanol (0.3 mg/kg). An intra-venous (IV) catheter was inserted, and initial anesthesia was induced by IV injection of sodium thiopental or propofol. An endotracheal tube was then inserted and a catheter placed in the urethra. The animal was placed in a stereotaxic frame and its head fixed in place with ear bars coated in topical anesthetic (5% lidocaine). The stereotaxic frame was mounted on an air table which was floated prior to polytrode insertion to minimize vibrations.

Following sedation and surgery, electrophysiological recordings were made in the visual cortex of cats with either 0.5 - 1.5% isoflurane and 70% N2O + 30% O2 (animal IDs C1, C2 and C3) or with continuously infused propofol (6-9 mg/kg/hr) and fentanyl (4-6 110 g/kg/hr) (C4 and C5). Heart rate and blood oxygenation were monitored with a pulse-111 oximeter (Nonin 8600V).

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Weight (kg)</th>
<th>Source</th>
<th>Anesthetic</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>M</td>
<td>7</td>
<td>7.0</td>
<td>HLLD</td>
<td>iso + N2O</td>
<td>α-BTX bupr</td>
</tr>
<tr>
<td>C2</td>
<td>M</td>
<td>5</td>
<td>5.1</td>
<td>HLLD</td>
<td>iso + N2O</td>
<td>α-BTX bupr, atrop, xyla</td>
</tr>
<tr>
<td>C3</td>
<td>F</td>
<td>1</td>
<td>3.2</td>
<td>UCD</td>
<td>iso + N2O</td>
<td>PB bupr, dobut</td>
</tr>
<tr>
<td>C4</td>
<td>F</td>
<td>1</td>
<td>3.4</td>
<td>UCD</td>
<td>prop + fent</td>
<td>PB bupivicaine</td>
</tr>
<tr>
<td>C5</td>
<td>F</td>
<td>1</td>
<td>3.5</td>
<td>UCD</td>
<td>prop + fent</td>
<td>PB</td>
</tr>
</tbody>
</table>

Mean arterial blood pressure was monitored with a Doppler blood pressure monitor (Parks Medical 811-B) placed on a shaved section of hind leg. Body temperature was maintained at 37°C with a homeothermic blanket (Harvard Apparatus). Eye movements were prevented either by continuous infusion of pancuronium bromide (C3, C4 and C5) or by retrobulbar injections of -bungarotoxin (Tocris) (C1 and C2). Pupils were dilated with tropicamide drops (0.5%). The eyes were refracted to a viewing distance of 57 cms with rigid gas-permeable contact lenses. After the craniotomy was made, the dura removed and a nick made in the pia with an ophthalmic slit knife. The electrode was then inserted perpendicularly into the cortex under visual control so that the upper recording sites lay \( \approx 100-200 \mu m \) below the surface. The craniotomy was filled with agarose gel (2.5%, Type III-A, Sigma-Aldrich, St. Louis, MO) in artificial CSF at 38-40°C. Recordings were made with 54-channel polytrodes (University of Michigan Center for Neural Communication Technology and NeuroNexus).

Polytrodes were either of 2-column (C2, C3 and C5) or 3-column (C1 and C4) design. Voltage signals were analogue bandpass filtered between 0.5 and 6 kHz, sampled at a rate of 25 kHz and digitized with 12-bit resolution (Blanche et al., 2005b). LFP recordings were obtained from 10 of the 54 channels, and fed in parallel to a separate set of amplifiers. These channels were numbered sequentially from 1-10, starting at the top of the electrode. On the 3-column electrodes the channels were 130µm apart, with the exception of channels 9 and 10, which were 65µm (C1) or 97µm (C3) apart. On the 2-column electrodes the channels were 150µm (C2 and C4) or 195µm (C5) apart, with channels 9 and 10 being 100µm (C2 and C4) or 195µm (C5) apart. LFP voltages were analogue bandpass filtered between 0.1 and 150 Hz, sampled at a rate of 1 kHz and digitized with 12-bit resolution. Experiments lasted up to 3 days. Data reported here were obtained from a total of 14 electrode penetration sites in 5 adult cats. Recording sites were in area 17 and receptive fields (not reported here) were typically within 10 degrees of the area centralis. Individual periods of spontaneous activity lasting from 5-60 minutes (average = 27 minutes) were recorded for each track with most tracks having multiple spontaneous activity recording periods. During these periods the cat viewed, either binocularly or monocularly through the dominant eye, through 3 mm artificial pupils, a field of uniform luminance (58 cd/m2) on a CRT monitor (Iiyama HM903DTB) with a refresh rate of 200 Hz, positioned 57 cms from the eyes.

**Cat visual stimuli**

Various sets of stimuli were used in cat visual cortex recordings and has been described elsewhere (Spacek, 2015; Swindale and Spacek, 2014). The approach is briefly described here (and largely adapted from previous publications). The receptive field (RF) was first identified in real-time using a mouse-controlled oriented bar. Drifting bars stimuli consisted of white and/or black bars on either a grey background with bars 10 or 6° long and 0.5 or 0.3° wide, drifting at 2.5 or 5°/s for 4s, for a total of 10 or 20°. Flashed gratings were composed of stationary, spatially sinusoidal gratings (of various sizes) displayed for 40ms each. Drifting gratings were similar to gratings and consisted
in various temporal (usually \( \leq 5\)Hz) and spatial frequencies. M-sequence stimuli (Shapley et al., 1991; Reid et al., 1997) were composed of 32 x 32 pixel movies with 65535-frames each presented for 20 or 40\(\text{ms}\) (summing all frames gave a grey image). Spontaneous activity was generally acquired while the cat viewed a blank grey screen.

Two types of natural scene movie stimuli were used: a (64\(\times\)64 pixel) video provided by Peter König lab using recordings from a camera mounted on a cat’s head as it behaved naturally (Kayser et al., 2003); and newer movies filmed by Martin Spacek using a digital camera (Canon PowerShot SD200) at 320\(\times\)240 pixel resolution and 60 frames per second (fps) of dense grass and foliage and other environments while also emulating sudden saccade-like movements. Movies were 1-5 minutes in duration and were presented either in entirety, up to 8 times in a single recording, or in short 4.5-5s clips that were repeated 200-400 times. Most stimuli were presented at 200Hz, with selected recordings at 66Hz. All stimuli were shown monocularly depending on which eye revealed the most neural activity (except full-screen flashes and some blank screen stimuli). During each track, stimulus recordings took several hours with varied type of stimuli were presented usually in randomly chosen order by the experimentalists (Martin Spacek and Nicholas Swindale).

**Mouse selection**

Mice experiments used in Chapter 4 were carried out by Dongsheng Xiao, Mattheiu Vanni and the author. Mice experiments used in Chapter 5 were carried out by the author. Animal protocols (A13-0336 and A14-0266) were approved by the University of British Columbia Animal Care Committee and conformed to the Canadian Council on Animal Care and Use guidelines. Animals were housed in a vivarium on a 12 h day light cycle (7 AM lights on). Experiments (Chapter 4 and Chapter 5) were performed towards the end of the light cycle (i.e. late afternoon/early evening). For recordings in Chapter 4, transgenic GCaMP6f mice (males, 24 months of age, weighing 2030 g; \(n=16\)), were produced by crossing Emx1-cre (B6.129S2-Emx1tm1(cre)Krj/J, Jax # 005628), CaMK2-tTA (B6.Cg-Tg(Camk2a-tTA)1Mmay/DboJ, Jax # 007004) and TITL-GCaMP6f (Ai93; B6;129S6-Igs7tm93.1(tetO-GCaMP6f)Hze/J, Jax # 024103) strains (Madisen et al., 2015). For recordings in Chapters 4 and 5 transgenic GCaMP6s mice (\(n=3\); Chapter 4; \(n=13\); Chapter 5) were produced by crossing Emx1-cre (B6.129S2-Emx1tm1(cre)Krj/J, Jax # 005628), CaMK2-tTA (B6.Cg-Tg(Camk2a-tTA)1Mmay/DboJ, Jax # 007004) and TITL-GCaMP6s (Ai94;B6.Cg-Igs7tm94.1(tetO-GCaMP6s)Hze/J, Jax # 024104) strains. The presence of GCaMP expression was determined by genotyping each animal before each surgical procedure with PCR amplification. These crossings are expected to produce a stable expression of the 3 calcium indicator variants (GCaMP3, GCaMP6s and GCaMP6f (Chen et al., 2013b) specifically within all excitatory neurons across all layers of the cortex (Vanni and Murphy, 2014). Control experiments (Chapter 4), assessing the specificity of spike triggered average maps, were performed in Thy1-GFP-M mice (\(n=6\); Jax # 007788). No method of randomization was used since all mice belonged to the same sample group. Samples sizes were chosen based on previous studies using similar approaches (Mohajerani...
et al., 2013; Vanni and Murphy, 2014; Chan et al., 2015).

**Mouse surgery**

Mice were anesthetized with isoflurane (1.5-2%) for induction and during surgery and a reduced maintenance concentration of isoflurane (0.5-1.0%) or urethane was used later during anesthetized data collection. In some cases, animals were allowed to wake up following isoflurane anesthesia for awake imaging (see Chapter 4, section Multimodal recording in awake mice). Throughout surgery and imaging, body temperature was maintained at 37°C using a heating pad with a feedback thermistor. For cortical recording experiments, mice were placed on a metal plate that was mounted on a macroscope. In order to minimize movement artifact (due to breathing and heartbeat), the exposed skull was fastened to a stainless steel head-plate with cyanoacrylate glue and dental cement. A 9 x 9 mm bilateral craniotomy (bregma 3.5 to -5.5 mm, lateral -4.5 to 4.5 mm) covering multiple cortical areas was made as described previously (Mohajerani et al., 2013). For sub-cortical experiments, mice were placed in a stereotaxic apparatus and an incision was made in the midline to expose the skull as in cortical experiments. A burr hole was then unilaterally drilled (usually in the right hemisphere) above the thalamic area (stereotaxic coordinates considering a 45 degree angle: between 1.7±0.3 mm posterior to bregma and 1.6±0.4 mm lateral to midline). The angular tilt relative to a perpendicular penetration to the cortical surface was estimated to be of less than 5° (Hunnicutt et al., 2014). In cases where the laminar probe was inserted (as opposed to a glass electrode) a craniotomy was only made for the probe insertion site and cortical GCaMP imaging was performed through intact bone.

**Mouse acute electrophysiological recordings**

For recordings in Chapter 5, GCaMP6s mice (13 males, 2-8 months of age, weight 20-35g) were used along with wildtype mice (C57/BL6; 3 males, 2-8 months of age, weight 20-35) for chronic recordings (1 mouse) and voltage-sensitive-dye recordings (2 mice). Transgenic GCaMP6s mice were produced by crossing Emx1-cre (B6.129S2-Emx1tm1(cre)Krj/J, Jax #005628), CaMK2-tTA (B6.Cg-Tg(Camk2a-tTA)1Mmay/DboJ, Jax #007004) and TITL-GCaMP6s (Ai94;B6.Cg-IGS7tm94.1(tetO-GCaMP6s)Hze/J, Jax #024104) strain (Madisen et al., 2015). They expressed calcium within excitatory neurons across all layers (Vanni and Murphy, 2014). GCaMP6s (Chen et al., 2013b) expression was validated by genotyping with PCR amplification. Surgical procedures for acute experiments are described in full elsewhere (Xiao et al., 2017). Briefly, mice were anesthetized with isoflurane (1.5-2%) for induction and during surgery with subsequent recording periods under reduced concentration of isoflurane (0.8-1.2%) or urethane (≈1mg/gram body weight). For acute experiments the skull was fixed to a head-plate to stabilize recordings and facilitate imaging. Extracellular recordings in mice were made with 64-channel polytrodes (A1x64-Poly2-6mm-23s-160-A64; NeuroNexus, Ann Arbor, MI) which have a 2-column (32 channels per column) staggered-format
with vertical and horizontal (inter-column-distance) of 46µm covering an ≈1450µm length of the probe. Voltage signals were acquired using a headstage amplifier (RHD2164, IntanTech, Los Angeles, CA) and USB interface board (RHD2000, IntanTech) at a sample rate of 25 kHz (16bit). Electrodes were inserted perpendicular to the surface of the cortex using a micro-manipulator (MP-225, Sutter Instrument Company) with some exceptions (noted in main text) required for simultaneous optical imaging of dorsal cortex. Cortical penetration was tracked using micro-manipulator coordinates and varied between ≈900µm to 1450µm (mean of 1256µm±157µm).

**Mouse chronic electrophysiological recordings**

Chronic tetrode implant recordings were performed in three mice (C57/BL6) with surgery protocols as described above (and in Xiao et al., 2017) with changes specific to tetrode implants. Specifically, tetrodes consisted of bundles of 16 wires (each 15µm in diameter) grouped in sets of 4, cut at an angle (to capture neural activity at different depths) and attached to an electronic interface board (EIB; Neurotek, Toronto, Ontario). The EIB size was 5.3mm x 9.8mm and used an industry standard Omnetics connector that protruded vertically ≈5mm. The EIB was mounted on a makeshift drive consisting of two screws attached to a 10mm x 3mm mounting board such that adjustment of the screws allowed for partial advancement of the EIB (and tetrodes) into tissue even after the mounting board was fixed in place. The total weight of the constructed tetrode was ≈1.2g. Following a small ≈1.5mm craniotomy, the EIB (with tetrodes protruding ≈1.0mm past the mounting board lower surface) was lowered into cortex using a micro-manipulator and the mounting board was cemented to the skull. Following surgery, the mouse was allowed to recover under a heat lamp and monitored daily for one week.

**Mouse awake acute electrophysiological recordings - external lab**

One additional awake visual cortex extracellular electrophysiology recording was obtained from a Ntsr1-Cre mouse. The recording was carried out by Martin Spacek and Laura Busse, at Ludwig-Maximilians Universitat in Munich, using animal protocols and experimental paradigms previously described (Erisken et al., 2014). Briefly, mice were chronically implanted with a head-post which allowed them to be placed on an air-suspended Styrofoam ball for habituation to head-fixation. Visual cortex recordings were subsequently made following habituation using a 32 channel linear silicon probe (Neuronexus, A1x32-5mm-25-177-A32) and while the mouse viewed natural scene movie stimuli identical to those presented to cats described above.

**Mouse sensory stimulation**

Different types of stimuli were used to confirm the correct insertion of the polytrode into sensory cortical areas for recordings presented in Chapter 5 (for recordings in Chapter 4, see Methods below). To identify barrel cortex penetrations, a whisker was attached to a piezoelectric device
(Q220-A4-203YB, Piezo Systems, Inc., Woburn, MA) and stimulated using single 1ms (or 10ms) square pulse. To identify visual cortex, a 1ms LED pulse of green (or blue) light was used. To identify auditory cortex, a 1ms cross-frequency noise chirp was used (note: in some experiments, single tones and frequency sweeps were also used). Stimulus trials varied between 30 to 100 per recording with inter-stimulus intervals of 3s to 10s. The location of retrosplenial cortex (RS) was inferred using Allen Mouse Brain Atlas (Lein et al., 2007) reported coordinates and relative coordinates for auditory, visual and barrel cortex ROIs where available. For all analyses reported in Chapters 4 and 5 the electrode insertion was confirmed using sensory stimuli as revealed in the electrophysiological responses (LFP average, CSD average, and/or single unit response) were used.

**In vitro slice recordings**

Slice recording (i.e., in vitro) data reported and analyzed in Chapter 2 came from in vitro experiments carried out previously and reported elsewhere Anastassiou et al., 2015. The data for that study was collected by two authors: C. Anastassiou and R. Perin. All figures and analysis in Chapter 2 were created by the author. Slice recordings were made at a lower temperature (~20°C) and as a result little to no spontaneous spiking was observed (see Anastassiou et al., 2015). The in vitro recordings thus contained lower noise, i.e. ~6µV - 9µV (post 200Hz high-pass filtering) than the noise observed in some in vivo recordings, i.e. ~9µV - 12µV reported herein (see Chapters 4 and 5).

**Optical imaging**

**Calcium imaging**

Mouse optical imaging recordings used in Chapter 4 were carried out by Dongsheng Xiao and Matthieu Vanni. Mouse optical imaging recordings used in Chapter 5 were carried out by the author. Images of the cortical surface were recorded through a pair of front-to-front video lenses (50 mm, 1.4 f:30 mm, 2 f) coupled to a 1M60 Pantera CCD camera (Dalsa) (Vanni and Murphy, 2014). To visualize the cortex, the surface of the brain was illuminated with green light (but not during image acquisition). Calcium indicators were excited with blue-light-emitting diodes (Luxeon, 470 nm) with bandpass filters (467-499 nm). Green emission fluorescence was filtered using a 510-550 nm bandpass filter or collected in a multi-band mode as described below. For single wavelength green epifluorescence 12-bit images at varying time resolution (20-100ms; i.e., 10-50 Hz) were collected using XCAP imaging software. In order to reduce file size and minimize the power of excitation light used, camera pixels were binned (8 x 8) producing a resolution of ≈68µm/pixel. These imaging parameters have been used previously for voltage sensitive dye imaging (Mohajerani et al., 2013) as well as anesthetized GCaMP3 imaging of spontaneous activity in mouse cortex (Vanni and Murphy, 2014) and awake GCaMP6 imaging in mouse cortex with chronic window (Silasi et al., 2016).
**RGB calcium imaging**

In some experiments (see main text Chapter 4, Chapter 5), a multi-wavelength strategy was employed to correct for potential green epifluorescence signals that were associated with non-calcium dependent events. The method was a variant of the spectral correction strategy described by others (Ma et al., 2016; Wekselblatt et al., 2016) where changes in green reflected light near the isosbestic point of hemoglobin are monitored and the calcium activity signal is accordingly corrected. The strategy employed here was related to previous work using blue excitation and reflected light (Sirotin and Das, 2010). Assuming that hemoglobin is the primary absorber in brain tissue *in vivo*, changes in blood volume or oxygenation affect both excitation and emission of light used for wide-field imaging (Ma et al., 2016). The strategy makes use of short blue wavelength reference light that is also near a hemoglobin isosbestic point. While others have used a strobed LED presentation with a subset of frames providing a green reflected light reference image (Ma et al., 2016; Wekselblatt et al., 2016), the approach was to take advantage of an RGB camera sensor to allow simultaneous acquisition of a shorter wavelength blue ≈447nm signal that correlates strongly with green reflected light signals. This strategy provides a short blue light reference without the need for strobing which can limit time resolution and potentially entrain some neuronal rhythms (Iaccarino et al., 2016) and is more technically demanding from a hardware synchronization standpoint. The strategy relies on the Raspberry Picams RGB sensor (Waveshare Electronics RPi Camera F) to independently resolve signals attributed to blood volume changes as blue reflected light, while simultaneously collecting green epifluorescence (GCaMP6). A Chroma 69013m multi-band filter (10mm diameter) was mounted over the image sensor allowing blue, green, and red signals to be simultaneously obtained in separate channels of the camera’s RGB sensor with less than 10% cross talk between channels. Two Luxeon LEDs were used: 1) Royal-Blue (447.5nm) LUXEON Rebel ES LED with added Brightline Semrock 438/24 nm filter to provide a short blue wavelength reflected light signal that is expected to report blood volume changes; and 2) a blue 473 nm Luxeon Rebel ES LED for excitation of GCaMP6 with a Chroma 480 nm/30 nm excitation filter. In preliminary analyses the short wavelength blue signal correlated positively with apparent blood volume artifacts that were revealed by parallel experiments using green reflected light imaging (r=0.93, see Fig 4.18). Given that the short-blue reflected light signals provided a surrogate indicator of green reflected light (i.e. they are highly positively correlated) this signal was used in a ratiometric correction strategy. While the shorter blue wavelength light will scatter more than a green strobed reflected light signal used by others (Ma et al., 2016; Wekselblatt et al., 2016), analysis of green reflected light and short blue reflected light indicated that two were highly correlated, suggesting that the major artifacts observed were associated with large blood volume changes in superficial cortical layers.
Mouse VSD imaging

VSD imaging was carried out as described previously (Mohajerani et al., 2010, Mohajerani et al., 2013; Vanni and Murphy, 2014). Briefly, either a unilateral craniotomy (1 wildtype C57/BL6 mouse; bregma 2.5mm to -4.5 mm, lateral 0mm to -6mm) or bilateral craniotomy (1 wildtype C57/BL6 mouse; bregma 3.5mm to -5.5mm, lateral 4.5mm to -4.5mm) was made with the underlying dura removed and RH1692 dye (Optical Imaging, New York, NY) (Shoham et al., 1999) dissolved in HEPES-buffered saline (0.621mg/ml) was added to cortex for 60-90min. VSD imaging began ≈30min following washing of unbound VSD. VSD data (12 bit monochrome) was captured with 6.67ms (150Hz) temporal resolution using a CCD camera (1M60 Pantera, Dalsa, Waterloo, ON) and EPIX E4DB frame grabber with XCAP 3.1 imaging software (EPIX, Inc., Buffalo Grove IL).

Mouse sensory stimulation and ROI mapping

For optical imaging recordings used in Chapter 4 sensory stimuli were used to confirm sensory cortical and sub-cortical areas using forelimb, hindlimb, whisker and visual stimulation (for stimuli used in Chapter 5 see above). To stimulate the forelimbs and hindlimbs, thin acupuncture needles (0.14 mm) were inserted into the paws, and a 0.2-1 mA, 1 ms electrical pulse was delivered. To stimulate a single whisker (C2), the whisker was attached to a piezoelectric device (Q220-A4-203YB, Piezo Systems, Inc., Woburn, MA) and given a single 1ms tap using a square pulse. The whisker was moved at most 90 µm in an anterior-to-posterior direction, which corresponds to a 2.6° deflection. A 1 ms pulse of combined green and blue light was delivered as visual stimulation. Averages of sensory stimulation were calculated from 20-40 trials of stimulation with an inter-stimulus interval of 10s.

Multimodal recording in awake mice

To initiate wakefulness isoflurane and oxygen were stopped and the anesthesia mask was removed. Calcium imaging data were obtained over the following 1 hour. The body temperature of mice was maintained with a heating pad. Awake calcium imaging of spontaneous activity was performed in the absence of visual and auditory stimulation. A behavioral monitoring camera was used to confirm that mice were awake and relatively unstressed with grooming and whisking occasionally observed. An analgesic, buprenorphine, was injected (0.075 mg per kg body weight intraperitoneally) 2-4h before awake calcium recordings. A second Dalsa 1M60 camera (150 Hz) or Raspberry Picams RGB sensor (60Hz) was used to capture body and whisker movements under infrared illumination.

Awake mice movement corrections

While relatively few large body movements were observed during awake imaging sessions, their impact on mapping was evaluated by generating [Ca] activity maps from periods of quiescence. To identify periods of movement or quiescence, the standard deviation of luminance fluctuation was
calculated for each pixel. This approach showed that most of the movements were localized on the facial (whisker and jaw) and forepaw regions. A region of interest was manually drawn for each frame and the sum of absolute value of the gradient was calculated by subtracting each frame from the previous one within this region (see Fig 4.19). This gradient profile within the region of interest was temporally smoothed at 0.1Hz and the median and standard deviation were calculated ($\sigma$). Periods of quiescence were identified as having a gradient lower than [median+$\sigma$/10], while periods of movement were higher than [median+$\sigma$]. To more selectively identify periods of quiescence isolated from any movement, an exclusion window of 10s was applied at the beginning and the end of each period of quiescence and only periods of more than 10s were used for comparison analysis. [Ca] activity maps (i.e. STMs; see main text) were then generated using spikes for periods of quiescence and compared with maps using all spikes with little overall differences (see main text).

**Histology**

For some recordings, pipettes (Chapter 4) were filled with dye (Texas red-dextran) or the rear of a laminar electrode shank (Chapter 4 and 5) was painted with fluorescent 1, 1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI, $\approx$10% in dimethylfuran, Molecular Probes, Eugene, OR). Dye-labeled pipettes and electrodes were not used until the dimethylfuran solvent had evaporated. At the end of each experiment, animals were killed with an intraperitoneal injection of pentobarbital (24 mg). Mice were transcardially perfused with PBS followed by chilled 4% PFA in PBS. Some mice brains were sectioned (coronal slices) (50$\mu$m thickness) on a vibratome (Leica VT1000S). Images of diI labeling with counter-stained DAPI were acquired using confocal microscopy (Zeiss LSM510) to reveal the electrode track and help identify the approximate subcortical location of recorded single units.

**Raspberry Pi imaging**

Raspberry Pi (RPi) + Pi Camera Module (Picam) imaging was employed to facilitate off-line hemodynamic corrections via the simultaneous acquisition of RGB data (see Methods above). The RPi and Picam required custom Python3.4 code for tracking single frame times. The custom code is described in the Appendix D.

**Analysis and computational methods**

**Coding toolboxes**

Except as otherwise noted in the main text or here, all analysis was carried out using custom Fortran or Python2.7 code developed as part of an electrophysiology and optical physiology toolkit available online (www.github.com/catubc/openneuron) and described in Appendix C. Event triggered analysis tools have been referenced in previous publications (Xiao et al., 2017) and are also
available online (www.github.com/catubc/picam; www.github.com/catubc/sta_maps).

**Single unit spike sorting**

The program SpikeSorter (Swindale and Spacek, 2014, Swindale et al., 2017 was used for all spike-sorting results presented herein. Briefly, mouse electrophysiological recordings were high pass-filtered at 1kHz (cat V1 recordings were already high-pass filtered), and single spikes were detected using a threshold of 4 to 5 times the median of the standard deviation of the absolute voltage values divided by 0.6475 (Quian Quiroga et al., 2004) and a dynamic-multiphasic event detection method previously described (Swindale and Spacek, 2015). Sorting was carried out by an automated method and followed by manual inspection of units. Units sorted in Chapter 4 had a minimum (max channel) peak-to-peak amplitude of 40µV. Units used in Chapter 5 had a minimum peak-to-peak threshold of 75µV.

**Single unit and imaging SNR exclusion criteria**

Single unit spikesorting results presented in Chapter 4 were subject to multiple exclusion criteria: only units with a peak-to-peak extracellular amplitude of at least 40µV, a minimum of 200 spikes, and a calcium cortical response (i.e. dF/F0 peak) of at least 1% were used. The reasons for these criteria were to improve single unit sorting isolation due to the lower-density of the extracellular polytrode used (i.e. 100µm vertical spacing, single column).

**Single neuron properties used for STMs**

STMs included in analyses were generated from a minimum of 200 spikes. Only STMTDs exhibiting fluorescence exceeding 1% dF/F0 (0.5% in some cases) were used for analysis. Sample sizes were not pre-determined but are consistent with previous experiments using similar methodology (Mohajerani et al., 2013; Vanni and Murphy, 2014; Chan et al., 2015).

**LFP-clustering recordings and unit yield**

For data used in Chapter 5, the average recording length was: 8.8±3.0hrs in cat recordings, 3.6±1.6hrs in acute mouse recordings, and 2.8hrs in the mouse chronic recording. Neuron yield per electrode track was: 99±38 in cat V1 recordings, 85±29 in acute mouse recordings, and 31 neurons in the chronic mouse recording. Average number of spikes per sorted unit was: 31807 in cat recordings, 11974 in mouse acute recordings, and 11190 in the chronic mouse recording. Neuron firing rates had log-normal distributions with long tails and medians of: 0.31Hz in cat recordings (86% of neurons fired <2Hz); 0.26Hz in acute mouse recordings (89% of neurons fired <2Hz); and 0.68Hz in the chronic mouse recording (87% of neurons fired <2Hz). Lastly, although continuous recordings of up to 7hrs (mice) or 14hrs (cats) were made, most of the analysis focuses either on the first 2-3 hours of a recording (mice) or 2-3 hours of continuous recording (cat) to limit complexities.
arising from spike sorting drift, polytrode damage to cortex and overall animal health deterioration.

**Clustering LFP events into LECs**

The approach was to convert 10-channel LFP data to an extracellular recording resembling a high-pass (i.e. spike) record and use SpikeSorter for event detection, alignment, feature-extraction, clustering and cleanup. Other spikesorting suites or other clustering methods (e.g. template matching) were not explored but they should be equally valid if the broad steps described herein are followed. After selecting 10 approximately equally-spaced channels of LFP from the recordings, the deepest recording channel was used to identify synchronized states: i.e. recording periods with a *synchrony index* (Li et al., 2009; Saleem et al., 2010) greater than 0.5 (i.e. periods where most of LFP power lies in the 0.1-4Hz band). In cat V1 recordings, synchronized state periods accounted for: 2.68±1.48hrs of total recording periods of 8.81±3.02hrs, ranging from 4% to 86% of total recording periods (Table 5.1). In mouse sensory cortex recordings, synchronized state periods accounted for: 2.7±1.46hrs of total recordings periods of 3.17±1.40hrs, ranging from 56% to 100% of the total recording periods (Table 5.2).

The synchronized state LFP record was next high-passed filtered (at 4Hz) to remove slower LFP fluctuations. It should be noted that even under anesthetic preparations cortical activity can become desynchronized for shorter (or longer) periods, e.g. contain many lower amplitude high-frequency activity. One option for removing such desynchronized periods is to concatenate the remaining synchronized periods together and perform spike sorting and LFP clustering on the concatenated records. A simpler approach, implemented here, was to manually mask desynchronized state periods on the basis of the synchrony index. This preserved the order of the LFP recordings relative to the high-pass (i.e. single neuron spiking) record and made it easier to compare LFP event times with neuron spikes at later stages of analysis.

The LFP record was then treated as if it was a spike recording by setting the sampling rate to a higher value (usually 50Khz) than the original 1Khz rate of the LFP record. This simulated a speeding *speeding up* of the LFP record 50 times so that LFP events that previously spanned 50-100ms now had a duration of 1-2ms and could be treated as single spike events by the spikesorting software (albeit with very large spatial extent). Standard sorting methods were employed starting with event-detection using a threshold of 5 times the standard deviation (STD) of the LFP signal divided by 0.6745 (Quiroga et al. 2004) using a detection filter with a temporal window (Swindale and Spacek, 2015) of 1ms (equivalent to 50ms realtime), a temporal lockout of 3ms (150ms realtime) and a 2mm spatial lockout (i.e. in a 150ms realtime period only a single LFP event could be identified). The events were next aligned to their mean using a least-squares criterion (described in Swindale and Spacek, 2015), principal components were calculated and events were separated using PCA into clusters called LECs (LFP event classes; see Chapter 5 text). LFP recordings had typically between 1-4 LEC types in cat V1, 3-4 LEC types in mouse visual cortex, and 1-2 LEC types in mouse barrel and auditory cortex (see Tables 5.3, 5.4). The LECs were
aligned using a centre-of-gravity measure (COG; Swindale and Spacek, 2015) which reduces RMS error between each event and the average template (rather than aligning to trough or peak) and all event times could then be exported with millisecond precision.

**LEC stability computation**

The internal stability of all LFP events in each LEC was determined by measuring the standard deviation of the FWHM of the first trough in each event (on the maximum amplitude channel of the LEC template). While not all LECs have a strong peak, all have troughs and single neuron spiking generally occurs shortly following the first trough. The FWHM of each event trough was computed (see G) and the distributions revealed that the vast majority of cat V1 LECs had a standard deviation <10ms.

**CSD computation**

Current-Source-Density (CSD) profiles were computed by taking the 2nd spatial derivative of the LEC templates (i.e. averages of all events in each LEC) across all LFP channels. For cat V1 recordings the entire 10-channels were used (as the polytrodes were completely inserted into cortex). For mouse recordings one column of the polytrode was selected (i.e. 32-channels) and the CSDs were computed on the subset of electrodes which were inserted into tissue (see above). An exclusion criterion was applied for very low amplitude CSDs: the maximum (absolute) values for current sources or sinks had to be 200A/m$^3$ resulting in rejection of 2 of 36 LECs in cat V1 recordings and none in mouse.

**Calculation of peri-LEC-event-triggered-histograms (PETH)**

LEC-triggered single neuron histograms (PETHs) were computed using previously described methods (Luczak et al., 2007, Luczak et al., 2009, Luczak et al., 2013; Bermudez-Contreras et al., 2013; Luczak and Bartho, 2012). Each spike time was replaced by a Gaussian to enable a more bin-width-independent computation of histograms. The gaussian width was 10ms instead of 20ms used in previous work as UP-state transition detection methods used herein were more precise than previously reported. Histograms were then computed from all events in each LEC. Peak latency (PL) for each neuron was chosen as the peak time of each neuron’s LEC triggered PETH (as opposed to the centre-of-mass used by previous methods which were less precise Luczak et al., 2007).

**Computation of dF/F0**

Raw imaging data were saved in a binary format and processed using Python2.7 code as previously described (Xiao et al., 2017). To compute dF/F0 values, F0 was first computed for each event by averaging the value of every pixel during the 3s period preceding the window of interest for the event (i.e. -6s to -3s; Chapter 5) or 3s period preceding the event (i.e. -3s to 0s; Chapter 6)
with both approaches yielding similar results. \(\text{dF/F0}\) was then computed by subtracting \(\text{F0}\) from each raw frame and dividing by \(\text{F0}\). As previously described (Xiao et al., 2017), other methods for computing \(\text{dF/F0}\) were tested including calculating \(\text{F0}\) as the average of the entire recording or by first band pass filtering the data (0.1Hz to 6.0Hz) with results largely similar across methods. ROI time courses (i.e. STMTDs in Chapter 4; ROI traces in Chapter 5) were defined as either the \(\text{dF/F0}\) at a pixel (Chapter 4) or the average activity in an ROI area (see Chapter 5). Spike-triggered-average Maps (STM) were defined as the maximum response calculated for each pixel within a time window of ±1 second of \(t=0\)ms.

**Computing VSD STMs**

VSD STMs and motifs (i.e. spatio-temporal dynamics; see Chapter 4) were computed as described above for GCaMP6. To be noted is that even during strong sensory stimuli, VSD signals for the RH1692 voltage sensitive dye peak at \(\approx 0.5\% \text{dF/F0}\). Thus, averaging over many VSD image frames resulted in STMs with \(\text{dF/F0}\) peaks between \(\approx 0.1\%\) to \(\approx 0.25\%\). Such STMs were nonetheless as averaging random frames (i.e. activity not related to the spiking of a single neuron) results in STMs with peaks \(< 0.01\%\).

**Computation of epoch correlation coefficients**

Changes in PLs over time were tracked by converting the PL order in each 30 minute epoch into n-dimensional (\(n = \# \text{ of neurons}\)) and computing the pairwise correlation coefficient against vectors in subsequent epochs.

**Sliding window PETH computation and controls**

Sliding window PETHs were computed using rasters from 30-minute sliding epochs in 1 minute increments. In keeping with methods previously described (Luczak et al., 2007, 2009; Bermudez-Contreras et al., 2013) a control was computed by dividing rasters into different groups and recomputing the PLs. However, in contrast to previous methods which divided all spikes (in an entire recording) into first vs. second half, spikes from each recording were divided into even vs odd events. Thus, single neuron spikes triggered by each LEC event (i.e. occurring within ±100ms of each LEC) were split into two groups taking each alternating event. The approach used here encompasses previous approaches as by comparing all possible epochs (spaced in 1 minute increments) it captures first vs second half and many other possible combinations. However, it additionally tracks the difference within each epoch between odd and even spikes. Given that PLs drift over time, it is not appropriate to implement the method applied in other studies. Because cortical neurons recorded spiked in tonic as well as burst patterns during UP-state transitions (not shown) other control metrics were tested, for example, by dividing PLs (as opposed to individual spikes) for each LECs into even/odd groups and recomputing the PLs using the window approach. The results of
these tests were similar to those presented using even/odd spike splitting.

**Computing precisely repeating spike triplets - triplet histograms**

Triplet histograms were computed as previously described (Abeles, 1982a, Luczak et al., 2007). Briefly, using 10ms-wide bins the inter-spike-interval between three cells’ spikes were binned and displayed in a 2D graph where the x-axis represents the inter-spike-interval histograms of all spikes of the first and second neuron and the y-axis represents the inter-spike-interval histograms of all spikes of the second and third neuron (see also main text).

**Stable neuron heuristics**

For several sections in Chapter 5, recordings were chosen and only stable units were selected for further analysis. Stable neurons were qualitatively selected as less likely to contain spike sorting errors. In particular, the selected neurons had extracellular templates with a peak-to-peak (max channel) amplitude 100µV. Additionally, the spiking rate for each neuron was qualitatively similar across the entire recording period to ensure that analysis based on spikes from different parts of the recording would not be biased due to substantially different firing rates (or a lack of spiking altogether).

**Removing UP-state-locked spiking**

For the computation carried out in Chapter 5 Figure 75 the goal was to determine whether co-occurring spikes during natural scene stimuli were evoked by stimulus - as opposed by UP-state transitions. Accordingly spiking during/near UP-state transitions was removed: for each LEC event across all LEC classes in the recording all single unit spikes that fell within a ±100ms window of the LEC time were removed. During the recording reported (see main text) the neurons considered had on average 7935 spikes and the procedure removed an average of 922 of their spikes.

**Spike-triggered-map (STM) temporal dynamics (STMTDs)**

For the analysis in Chapter 4, STMTDs were defined as the time course of activity of the maximally (or minimally) activated pixel in a region of interest. The ROI chosen was the left-hemisphere barrel cortex and tracking the activity of the maximally (or minimally) activated pixel resulted in a 1-dimensional trace of usually 180 data points (i.e. 6 sec x 30Hz). The STMTD traces for all neurons were analyzed together using PCA and the resulting clusters were separated using k-means clustering algorithm (n=3). Putative cell classification into inhibitory and excitatory cell types was based on the full-width-half-max of each unit’s positive and negative phases (Connors and Gutnick, 1990; Pape and McCormick, 1995).
Computation of seed pixel correlation maps (SPM)

SPMs were computed by subtracting the contribution of global and illumination fluctuations from the signal of each pixel (Vanni and Murphy, 2014), also known as global-signal-regression. The spontaneous activity recording sequences were then temporally band-pass filtered (0.3-3Hz). Cross-correlation coefficient r values between the temporal profiles of one selected pixel and all the others were calculated (White et al., 2011; Mohajerani et al., 2013; Vanni and Murphy, 2014). Similarities between STM and SPM maps were performed by measuring the r-value Pearson correlation coefficient between each pair of pixels. To compare the STM with anatomical database, brain imaging stacks of 140 slices were downloaded from the Allen Mouse Brain Connectivity Atlas providing AAV-virus tracing database (http://connectivity.brain-map.org/, (Oh et al., 2014). For each slice, the first dorsal 300 µm of brain fluorescence in the z-axis (i.e. depth) were summed to generate partial maximum z-projection maps similarly to previous studies (Mohajerani et al., 2013).

Deconvolution

Pixel-wise calcium imaging deconvolution was done using a method described (Pnevmatikakis et al., 2016) and the code provided by the authors on Github (https://github.com/epnev/ca_source_extraction). Briefly, the method uses an autoregressive approach to estimate the calcium transient as an impulse response from the data itself (Pnevmatikakis et al., 2016). Using this approach the time course of calcium transients is deconvolved via efficient non-negative, sparse, constrained deconvolution. As explained in the main text (Fig 4.3) deconvolution did not substantially change the STMs otherwise obtained.

Cell spiking mode determination

Given that single neurons can fire in both burst and tonic modes, it was determined whether these different modes could yield substantially different STMs. The methodology employed was previously described for defining main spiking modes of a thalamic neuron (Sherman and Guillery, 2006, Fig 6.5, pg 236). Briefly, the method requires determination of the distribution of each spike’s inter-spike-interval (ISI) between the previous spikes (always positive; x-axis) and following spike (always negative; y-axis). The distribution is then plotted using logarithmic scales and clustered (Fig 4.11). Spike groups occurring in approximately each quadrant indicate different spiking modes: first spikes in a burst (bottom right), spiking occurring during a burst (bottom left), last spikes in a burst (top left), and tonic spikes (top right). The vast majority of cortical cells recorded in barrel cortex did not exhibit multiple classes of spiking modes and only a few of thalamic cells recorded showed clear bursting modes while also passing minimum thresholds (see Methods: Single unit activity analysis).
**Single-spike STM-space analysis**

As the STM method averages over the STMs for all spikes from a single neuron, it was important to determine whether single spike STMs could be naturally clustered by similarity. The overall aim of the method was thus to group single spike STMs by similarity in a high-dimensional space and to determine whether natural clusters occur or whether spontaneous activity could be separated from manually partitioned sub-grouping of STMs. First, each 256 x 256 STM was subsampled to 64 x 64 pixels and converted to 4096-Dimensional vector. The next step was to compute distributions of the STMs of all spikes (which have high variability) and evaluate them in a high dimensional space (or a reduced space using PCA). The lack of obvious clusters (in 2D) indicated that there were no sub-groups of STMs present in the data which are removed by the STM averaging procedure. Next, STMs were grouped by similarity into 4 (or more) partitions to reveal active sub-networks present during single spiking. While inter-spike-interval (ISI) distribution were largely similar for the sub-grouped networks, the resulting 4 sub-networks had substantial diversity indicating that (on average) spiking occurred during different types of active cortical networks with only one of these sub-networks resembling the all-spike average STM (see Chapter 6, Fig 4.14D, 4 sub-network STMs and sum at the bottom). Next, spontaneous STMs - i.e. STMs obtained from all spontaneous data without consideration of spiking - were converted to an STM-space representation. The spontaneous STMs were grouped into 4 sub-networks (similar to spike triggered STM partition) by re-using the spike generated sub-network centres (Fig 4.14B,F). This ensures that the spontaneous sub-networks are similar to the spike-triggered sub-networks. The resulting spontaneous sub-networks are similar - but not identical - to the cell spike triggered sub-networks (Fig 4.14H; note that the sum is mostly noise as expected when summing over all activity). Importantly, when subtracting the spontaneously active sub-network STMs (Fig 4.14H) from the spike-triggered sub-network STMs (Fig 4.14D), the results yield STMs which represented mainly single cell spiking components and are very similar to the overall average STM.

**Purity and completeness spike-sorting metrics**

In addition to reporting false positives (FP - incorrectly assigned spikes in a single unit cluster) and false negatives (FN - missed spikes in a single unit cluster) for spike sorting data, two metrics - *purity* and *completeness* - were also developed which better describe spike-sorting error given ground-truth data from *multiple* neurons. There are very few experimental studies capturing intracellular and extracellular data simultaneously and almost none where two or more neurons are recorded from simultaneously. One *in vivo* anesthetized rat hippocampus study successfully patched 3 hippocampal cells simultaneously with extracellular waveforms (Henze et al., 2000; Note: 3 cells were obtained from ≈ 30 animals). Because it only considered single neurons at a time, this study quantified sorting errors using the traditional *confusion* matrix approach, i.e. characterizing false positives (e.g. spikes in a unit not belonging to the intracellularly recorded neuron) and false
negatives (e.g. spikes from a cell that were missed by the sorting algorithm). When considering more than one cell, FP and FN rates are not as useful because errors don’t include just missed spikes or sorted noise, but more complex over-splitting and under-splitting errors which traditional confusion matrices do not capture.

The purity of a sorted unit $U_i$ is defined as the largest number of spikes in the unit that come from a single ground truth neuron divided by the total number of spikes in that unit. For $n$ neurons, the purity of a sorted unit $U_i$ is thus computed by searching over all cells $C_j$ for the maximum match (i.e. intersection) of spikes:

$$purity_i = \frac{\max|U_i \cap C_j|}{|U_i|}, 1 \leq j \leq n \quad (1)$$

The purity metric properly takes into account errors from under-splitting units, i.e. presence of spikes from multiple cells in a unit, while also providing a measure of overall error relative to the cell where most of the spikes come from. However, this is not sufficient to capture all the important information given ground truth spiking rasters. For example, a unit with 100 spikes, 90 of which come from a unique neuron that fires 900 spikes has 90% purity, but only captures 10% of the overall activity of that neuron. The completeness of a sorted unit is thus introduced to additionally capture how much of the overall spiking of the principally identified neuron was captured by the sorted unit. The completeness of a sorted unit $U_i$ is defined as the largest number of spikes in the unit that come from a single ground truth neuron divided by the total number of spikes in the cell.

$$completeness_i = \frac{\max|U_i \cap C_j|}{|C_j|}, 1 \leq j \leq n \quad (2)$$

The completeness metric thus accounts for how ”complete” a sorted unit captures the spiking activity of its best matching cell. For a perfectly sorted recording, both purity and completeness metrics = 1 for all sorted units.

Hyper-angle computation

The angle between two N-dimensional vectors $\mathbf{u}$ and $\mathbf{v}$ is:

$$\theta = \cos^{-1}\left(\frac{\mathbf{u} \cdot \mathbf{v}}{|\mathbf{u}||\mathbf{v}|}\right) \quad (3)$$

False discovery rate correction - Benjamini-Hochberg

The false discovery rate (FDR) arises as a type of error from consideration of multiple comparisons. For example, for a significance p-value set at 0.05%, on average, one out of 20 comparisons will yield a statistically significant result regardless of the data types and distributions (includ-
ing noise). This error was corrected using Benjamini-Hochberg (BH) implementation in Python (\texttt{statsmodels.sandbox.stats.multicomp.multipletests} using the \texttt{fdr_bh} method). In con-\texttt{trast to the Bonferroni correction which controls the overall family-wise error rate by dividing the p values of all comparisons by the \# of tests (and is very conservative), BH controls for the false discovery rate, i.e. the ratio of false positives among the data considered. For the example provided above, BH controls for the one false positive value that would occur in 20 tests for an FDR (p value) set to 0.05\%. 


Spike Sorting Algorithm Testing
Using In Vitro and In Silico Datasets

Electrophysiology, i.e. measuring the electrical activity of a cell or population of cells, is the oldest and arguably still the most common method for investigating neural activity in the nervous system. Most of the results in this thesis rely on tracking single neuron spiking activity as recorded in extracellular cortical and subcortical areas. Isolating and grouping spiking activity of neurons from extracellular recordings is done using spike-sorting: i.e. methods for assigning spikes or events to individual neurons (also known as units). Yet, while high-density electrodes and spike-sorting algorithms have been available for many years, adequate experimental or simulated ground truth datasets to test and validate spike sorting algorithms have been limited. The limitations arise largely due to the difficulty in capturing true spiking activity intracellularly (e.g. using patch clamping methods) while also recording extracellularly from very close by (i.e. <50-100µm). Yet, it is important to quantify how good spike sorting methods are using independently validated datasets.

Accordingly, this chapter is dedicated to generating in vitro and in silico datasets for testing spikesorting algorithms and confirming that the methods (and in particular SpikeSorter used herein, Swindale and Spacek, 2014) are adequate for future research. Biophysically simulated datasets can provide ground truth spiking activity for many neurons (1000s or more) while also generating extracellular voltage records that can be spike sorted. While simulations have some inherent limitations (e.g. cannot yet fully emulate extracellular space or electrode damage to tissue), single neuron and network models have become sufficiently accurate to provide a very good approximation of in vivo extracellular recordings (see text below).

Accordingly, novel in vitro (i.e. cortical slice) and in silico (i.e. simulated) datasets are provided and analyzed to assess the general performance of sorting methods, as well as different sorting suites and multiple operators, and to assess the role of multi-channel probe configurations on spike sorting quality for electrodes similar to those used in Chapters 4 and 5. Simulated datasets were sorted by up to 7 different operators using 4 different spike sorting suites including SpikeSorter, KlustaKwik (Kadir et al., 2014), SpikingCircus (Yger et al., 2016) and an earlier development version of Kilosort (Pachitariu et al., 2016). Lastly, an ongoing cloud-based effort is presented for providing ground-truth datasets to the spike sorting development community. The general findings of this chapter are that: (i) simulated datasets are statistically similar to in vivo and in vitro recorded data, i.e. they have similar extracellular voltage signal standard deviations and similar neuron yield to in vivo recordings; (ii) operator skill may be more important than spike sorting suite algorithms and features; (iii) higher density probes provide much lower error rates; and (iv) with respect to single
neuron sorting quality, channel spacings <20µm may not provide better unit yield or sorting quality for neuron soma diameters of 20-30µm (or larger).

Perhaps most relevant for this thesis, the spike sorting software used here, i.e. SpikeSorter (Swindale and Spacek, 2014), provides sorting results that are quantitatively as good as other sorting software suites.

Results presented in this chapter from in vitro and in silico biophysically detailed simulations have been published or have been presented at conferences: Mitelut et al., 2014; Mitelut et al., 2015; Hawrylycz et al., 2016; Vyas et al., 2016; Jun et al., 2017a; Jun et al., 2017b; Gratiy et al., 2017. Unless otherwise noted, all analysis, figures and simulations in this chapter were prepared by the author (the exception of Fig 3.18, which is adapted from Jun, Mitelut et al., 2017a).

Electrophysiology and single unit spike sorting

While extracellular recordings have been made for many years, it continues to be unclear what the total extent of the LFP is (see Introduction; also Katzner et al., 2009; Yoshinao and Charles, 2011; Buzsáki et al., 2012). What is better understood, however, is that extracellular recordings capture the somatic action potential (AP) of nearby (<50µm) single neurons, i.e. the transmembrane current activity occurring in the soma, axon hillock and initial segment of a neuron during an action potential. More importantly, somatic action potentials of nearby neurons have sufficiently large voltage amplitudes and can be separated and analyzed using a process called ”spike sorting” which uses signal processing techniques to assign each spike to a set of unique cells (or units; Lewicki, 1998). The term ”unit” is used to refer to neurons isolated from extracellular recordings as in the absence of ground-truth confirmation, the identity of the isolated neuron is only putative. There are many spike-sorting approaches aimed at sorting recordings from high-density electrodes (for an older review see Lewicki, 1998; Lewicki, 1994, Gray et al., 1995, Fee et al., 1996, Zouridakis and Tam, 1997, Harris et al., 2000, Zouridakis and Tam, 2000, Hulata et al., 2002, Nguyen et al., 2003, Shoham2003, Quian Quiroga et al., 2004, Litke et al., 2004, Pouzat et al., 2004, Hazan et al., 2006, Bar-Hillel et al., 2006, Wood and Black, 2008, Wolf and Burdick, 2009, Gasthaus et al., 2009, Calabrese and Paninski, 2011, Swindale and Spacek, 2014, Pachitariu et al., 2016, Yger et al., 2016, Jun et al., 2017a). As spike-sorting algorithm development can be a challenging endeavor with several different approaches employed, it is not reviewed at length here. An example is provided, however, showing the steps involved in one recently developed spike sorting approach (Swindale and Spacek, 2014). There are several steps including: identifying events, separating events from noise; clustering the events using principal-component-analysis (PCA); and merging and cleaning of clusters using both automated and supervised methods (see Fig 3.1 as an example of steps involved).

Remarkably, despite the central importance of spike-sorting to single neuron electrophysiology, adequate ground-truth datasets to test spike-sorting algorithms are very limited (see also below)
with most sorting suites relying on repeated sorts from the same in vivo recording (e.g. units are sorted and added back into the extracellular record for resorting - leading to potentially biased results).

There are several challenges to obtaining ground-truth dual-recording datasets in vivo which require a single neuron to be patched (or otherwise recorded directly) while a nearby (<50µm) extracellular probe captures its activity. First, despite many advancements in single neuron patch clamping (Sakmann and Neher, 1984) or sharp electrode recording methods, it remains very challenging to make even single neuron recordings in vivo (i.e. without a nearby extracellular electrode) for extended periods of time. Accordingly, the few simultaneous patch clamp (or juxtacellular Neto et al., 2016) and extracellular recordings datasets rely on cortical slices and usually capture only a single neuron at a time for < 1 hour. Second, and most challenging, is targeting the single neuron patch clamp very near (i.e. <50-100µm) from an extracellular electrode. This is challenging because advancing the patch pipette is usually done without visual information (i.e. blindly) and single neurons are not easy to specifically target, even in slice, let alone when they must be very close to another extracellular probe. This results in at best in vitro dual-recording datasets that contain at most 1 neuron patched at one time (though most attempts fail, e.g. see discussion in

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**Figure 1: Steps involved in spike-sorting.** Steps involved in one approach to spike sorting. (Adapted from Swindale and Spacek, 2014 with permission).
Additionally, dual-recordings in vitro result in lower background activity than observed in vivo and very little to no spontaneous activity in the patched neuron or other nearby cells (see Anastassiou et al., 2015).

In this chapter the lack of ground-truth datasets is addressed through the use of novel in vitro and simulated datasets. The novel in vitro datasets come from single neuron recordings and are used for testing both single neuron and quasi-multi neuron recordings (i.e. multi-neuron recordings generated by concatenating single neuron recordings). The simulated datasets are obtained using the Blue Brain Project (BBP) single neuron models and the Allen Institute for Brain Science network models and provide extensive ground-truth spiking datasets are assessed against several sorting suites and multiple operators. Additionally, the role of polytrode channel layouts (i.e. electrode spacing) on spike sorting quality is briefly investigated in simulated datasets.

In the sections below 7 spike detection and sorting tests are applied to multiple ground truth datasets starting with in vitro recordings where spike detection, minimum neuron separation distance and multi-operator sorting tests. Next, biophysically detailed multi-compartment neuron and network models are discussed and novel in silico, i.e. simulated, datasets are generated and sorted by multiple operators and across multiple electrode configurations. The chapter ends with a brief discussion of the realism of simulated datasets and a cloud-based extracellular sorting application currently being developed.

**Using a novel in vitro extracellular-intracellular dataset for spike sorting validation**

**Existing spike sorting ”ground-truth” datasets**

As mentioned above, because of the importance of precisely identifying single neuron spikes to many fields of neuroscience, there have been significant efforts to develop high-density electrodes and spike sorting solutions. Yet, there are very few ground truth datasets for evaluating the performance of sorting algorithms or that test spike sorting on high-density electrode layouts (e.g. Jun et al., 2017b).

An older study (Henze et al., 2000) recorded several rat hippocampus neurons using intracellular and bundled wire extracellular tetrodes. That study resulted in ~3 neurons sufficiently isolated (out of 30 rat experiments) and the results were limited to analysis of spike sorting quality in single neuron preparations. Another substantial limitation of the study was the use of wire tetrodes which, although still used by some labs, are being replaced by high-density electrodes which have dozens to hundreds of channels.

A more recent in vivo study recorded single neurons using juxta-cellular electrode and high-density extracellular probes (Neto et al., 2016). While the probes were high-density, due to the experimental challenges, out of the several dozen recorded neurons, only 1 neuron had sufficiently
large PTP amplitude (i.e. $>100\mu V$) to be useful for high density sorting algorithm testing (e.g. see comments in Jun et al., 2017a).

Another recent effort used simulated datasets consisting of up to 10 biophysically detailed neurons (from up to three unique neuron morphologies) connected to other single compartment cells with LFP-like noise being added from in vivo recordings (Hagen et al., 2015). While these simulated recordings can generate a lot more data, they are limited in the variability of their neuronal morphology and electrical behaviour.

Additionally, only a few neurons are captured for each simulated polytrode at one time, synchronous spiking is largely absent (i.e. neuron connectivity was not implemented), and the method cannot be expanded further (i.e. the number of neurons cannot be increased; private communication with E. Hagen).

**Dataset acquisition**

Data used in this section comes from in vitro experiments carried out previously for a previous study (Anastassiou et al., 2015) aimed at evaluating extracellular spike waveform variability. The data for that study was collected by C. Anastassiou and R. Perin. All figures and analysis in this section were created by the author.

Simultaneous extracellular and intracellular recordings of single neuron activity continues to be a very challenging endeavor. The findings reported herein use data from 10 individually whole-cell patched excitatory neurons which were collected over $\sim$1 year of experimental efforts on a 12-patch pipette system (Perin and Markram, 2013). The experimental methods for obtaining these recordings are described in detail elsewhere (Anastassiou et al., 2015). Briefly, recordings in rat somatosensory cortex slices were carried out using whole cell patching with extracellular electrodes inserted nearby (i.e. $\sim$50$\mu m$; Fig 3.2). Extracellular potentials were recorded an H32 extracellular probe containing 4-shanks and 8-channel per shank (NeuroNexus, Ann Arbor, MI). Each shank had eight recording sites ($160 \mu m^2$ per site, 1-3 M$\Omega$ impedance) and inter-shank distance was 200 $\mu m$. Recording sites were staggered to provide a two-dimensional arrangement ($\sim$30$\mu m$ vertical staggered separation; Fig 3.2A). Both excitatory and inhibitory neurons were targeted and in one case two neurons were simultaneously patched. Recordings lasted $\approx$1hour each.

The patched cells were stimulated using constant and ramping direct-currents (Fig 3.3) as well as alternating-currents (not analyzed here). Extracellular spike peak-to-peak (PTP) amplitudes ranged from $\sim$40$\mu V$ to $>125\mu V$. Under strong current stimulation some neurons fired repeated spikes and the extracellular PTP amplitudes could decrease by up to $\sim$50%.

**Results of sorting in vitro datasets**

Spike detection and sorting methods were applied to the original in vitro data in three tests: an event detection-only test (Test #1), a spike sorting test for minimum-distance spatial separation (Test
### Test no. 1

The first test using *in vitro* datasets was aimed at qualitatively characterizing how lower event detection thresholds affect the percentage of correctly identified spikes from a cell while increasing the False Positive (FP) rate. FP was defined as the number of incorrectly detected events per second per electrode (another way to normalize is to divide by the SNR or standard deviation of the electrophysiological signal, see discussion below). However, this first test used FP rate as defined in this more limited way in an attempt to broadly quantify the relative error rate differences across units of different PTP amplitudes as a function of event detection threshold. Accordingly, for this event detection-only test, no spike-sorting was done and only an event-detection algorithm was run in order to see how the ratio of neuron events vs. noise events increase for lower thresholds. The test was also performed on each neuron’s extracellular record without mixing data from other neurons.

Using all extracellular spikes from 9 (selected) cells (Fig 3.4) and a threshold of 3.2 (see above, #2) and a full spike-sorting test on all spikes from all cells (>27,000 total spikes) for three different operators (Test #3). Two recorded neurons from this dataset are shown in Fig 3.3 including their intracellular traces, their intracellular and extracellular PTP amplitudes and their extracellular templates.

A couple of important notes should be made. First in the absence of current driving, there was no observable spiking in the cold slices in these experiments (see Anastassiou et al., 2015). Also all spikesorting results (e.g. Test 2 and 3) were obtained following completed spikesorting of the data by a human operator and thus contain subjective splitting and merging decisions.
Figure 3: Characterizing extracellular/intracellular datasets. A. Example of patched neuron membrane voltage during multiple DC ramp current injections. B. Intracellular peak-to-peak amplitudes of neuron in (A) computed using the second derivative of $V_m$ reveals within ramp ($\approx$1.4min duration) spike-amplitude adaptation and longer time-scale adaptation (i.e. following 10 current ramps). C. Extracellular peak-to-peak amplitude showing extracellular spike-amplitude adaptation. D. 50-extracellular spikes aligned to maximum amplitude channel on single shank of H32 probe. E-H. Same as A-D for a different cell with lower PTP amplitude. (Note: cell id numbers correspond to original recording date and were left in for reference purposes).
also Methods) resulted in spikes from the largest PTP amplitude neurons (n=5) being detected very cleanly: no events from other units were present in those detected units (Fig 3.4B points at bottom of graph, i.e. FP=0). This threshold value was selected specifically as the approximate value FP rates began to rise significantly. As expected, decreasing the detection threshold (Fig 3.4C-E) results in additional spikes being detected, for example, spikes detected for the light-blue unit (46.0µV PTP amplitude) increase from ∼20% (3.2 threshold) ∼30% (3.0 threshold). But the cost of those additionally detected events is that there error rate went from ∼0 (3.2 threshold) to ∼30 events per minute of recording (3.0 threshold). Importantly, as the threshold values drop there is a substantial increase error rates for all neurons including (up to hundreds of incorrect events per minute of recording).

A few additional points should also be made. While these FP rates are provided pre-clustering (i.e. the detected events have not been clustered and cleaned; see spike sorting section above), they nonetheless warn of an uneven trade-off where increasing the number of neuronal events (i.e. spikes) passing threshold may lead to exponential increases in noise for clustering stages. While some of the noise (i.e. background activity or spikes from other neurons) may be removed by subsequent sorting stages, there is no (analytical) limit on how much noise can be removed by a sorting process. This is an expected result due the (gaussian) distribution of extracellular potential values (not shown) and these elementary tests characterize this distribution using real tissue recorded data in a spike sorting toolbox using real event detection methods. Second, decreasing thresholds adds potential errors (i.e. increases FP rate) even for high-amplitude units (e.g. red, orange units in Fig 3.4). Lastly, it should be noted that all in vitro recordings were done in relatively cold slices (i.e. ∼20°C) and there was largely no spiking observed in the record other than the single patched neuron. This is not the case in vivo where a single electrode can detect a few nearby cells (though electrode contact resistance can affect this number). Additionally, in cold slice, due to de-afferentiated and disconnected tissue, the standard deviation of the signal was lower than in vivo and possibly skewed some of the results towards higher-error rates due for such in vitro preparations.

In sum, spike detection varied as expected (i.e. lowering threshold values increased errors) with a strongly nonlinear increase in FP rates near a specific threshold (3.2 here; note: for other recordings, this thresholding value may depend on the STD of the original raw voltage signals and the latest version of SpikeSorter uses an automated thresholding method based on voltage distributions on each channel).

**Test no. 2**

The second test carried out was aimed at arguably the most elementary question that can be asked of a spike sorting algorithm: what is the closest spacing of two cells that still allows their spikes to be separated using spike-sorting methods? The ideal experimental data for investigating such a question would come from simultaneously patched, nearly electrically and morphologically identical, physically neighbouring neurons while also recording their extracellular potentials using
Figure 4: Detection threshold vs. false positive rates. A. Peak-to-peak extracellular potential amplitude (on the maximum amplitude channel) of 11 in vitro recorded single cells. B. False-positive rate (FP; computed as # of incorrectly clustered events per second per electrode) vs. percent correct spikes detected for an event-detection run using a threshold of 3.2 (see main text) showing very low FP rate for higher amplitude units. C-E. Same as B for lower detection thresholds (Adapted from Mitelut et al., 2014 with permission).

A high-density electrode <50µm away. Not only that, but such an experiment would need to be reproduced for many different extracellular electrode penetrations to capture different relative locations of the two neurons in the extracellular record (i.e. different angles between the neurons and the face of the electrode). Unfortunately such data is not only not available but may not be obtainable for a long time given the current state of art of single neuron patching.

Given that such an ideal test is not possible, a modified approach using available in vitro data was implemented herein where a hybrid spike recording was made using an original single neuron record plus a spatially shifted spiking record based on a linear interpolation procedure. The added neuron spikes essentially simulate an extracellular space shift in the 2-dimensional plane perpendicular to the extracellular probe. In particular, voltage values from spikes from a single
neuron were \textit{linearly} interpolated (only in the vertical, i.e. y-direction) to simulate spikes from a neuron at a slightly shifted location on the probe (Fig 3.5A). For example, spatially interpolating a voltage value on channel \( c \) at time \( t \), \( 10\mu m \) towards channel \( c-1 \) would require an interpolation:

\[
V_t^c \Rightarrow V_t^{c-1} \frac{10}{30} + V_t^{c+1} \frac{20}{30}
\]  

(4)

The linear-interpolation method was implemented for each neuron by first identifying 50 sample points (@20Khz sampling rate) for every spike’s multi-channel extracellular waveform. Each spike’s waveform was then interpolated using equation (3.1) and then added back - at a random, non-overlapping location - to the original extracellular recording. The final extracellular record thus contained the original neuron with \( n \) spikes and the shifted neuron record also with \( n \) spikes for a total of 2\( n \) spikes. This shifted-spiking record poses a more difficult sorting task: both “neurons” to be sorted have very similar extracellular spike waveforms (but for the minor scaling) which is not expected of \textit{in vitro} or \textit{in vivo} recordings making the recording more challenging for sorting algorithms to analyze.

Using spatial shifts of 15\( \mu m \), 10\( \mu m \) and 5\( \mu m \) all spikes from 8 (selected) neurons were \textit{interpolated} using the method described above (Fig 3.5). However, the approach was slightly different from Test \#1 above. First, only neurons that could be well isolated after the maximum shift condition, i.e. 15\( \mu m \), were chosen: i.e. neurons that had >50% correct spike identification. The reason was that neurons with higher errors would be very poorly sorted for smaller spike shift conditions (and not useful herein). Second, the goal of the test was not to identify the number of neurons as those were fixed at 2 for each dataset. Rather, the goal of the test was to detect as many of spikes as possible and separate them into two clear units (i.e. clusters). All tests were carried by the author and involved full spike-sorting, i.e. event detection + clustering + operator cleanup. The FP value was an average of the FP rate for both sorted neurons (i.e. the average of the percentage of incorrectly identified spikes in each of the two units) and an average of the true positive (TP) rate (i.e. the average of the percentage of correctly identified spikes in each of the two units).

The results show that for a simulated vertical spatial displacement of 15\( \mu m \), even pairs of neurons with a PTP amplitude of \( \sim 45\mu V \) can be relatively well separated from each other (Fig 3.5B). This is a promising result suggesting that neurons that are vertically aligned (i.e. one on top of the other) relative to the face of a high density extracellular probe can be well isolated. However, decreasing the spatial separation to 10\( \mu m \) substantially increases error in sorting of pairs of neurons with PTP <50\( \mu V \) (Fig 3.5C) and one neuron with slightly larger PTP (Fig 3.5C - cyan colour, 56.6\( \mu V \) PTP) which had a more limited extracellular waveform making it more challenging to sort to begin with. Decreasing the spatial separation to 5\( \mu m \) increases the false positive rate as well decreasing the percentage spikes correctly identified for most neurons (except the largest PTP amplitude neuron) making most spike sorted data essentially unusable for analysis purposes.

These findings are encouraging as even in the extreme, i.e. 5\( \mu m \) simulated shift, case the
neuron with PTP value of $>110\mu V$ was relatively well isolated. This provides general support for the stability and isolation for neurons with PTP values $>110\mu V$ even when very similar neurons nearby provide confounding spikes. However, while somata cannot overlap in space, given a nearby extracellular probe insertion pairs of neurons can have virtually any relative position (i.e. even $<5\mu m$) to the face of the probe. That is, pairs of neighbouring neurons are not generally aligned perpendicularly to the face of the electrode: i.e. their somata will usually not be on top of each of each other and at the exact same distance from the face of the electrode. Thus, the distance of pairs of neurons to the electrode can be arbitrarily along a given axis, and certainly $<5\mu m$. This is a significant caveat not only to this pair-wise study presented here, but the very concept of pair-wise neuron separation and reflects the real complexity of properly testing spike sorting algorithms using real patch clamp data.

These considerations caveat the results presented here and suggest other, perhaps more comprehensive studies including in silico recordings (see next Section) are required to address the complexity of what is likely the most fundamental spike sorting test that can be carried out.

**Test no. 3**

In the last test using in vitro data, three operators sorted the combined (i.e. randomly concatenated) extracellular spiking data from 10 neurons (Fig 3.6). For this test, the multi-channel waveforms for spikes from each neuron were extracted from the original (single neuron) record as 500-1000 sample points (i.e. 10-20ms sections) and randomly concatenated with other neurons and random length noise from the original recordings. This resulted in a final recording that contained all spikes from the original 10 neurons in random temporal order. To note is that although some neurons were located on the same electrodes, they did not temporally overlap (i.e. no two spikes were added together at a point in time).

The error rates are reported as purity and completeness metrics (see Methods). Briefly, purity identifies the proportion of spikes in a sorted unit that come from the principally identified neuron (i.e. most number of spike matches), and completeness reflects the proportion of spikes from the identified principal neuron that are captured in the sorted unit. Thus, the ideal sort should have large purity and high completeness values.

Neurons in this in vitro dataset had spiking rates of 3Hz-12Hz (Fig 3.6A). Clustering of the extracellular spikes using PCA in SpikeSorter (following event detection and feature selection) showed in many cases clear grouping (see Fig 3.6C). Three operators of varying skill (Fig 3.6D: grey: $\approx$1 year sorting experience, green: 5 years sorting experience, and blue: 10+ years of sorting experience), sorted the data using SpikeSorter (Swindale and Spacek, 2014: Fig 3.6D-F - grey and blue) and KlustaKwik (Kadir et al., 2014: Fig 3.6D-F - green) sorting suites (Fig 3.6D-F). The purity values were high for all sorts ranging from 88 to 93%. This meant that on average, each sorted unit contained between 88% to 93% of spikes coming from a single neuron. Completeness rates varied substantially more: 53-70% indicating that on average each sorted unit captured between
Figure 5: Spatial separation test. A. Conceptual single neuron separation showing extracellular waveforms from a single neuron in the original and vertically shifted condition. B. 15µm cell separation test showing the % spikes correctly identified (i.e. true positive, TP) vs. false positive % for 8 neurons (see main text also). C,D. Same as B for different spatial separation tests. (Adapted from Mitelut et al., 2014 with permission).
53% to 70% of its principal assigned neuron’s spiking activity. Considering the number of units sorted (Fig 3.6E-left) there is also variability: one operator found all 10 units whereas the other two (blue and green) only found 8 units further suggesting that those operators did not set sufficiently low thresholds. Thus investigating multiple threshold settings may be required as part of spike sorting strategies. However, it is also important to note that the grey operator (i.e. the author) was aware of the presence of 10 units and was therefore biased during sorting by this extraneous information. Interestingly, however, despite finding 20% less units (i.e. 8 instead of the original 10), one operator (green) identified >17,000 spikes whereas the grey operator found all 10 units but only identified ~16,500 spikes. It is unlikely that this was a chance result as the green operator’s results were also very good when considering other tests including multiple operators and spike sorting tests using simulated data (see below) suggesting the green operator’s skill was the main reason for these higher sorting metrics.

Finally, a single value metric (SVM) was used to reduce the overall sorting quality to one value. The SVM was defined as the sum over the product of purity and completeness values for all units $i$:

$$\Sigma_i(purity_i \ast completeness_i)$$

The SVM revealed a ranking that was as expected: the grey operator who identified all 10 units had the greatest overall SVM metric, followed by the green operator who identified more spikes than everyone else with the blue operator being ranked last.

Summary

Although very challenging to obtain and thus rarely available, simultaneous extracellular-intracellular in vitro datasets allow for the elementary testing of spike-sorting detection and sorting methods. Applying spike detection and sorting algorithms to a unique 10 cell dataset (Anastassiou et al., 2015) several novel findings were made:

- false-positive rates increase with decreasing spike detection threshold values which suggests attempting to sort lower-amplitude units becomes significantly more challenging to mixing of other units’ spikes and background noise;

- while higher amplitude neurons (e.g. >50µV PTP amplitude) can be separated from biophysically similar and nearby (i.e. ≥10µm distance) neurons, neurons that are farther from the electrode (and pairs of neurons not lying perpendicular to the face of the probe) are likely challenging to sort. Additionally, the minimum-distance test is not easy to interpret or apply and better spatially diverse metrics need to be investigated);

- varying event detection threshold values should be carried out during each sort (or animal recording) and automated methods for determining optimal event detection threshold should
Figure 6: In vitro datasets multi-operator sorting results. A. Firing rate distributions of in vitro recorded (excitatory) neurons used for spike sort testing. B. Example of extracellular traces (50ms) on a single shank of the H32 extracellular polytrode. C. Examples of cluster separation for units sorted from the dataset. D. Purity and completeness metrics from three operators who sorted the datasets. E. Number of units sorted (left) and number of spikes sorted (right) for three operators. F. Sorting quality using a single-value-metric (see main text) for the three operators. (Adapted from Mitelut et al., 2015 with permission).

be eventually implemented (see e.g. latest version of SpikeSorter, Swindale and Spacek, 2014);

- purity values across operators sorting in vitro recordings were similar and quite high (∼90%), however, completeness values varied much more suggesting that operator skill plays a significant role in spike sorting.

Using novel in silico datasets for spike sorting validation

While the in vitro recordings discussed above provide ground-truth (i.e. spike times) for single neurons recorded extracellularly, the datasets are limited in a number of ways. First, the datasets contain only single neurons (that need to be combined artificially to generate multi-neuron record-
dings) and were acquired in "cold" (i.e. 20°C) cortical slices which have no background spiking activity from other neurons or the patched neuron. Additionally, the recordings were obtained using only one type of electrode configuration (i.e. the Neuronexus H32 layout) and cannot be adapted to other more common multi-column high-density electrodes.

In order to provide more complex and flexible datasets, biophysically detailed network models were employed to generate simulated extracellular recordings on H32 probes and higher density (e.g. 20µm spacing) electrode layouts. In this section a brief review of single neuron and network modeling is provided before discussing biophysically detailed in silico datasets and spike-sorting results across several tests and electrode layouts. Data generated and analyzed here comes from in silico simulations prepared for a number of studies: Mitelut et al., 2014; Mitelut et al., 2015; Hawrylycz et al., 2016; Jun et al., 2017a; Jun et al., 2017b. All simulations and figures were generated by the author (the exception is Fig 3.18 based on data generated by the author and sorted by J. Jun; see also Jun, Mitelut et al., 2017a).

Background - computational models of neurons and networks

One approach to investigating brain function has been to build comprehensive forward models (Buzsáki et al., 2012) - i.e. models that take into account all or most of the known single neuron biophysical properties to build single neuron and cortical network models. The network models are constrained using results for pair-wise neuron and network connectivity experiments and large ensembles of connected neurons can be used to simulate neuronal activity at multiple spatial and temporal scales.

Developing models of single neurons that account for synaptic input to compute the somatic response, i.e. somatic action potentials, has been an ongoing project for many decades (see Fig 3.7 for morphology and synapse model for a mouse V1 neuron). In very early studies only the somatic compartment was considered with synaptic input directly affecting the soma (i.e. McCulloch and Pitts neurons; McCulloch and Pitts, 1943). The Hodgkin and Huxley model of the action potential (Hodgkin and Huxley, 1952) introduced more complex nonlinear properties of membranes but it was not until Wilfrid Rall’s adoption of cable theory to model single neurons (i.e. compartmental modeling; Rall, 1964) that the contribution of distant dendritic processing (including both passive and active membrane properties) was taken into account. Modern computers further advanced modeling with arguably the most successful and commonly used single neuron modeling software, i.e. NEURON, (Hines, 1986; Hines and Carnevale, 1997) eventually being adopted to parallel computer hardware (Migliore et al., 2006) and used in large scale cortical simulations such as the Allen Institute and the BBP.

In parallel to theoretical model development, single neuron recordings have lead to the establishment of databases for 3D neuron morphologies (i.e. www.neuromorpho.org; Ascoli et al., 2007) which at the time of writing contains 62,304 single neuron reconstructions, from 206 different brain regions from dozens of species contributed by 278 groups around the world. Other databases (e.g.
ModeDB; McDougal et al., 2017) provide complete models of single neurons, i.e. they provide both morphology and descriptions of membrane conductances in models that can be readily simulated (over 1100 published articles as of the time of writing).

However, the development of good single neuron models requires - at a minimum - in vitro or in vivo patching of single neuron somata (and dendrites if possible) and the recording of somatic action potentials under different DC and AC current loads (actually, fitting neurons using extracellular data is better, i.e. more constraining, Gold et al., 2007; however, ideal fitting requires measuring activity at all dendrites which is not currently possible using available experimental tools). Subsequently fitting multi-compartment single neuron models, i.e. finding the optimal parameters of active and passive membrane conductances and distributing them along the dendritic and somatic compartments to replicate in vivo and in vitro responses of single neurons - is challenging. Only very recently has single neuron model fitting become standardized and automated led in large part by the BBP and the Idan Segev group (Hay et al., 2011, Hay et al., 2013). These efforts resulted in the development of "evolutionary" algorithms that can even generate single neuron models with complex behaviours such as Ca$^{2+}$ spikes and back-propagating action potentials.

While initial single neuron modeling software suites (e.g. NEURON) were developed to compute the intracellular and transmembrane currents for a given description of a neuron, computing
extracellular potentials at an arbitrary location in space from single neuron intracellular activity is a more recent addition (e.g. the addition of the extracellular mechanism in NEURON). The most common approximation for computing the extracellular potential of multi-compartment neurons is the line-source-approximation (LSA) which can approximate the potential at any location in space from single neuronal compartments with specific transmembrane currents and known lengths (Fig 3.8; Plonsey, 1974; Holt and Koch, 1999; Gold et al., 2006, 2007).

Computing the extracellular potential (using Ohm’s law: $V=IR$) at a point in space based on multi-compartment single neuron models requires the computation of the voltage from transmembrane currents of every 3-dimensional (cylindrical) compartment of a (simulated) neuron. The LSA reduces the distribution of transmembrane current from the surface of each (cylindrical) compartment to a line segment that passes through the centre of each segment. Thus, for each neuron, the extracellular potential $V$ at electrode site $i$ is computed by summing over the transmembrane currents.
current from each simulated neuron segment $j$:

$$V_i = \Sigma_j (R_{ij} I_j)$$

(6)

where $R_{ij}$ is the resistance:

$$R_{ij} = \frac{1}{4\pi r}$$

(7)

and has to be computed for every distance $r$ along the neuron segment (i.e. by integrating over segment). The LSA simplifies this computation as the integral can be computed analytically (for more details see Holt and Koch, 1999; Gratiy et al., 2017).

Using this approach it is possible to generate simulated extracellular recording data for single neurons to test our understanding of active and passive channels in neuron models (Holt and Koch, 1999; Gold et al., 2006, 2007).

Over the past several years, the Allen Institute for Brain Science has been developing methods to characterize and catalog morphologies and physiological properties of large numbers of single
neurons from mouse V1. In parallel with these efforts, the modeling group at the Allen Institute has developed Python-based interfaces to NEURON (which is a C/C++-based low-level simulation environment) to describe and simulate network models of V1 at different levels of granularity (i.e. detail; Hawrylycz et al., 2016). The lowest-level, most detailed network models use multi-compartment single neuron models (with passive and active) dendritic conductances that can be synaptically connected and driven by background and in vivo-like inputs recorded from LGN during awake and anesthetized mouse experiments (Fig 3.9; Gratiy et al., 2014, Gratiy et al., 2015, Gratiy et al., 2017).

The initial biophysically detailed Python-based network model (Gratiy et al., 2014) did not contain an extracellular potential calculator. Accordingly, some of the in silico datasets presented and analyzed here required a temporary solution (i.e. a Fortran-based calculator implemented by the author; see results below; see also Mitelut et al., 2014). However, over the past two years a Python-based simulator and extracellular potential calculator has been developed (Gratiy et al., 2015, 2017) and several simulated datasets have been developed and used to test spike-sorting algorithms (Mitelut et al., 2015, Jun et al., 2017a) and the next-generation of high-density extracellular electrode layouts (Jun et al., 2017b).

Currently, a multi-purpose, cortical area-independent interface that uses a graph theoretic approach to describe cortical networks (i.e. using nodes and vertices) has been developed (Fig 3.10; Gratiy et al., 2017 in preparation). This most recent version contains a simulator component - Bionet (main author: S. Gratiy; Gratiy et al., 2017) in addition to a network description building tool - Netbuilder (www.github.com/netbuilder; author: Y. Billeh) and an OpenGL based 3D visualization tool - Biovis (www.github.com/catubc/biovis; author C. Mitelut; see Appendix). The approach first requires a description of a network including morphological types, synapse types and connectivity matrices; and then enables simulations of the network using the provided connectivity in addition to time-varying synaptic inputs (e.g. LGN-like inputs simulating thalamic input to V1).

**Results of sorting in silico datasets**

Spike sorting methods were applied to in silico recordings in four tests: a single operator sorting test using a 500 neuron patch recorded on an extracellular H32 probe layout (Test #4); a 3-operator sorting test of a ∼3,200 neuron patch simulation recorded on the H32 probe (Test #5); a 7-operator sorting test of a ∼3,200 neuron patch simulation recorded on a 30-channel high-density probe (Test #6), and a single operator sorting test of 1-column vs 2-columns using a 30-channel high-density probe (Test #7). The simulations were run using early development versions of BioNet (Gratiy et al., 2014, 2015) using hundreds to several thousand neuron networks. The single neuron models came from 12 unique neuron morphologies (7 pyramidal and 5 basket cells) from BBP-related studies (Hay et al., 2011, Hay et al., 2013, Hu et al., 2009, Norenberg et al., 2010). There were multiple electrode layouts simulated but only two are discussed in this thesis: the H32 probe (used for in vitro recordings previous section) and a 2-column, 30-channel high-density electrode (20µm
Figure 10: Bionet network examples. A. Examples of an instantiated networks showing only the somata location in a mouse V1 column with additional extra-columnar L4 neurons (note: L1 neurons have brighter colours, all other layers are dimmed). B. An example of a vertical slice (width ≈100µm) showing neurons in L4, L5 and L6. (Data generated using netbuilder and visualized using biovis).

Spacing in the x- and y-axes) which was based on a new generation of high-density electrodes - i.e. IMEC probes - that were in development (soon to be available to the public, see Jun et al., 2017b).

The simulations were run on supercomputer clusters through the Neuroscience Gateway (NSG) at the University of California, San Diego - Super Computer Centre. Many different simulations were run (only some of them are presented in this chapter) ranging from 30 seconds to 10 minutes in duration and using electrode layouts with 30 to as 7,500 sites. The simulations generated up to several hundred thousand single spikes (see Appendix A for more details). Several of those simulations were selected for spike sorting by multiple operators using different spike sorting suites and approaches. The overall findings are that electrodes with higher channel count and higher spatial density substantially improve sorting quality and quantity. However, operator skill was also a significant factor for sorting quality. Surprisingly, 2-column high-density probes may only marginally improve sorting quality compared to single-column configurations suggesting that future hardware development should focus on lower footprint, possibly multi-shank (3D), but single column electrodes.

The simulations, analysis of spike-sorting rasters and figures presented in the remaining sections of this chapter were all generated by the author.
Figure 11: Simulated neuron morphologies and physiology. A. Examples of an intracellular trace (100ms) from a biophysically detailed actively spiking single neuron. B. Partial morphology and soma (black) of a neuron near an 8-channel extracellular electrode (red). C. Intracellular traces (blue) and spike rasters (black) of neuron in (A) over the entire 60sec simulation period. D. Extracellular traces of all spikes of the sorted neuron. E-H. Same as (A-D) but for a different neuron. (Adapted from Mitelut et al., 2014 with permission).

Test 4

This 4th spike sorting test relied on in silico datasets created using an early Python-based network simulator (Gratiy et al., 2014) and was previously presented (Mitelut et al., 2014). It was largely carried out as a proof of principle to show that the simulations can generate realistic test datasets for spike sorting purposes (Figs 3.11, 3.12).

The test relied on a simulation of 500 biophysically detailed and synaptically connected neurons based on 7 morphologically and functionally detailed multi-compartment, biophysically detailed models (Hay et al., 2011, Hay et al., 2013). The simulations were run on a dual-Xeon CPU workstation with 32 cores and 128GB of ram and required ~48 hours for simulating the activity and saving transmembrane currents to disk (using a ~1TB of space on solid-state-drive) with the computation of extracellular waveforms requiring an additional ~168 hours to compute (using an offline Fortran calculator). The simulated dataset was 60 seconds long and contained >37,000 extracellular spike waveforms. The data was analyzed by the author using SpikeSorter and revealed that the extracellular waveforms had typical characteristics of in vivo recoded spike waveforms (Fig 3.11D, H).

The result of this test is summarized in Figure 3.12B as unit detection accuracy vs. PTP extracellular amplitude. Detection accuracy (a metric identical to purity) for all neurons sorted

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Figure 12: 8-channel probe simulated traces and sorting results. A. Examples of simulated extracellular traces recorded on a single shank of an H32 probe (8-channels). B. Unit detection accuracy (i.e. TP rate; see also main text) as a function of unit PTP amplitude. Note that some neurons are outliers (arrow) despite having large PTP. (Adapted from Mitelut et al., 2014 with permission).

revealed an expected asymptotic relationship with accuracy increasing with increasing PTP amplitude. However, a few neurons seem to deviate substantially from this asymptotic relationship (e.g. Fig 3.12B - small green cluster with >300µV amplitude but <25% detection accuracy) suggesting either a combination of similarity in extracellular waveform or over-splitting of units due to spike-amplitude adaptation during burst spiking (a mechanism present in the BBP single neuron models). These initial simulation and sorting results validated the use of simulated extracellular data by confirming the expected dependence of sorting on extracellular PTP amplitude which was observed for in vitro datasets previously tested.

Test no. 5

The next test was a 3-operator sorting test based on simulated data for the H32 probe layout. This test was the same as Test 3 (which used an in vitro dataset; see above) but using simulated datasets that contained several thousand simulated neurons for which ground-truth rasters were known (Fig 3.13).

The activity of 3,198 inter-connected neurons were simulated and a 4 minute recording was generated. The neurons had spiking rates of 5Hz-10Hz and clustering of data (using SpikeSorter selected features and PCA) revealed clear clusters in most cases (Fig 3.13C). Three operators (same as Test 3) sorted the data using SpikeSorter (Swindale and Spacek, 2014: Fig 3.13D-F - grey and blue) and KlustaKwik (Kadir et al., 2014: Fig 3.13D-F - green). The purity values were 81% to 85% (somewhat lower than the in vitro sorts of 88% to 93%). Completeness rates were: 55-67%, similar to in vitro results (53% to 70%). Considering the number of units sorted (Fig 3.13E - left) there is substantial variability: one operator found 27 units (blue) whereas another operator identified 35 units (green). This ~30% increase in additional units (over the blue operator) is substantial.
Figure 13: In silico datasets multi-operator sorting results. A. Firing rate distributions of 3,198 in silico excitatory and inhibitory neurons used for spike sort testing. B. Example of extracellular traces (50ms) on a single shank of the H32 extracellular polytrode. C. Examples of cluster separation for units sorted from the dataset. (Note clusters were separated using a basic k-means algorithm for display purposes only). D. Purity and completeness metrics from three operators who sorted the datasets. E. Number of units sorted (left) and number of spikes sorted (right) for three operators. F. Sorting quality using a single-value-metric (see main text) for the three operators. (Adapted from Mitelut et al., 2015 with permission).

and (as discussed below) strongly suggests that operator skill is very important in sorting. As expected, the number of spikes clustered also varied with each operator: from \( \sim 45,000 \) spikes (grey operator) to \( \sim 64,000 \) spikes (green operator) constituting an increase of 42% more spikes across results. This is a significant difference which suggests substantial room for improvement in sorting (for the grey operator) either in subjective decision making (e.g. setting thresholds or splitting clusters) or cluster cleanup and is briefly discussed further below. Finally, the SVM value (i.e. the sum of purity x completeness for all units; see discussion above) revealed a \( \sim 22\% \) improved performance of one operator (green) over the lowest performing operator (grey). This suggests that while the best operator (green) found 30% more units and 42% more spikes, a portion of the spikes
and units were not correctly identified resulting in a lower overall SVM than expected.

**Test no. 6**

The spike sorting tests discussed so far used older electrode layouts (i.e. the H32 polytrode). The tests below (Test 6 and Test 7) were carried out on high-density extracellular electrode layouts. These electrode layouts were based on real electrodes being developed in a multi-year collaboration between the Allen Institute, University College London, Howard Hughes Medical Institute (Janelia Farms) and Interuniversity MicroElectronics Center (IMEC) in Belgium. (Note: at the time of writing, the manuscript describing this largely electronics engineering effort are under review, see Jun et al., 2017b).

The simulations used in Test 6 relied on a similar 3,198 neuron network patch (used in Test 5) but on a 2-column 20µm spacing (x- and y-axes) 2nd generation IMEC electrode layout (Fig 3.14). (Note: additional simulations testing variations on this IMEC-polytrode layout in support of Jun et al., 2017a were carried out but are not presented here. The simulations had similar firing rates, voltage traces, cluster distributions and unit templates as in vivo recordings; Fig 3.15). The extracellular simulation used a previous version of the Allen Institute biophysically detailed model (Gratiy et al., 2015) and was run using the Neuroscience Gateway service (ssh access) established.
Figure 15: High-density simulated extracellular recordings. A. Firing rate distributions of 3,198 in silico excitatory and inhibitory neurons used for spike sort testing. B. Example of extracellular traces (50ms) on the 2-column IMEC polytrode. C. Examples of cluster separation for units sorted from the dataset. (Note clusters were separated using a basic k-means algorithm for display purposes only). D. Example sorted units from simulated data. (Adapted from Mitelut et al., 2015 with permission).

at the San Diego Super Computer Centre (Sivagnanam et al., 2013; see also Appendix A). The simulations provided 4 minutes (240 seconds) of extracellular data on a 30-channel version of the IMEC polytrode sampled at 20Khz.

The datasets were sorted by 7 different operators using 4 different spike sorting suites: 2 operators used SpikeSorter (Swindale and Spacek, 2014), 1 operator used a Python-based version of SpikeSorter with some modifications (https://github.com/spyke/spyke), 2 operators used KlustaKwik (Kadir et al., 2014), 1 operator used SpikingCircus (Yger et al., 2016) and 1 operator used a development version of Kilosort (Pachitariu et al., 2016). An additional operator relied on a development version of JRClust (Jun et al., 2017a), but the results were not incorporated into this dataset as JRClust was not fully developed at the time (i.e. the results were quite poor). In order to encourage participation, the agreement with the operators was that names and suites used were not to be identified.
The results are presented in Fig 3.16. Purity values (i.e. the average purity across all units sorted by each operator) ranged from 83% (cyan operator) to 99% (red operator) and completeness values ranged from 52% (grey operator) to 74% (pink operator) (Fig 3.16A). The number of sorted units, however, varied widely from: 22 units (cyan operator) to 61 units (green operator) with other operators identifying different numbers of units in that range (Fig 3.16B). Similarly, the number of spikes sorted also varied from $\approx 47,000$ spikes (cyan and red operators) to $> 91,000$ spikes (green operator). The SVM sorting metric found the expected rank of sorters based on the number of units and number of spikes. In particular, the SVM correctly identified the best (green) and worst (cyan) operators, with the worst operator doing more than 2 times worse than the best operator and lagging substantially behind all other operators.

The variable findings with respect to the number of spikes and number of units are of significant concern as it suggests substantially different units and spikes are identified by different operators and suites. What’s quite surprising is that the best (green) and worst (cyan) sorts were carried out using the same spike sorting suite (name not disclosed). It is also of some concern that the distribution of number of spikes and number units is not only broad, but no 2 operators identified the same number of units. This suggests that highly variable sorting results are likely present for in vivo sorts adding substantial variability to results reported in publications. This conclusion cannot be understated especially as the number of spike sorting suites continues to increase with time.

A final caveat should be noted: while the best performing operator (green) identified substantially more units ($\approx 20$) than other operators, the low PTP amplitude units identified by this operator had substantially lower purity and completeness values (not shown). For in vivo applications, it cannot be known whether the addition of such lower-quality units helps with analysis (e.g. by increasing the clusterability of firing-rate state space distributions - e.g. Mazor and Laurent,
Figure 17: Sorting results - 2-column vs 1-column electrode layouts. 2-column IMEC probe layout vs a 1-column version of IMEC probe (using the same vertical density). B. Spike sorting results reveals only minor spike sorting differences between the two layouts (see also main text; adapted from Mitelut et al., 2015 with permission).

2005) or whether the addition of these units would confounds analysis such as in single visual neuron properties. Thus, it is unclear what the usefulness of such units is without further tests and in vivo applications. However, it is important to note that this question could be pursued further via simulated datasets and ground-truth for low-PTP amplitude units.

Test no. 7

The last test presented here is aimed at addressing a way for reducing electrode damage which continues to be a concern and active efforts to minimize damage are studied (e.g. Kozai et al., 2010). This final test was carried out to determine whether narrower electrodes would result in a loss of sorting precision. Specifically, the 2-column IMEC probe was tested against a 1-column version of the probe. This test was implemented in a simple manner by sorting simulated data acquired on the 2-column IMEC probe layout and comparing with sorted data from the same simulation but ignoring (i.e. removing) one of the columns from the extracellular voltage record.

Surprisingly, sorting the data using the 1-column version of the probe yielded very similar sorting error rates (Fig 3.17). An interpretation of this result is that given the size of the somas in the simulations (i.e. ~20μm to ~35μm diameter) from the perspective of single-unit spike sorting, electrode density may reach a saturation point beyond which increasing vertical density (or even horizontal density) provides limited benefits.

Thus, this test suggests that future polytrode development efforts should focus on minimizing polytrode size (i.e. width) to minimize damage to cortex which may be a more important goal in electrode development. In fact, polytrode fabrication techniques have already reached extraordinary densities (e.g. 1μm inter-site-spacing and 30μm electrode thickness; private communication Ed Boyden lab).
Figure 18: Sorting results are similar across *in vitro*, *in vivo* and *in silico* datasets.
A. Definition of false negative and false positive metrics used for comparing spikesorting results across *in vitro*, *in vivo* and *in silico* datasets. B. The number of sites on which each sorted neuron template has a voltage peak (V_p) amplitude > than 3 x the RMS of the signal (i.e. V_{rms}). C. Data from (B) reveals no statistical difference between sorting real vs. simulated data. D. False positive rates across different dataset types show similar trends. Inset shows the distribution of simulated units as a function of PTP amplitude (i.e. V_p) divided by the noise RMS (i.e. V_{rms}). E. False negative rates are also similar across the datasets. F. There are no statistically significant differences between real and simulated data. (Adapted from Jun et al., 2017a with permission)

Ongoing efforts for spikes sorting suite development

As part of an ongoing effort to develop independent datasets for spikesorting tests several dozen biophysically detailed network simulations have been generated and analyzed over the past three years. Several of those datasets have been analyzed and presented in the sections above (Tests #1-7). Simulated datasets reproduce spatio-temporal aspects of high-passed extracellular potential data (i.e. spike records) and confirm (as expected) spike detection and sorting accuracy decay substantially with decreasing spike PTP height (i.e. single neuron distance from electrode). Simulated recordings also have signal noise standard deviation of 8-11µV similar to *in vivo* recordings (though simulations with higher firing rates can have slightly higher standard deviations). Over the past 1.5 years many of the simulated datasets were made available on the author’s freely accessible website (www.spikesortingtest.com; see also Appendix ). The website serves as a cloud-based validation tool for testing spikesorting algorithms. Users are able to download simulated datasets, sort them using their preferred spike sorting suite and upload the sorting results. They receive a report usually in 30-60 seconds. The website was implemented using multiple cloud computing tools including Django, and runs on Python and Fortran modules on the server side.
Lastly, a new spikesorting toolbox (JRClust; Jun, Mitelut et al 2017) has been developed over the past two years which implements a novel signal transformation approach, clustering method and automated drift tracking and seeks to increase sorting automation (i.e. reduce operator input). JRClust relies largely on large simulated datasets for testing and uses graphics-processing-unit (GPU) based code to achieve faster than real-time sorting even in recordings with more than 100-channels. Importantly, JRClust was used to sort more than dozen simulated datasets and it was shown that spikesorting results of simulated datasets were statistically indistinguishable from sorting the available hybrid-in vivo (12 neurons) and in vitro (4 neuron) datasets (Fig 3.18). In particular, it was shown that the physical extent of each neuron’s template, i.e. the number of sites on which each neuron spike has a voltage peak (V_p) amplitude > than 3 x the RMS of the signal (i.e. V_rms) was very similar between the different datasets (Fig 3.18B, C). Second, false positive (Fig 3.18D) and false negative (Fig 3.18E) rates are similar across the datasets and there are no statistically significant differences(Fig 3.18F).

**Discussion**

The general findings of sorting *in silico* datasets are that:

- most operators and sorting suites have high *purity* (i.e. low error) rates and moderate *completeness* (i.e. missed spikes) rates;
- the number of spikes and units sorted vary substantially across operators with as much as three times less units (e.g. 61 vs 22) sorted by one operator vs another even when using the same sorting suite;
- simulated datasets are a good representative of *in vivo* and *in vitro* recorded data as they have similar signal-to-noise ratio of extracellular voltage signal, similar extracellular template size distribution and similar neuron yield;
- as expected, higher density probes provide lower error (e.g. higher *purity*) rates than lower density probes;
- electrode density may saturate beyond a value (e.g. <20µm) and further increases in channel density may not be useful for unit isolation while potentially being more damaging to tissue.

Large-scale simulations (i.e. containing many neurons and electrode sites) can provide previously unavailable ground-truth data making it possible to test both novel spike sorting methods and novel electrode layouts. The availability of large ground-truth datasets may also enable the analysis of more complex issues such as the role of operator skill - including identifying and optimizing automation based on subjective decisions. The ability to generate arbitrary electrode layout may even be used to test the limits of extracellular electrophysiology, i.e. determine what electrode
Figure 19: Allen Institute active conductance neurons have small spatial propagation. Example extracellular potentials (\(~40\) channels) showing that single neuron spikes are largely localized to single channels and do not propagate to nearby channels making them more challenging to sort using sorting algorithms (Note: only spikes from the first 500 simulated neurons are highlighted due to limitations in visualization in SpikeSorter.

layouts are required in order to capture all - or the vast majority of - neuronal spiking in a patch of tissue.

However, modeling extracellular voltages has not been perfected. For example, while data presented in this chapter comes from BBP single neuron models, the Allen Institute has also released their own mouse V1 neuron models that also contain active conductances. While arguably more appropriate for use with the Allen Institute network models, unfortunately, the Allen Institute single neuron models generated extracellular spikes that were difficult to sort due to very small extracellular waveforms (Fig 3.19). That is, the extracellular waveforms for each spike was confined mostly to one channel resulting in an extracellular record that looked more like it was obtained from multiple single neuron (extracellular) recordings rather than using high-density electrodes.

There are several important remaining challenges in spike sorting including: capturing low-amplitude neurons, increased automation of drift correction and improved overall sorting automa-
tion (i.e. decreased human supervision). Because of the approximately gaussian distribution of extracellular potential values around $V_e=0\mu V$, it will continue to be challenging to capture lower amplitude neurons. Even though lower noise hardware is being developed, this will not overcome the $1/r$ dependency of extracellular signals. Thus, distant neurons will always contribute noise to recordings of closer, potentially more sortable units (e.g. 50\mu m to 100\mu m from an electrode). Automation will continue to be a challenge - but likely only for a smaller proportion of neurons, i.e. those neurons that have very similar (usually low-amplitude) templates and which will need to be compared manually. Drift correction may in fact be the easiest to tackle as ground-truth datasets can be used to improve tracking of electrode movement (see Jun et al., 2017a for an implementation of drift correction). One important result from this chapter is that spike sorting results using SpikeSorter - the main sorting toolbox used in this thesis - is at the same level as other sorting tools. Overall, however, more discussions need to be had in the neuroscience community and further standards need to be developed where neuron yields are evaluated against neuron quality and decisions are made about what types of error rates are acceptable. It is also important to emphasize the independence of spike sorting development and data generation/ground truth (see e.g. approach above and also Neto et al., 2016). Importantly, there is also a need for the development of some standards of analysis where independence is preserved between data generation, i.e. between experimentalists who patch neurons or developers who run simulations, and sorting suite developers.
Optical Mapping of Spontaneous Single Neuron Activity

“Using voltage sensitive dye imaging, we previously established a close link between ongoing activity in the visual cortex of anaesthetized cats and the spontaneous firing of a single neuron...We suggest that dynamically switching cortical states could represent the brain’s internal context, and therefore reflect or influence memory, perception and behaviour.”

Tsodyks et al., 1999

Spontaneous neural activity is present across all spatial and temporal scales. For example, single neuron spiking can be studied during stimulus-free periods using intracellular or extracellular recording methods while the activity of large populations of neurons across entire cortical areas (e.g. visual cortex) can be studied using recent optical imaging methods (e.g. GCaMP6 or VSDs) or functional-magnetic resonance imaging (fMRI).

Over the past couple of decades, a number of studies sought to relate spontaneous and stimulus evoked neuronal activity across spatio-temporal scales. The studies have been generally carried out by simultaneously recording single neuron activity along with activity of large populations of neurons making it possible to correlate activity at the micro-scale (e.g. spiking neurons) with macro-scale neuronal activity. Two early studies (Tsodyks et al., 1999; Kenet et al., 2003) related spontaneous and stimulus evoked neuron spiking (using extracellular electrodes) to ongoing dorsal cortex activity (using VSDs over ≈2mm x ≈6mm regions) in cat V1. These studies made a number of findings including that stimulus evoked and spontaneous neuron spiking correlated with the same pattern of cortical activity of large numbers (i.e. millions) of neurons as observed in VSD. Additionally, it was shown single neuron firing rates depended on the spatial pattern of ongoing population activity in cortex suggesting that the population activity modulates the firing rate of a single neuron. Interestingly, the studies identified the presence of “dynamically switching cortical states” (e.g. activated patterns that mimicked ocular dominance columns) which emerged spontaneously suggesting “dynamically switching cortical states could represent the brain’s internal context” (Kenet et al., 2003). In other words, the spontaneously occurring activity patterns were reminiscent of stimulus evoked patterns hinting that ongoing cortical activity may function as an activity-space repertoire from which stimulus representations are selected.

These findings suggest that functional studies across spatial scales is not only possible, but can yield insightful results that are otherwise not obtainable. They also confirm a strong link across spatial scales and that spontaneous activity, even at large spatial scales, can yield insight about how information is processed in the brain.
These pioneering studies suggest new directions for inquiry about micro-to-macro scale neuronal interactions - and also about how such findings can be extended using more recent experimental methods. For example, do other imaging methods, e.g. based on [Ca] reporters, confirm or extend these findings in other cortical areas and in other animals that do not have cortical structures such as ocular dominance columns? Recently completed mouse anatomical connectivity database (i.e. Allen Mouse Brain Atlas) document with extraordinary detail cortical and subcortical monosynaptic connections. Can additional multi-scale functional studies use this information and target large areas of cortex and even subcortical neurons to define functional relationships across large areas of cortex? The work in this chapter expands on the pioneering cross-scale functional connectivity studies discussed above to anesthetized and awake mice recordings using the latest transgenic [Ca] reporters, i.e. GCaMP mice. The work also relies on high-density (i.e 64-channel) extracellular probes which not only decrease the single neuron spiking detection error rates (i.e. improve spike sorting) but also yield simultaneous spiking from dozens of cells while providing spatial information across multiple cortical laminae and subcortical nuclei. Wide-field optical mapping (WFOM; Ma et al., 2016) techniques are used to simultaneously capture calcium activity across large areas of mouse dorsal cortex including visual, barrel, motor and medial cortical structures and relate them to the spiking of single neurons. Methods for computing ”spike-triggered-maps” (STMs) using GCaMP6 (with preliminary findings using VSD reporters) are also explored.

The general findings of these investigations are that neurons from different cortical depths (but within the same area) yield similar dorsal cortex GCaMP6 STMs conforming to the expected mono-synaptic anatomical connectivity. However, subcortical neurons - from the same or different thalamic nuclei - exhibit substantial differences to their expected mono-synaptic connectivity maps as well as temporal dynamics. In particular, subcortical neurons are more likely to be co-activated during dorsal cortex activity of areas that are not known to be monosynaptically connected based on mouse brain connectivity information (e.g. Lein et al., 2007; Oh et al., 2014). The work presented in the first section of this chapter (i.e. barrel cortex and thalamic neuron mapping) have been published (Xiao, Vanii, Mitelut et al 2017). The majority of experiments in this section were carried out by Dongsheng Xiao and more than half of the analysis and figures were prepared by the author. For clarity all figures in the first section of this chapter will be captioned with initials of the author who made them as follows: CM: Catalin Mitelut, DX: Dongsheng Xiao, MV: Matthieu Vanni.

The findings presented in the second section (i.e. visual and auditory cortex neuron GCaMP6 mapping) have been partially presented at a conference (Mitelut et al., 2016). All experiments and analysis presented in the second section were carried out by the author. The findings presented in the third section (i.e. auditory and visual cortex neuron VSD mapping) have not been presented before. All experiments and analysis presented in the third section were carried out by the author.
GCaMP6 mapping of spontaneous activity of barrel cortex and thalamic neurons

Summary

Understanding brain function requires knowledge of cortical operations over wide-spatial scales from single neurons to large populations. In order to investigate the relationship between spontaneous single neuron spiking and mesoscopic cortical activity, in vivo, wide-field imaging and sub-cortical and cortical cellular electrophysiology were carried out in GCaMP mice. A rich set of cortical activity motifs were identified in spontaneous activity in anesthetized and awake mice. Using genetically targeted indicators of neuronal activity, mesoscale spike-triggered averaging allowed the identification of motifs (i.e. spatio-temporal patterns) that were preferentially linked to individual spiking neurons. Single thalamic neuron spiking correlated with cycles of wide-scale cortical inhibition and excitation. In contrast, single cortical neurons correlated with mesoscale spatio-temporal maps expected for regional cortical consensus function. The approach can define network relationships between any point source of neuronal spiking and mesoscale cortical maps.

Background

Neural activity ranges from the microscale of synapses to the macroscale of brain-wide networks. Mesoscale networks occupy an intermediate space and are have been widely studied in cortex forming the basis of sensory and motor maps (Bohland et al., 2009). These networks are largely defined by co-activation of neurons and have been evaluated with a variety of statistical approaches that capitalize on detecting synchrony. The study of large scale networks (meso-to macro-scale) has been mostly restricted to functional magnetic resonance imaging (fMRI), or magnetoencephalography that can capture whole-brain activity patterns (de Pasquale et al., 2010; Kahn et al., 2011; Logothetis et al., 2012), but lack high spatial and temporal resolution and sensitivity. To overcome these limitations, alternative approaches including mesoscopic intrinsic signal, voltage, glutamate, or calcium sensitive indicator imaging have been employed (Kleinfeld et al., 1994; Kenet et al., 2003; Ferezou et al., 2007; Chemla and Chavane, 2010; Chen et al., 2013b; Mohajerani et al., 2013; Stroh et al., 2013; Vanni and Murphy, 2014; Carandini et al., 2015; Chan et al., 2015; Madisen et al., 2015; Wekselblatt et al., 2016; Xie et al., 2016). New preparations using large scale craniotomies (Kim2016b) and large format imaging systems (Tsai et al., 2015; Sofroniew et al., 2016) provide the ability to link mesoscale activity patterns to individual neurons. However, these measures are restricted to superficial layers of cortex and cannot assess functional connections to sub-cortical structures. While developments in fiberoptic technology allow local optical functional assessment of brain activity in sub-cortical structures (Hamel et al., 2015; Kim et al., 2016), they cannot simultaneously resolve cortex over large fields of view. Although the evolution of imaging has revealed new aspects of cortical processing in identified neurons (Harvey et al., 2012; Chen et al., 2013a;
Chen et al., 2013b; Fu et al., 2014; Guo et al., 2014), the electrically recorded action potential is still a signal of prominence given its temporal precision and ability to reflect the output of neuronal networks (Buzsáki, 2004).

Extracellular recordings of single units were made in cortex, thalamus, and other sub-cortical sites with simultaneous mesoscopic functional imaging in transgenic mice expressing the calcium indicator GCaMP (Zariwala et al., 2012; Vanni and Murphy, 2014; Silasi et al., 2016). While slower than protein-based or small molecule voltage sensors, GCaMP imaging offers a high signal to noise ratio and is associated with supra-threshold activity which in some cases is a more direct reflection of spiking activity. This work extends pioneering studies investigating the relationship between single neuron spiking and local neuronal population activity assessed by voltage-sensitive dye imaging. Specifically, spike-triggered averaging (STA) has been previously used to identify the local activity profile related to the spiking activity of a single neuron within a population (Arieli et al., 1995) and it was further demonstrated that this activity profile could reveal the instantaneous spatial pattern of ongoing population activity related to a neurons optimal stimulus in the visual cortex of anesthetized cats (Tsodyks et al., 1999). The work presented herein extends these approaches and also exploits the main advantage of mesoscopic imaging allowing the simultaneous measurement of brain activity in multiple regions across most of cortex simultaneously rather than activity surrounding the recording site. This multi-scale strategy can help define temporal relationships between the activity of single neurons at the microscopic scale and mesoscale cortical structural projection maps (Zingg et al., 2014; Madisen et al., 2015). Furthermore, multisite silicon probes used here enable the assessment of long-distance activity relationships between multiple subcortical single neurons and mesoscale cortical population activity. Spontaneous activity in awake and anesthetized mice was exploited as a source of diverse cortical network activity motifs (Mohajerani et al., 2010; Mohajerani et al., 2013; Chan et al., 2015). Application of spike-triggered averaging in spontaneous cortical (calcium) activity linked the activity of single neurons to mesoscale networks. Single thalamic neuron spiking was found to functionally link to more diverse sensorimotor maps whereas cortical neurons spiking was largely associated with consensus cortical maps. Thalamic neurons were found to both predict and report (firing before and after) specific cycles of wide-scale cortical inhibition and excitation, while cortical neuron firing was usually associated with excitation (and in some cases multi-second refractory depression). These results are consistent with an active computational role of thalamus in sensory-motor processing (Theyel et al., 2010; Hooks et al., 2013; Petrus et al., 2014; Sheroziya and Timofeev, 2014; McCormick et al., 2015), as opposed to merely serving a relay function. The findings are also consistent with a diverse role of the thalamus in feed-forward sensory processing. Thalamocortical transmission can dynamically and differentially recruit local cortical excitation and inhibition based on thalamic neuron firing patterns and where thalamocortical feedforward inhibition is a critical feature(Galarreta and Hestrin, 1998; Swadlow and Gusev, 2001; Gabernet et al., 2005; Cruikshank2007; Hu2016). This spike-triggered cortical and subcortical neuron mapping technique, exploiting mesoscopic calcium imaging, can be extended to
Figure 20: Experiment setup: multichannel electrode recordings and spike-triggered-averaging (DX, CM). A. Set-up for simultaneous wide-field calcium imaging and single unit recording using a glass pipette or laminar silicon probe. B. (i) Top view of wide-field transcranial window and (ii) cortical atlas adapted from the Allen Institute Brain Atlas. C. Example of (i) cortical and (ii) subcortical pairs or spike recordings from separate channels showing cluster isolation in the first two principal components. D. Procedure for generating a spike triggered average map (STM) for a unit located in barrel cortex.
any brain location where electrodes can be placed to identify functionally linked cortical mesoscale networks.

Results

Linkage of individual spiking neurons to specific mesoscopic cortical maps

Wide field of view mesoscale cortical imaging was used in GCaMP transgenic mice (Madisen et al., 2015) in combination with cellular electrophysiology recordings to derive cortical networks that reflect activity at targeted point sources of neuronal spiking throughout the brain. Cortical and sub-cortical neuron spiking activity was recorded electrically while simultaneously imaging cortical
mesoscopic activity across a \( \approx 9 \text{mm} \times \approx 9 \text{mm} \) bilateral window that encompassed multiple areas of the mouse dorsal cortex including somatosensory, motor, visual, retrosplinal, parietal association and cingulate areas (Fig 4.1A,B). Spectral decomposition of the mesoscopic spontaneous activity using GCaMP6 revealed the presence of information below 10 Hz that was distinct from non-specific green light reflectance (Fig 4.2). Given the slow \( \text{Ca}^{2+} \) binding and unbinding kinetics of GCaMP6, imaging dynamics are expected to be prolonged compared to actual spike records. Additionally, deconvolution was employed as described elsewhere (Pnevmatikakis et al., 2016) to possibly improve the time course of raw calcium signals relative to the spike-triggered-mapping techniques used herein (Fig 4.3). While deconvolution improved the temporal dynamics of the decay of the calcium signal, spike triggered analysis was only marginally affected and deconvolution was not implemented throughout.

Spiking signals were initially recorded in multiple brain areas using glass electrodes (n=8 mice) to minimize obstruction of cortical imaging and reduce potential for damage from electrode placement. Subsequently, laminar probes (16 channel with 0.1 mm contact spacing) permitted the resolution of more spiking neurons simultaneously, and facilitated the recordings in multiple sub-cortical regions (n=16 mice). Given the invasive nature and the long duration of recordings, initial data was obtained from urethane (n=4) or isoflurane (n=12) anesthetized adult mice, with additional awake recording periods (n=12, see Methods). The spike triggered average maps (Fig 4.1D,E) obtained under both these conditions were qualitatively similar and this observation was consistent with previous work using VSD imaging (Mohajerani et al., 2013). To perform these assessments single neuron spikes were identified from extracellular recordings using spike sorting methods based on clustering of principal components distributions of spike signals on sets of adjacent channels (Swindale and Spacek, 2014) (Fig 4.1C).

Initial characterization of GCaMP6 (Chen et al., 2013b) suggested a calcium excitation rise time of \( \approx 100 \text{ms} \) and a minimum decay time of \( \approx 150-200 \text{ms} \); accordingly, analysis of dynamics is usually limited to below 10 Hz. Additionally, \([\text{Ca}]\) time-frequency power analysis on the imaging data was used to evaluate power at different frequencies in the GCaMP6 signal and compared to the green reflectance signal - which is not expected to carry neuronal activity information. This analysis showed the presence of \([\text{Ca}]\) activity power up to 8-10Hz which was substantially higher (5-10 times) than the power in the reflectance signal - validating the use of fluorescence signals up to this frequency limit (Fig 4.2).

Spike triggered average maps (STM) were computed from simultaneously acquired wide-field calcium imaging and single neuron recordings to investigate how single neuron spiking activity at a specific cortical or sub-cortical location was related to regional cortical activity (Fig 4.1D; see also Methods). For each individual spike, cortical imaging frames were considered from 3s before to 3s after the spike. The frames were normalized as \( \text{dF/F}_0 \) by subtracting and dividing the average calcium activity during the 3s preceding the spike (see also Methods for alternative \( \text{dF/F}_0 \) calculations). Next, all spike triggered frame stacks were averaged into a multi-frame motif.
Figure 22: Deconvolution does not substantially change STMs (DX). A. Top: Original (dF/F0) calcium image and time course of calcium dynamics in a region of interest (ROI). Bottom: deconvolved calcium image and time course in the ROI. B. Top: spike triggered average map (STM) and STM temporal dynamic (STMTD) of original data in ROI. Bottom: spike triggered average map (STM) and STMTD of deconvolved data in the ROI for spikes from the same neurons. (Note: neurons were recorded in thalamus under anesthesia).

ranging from -3sec to +3sec centred on spiking (e.g. 6sec x 30frames per second = 180 frames; see also Fig 4.16A for a full-temporal resolution motif example). The STM - computed as a single image representation - was then defined as the maximum (or peak) activity at each pixel within the time window of ±1 second of the single neuron spiking. This pixel-specific peak-value method better captures correlated activity than merely averaging over the ±1 second interval which smooths out highly activated but short duration - activity increases. The approach revealed that the activity recorded from a single right barrel cortex neuron, for example, yielded an STM showing strong and specific GCaMP signal in barrel and motor cortices of both hemispheres (Fig 4.1E(i)). STMs calculated by averaging calcium activity centred on spiking activity accordingly revealed spatial specificity correlating with neuron spiking that was not present when compared with calcium activity in reference region (e.g. hind limb; Fig 4.1E(ii)) or random spike averaging (Fig 4.1E(iii); see also Methods).
Kinetics of spike-triggered mapping

By computing spike-triggered calcium image averages, the contribution of neurons which fire out of phase is substantially reduced. Analysis of STM time dynamics indicate slower time to peak (100ms) than postsynaptic potentials evoked by a single synaptic connection (time to peak 20ms) (Bruno and Sakmann, 2006). Slower dynamics are expected given the kinetics of GCaMP6 (Chen et al., 2013b) and deconvolution (Pnevmatikakis et al., 2016) can be used to take into consideration the slower kinetics and compensate accordingly. Using this approach, a significant acceleration of raw data was observed but very modest effects on STMs indicating that STMs may already represent accelerated activity relative to GCaMP6 kinetics perhaps due to the statistical nature of spike-Ca2+ transient temporal convergence. It is also possible that slower dynamics reflect sequences of spiking activity propagating through specific polysynaptic circuits. This speculation was supported by the similarity in time dynamics during cue-triggered recall of learned temporal sequences shown in other studies (Xu et al., 2012). The kinetics of imaging can be improved in future studies using faster sensors such as organic voltage sensitive dyes (Shoham et al., 1999; Mohajerani et al., 2013), or genetically encoded voltage (Carandini et al., 2015; Gong et al., 2015; Abdelfattah et al., 2016) or glutamate sensors (Xie et al., 2016) - but at the cost of lower SNR data and more ambiguous interpretations of activity (e.g. glutamate activation; see also Chapter 4, last section on VSD STMs).

Validation of GCaMP6 correlation with single neuron activity

The specificity of STMs computed in GCaMP6 mice (reflecting underlying neuronal activity) was confirmed by imaging Thy-1 GFP-M mice (n=6 mice) that lacked calcium-dependent neuronal fluorescent signals. Thy-1 GFP-M mice failed to produce functional maps using the same STM procedure outlined above (Figure 4.4A,B). Additionally, the stability of STMs as a function of the number of neuron spikes was measured by quantifying the similarity of STMs generated from the total number of spikes vs subsets of spikes. Stable STM maps were generally observed using at least 256 spikes (Fig 4.4C-E). The stability of STMs was also confirmed by comparing the maps generated by splitting a unit’s spikes into two halves, or into odd and even groups which yield similar STMs.

Thalamic neurons show more diverse STMs than cortical neurons

The next step was to apply the STM method to multiple recordings in cortex and thalamus and identify mesoscopic networks associated with single neurons. First, the anatomical location of single neurons was confirmed by labeling probes with Texas red-dextran or DiI as to visualize tracks and approximate the location of each sorted neuron on the electrode (Fig 4.5A, subcortical track and nucleus identification from 4 different mouse experiments, see also Figure 4.4A,B). Spiking cortical neurons had very similar STMs in each cortical recording and were linked to consensus
Figure 23: Control recordings and convergence of STMs with # of spikes (DX) A. Simultaneous calcium and spiking activity recording in GCaMP6f mouse and STM yielded from single unit recorded in barrel cortex.
Figure 23: (continued from previous page) B. Simultaneous GFP fluorescence and spiking activity recording in Thy-1 GFP-M mouse and STM yielded from single unit recorded in barrel cortex resulted in no clear regional map. C. STMs generated from a subset of spikes (2, 2048, on the left) randomly chosen in one experiment. Correlation coefficients (r-value, on the right) between STMs were used to evaluate the consistency of mapping. In this example, STMs generated by more than 64 spikes generated a correlation >0.9 and were very similar between the pairs of SPMs made using the same number of spikes. D. Distribution of correlation values between pairs of STM for an increasing number of spikes. No significant change in r-value distribution was observed for 512 spikes in comparison to 256 spikes (Mann Whitney test, P=0.126, U=948.5, 256 spikes group n=58, r-value=0.97±0.01, mean±SD; 512 spikes group n=40, r-value=0.98±0.01, mean±SD). E. STMs and profile of responses computed using spikes divided into halves or even-odd sets. (Note: neurons in examples come from recordings performed under anesthesia).

local and long range cortical networks (Fig 4.5B-left: single STM examples of cortical STMs; right: contours of all single neuron STMs recorded in each recording shows substantial overlap across all depths). Consensus cortical networks have been discussed previously (Mohajerani et al., 2013) and are defined by using correlated activity mapping techniques (i.e. seed pixel analysis: each pixel’s correlation value with every other pixel is computed over the entire recording). Consensus maps reflect major mono-synaptic intra-cortical axonal projections and generally demarcate either single sensory areas (e.g. visual cortex) or sensory areas and their major mono-synaptic projections (e.g. barrel and motor cortex; see also Mohajerani et al., 2013). Barrel cortex neurons have STMs that substantially or completely overlapped with regional GCaMP signal changes in barrel and motor cortex, as well as showing signals in homotopic areas of both hemispheres, consistent with the previously observed pattern of long distance mono-synaptic connections (Ferezou et al., 2007; Mohajerani et al., 2013; Guo et al., 2014; Vanni and Murphy, 2014; Chan et al., 2015). The similarity across cortical neuron STMs can be observed in “contour” maps of each STM (computed as the border of full-width-half-max value STM) which largely overlap with each other (Fig 4.5B,C-contours).

The patterns observed in thalamic neuron STMs also supported the presence of a functional link between specific thalamic nuclei and consensus cortical projection areas (Hunnicutt et al., 2014; Oh et al., 2014; Zingg et al., 2014), but also showed additional variability and complexity to cortical neuron STMs. Thalamic neurons were associated with bilateral hemispheric signals within multiple primary sensorimotor and higher order brain areas (Fig 4.5C; Note: some unilateral STMs are observed generally in contralateral (to probe) areas and it is likely this is due to electrode damage in the ipsilateral hemisphere rather than unilateral mono-synaptic thalamic neuron connections to cortex - which have not been previously described).

The use of multichannel probes enabled the recording of multiple single neurons across multiple cortical layers and multiple thalamic nuclei simultaneously. Accordingly, additional relative (and absolute) laminar location analysis could be carried out. Cortical neurons in the same cortical area -
Figure 24: Topographic properties of neurons and neighbouring similarity metric (CM, DS). A. Electrode track for sample recordings (Blue channel: DAPI, yellow: DiI). B. STMs and overlay contours of neurons recorded in barrel cortex with each color representing one neuron’s STM border (see main text and Methods). C. STM and overlay contours of neurons recorded in thalamus in the electrode track presented in panels A and B. Color bar on the right side indicates the depth of each recording site. D. Diversity of overlap of STMs between neurons on neighbouring laminar electrode channels. (i). Example of overlapping STMs (red area) between two cortical neurons recorded on adjacent channels. (ii). Example of overlapping STMs for neighbouring pairs of neurons recorded subcortically showing differences across depth.
Figure 24: (continued from previous page) (iii). Average neighbouring cortical neuron map overlap (blue: 93%) and neighbouring sub-cortical neuron overlap (78%) show significant differences (Mann Whitney test, p<0.0001, U=617408.0, mean percentage overlap of cortical STM pairs=92.77±0.23%, mean±SEM, n=966; mean percentage overlap of sub-cortical STM pairs=78.11±0.61%, mean±SEM, n=1936). These results are from awake mice (except top row, Mouse #1).

but from different depths - exhibited substantial similarity (i.e. overlap) and there were only subtle topographic changes in STMs across depth: static STMs were largely similar between and within superficial and deep layers (see contour maps in Fig 4.5B; see also Fig 4.5D(iii) overlapping analysis results). Subcortical neurons, had more diversity even within the same sub-nucleus in thalamus (see examples in Fig 4.5C where neurons from neighbouring electrodes could have different STMs; see also examples in Fig 4.7 SUA STMs). To quantify the difference of variability of STMs across depth for both cortical and thalamic recordings the percentage overlap of static STMs (Fig 4.5D; red areas) was computed for all pair-wise neighboring channel neurons (i.e. 100µm apart). STMs derived from neighboring electrode contacts in cortex were largely similar (mean of 93% across all cortical recordings) whereas sub-cortical STMs were more varied (mean of 78% Fig 4.5D(iii)).

Additional methods of generating event triggered maps were explored including using events from MUA rasters or using LFP amplitude to trigger (i.e. scale) imaging frames. MUA-triggered STMs were computed using thresholded spiking (i.e. 4 times standard deviation of the signal divided by a scaling factor Swindale and Spacek, 2014). Additionally, band-passed LFP triggered STMs were computed for Delta (0.1-4Hz), Theta (4-8Hz) and Gamma (25-100Hz) bands (see also Methods). Briefly, LFP triggered STMs were computed using data from 60 second recording periods by scaling (i.e. multiplying) each imaging frame by the average LFP amplitude (at each recording depth) at that point in time. Thus, imaging frames during which the average LFP amplitude was large and positive contributed substantially to STMs, whereas frames where the average LFP values were closer to zero did not (negative LFP values were clipped). (Note: negative LFP values were also explored with somewhat different results - not shown here).

The MUA-triggered method revealed that both cortical and subcortical MUA triggered STMs were largely similar to single-unit STMs and did not vary substantially across laminar depth (Figs 4.6, 4.7)). An additional method was implemented (see Xiao et al., 2017 reviewer comments online) where a normalized MUA-triggered STM was subtracted from single-unit STM with the hypothesis that such methodology would reveal unique STMs not present in single neuron STMs. Unfortunately the resulting STMs appear largely as very noisy maps (i.e. maps with <1% dF/F0 values that have no ROI specificity). This is due to the similarities between single-unit and MUA STMs before subtraction which results in cancellation of the vast majority of high-SNR calcium signal. The LFP-band triggered STM methodology revealed STMs that were substantially different from single cell and MUA triggered STMs but stereotyped across depth (with some minor differences;
Figure 25: LFP and MUA triggered STMs: cortical neuron examples (CM). STMs were computed at different depths of the electrode using single cell spikes, Multi-Unit-Activity (MUA) and LFP amplitude. Single unit STMs were computed (see Methods) for up to three representative cells at each depth. MUA STMs at each electrode were computed using all spiking activity over a threshold (\textit{math}>4 times the standard deviation of the high-pass record divided by a scaling factor - see main text). LFP triggered STMs were computed by scaling each image by the average LFP amplitude - and averaging over a 60 second recording period. Thus, imaging frames where the average LFP amplitude was large and positive contributed substantially to the STM, while frames where the average LFP values were low did not. The various band-passed LFP values used were delta: 0.1-4Hz, theta: 4-8Hz and gamma: 25-100Hz. Single cell STMs at each depth and across depth have similar motifs to each other and MUA triggered STMs. LFP triggered STMs are substantially different from single cell and MUA triggered STMs and across different LFP frequency bands (see main text).
for example, see Fig 4.6 Theta band-triggered STMs change from left barrel cortex activation to left limb cortex areas with depth). The LFP-triggered STMs were also different across LFP frequency bands. However, it is important to note that LFP amplitude- (or power-) triggered maps had very low dF/F0 values. That is, most LFP-amplitude triggered STMs had peak dF/F0 values \( \mathit{math}<0.05\% \) dF/F0 whereas single neuron or MUA-triggered STM had peaks of 1-5% dF/F0. This indicates that the LFP-triggered method requires averaging over highly-variable dynamics (e.g. the LFP signal might vary substantially more than the GCaMP6 signal). Nonetheless, LFP-triggered STMs appear stable across depths and are highly unlikely to result from averaging random activity. In fact, LFP-triggered STMs reveal anatomically discrete maps that are stable across multiple depths in cortex and subcortical areas with only small - but systematic - differences across layers (Figs 4.6,4.7). Whereas LFP-amplitude triggered STMs for delta band activity were similar to single neuron STMs, STMs associated with higher frequency bands showed different patterns suggesting a different type of functional connectivity present at higher LFP frequencies across spatial scales (Figs 4.6,4.7). As LFP contains mostly synaptic activity and only limited spiking activity (i.e. spiking only from within a region with radius of \( \approx 250\mu \text{m} \); Buzsáki et al., 2012) LFP-triggered STMs likely represent average synaptic activity from large neighbouring regions thus blurring the overall effect of local LFP activity. However, because delta band activity is where most GCaMP6 functional imaging indicator power is located (Chan et al., 2015), higher frequency components are closer to hemodynamic and other noise sources making interpretation of the results above 4Hz challenging. Other, higher temporal resolution imaging methods (e.g. VSD) will likely yield additional insight into the relationship between mesoscale maps and LFP activity across layers and in subcortical areas and is a topic to be investigated in future work.

**Sub-cortical neuron STMs are more diverse than cortical neuron STMs**

To quantitatively determine how single cortical and sub-cortical neuron STMs relate to intra-cortical networks, single neuron STMs were compared (using cross-correlation) against a cortex-wide library of seed pixel correlation maps (Mohajerani et al., 2013; Vanni and Murphy, 2014; Chan et al., 2015) generated iteratively for all pixel locations in the same spontaneous activity recording (Fig 4.8A,B). To create SPMs, the cross-correlation coefficient \( r \) values between the temporal profiles of one selected pixel and all the others within the field of view were calculated (Mohajerani et al., 2013; Vanni and Murphy, 2014; Chan et al., 2015; see also Methods). To evaluate the similarity between static STMs and SPMs, the correlation coefficient between pixels of both types of maps was computed for all possible SPMs in the library. The best matching SPM was selected, i.e. the SPM with the highest correlation between a given STM and the library of SPMs. The library of SPMs is expected to reflect cortical consensus activity motifs (areas undergoing temporally-correlated activity) and can be largely attributed to underlying intra-cortical axonal projections (Mohajerani et al., 2013). Single cortical neuron derived STMs were more similar to SPM correlation maps (Fig 4.8A), i.e. the correlation values were high between SPMs and cortical STMs (Fig 4.8C). In
Figure 26: LFP and MUA triggered STMs: subcortical neuron examples (CM). Thalamic STMs: Spike vs. LFP. STMs were computed at different depths of the electrode using single cell spikes, Multi-Unit-Activity (MUA) and LFP amplitude (see also Fig 4.6).
In contrast to cortical STMs, single thalamic neurons at some depths have more varied motifs (e.g. electrodes 4, 11, 12, 15), while MUA triggered STMs appear similar across large thalamic regions (e.g. electrodes: 4-16). LFP triggered STMs are different from single cell and MUA triggered STMs and across different LFP frequency bands (see main text).

Contrast, thalamic STMs were more complex and corresponded to more unique distributions of cortical patterns and had lower correlations with the cortical consensus SPM library (Fig 4.8B,C). For example, VPM, VPL and CP neurons can functionally link to multiple cortical areas and this diverse connectivity was not always present in SPMs (i.e. SPMs made from seeds in BCS1, HLS1, RS or other areas). It is possible that sub-cortical neuron STMs neurons are the super-position of 2 or more cortical networks defined by SPMs. For example, some subcortical neuron STMs can be better described using pairs of SPMs which when added together provide a more complex map that is arguably more similar to the single subcortical neuron STM (see Fig 4.8D for examples neuron STMs constructed from combination of SPMs).

To better understand the underlying structural circuit basis of distinct thalamic STMs, thalamic neuron location and projections were analyzed using the Allen Mouse Brain Connectivity Atlas (Oh et al., 2014) (Fig 4.9; see also Mohajerani et al., 2013). The 3D-atlas data was used to match the composed anatomical 2D maps with 2D static STMs. As expected, STMs of spiking cortical neurons corresponded with underlying structural axonal projections, i.e. monosynaptic connections (see BCS1 example in Fig 4.9, also Mohajerani et al., 2013). In contrast, not all sub-cortical neuron STMs had monosynaptic projections from sub-cortical to cortical areas that accounted for their cortical mesoscale patterns. There are such examples coming from non-thalamic neurons: e.g. while hippocampus (HPF) does not have a strong direct anatomical link to RS, one identified HPF neuron had an STM that was dominated by RS co-activation. Another example showed that a caudoputamen (CP) neuron had a strong hindlimb/forlimb map (BCS1/HLS1) yet there was no established direct monosynaptic links between CP and such areas suggesting that sub-cortical STMs could represent polysynaptic links to cortex (Humnicutt et al., 2014; Oh et al., 2014).

Cortical and sub-cortical neuron firing is tuned to cortical network dynamics spanning millisecond to multi-second time scales

After the static STM spatial analysis considered above, the next step was to consider time dynamics and determine whether single neuron STMs had inhibition-activation patterns that revealed additional, novel information about micro-to-mesoscale functional connectivity (Figs 4.10-4.15). STM Temporal Dynamics (STMTDs) were computed by identifying regions-of-interest (ROIs) with high activation and tracking the time course of the maximally activated/depressed cortical pixel within each ROI from 3s before to 3s after spiking. As all extracellular recordings were in right barrel cortex and predominantly right subcortical structures, the left barrel cortex (LBCS1) was used.
Figure 27: STMs vs SPMs (MV,DX). A. Cortical STM (left) and the best fitting SPM (right) according to correlation coefficient (cc) values for different neuron locations (left of panel). Similarity was calculated by measuring the r-value Pearson coefficient between each pair of map pixels. Group data from 12 GCaMP6f mice are reported in panel C. B. Sub-cortical STM (left) and the most similar SPM (right). Cell #2 and #4 were from GCaMP6s mice. Cells #3, 5-7, 11 were from GCaMP3 mice. Other cells were from GCaMP6f mice. These examples were performed under anesthesia. C. Distribution of r-values (Mann Whitney test, p < 0.0001, U = 5227, sub-cortical group n = 246 r-value = 0.64 ± 0.18, mean ± SD; cortical group n = 168 r-value = 0.85 ± 0.04, mean ± SD). D. Examples of sub-cortical STMs compared with pairs of SPMs for seed indicated by a and b.
Figure 28: STM comparisons with Allen Brain Atlas projection maps (DX). A. From top to bottom, example STMs from neurons recorded in BCS1, VPM, VPL, HPF and CP, respectively. B. Example projection maps (2D surface and 3D) reconstructed from Allen Brain Atlas with injection sites (Oh et al., 2014) in the same region as the recorded neuron (in A). For example, for a spiking neuron recorded in BCS1 anterograde labeling of GFP emanating from an injection site in BCS1 shows mono-synaptic projections to motor cortex and is present across cortex in the 2D surface plot of cortex. This projection pattern for BCS1 matches the STM map well and is consistent with previous work (Mohajerani et al., 2013). In contrast, for sub-cortical injections of GFP tracer in HPF there was less overlap between STMs and projection maps perhaps suggesting STMs represent polysynaptic as opposed to merely monosynaptic pathways. (Website: 2015 Allen Institute for Brain Science. Allen Mouse Brain Connectivity Atlas. Available from: http://connectivity.brain-map.org.)
as the primary ROI as it contained the most activated and depressed calcium activity patterns. It should be noted that ipsi-lateral cortex (e.g. left barrel cortex in a left barrel cortex electrode penetration) was also highly activated, but even for cortical neuron generated STMs, it showed substantially less consistent activation than the its opposite hemisphere homotopic area (likely due to damage done by the electrode in the ipsilateral hemisphere).

As observed for static STMs, the cortical calcium dynamics associated with cortical neurons were relatively homogeneous with a peak in activity within \( \approx 100 \text{ms} - 200 \text{ms} \) following spiking and a return to baseline (Figure 6A). However, some cortical cells (\( \approx 20\% \)) participated in multi-second depression dynamics (see distribution of profiles in Figs 4.10, 4.17B,C and 4.20A). In contrast, STMTDs obtained from thalamic neuron recordings were more varied and were dominated by depression dynamics (\( \approx 80\% \) of cells) lasting up to 3 seconds (Fig 4.10B, 4.17B,C and Fig 4.20B).

**Bursting vs. tonic spiking modes reveal similar STMTDs for both cortical and subcortical neurons**

The results above indicate that averaging GCaMP cortical motifs from all spikes of a single neuron produces converging STMs and STMTDs for both cortical and subcortically recorded single neurons. However, thalamocortical synapses are known to be prone to synaptic depression and burst pattern firing may yield altered cortical responses and STMs (Castro-Alamancos, 1997; Gil et al., 1997). It is thus conceivable that averaging all of single neuron’s spikes over a period of several minutes (or longer) could mask spatial heterogeneity observed when averaging over (functionally or otherwise defined) sub-groups of spikes. The question is thus whether averaging over all of a neuron’s STMs is a correct (or representative method) of identifying a single neuron’s contribution to (or correlation with) ongoing cortical activity (see also reviewer’s comments in Xiao et al., 2017).

Therefore, additional analyses were performed where spike triggered STMs and STMTDs were computed using only sub-groups of spikes either representing spiking modes (bursting vs tonic) or motif similarity in a high-dimensional space with the goal of determining whether during different types of active cortical dynamics single neurons contribute different types of STMs or temporal dynamics (Figs 4.11-4.15).

Arguably the most obvious method for separating spontaneous neuron spiking into groups is to partition them into bursting versus tonic modes (Figs 4.11-4.13; see Methods). The first methodology implemented was previously described for defining the main spiking modes of a single (thalamic) neuron (see Sherman and Guillery, 2006, Fig 6.5, pg 236). Briefly, the method requires computing the distribution of each spikes inter-spike-interval (ISI) between the previous (x-axis) and following (y-axis) spike (Fig 4.11). The resulting pre- and post-spike 2D distribution is then plotted using logarithmic scales. Naturally arising groups are then clustered as the main spiking modes of the neuron. In particular, spike groups occurring in approximately each quadrant of the plot indicate different spiking modes: first spikes in a burst (bottom right), burst spiking (bottom left), last spikes in a burst (top left), and tonic spikes (top right). The vast majority of cortical
Figure 29: Cortical and Subcortical Motifs and STMTDs (CM, DX). A. Top: right hemisphere barrel cortex neuron motif and STMTD in a left hemisphere barrel cortex ROI. The maximally activated pixel in the ROI (red arrow) is tracked over time and reveals dynamics which rise quickly at spike time $t=0$ and decays in $\approx 1$sec followed by a further 1-2sec cortical depression (red curve in right plot).
neurons recorded in barrel cortex did not exhibit multiple classes of spiking modes, however 2 examples are provided where some spike-mode clustering is present (Fig 4.11A,B). Despite the apparent spike-mode differences, the spike sub-groups representing different spiking modes yielded very similar motifs (Fig 4.11 A(ii),B(ii)) and similar STMTDs in the ROI of highest activation, i.e. left barrel cortex (Fig 4.11A(iii),B(iii)). Two additional examples of thalamic neurons are provided which show spiking modes (Fig 4.11C,D). However, much like cortical neurons the thalamic neurons identified had STMs and STMTDs that were largely stable across the clustered spiking modes.

The isi-based methodology for identifying spiking modes did not reveal clear spiking modes for the 3 cortical and 4 thalamic cells provided as examples (see Fig 4.10) nor for additional examples considered (see examples in Fig 4.11). An additional methodology was implemented for previously considered neurons (i.e. neurons in Fig 4.10): bursting mode initiation was identified as spiking that is preceded by at least 500ms of silence (Figs 4.12,4.13). Using this heuristic to define spiking modes, single-neuron motifs and STMTDs were computed across all-spikes vs heuristically defined bursting mode spikes. The results were similar to the findings above: both motifs and STMTDs are similar across all-spike and heuristically defined bursting modes for both both cortical and thalamic cells examined.

**STM-space grouping and decomposition reveals single neurons participate in stereotyped STMs that are present during spontaneous neural activity**

The next step was to determine whether single spike STMs (rather than STMs generated as an average of STMs over all of a neuron’s spikes) fell into distinct clusters or groups - possibly indicating underlying spiking modes or network changes that could not be captured by traditional spiking mode analysis. The method used was to compute the STM for *each spike*, convert it to a high dimensional vector and determine whether some spikes gave rise to GCaMP STMs that were similar to each other and could be clustered or grouped (see Methods). However, none of the single neurons considered showed evidence of naturally occuring clusters or groupings (not shown; but see Fig 4.14B for single spike motif distributions using PCA; see also Methods). This suggests that single neurons fire spikes during ongoing or ”spontaneous” mesoscale activity (i.e. activity not necessarily associated with our single neuron activity) and that single neuron spikes do not associate only with specific mesoscale activity patterns.
Figure 30: Firing modality-defined STMs and STMTDs (CM)
Figure 30: (continued from previous page) A(i), B(i): Cortical cell spiking modes determined by grouping the distribution of each spikes inter-spike-interval between previous (x-axis) and following (y-axis) spike. The four quadrants indicate different firing modes (see also main text and Methods). Cortical cells (barrel cortex) generally did not have clear spiking modes but two examples are provided where clusters were present and spikes were grouped accordingly. A(ii), B(ii): Six-second motifs generated using spikes from different spiking modes in part (i) are largely the same for cortical cells. A(iii), B(iii): STMTDs of left-hemisphere barrel cortex tracked across time for all spiking modes were largely similar. C, D: Same as in A, but for thalamic cells where bursting modes are more readily found.

Figure 31: Heuristically defined bursting reveals similar STMTDs for cortical neurons (CM). Left: Spatio-temporal motifs for the 3 cortical cells presented in Fig 4.10 considering contributions of all spikes from each cell (top motif) versus just the bursting condition for each cell (i.e. only spikes that are preceded by a >500ms silent period; see also Methods). Right: The time course of the peak signal in the left hemisphere barrel cortex (see Figs 4.10,4.11 also) for all spikes (blue) and first spikes in a burst (red curves). Both the motifs and time course curves are similar for both conditions.

As natural clusters are not present in STM-space, an alternative ”partitioning” approach was implemented to determine how sub-groups of similar-STMs compared to the overall average. The method essentially splits single spike STMs into groups of similar ongoing ”network” activity that are present during active single neuron spiking. The hypothesis was that even though single spike STMs are diverse and very different from the average neuron STM (i.e. average over all spikes), perhaps when grouped by similarity the average ”sub-group” STM would be similar to the average STM for all spikes and/or reveal further information about the types of ongoing activity that correlates with single neuron spiking. However, the results were similar to those above: subgroups
Figure 32: Heuristically defined bursting confirms similar STMTDs for subcortical neurons (CM). Left: Spatio-temporal motifs for the 3 of the 4 cortical cells presented in Figure 6 considering contributions of all spikes from each cell (top motif) vs. just the bursting condition for each cell (i.e. only spikes that are preceded by a >500ms silent period; bottom motif). Right: The time course of the peak signal in the left hemisphere barrel cortex (see Figs 4.11-4.12 also) for all spikes (blue) and first spikes in a burst (red curves). Both the motifs and time course curves are largely the same for both conditions. The bursting condition for the 4th cell in Fig 4.10 had an STM with a peak dF/F0 value of ≈0.25% and was excluded from comparison here.

of spikes gave rise to different STMs (Fig 14.4D) further supporting the hypothesis that single neuron spiking occurs during varying ongoing cortical activity. Given that STM sub-grouping revealed different patterns of calcium activity during single neuron spiking, the next step was to determine whether it is possible to remove the ongoing or "spontaneous" activity contributions from single spike STMs. The goal was to isolate the contribution (or "correlation") of single spikes to observed mesoscale GCaMP activity.

The first step was to replicate the grouping method above and generate several (e.g. 4) functional sub-network partitions from the STM distributions (Fig 4.14B). The resulting 4-partition STMs were substantially different from each other and the all-spike STM average (with the exception of STM resembling the all-spike average STM; Fig 4.14D; all spike average is the bottom STM). The inter-spike-interval (ISI) distributions during activation of these sub-networks was also similar across all partitions and had a poissonian distribution - further confirming that the similarity-based partitioning method captured similar patterns of spiking and did not selectively group bursting vs tonic periods of activity (Fig 4.14C). The sub-network partition STMs thus contained spiking contributions to ongoing cortical activity particular to each STM-space partition. The next step was to remove the spontaneous component (i.e. component not specifically related to spiking)
Figure 33: Single neurons participate in stereotyped network activity patterns during all spontaneous activity (CM). A: Five examples of STMs generated from 5 single spikes from a single neuron reveal substantial variability during spiking and high activation (peak dF/F0 > 5%). B: Distribution of all single spike STMs (3779 spikes for example neuron) from a single cortical cell visualized in 2-Dimensions using PCA does not reveal natural clusters and is partitioned using neighbouring distance (i.e. K-Means, n=4) into 4 sub-networks (coloured dots) each with a distinct centre (larger colour dots). C: Spike rasters for the 4 sub-networks reveal no natural spike-timing related clusters: spikes in each sub-network are distributed in time inter-spike-interval (ISI) distributions are similar for all 4 sub-networks (green, red, blue and magenta colours) and the all-spike condition (black colour). D: STMs generated from the 4 sub-networks reveal substantial differences in the sub-network dynamics (top 4 STMs) with the sum of all STMs providing the average STM pattern (bottom STM) (Note: partitioning the data randomly does not reveal these sub-networks but patterns similar to the all-spike STM). E: Same as (A) but STM examples are from all possible spontaneously occurring STM during the recording (9439 possible STMs in a ≈5.2 mins recording at 30Hz). F: Same as in (B) but STM distributions are for all spontaneous STMs with the centres of the sub-networks obtained from the single-cell STM sub-networks in (B). G: Same as in (C) but for all spontaneous STMs. The ISI histograms peak at ≈33ms (i.e. a single frame-interval) indicating that spontaneous STMs group naturally into sub-networks and are dominated by bursts of similar STMs each separated by single frames. H: Same as in (D) but for the 4 sub-networks generated from spontaneous STMs (Note: as expected the sum of all spontaneous STMs is ≈0.0% dF/F0, see bottom STM). I: Subtracting the nearest spontaneous sub-network STMs from spiking generated sub-network STMs reveals that single cell STM contribution is largely uniform despite spiking occurring during vastly different activated functional networks (see D).
by finding the most similar types of STMs time considering STMs from all spontaneous activity periods. Thus, the same partitioning method was used but this time spontaneous calcium activity was used to generate STMs: essentially converting every frame during into an STM (Fig 4.14.E-H). The distribution of spontaneous STMs also has substantial variability (Fig 4.14F). The spontaneous motifs were then projected to STM-space but were partitioned based on the spike-triggered STM sub-network centres derived previously (Fig 4.14B). This guaranteed that spontaneous activity STMs would be the closest (i.e. most similar) to our spike-triggered STM partitions and allowed for matching each spiking partition STM to an all-frame-partition STM. Because spontaneous STMs were computed based on single frames, the spontaneous activity motifs showed a strong ISI distribution peak at ≈33ms which is equivalent to the single inter-frame-interval of the imaging system (i.e. 30FPS; Fig 14.4G). This confirms that neighbouring frames were substantially more likely to be in the same region of STM-space and are thus grouped together. This is expected as transitions in GCaMP6 dynamics usually last several frames at a 30Hz sampling rate. Importantly, the sum of the 4 spontaneous STMs yield an STM with an approximately 0% dF/F0 value - which is the expected result when averaging all spontaneous motifs (or frames) during a recording (Fig 4.14H bottom STM). The last step was to remove the spontaneously activity STMs (Fig 4.14H) from the single cell spike-triggered sub-networks (Fig 4.14D). The resulting difference STMs are very similar to the average STM computed using all spikes.

These results suggest that single spike STMs contribute (or correlate with) a stereotyped pattern of mesoscale activity that can be recovered by removing very similar STM cortical patterns that occur during non-spiking periods. In other words, single neuron spikes correlate with (or participate in) activated functional networks that ride on top of other types of neuronal activity.

The STM partitioning method was next applied to two cortical and two thalamic neurons with similar results (Fig 4.15). Additionally, a 12 sub-group partition was carried out (Fig 4.15C) and much like in the 4-partition cases, subtracting spontaneous activity STMs resulted in difference STMs that were largely similar to the all-spike averages. While not investigated further here, it would be interesting to determine where along the continuum of partitions (i.e. from 1-partition (all spikes) to all possible partitions (single spike partitions) this methodology breaks down. Such an investigation could use correlation r-values between sub-partitioned STMs obtained after spontaneous STM removal and the total-spike STMs and characterize the r-value curves to determine whether they are linear, contain non-linearities or show plateau or asymptotic behaviour.

In sum, using various methods for dividing single neuron spikes and STMs it was shown that all-spike STMs are either preserved or can be recovered from the data. This supports the averaging based STM methodology used here as representative of a single neuron’s contribution to ongoing cortical activity. The findings further suggest that: (i) cells fire during many different ongoing cortical states; and (ii) that despite the high degree of variability single cell spikes appear to correlate with (or possible contribute to) similar overall dorsal cortex activity patterns (as observed in STMs).
Figure 34: Cortical and subcortical examples of STM-space grouping and decomposition (CM). A. Two examples of single cortical neuron STMs recovered using the partitioned sub-network approach: i) 4 spike-triggered sub-network STMs; ii) spontaneous sub-network STMs; iii) difference between cell-triggered and spontaneous motifs reveal single cell contributions to active sub-networks. B. Same as (A) but for two thalamic neurons. C. Same as in (A) but partitioning data into 12 sub-networks also reveals that average STMs are largely recoverable from active sub-networks.
Figure 35: Example of STM vs. Variance STM (CM). A. Spatiotemporal motif of spike triggered average map of a cortical neuron showing activation near t=0 and following for approximately 300ms. B. Spatiotemporal dynamic of spike triggered variance map of the same neuron showing variability increases post spiking (i.e. t=0) but at partially different ROIs including the ≈ RS region (medial-posterior) not present in STMs. Note that the amplitude of the variance is small (dF/F₀ from -0.2% to 0.2%).
An additional test was performed comparing spike-triggered [Ca] motifs against spike-triggered [Ca] variance maps. Because of the high variability across single-spike STMs (and the corresponding 180-frame motifs) it was important to consider how variance changes over time (Fig 4.16; see also Methods). While spike triggered activity maps were of interest because they showed spike-time locked responses and revealed distinct spatial patterns, variance maps do not have the temporal specificity of STMs. An example is provided where an STM is compared to the variance map where both the spatial and temporal differences are clear (Fig 4.16). The variance map does have spatial specificity that is present throughout the recording period but increases following spiking. Interestingly, the RS region (medial-posterior association cortex) shows increased variance across all times suggesting that this association area is highly activated and depressed during single neuron spiking - but that such variance is averaged out in STMs (likely due to the methodology). It should be noted, however, that the variance map dynamics are much smaller ($dF/F_0 \approx 0.2\%$) when compared to the STM range ($dF/F_0 \approx 4.4\%$).

**STMTD clustering suggests novel single-neuron physiological properties**

The next step was to investigate whether cortico-cortical and thalamocortical temporal dynamics revealed by the functional mapping approach could be further characterized by considering all animals and recordings (Fig 4.17). Because single neuron imaging motifs (i.e. 6 second, 180 frame stacks, 256 x 256 pixels) contained time-varying activation in multiple regions, the approach taken was to track cortical dynamics in only one specific ROI: contralateral (i.e. left) barrel cortex (L-BCS1; Fig 4.17A; Note: alternative ROI methods were explored but were found to be too noisy-not shown). L-BCS1 was chosen as the homotopic region corresponding to the electrode insertion site (i.e. the electrode was always inserted into right hemisphere barrel cortex and right hemisphere sensory thalamus) and L-BCS1 was activated for most STMs. For this analysis data across all animals was combined thus covering a variety of individual track penetrations. Rather than choosing a specific (i.e. absolute) pixel location for analysis across all data, the location of the maximally activated (or depressed) cortical pixel was selected for each neuron and its activity was tracked over time. This method was viewed as a more neutral means of comparing dynamics across all neurons which could account for variations in recordings: including variations in probe insertion angle, animal variability, unit yield and variable probe damage (Fig 4.17A - red dots).

The method employed to track dynamics at a specific ROI pixel was identical to the STMTD approach described above. After generating all STMTDs, each STMTD was converted to a high-dimensional vector (i.e. 180-Dimensions for a -3sec to +3sec period, i.e. 180 frame stack). The distribution of all 428 STMTDs from both cortical and subcortical cells was then plotted together and visualized using PCA (Fig 4.17B; see also Methods). The time courses fell into 3 broad patterns and were clustered using a K-means algorithm (with n=3). Based on their temporal relationship to spikes, the three patterns had specific characteristics: pattern #1: spike-triggered-excitation; pattern #2 spike-triggered inhibition; pattern #3 inhibition triggered spiking followed by inhibition.
The distribution of these 3 patterns was not evenly divided in cortical and thalamic neuron populations (Fig 4.17(ii)). Specifically, ≈80% of cortical neurons were associated with a purely cortical excitation profile (pattern #1) whereas 20% correlated with spiking-triggered inhibition (pattern #2). In contrast, only 20% of thalamic neurons associated with post-spiking cortical excitation (pattern #1), while 80% of were associated with cortical inhibition patterns (45% with pattern #2 and 35% with pattern #3). Notably, the purely inhibitory pattern (i.e. pattern #3) was only identified for subcortical neurons. Neither cortical cell depth, subcortical cell location (e.g. VPM vs VPL), nor cell-type classification (inhibitory and excitatory types; Connors and Gutnick, 1990; Nowak et al., 2005)) revealed any significant correlations between the pattern clusters and cell-classification (not shown).

These findings using STMTD clustering suggest that single neurons have unique - likely discrete - properties that relate their spiking to mesoscale activation patterns. As such, STMTD classification might be a novel intrinsic single-neuron physiological property and that such properties are distributed across all neurons in cortex (and subcortical regions). Future work with significantly larger datasets (from multiple cortical and subcortical areas) could further expand these findings and determine whether additional classes of temporal dynamics are present in cortical cells or whether a continuum of spatio-temporal relationships is involved.

**STM hemodynamic corrections are small compared to SNR of calcium activity**

Because increases in blood volume are expected to decrease both excitation and emission light, it has been previously suggested that the relative contribution of hemodynamic activity vs neuronal activity needs to be accounted for (Wekselblatt et al., 2016; Ma et al., 2016). Here additional experiments were done using a multi-wavelength approach similar to corrections performed in recent studies (see Methods). Briefly, previous approaches implemented a strobed light presentation using alternating blue and green lights to capture GCaMP epi-fluorescence (blue illumination) and green reflectance (dim green illumination) in alternating frames. The approach here was similar but methodologically simpler to implement: short blue reflected light was simultaneously monitored (with green epi-fluorescence) as a reference signal for fluorescence changes due to hemodynamics. This method was implemented as there was some concern the strobed light approach looses some time resolution as well as requiring camera timing signal programming which are not trivial to implement for all acquisition systems. Additionally, flashing (strobing) lights could potentially stimulate the visual system of mice (although blocking the excitation/reflectance lights from reaching eyes while also preserving access to the eyes for visual stimulation is a potential solution). Accordingly, the method developed here (Fig 4.18A,B) relies on using a color RGB camera (Picam) (Murphy et al., 2016) which allows for simultaneous acquisition of a short blue light reflected signal (447 nm LED and 438/24 nm filter near an isosbestic point for hemoglobin), and a green epi-fluorescence signal (GCaMP).

In a control experiment it was shown that the short blue reflected light signal (i.e. 438 nm
Figure 36: STMTD clustering suggests STMTD dynamics represent discrete single neuron physiological properties (CM). A. Example of two STMTDs from pixels within L-BCS1 (left barrel cortex) from a single mouse recording (both cortical and subcortical neurons STMTD pixel locations shown). Each recorded neuron STMTD has a slightly different maximum pixel amplitude location, but all fall within the L-BCS1 region. B. STMTD PCA distribution from all 428 cortical and subcortical neurons recorded from all mice separated using KMEANS (n = 3). C (i). STMTD (±SD) classifications from (B) with the number of neurons from cortex and thalamus used for the average are presented in title. (ii) Distribution of STMTD classification between cortical (clear) and subcortical (hashed) neuron generated STMTDs.
reflected light) was strongly correlated with 532 nm green reflected light \((r = 0.93; \text{Fig 4.18(D)})\). For the examples provided (Fig 4.18C-G) the short blue reference signal correlated significantly with apparent blood volume artifacts revealed by parallel experiments using green reflected light imaging. Consequently, the ratio of green over blue signal greatly reduced the blood volume hemodynamic response. To determine whether the short blue correction strategy was effective, data from GFP-m mice was examined where green fluorescence signals are not expected to be the calcium dependent as in GCaMP6 mice (Fig 4.18E). The ratio of \(dF/F_0\) green signal to blue reflected light signal \(dF/F_0\) could then be used to reduce nonspecific signals observed in GFP mice. Consistent with previous work (Ma et al., 2016; Wekselblatt et al., 2016) blood volume artifacts were greatly reduced using this strategy. This approach was then applied to GCaMP fluorescence data. Although the approach was effective at removing smaller non-specific signals in GFP mice, GCaMP6 mice have a much larger activity-dependent signal and only a relatively small apparent contribution of blood volume to cortical and subcortical STMs was observed consistent with previous work (Vanni and Murphy, 2014; Murphy et al., 2016; Silasi et al., 2016) (Fig 4.18F,G). Furthermore thalamic STMs and STMTDs still indicated cases where thalamic spiking was associated with cortical inhibition. These comparisons were all performed after signals were converted a \(dF/F_0\) value and using a green/blue weight of 1 (Fig 4.18C). Weights >1.0 yielded some aspects of the kinetics that were over-corrected. This additional correction dampens non-specific fluctuations associated with blood volume changes which were aggravated in the awake state. Overall, the approach supports a short blue reflected light approach: shot blue light signals can be used as a surrogate for a green reflected signal without the need for alternating cycles of light. Thus the short blue reflected light signal is an equivalent strategy similar to previous approaches (Sirotin and Das, 2010; Ma et al., 2016; Wekselblatt et al., 2016).

After implementing the corrective strategy to additional RGB Picam recordings, only minor changes were observed in spike-triggered map activity consistent with control investigations of functional connectivity or task-related connectivity in GFP animals in experiments done previously (Vanni and Murphy, 2014; Murphy et al., 2016; Silasi et al., 2016). While the multi-wavelength correction strategy yielded some changes to the STMs, the corrections only altered cortical or thalamic maps or dynamics peaks by less than 10% \(dF/F_0\). Furthermore, notable features, such as some areas showing apparent cortical inhibition (reductions in calcium activity) were preserved in the corrected maps. Given that the correction does not have a large impact and failed to change the appearance of the maps, the original figures were left intact and not modified further (Note: originally acquired data also cannot be corrected offline as it was captured using a single wavelength).

**STMs are not affected by (minor) body movements**

The primary goal of this study was to assess cortical functional connectivity based on coincidence between individual neuron spiking and ongoing spontaneous activity in awake and anesthetized
Figure 37: STM hemodynamic corrections are small compared to SNR of calcium activity (MV)
mice. However, in awake (head-fixed) mice neuronal activity is rarely entirely spontaneous and periods of volitional movement are interspersed within largely quiescent but longer intervals. Observations indicated that limb twitches as well as tail and facial movements were present in the recordings. In order to evaluate the impact of body movement, behavioural video recording was used to track movement. STMs were then generated from spikes during periods of quiescence and compared with STMs from all spikes (4.19). Periods of quiescence and movement were identified by measuring behaviour collected simultaneously with neurophysiological data (see Methods, Fig 4.19A). STMs generated from periods of quiescence did not differ from those generated using all spike (see Methods, Fig 4.19B). This suggests that movement either minimally contributes to STMs or contributes diverse patterns which are generally averaged out by the methodology. The analysis also indicated that periods of high movement were relatively rare in awake head-fixed mice under the conditions employed here and contributed negligibly to overall maps. Therefore, brain imaging activity obtained in awake states is mostly indicative of a quiescent, awake state and is not primarily movement-related activity.

**STMTD diversity is substantially greater in subcortical than cortical neurons**

Lastly, all neuron motifs across all recordings were compared to assess large trends across the two types of data. Because of the large number of neurons across all recordings it is not possible to plot all motif or STM profiles side-by-side for such a comparison (i.e. the motifs would be small and
Figure 38: Body movement contributes minimally to STMs (MV). A. Image insets: pictures of the frame average and standard deviation showing the location of movement over one entire recording. Yellow box: region of interest used to quantify movement. Graph: Black curve is movement density calculated for each frame by measuring the average of the absolute gradient within the region of interest (yellow box). Standard deviation (std) and median of the profile were calculated and period of quietness and movement are identified by selecting periods of time below \([\text{median+std/10}]\) (green) and above \([\text{median+std}]\) (red) respectively. B. Motifs generated for all spikes (top, black curve) and spikes only from quiescent periods (bottom, green curve). C. Maximum and positive peaks amplitude for motifs from all and quiescence periods showing no overall change trends (paired t-test: \(p=0.108\) and 0.431 respectively, \(n=31\)).
illegible). Accordingly, a method was devised to convert the 180-frame, 256x256 pixel image stacks for each neuron to several 2-Dimensional (time vs. amplitude) STMTDs which capture the most activated (and easy to identify) ROIs for comparison across all data (Fig 4.20). Thus, STMTDs for 8 major ROIs across all mice and recordings were plotted by aligning the peak activation (red) or peak depression (blue) in various ROIs (Fig 4.20). This allows for a comparison of temporal relationships for all data that is initially 3-Dimensional using only 2 dimensions. As observed for individual neuron STMTDs, cortical neurons STMTDs generally peaked in excitation in barrel and motor cortex (BCS1 and M1) and were followed by a return to baseline or depression in barrel cortex (Figure 4.20A). In contrast sub-cortical neurons were linked to diverse cortical activity profiles, in particular longer depression across multiple cortical ROIs (Figure 4.20B).

Discussion

Using wide-field spontaneous calcium imaging data, mesoscopic cortical maps defined by the spiking activity of individual cortical and sub-cortical neurons were characterized. The results demonstrate that STMs reveal functional cortical architecture related to the activity of individual cortical and subcortical neurons. STMs for cortically recorded neurons reflect the cortical state in mono-synaptically connected areas during spiking activity. STMs of sub-cortical neurons have high variation than maps attributed to spiking cortical neurons. For example, sub-cortical STM patterns for neighboring neurons were more diverse than those of neighbouring cortical neurons, and were less likely to match intra-cortical consensus activity patterns defined using seed pixel correlation mapping. Sub-cortical-neuron derived STMs revealed multiple areas of activation and multimodal kinetic behavior, while intra-cortical spiking neuron networks were simpler in structure and kinetics. Furthermore, spiking sub-cortical neurons reflected diverse cortical multi-phasic excitation-inhibition timing patterns that were reflected in dynamic STMTDs. In contrast, most spiking cortical neurons were linked to a single phase of cortical excitation.

Event triggered mesoscale mapping

Previously, spike-triggered averaging of local field potentials has been used to investigate of how single neurons in visual cortex were linked to on-going state-dependent activity (Nauhaus et al., 2009), however, this work only examined such correlations locally (i.e. within visual cortex) and did not assess regional connectivity using imaging or investigate differences with individual sub-cortical neurons. Other similar applications where single neuron spiking was recorded and related to spontaneous activity using calcium imaging have been restricted to in vitro brain slices (Aaron and Yuste, 2006)). The study presented here extends previous in vivo work that assessed spike-triggered mapping using voltage sensitive dye imaging (Arieli et al., 1995; Tsodyks et al., 1999) to encompass a larger spatial scale, higher density electrode arrays, awake recordings, and selective genetically encoded indicators of activity. While being important seminal findings (Arieli et al., 1995; Tsodyks
Figure 39: ROI-specific dynamics reveal cortical stereotypy and subcortical diversity (CM). A. Top: Normalized STMTDs (as in Fig 4.17) from multiple ROIs (HLS1, FLS1, BCS1, RS, V1, M1, PTA and ACC, see Table 1) for 255 cortical cells. Each horizontal line represents a single neuron’s STMTD in each of the eight ROIs normalized to the overall maximum or minimum activation. Bottom: average (± std) of STMTD within each ROI for all neurons. B. Same as A, but for all thalamic neuron generated STMTDs reveals thalamic STMTDs are more diverse, less temporally precise, and contain longer depression epochs. (Note: these results are from awake mice).

et al., 1999), previous spike-triggered averaging work was largely confined to the visual system, performed under anesthesia, and was unable to define how multiple brain areas interact. The approach here is most analogous to event-triggered MRI imaging from the standpoint of larger spatial scale (Logothetis et al., 2012) where it was observed that during hippocampal ripple states that cortex exhibited net positive bold responses and thalamus net negative BOLD responses. This anti-correlation is consistent with observations of thalamic spiking activity in the current study corresponding with cortical temporal dynamics exhibiting slow depression of calcium signals and may point to a larger coordinated network involving other brain structures.

The current study has advantages over MRI signals which lack temporal resolution and can be
more difficult to relate to neuronal activity than GCaMP signals that are isolated within excitatory neurons of GCaMP6f mice using specific promoters (Chen et al., 2013b; Madisen et al., 2015). Unique to the approach presented here is the ability to assess the functional connectivity and temporal dynamics between specific sub-cortical neurons and areas of cortex not predicted by previous knowledge such as linkages between thalamic neurons and cortical state as defined by GCaMP signal dynamics. The approach provided here can be further refined when more selective cre-dependent CGaMP6f transgenic mice are available to allow for the expression of calcium indicators in particular neuron types (Madisen et al., 2015). Furthermore, 2-photon microscopy could be used to provide information about behavior of individual cells within the context of larger maps (Chen et al., 2013a; Guo et al., 2014; Okun et al., 2015). Because of the high sensitivity of the indicator and the possibility of measuring the activity of dozens of single-units using multiple electrode channels simultaneously, a large number of functional connections can be mapped in only a few minutes of recording. Although only a single electrode shank was used here, future experiments could further increase the yield using higher density electrodes and multiple shanks to collect spikes from more neurons simultaneously.

Cortical and sub-cortical neuron derived maps reflect different functional roles

The results indicate that neocortex contains discrete subdivisions where individual spiking cortical neurons generally belong to spatial-temporal maps that follow a consensus function that can be defined using correlation as in previous work (Mohajerani et al., 2013; Chan et al., 2015). In contrast, single thalamic neurons tend to fire when cortex is in more kinetically-diverse states. The more diverse dynamics between thalamic neurons and cortical mesoscopic networks indicate that sub-cortical thalamic neurons play an instructive role with respect to cortical state, particularly with respect to feed-forward cortical inhibition (Stroh et al., 2013; Urbain et al., 2015), whereas cortical neurons may serve as relay endpoints or amplifiers (Douglas et al., 1995). A better understanding of these dynamics may yield insight into how disorders, such as epilepsy, and dementia, emerge when interactions between brain areas are disrupted (Paz et al., 2013; Busche et al., 2015; McCormick et al., 2015). The diversity in sub-cortical spiking derived maps may also reflect differing receptive field properties in thalamus and cortex based on varying types of functional convergence described previously (Miller et al., 2001). Indeed, in the somatosensory whisker barrel system, evidence for "ensemble convergence" has been described where input from the thalamus can extend outside of the boundaries of the corresponding cortical receptive field (Simons and Carvell, 1989; Linden and Schreiner, 2003). The larger diversity of maps derived from the spiking of different thalamic neurons may be expected because of the smaller size of thalamic nuclei compared to the cortex and the recording of thalamic neurons from more varied structures. Another potential source of variance may arise from the diversity of thalamocortical impulses that can be comprised of patterns of activity ranging from tonic, 'relay' transmission consisting of high regular rates of firing to burst-like activity where firing rates are low and interspersed with high-frequency events (Steriade
and Llinás, 1988; McCormick and Feeser, 1990; Sherman and Guillery, 1996). Thalamic bursting can powerfully activate neocortical circuits and has been suggested to serve a "wake-up" signal to sensory cortices (Sherman and Guillery, 1996; Swadlow and Gusev, 2001). While segregating recordings into various firing configurations did not reveal substantially different STMs or STMTDs, it may be that other properties (not accessible here) such as specialized synapses may account for map diversity. Interpreting these results is also caveatted by the mesoscale resolution and calcium dynamics present in the recorded data.

**Applications of spike-triggered mapping**

Mapping the functional connectivity of spiking neurons is important for understanding brain function and finding therapeutic targets for brain stimulation or brain machine interfaces. Identification of networks linked to individual neurons may help reveal the mechanism of brain machine interfaces where key signals are often attributed to only a small number of neurons (Stanley et al., 1999; Serruya et al., 2020; Taylor et al., 2002; Guggenmos et al., 2013). Other applications include understanding of how small groups of epileptic neurons (Paz et al., 2013) are coupled to brain networks leading to seizure propagation. Given that reciprocal connections between mesoscale structures are widespread, the cortical maps associated with a spiking neuron in a sub-cortical structure such as the sub-thalamic nucleus may provide clues as to how cortical activity can be manipulated to affect a sub-cortical target. This hypothesis can be tested by recording sub-cortically using electrode arrays while stimulating regions of cortex that show coincident STMs using Channelrhodopsin-2 or other opsin-activity sensor pairs (Lim et al., 2012; Rickgauer et al., 2014; Zou et al., 2014; Abdelfattah et al., 2016; Kim et al., 2016).

**Extension to behaviorally driven activity**

The same approach applied here could be extended to generating STMs during specific behaviors. However, major shifts in area map boundaries as defined during spontaneous activity are not expected, as these are largely determined by anatomy (Mohajerani et al., 2013; O’Connor et al., 2013; Oh et al., 2014; Zingg et al., 2014) and in the case of sub-cortical neuron maps (HPF for example) poly-synaptic connections. During behavior more nuanced changes in the weighting, timing, and frequency-dependence of STM networks might be present during an active task. It is also possible that specific behaviors will reveal the superposition of multiple cortical motifs associated with progression through a task. STM mapping of cortex would be particularly interesting in the context of rhythmic whisking-related centers within the medulla and thalamus and their linkage to cortical maps within barrel-motor areas (Moore et al., 2013; Deschnes et al., 2016; Sreenivasan and Petersen, 2016).
Summary
In sum, single neuron spiking activity reliably reflects mesoscale activity transitions within mouse cortex. STMs together with connectomic information (Hunnicutt et al., 2014; Oh et al., 2014; Zingg et al., 2014; DeNardo et al., 2015) may help bridge the gap between single neuron function and larger networks. The findings presented herein reveal that thalamic neurons interact with cortex during specific state transitions that are reflected by typical consensus cortical neuron behavior. The presence of such long-range relationships in spontaneous activity may suggest new opportunities and routes by which brain stimulation and inhibition can be applied to affect synaptically connected areas.

GCaMP6 mapping of spontaneous activity of auditory and visual cortex neurons

Background
The findings provided above (also published as Xiao et al., 2017) related primarily to barrel cortex and thalamic neurons. There were a number of limitations in that study including that the cortical neurons recorded come solely from barrel cortex and the use of low density electrodes (single column, 16 channel 100µm vertical spacing electrodes) limited the spatial analysis and spike sorting quality. In this section additional data is presented to (briefly) investigate whether the STM methods implemented in the first section can be applied to auditory and visual cortex neurons. The data also comes from higher-SNR GCaMP6s mice recordings and was obtained using high-density extracellular probes in visual and auditory cortex. Part of the work in this section was presented previously (Mitelut et al., 2016). All experiments and analysis in this section were carried out by the author.

Results
Using methodology described above (and in Xiao et al., 2017) cortical neuron STMs were computed for recordings from the visual and auditory cortices of 2 GCaMP6s mice (Fig 4.21-23). The data was acquired using a Raspberry Pi 3 camera with custom Python code used for tracking single frame times for offline alignment with the imaging system (see Appendix ). The findings confirm that all cortical neurons (with >25 spikes in a recording period) can generate high-SNR STMs. In particular, stable STMs (i.e. qualitatively discrete maps, many of which were similar to consensus map) were obtained using as little as 25 spikes to as much as several thousand. These findings (discussed in detail below) confirm that cortical neurons excluded in the previous section (due to low SNR) were likely due to experimental setup challenges and not cortical physiology.

Auditory cortex neuron motifs showed diversity across depth and almost all contained pre-spiking inhibition in medial-posterior areas prior to spiking (Fig 4.21-left). This depression com-
Figure 40: Single neuron STMs - auditory and visual cortex.
Figure 40: (continued from previous page) Left: single auditory cortex neuron motifs plotted by depth starting with most superficial neurons (top) and normalized to dF/F0 peak (left values) reveal \( \approx 500\text{ms} \) cortical depression precedes most spiking. The auditory cortex motifs reveal the involvement of a medial area being co-activated with auditory cortex areas (lateral-posterior activated areas. Right: single visual cortex motifs plotted by depth reveal substantially stereotyped STM patterns across all layers of cortex.

Figure 41: Auditory cortex neurons - firing rates and STM-space distributions. A. Firing rate distributions for recorded auditory cortex neurons. B. Peak dF/F0 for auditory cortex neurons. C. STMTD-space distributions (shown using PCA) for all auditory cortex neurons (viridis colour scheme) against controls using time-scrambled (red) and the origin (i.e. 0-vector; black) show diversity of activation dynamics.

ponent is larger than those observed in barrel cortex where almost no pre-spiking inhibition was observed for cortical neurons. This longer pre-spiking depression suggests auditory cortex neurons may form an additional STMTD class of their own - possibly suggesting that neurons in different cortical areas may have different temporal relationships with dorsal cortex. Interestingly, the medial cortex area where pre-spiking depression occurs is also co-activated with the auditory cortex area following spiking. This suggests that auditory cortex neurons may have mono-synaptic corrections with medial cortex neurons - a functional relationship not fully characterized previously (to the author’s knowledge).

In contrast, STMs for visual cortex neurons were very similar across all layers. Assuming that
the recordings were correctly acquired and were not dominated by aberrant activity in Cre-dependent GCaMP6s mice (Steinmetz et al., 2017), these findings suggest that visual cortex neurons form part of functional networks that - at the mesoscale - appear to engage the entirety of visual cortex during excitation and depression. However, it must be noted that both of the recordings (i.e. auditory and visual) reported here come from urethane anesthetized mice where large numbers of UP-state transitions underlie most spiking activity. Accordingly, it is expected that single neurons during such synchronized cortical states (see Chapter 5 for discussion of such states) spike simultaneously with large parts of cortex.

Firing rate and dF/F0 distributions did not reveal depth dependent trends in the auditory cortex neurons (Fig 4.22A,B). And distributions of STMTDs for each neuron motif (see Fig 4.17) against randomized STMTD traces qualitatively confirmed the variability observed in the auditory cortex motifs. Visual cortex neuron firing rates and dF/F0 distributions were somewhat higher indicating a potential issue with the cortical recording in that mouse (i.e. visual cortex neurons should fire more sparsely than other areas). The STMTD distributions were strikingly narrow reflecting the qualitative similarity observed in the STMs (Fig 4.21-right).

While no clear clustering was observed in the distributions of the STMTDs, this was expected as the neurons were recorded in cortex during anesthetized states (i.e. barrel cortex neuron STMTDs fell two classes with one class constituting \(\approx 80\%\) of the total dynamics).

Summary

In sum, the single-neuron spike-triggered cortical motifs of auditory and visual cortex neurons confirm that the STM methodology applies to all cortical recorded neurons (with \(>25\) spikes). The better temporal resolution of the imaging system (i.e. software) used here coupled with the much higher-density extracellular probes validate the general applicability of the spike-triggered-mapping procedure discussed in the previous chapter. Interestingly, the variability in GCaMP6s auditory cortex motifs across depth not observed in visual cortex motifs suggests future avenues of investigating mesoscale maps and differences across areas of cortex.

VSD mapping of spontaneous activity of auditory and visual cortex neurons

Background

The findings provided in the two previous sections (also published Xiao et al., 2017, Mitelut et al., 2016) were obtained from recordings in GCaMP6 mice. The limitations in GCaMP6 imaging are discussed above and include the relatively slow rise (\(\approx 100\text{ms}\)) and decay time (\(\approx 100-200\text{ms};\) Chen et al., 2013b) of calcium dynamics. Additionally, intracellular \([\text{Ca}]\) is a partially biased reporter of spiking activity: \([\text{Ca}]\) increases exponentially during bursting as opposed to tonic spiking activity.
In this section additional results are provided from spike-triggered mapping explored in VSD preparations. VSDs that report membrane depolarizations have been used for over 40 years (Cohen et al., 1974) and in contrast to intracellular calcium sensors such as GCaMP6, membrane potential reporters such as VSDs are ideal for reporting the state of a neuron as they have fast responses and can report sub-threshold activity (Peterka et al., 2010). However, most VSDs currently in use (and the one used herein) suffer from low-SNR issues: the dF/F0 signal generally peaks around 0.5% which is low when compared to GCaMP6 signals that can reach up to 50% dF/F0 values. However, averaging over many events can improve SNR substantially.

The data presented in this section comes from recordings used for analysis in other chapters (see Chapter 5) and is presented as a brief - but complementary - section that shows the power of spike-triggered mapping at the mesoscale can overcome the lower SNR of VSDs. Additionally, high-density probes are used and it is shown that large-dorsal cortex craniotomies can yield meaningful VSD STMs (i.e. such crainotomies are not required for GCaMP6 recordings that were carried out through the intact skull).

The experiments and analysis in this section were carried out by the author.
Figure 43: Single neuron VSD STMs - visual cortex. Visual cortex VSD motifs aligned by depth (top: more superficially recorded neurons) reveal variations in spatial patterns and temporal activation times with strong co-activation of medial cortex often dominating the mesoscale motif.
Results

Using the spike-triggered-averaging method described above, VSD imaging (150FPS, i.e. 6.7ms resolution) motifs were computed for single neurons recorded in the visual and auditory cortex of 2 mice. Additionally, motifs from mouse subcortical neurons (unspecified area) are reported. All neurons recorded in visual cortex which had >100 spikes during a recording period generated motifs with temporal and spatial specificity (Fig 4.24-26; partial selection of neurons). The visual cortex neuron motifs showed activation from ≈-100ms to +100ms following spiking. Overall, neurons recorded across all layers showed medial co-activation with some visual cortex specific activation (with left hemisphere activation being stronger than right hemisphere likely due to electrode damage to the right hemisphere).

The VSD activation patterns in visual cortex show substantial variation when compared to the GCaMP6 patterns (Fig 4.21-right) which were stereotyped in time and spatial dynamics (Fig 4.24). In addition, the vast majority of visual cortex neurons show substantial co-activation of medial (anterior and posterior) cortex during visual cortex spiking. Because VSDs report sub-threshold activity, VSD imaging provides additional details about the preferred depolarization profile of single visual cortex neurons during large meso-scale depolarization events (e.g. UP-state transitions) that occur during anesthetized and/or synchronized state recordings explored here. However, the diversity present suggests that large-scale investigations of single-neuron VSD maps in visual (and other cortical areas) may reveal a much wider range of preferred spatio-temporal mesoscale activity patterns (i.e. motifs) than those observed in GCaMP6 mice. Such patterns may constitute a comprehensive representation-space of mono-synaptic connectivity between single neurons and dorsal cortex. VSD activation patterns in mouse auditory cortex (single hemisphere preparation; see also Methods) also indicate differences across cortical depth. Auditory cortex VSD STMs also confirm the involvement of a medial cortical area during spiking that was observed in GCaMP6 STMs (4.21-left). This co-activated medial area also seems to often lead the auditory area activation suggesting a possible causal relationship.

In contrast to cortically recorded neurons - but consistent with GCaMP6 findings in thalamic neurons - subcortical neurons (likely recorded from auditory thalamus, i.e. medial geniculate nucleus and surrounding nuclei) had a wider range of motifs (Fig 4.26). There were several striking patterns not observed in GCaMP6 motifs including: peak motif activation that was offset from t=0 by up to 30-60ms; motifs that had anterior-posterior oscillation cycles on top of which auditory cortex-like motifs occured; and even anti-motifs where medial depression occurs during auditory cortex activation. There are also some purely depressive motifs which were also observed in thalamic neuron GCaMP recordings (Fig 4.17C). Two example VSD motifs were expanded to full-temporal resolution (150FPS, 6.7ms per frame; Fig 4.27 and 4.28).
Figure 44: Single Neuron STMs - Auditory Cortex Neurons. Auditory cortex VSD motifs aligned by depth (top: more superficially recorded neurons) reveal similar spatial patterns but different temporal profiles.

Summary

In addition to GCaMP6, VSD optical mapping of single neuron spiking activity is a viable method for investigating functional connectivity across spatio-temporal scales. The VSD single neuron motifs reveal additional insight and constitute a complementary method for investigating cortical function.
Figure 45: Single Neuron STMs - Subcortical Neurons. Subcortically recorded neurons arranged by order of depth show substantial diversity.
Figure 46: Example (#1) subcortical neuron VSD STM - complete motif. Example of single subcortical neuron STM computed at full acquisition resolution (i.e. 150FPS, 6.7ms per frame) shown from -1sec to +1sec following spiking reveals large-scale cortical depression with no clear cortical activation patterns centred on spike time, i.e. t=0ms.

Discussion

This chapter explores single-neuron triggered meso-scale calcium and VSD activity patterns. The findings of spike-triggered-mapping are discussed at length above (see Discussion in first section).

Future work should focus on a number of improvements in both experimental acquisition and analysis. First, cortical states were not fully explored in these datasets and a greater effort should be made to compute maps as a function of synchrony index (see Saleem et al., 2010; see also Chapter 5). Higher spatial resolution could be implemented using two-photon microscopy to compute spike-triggered-maps and higher signal-to-noise ratio VSDs could reveal substantially more interesting
Figure 47: Example (#2) subcortical neuron VSD STM - complete motif. Example of single subcortical neuron STM computed at full acquisition resolution shows spiking correlates with the onset of a posterior depression pattern which evolves into a partial auditory-cortex neuron-like motif before returning to the anterior-posterior depression/activation shape.

dynamics present at the mesoscale. Lastly, blood volume corrections (e.g. Ma et al., 2016) need to be implemented in a more formal manner to address potential confounding results.
Multiple Classes of UP-state Transitions and Non-Stationary Single Neuron Firing Order Revealed by Local-Field-Potential Event Clustering During Slow Oscillations in Mouse and Cat Cortex

“Most of the information [in cortex] turns out to be encoded by the firing rates of the neurons, that is by the number of spikes in a short time window.”
Rolls and Treves, 2011

“When rephrased in a more meaningful way, the rate-based view appears as an ad hoc methodological postulate, one that is practical but with virtually no empirical or theoretical support.”
Brette, 2015

“... transient, sequentially organized packets of activity could constitute a basic building block of the cortical code. ...cortical activity is composed of coherent and structured packets of population activity lasting a few hundred milliseconds. ... the fine temporal structure of packets is largely conserved across spontaneous and stimulus-evoked conditions, and across different brain states, and ... variations on a common sequential structure can encode information about sensory stimuli.”
Luczak et al., 2015

Understanding how spiking cortical neurons represent information is one of the most central questions in all of neuroscience. The rate coding, also known as the "firing rate", doctrine has dominated theoretical and experimental work since the early part of the 20th century and suggests that neurons represent information through their firing rates (Adrian, 1926). Yet an increasing number of both theoretical and experimental studies over the past two decades support spike timing based codes (or the relative coordination of spikes) as important to information processing especially during stimulus presentation (Abeles, 1991; Izhikevich, 2005; Singer, 1999; Thorpe et al., 2001; Deneve, 2008). This recent work has prompted some to argue that firing rates have a
merely correlational role in information processing and only spike timing neural codes can be causal (Brette, 2015).

One particular line of studies that supports the importance of spiking timing has hypothesized that the cortical circuits and neuronal mechanisms engaged by slow oscillations (Steriade et al., 1993a,c; Steriade and Amzica, 1998) occurring during slow-wave sleep (SWS), quiet awake and anesthetized states in mammalian cortex - could be the unifying paradigm for the study of cortical function (Sanchez-Vives et al., 2017, see also Neske, 2016). This hypothesis is supported by several recent studies from Arthur Luczak (e.g. Luczak et al., 2007, 2009; Luczak and Bartho, 2012; Luczak et al., 2013, 2015) discussed at length below, but was initially articulated almost two decades ago when it was suggested that ”sensory stimuli trigger K-complexes by addressing the same cortical machinery that produces spontaneous K-complexes during the slow (<1 Hz) oscillation... sensory evoked K-complexes may be the exception rather than the rule” (Steriade and Amzica, 1998). As such, studying spontaneous activity during slow-oscillations and what are now called UP-state transitions (aka "K-complexes" Steriade et al., 1993a,c) in active cortical areas (e.g. rat somatosensory cortex) revealed that some (i.e. high-firing rate ) neurons are activated in specific firing orders (i.e. "packets"; see also Luczak et al., 2015) during both spontaneous and stimulus evoked periods. Such findings of a cortical neuron firing order pose a significant challenge to firing rate coding theories which argue that only the firing rate of a neuron is important for the representation and relaying of information (Adrian, 1926; Shadlen and Newsome, 1994; Rolls and Treves, 2011).

This chapter is aimed at extending previous findings of firing order to the visual cortex of cat and mouse - areas not previously investigated by others likely due to the sparse firing properties of visual cortex neurons. A novel method is developed similar to spike sorting which can identify and classify UP-state transitions in cat V1 and mouse sensory cortex using local field potential (LFP) from extracellular recordings. Additionally, it is also shown that >90% of all neurons exhibit strong peaks in spiking during LEC-defined UP-state transitions even in visual cortex - many more neurons than in previous findings in rat somatosensory and auditory cortex. Perhaps most interestingly, it is shown using multiple methods that some neurons change their relative firing orders over periods of many minutes (e.g. 30-120minutes or longer).

These findings provide a novel, single-neuron independent and temporally precise definition of UP-state initiation that identifies multiple types of UP-states and enables the study of single neuron latencies during UP-state transitions across all cortical areas.

There are a few notes to be made before discussing the results. First, in addition to cat experiments (see below), approximately 27 mice (mostly GCaMP6s) were used to carry out simultaneous electrophysiology and widefield optical imaging experiments (all experiments carried out by the author). These experiments were specifically made to investigate issues arising within this project (see below). Unfortunately, a May 2017 bioRxiv pre-print article (Steinmetz et al., 2017) claims that all (or most) Cre-Emx1 dependent GCaMP6s mice have large amplitude aberrant epileptiform
Figure 48: Power spectrograms and stimulus annotations - Cat C3. 4 tracks from one isoflurane/N₂O anesthetized cat (C3) with power spectrograms (middle), stimulus annotations (right) and synchrony index (left) reveals all tracks have prolonged synchronized state periods (blue trace sections in synchrony index) compared to desynchronized states (black trace sections). A synchrony index threshold value of 0.5 was used to define most synchronized state recording periods (dashed black line in synchrony index traces; see Methods for stimulus descriptions; see also main text).
LFP electrical activity events in cortex. As the current chapter focuses on identifying and grouping stereotyped, large amplitude electrical LFP events occurring during UP-state transitions in cortex, the grouped events are likely to overlap with aberrant electrical activity events reported in this recent article. Accordingly, until the issue of Cre-EMX1 dependent GCaMP6s aberrant electrical activity is resolved (i.e. the publication passes peer review and the findings are validated), several of the sections in this chapter have been limited to cat electrophysiology recordings with only limited examples from GCaMP6 mouse recordings (as indicated in the main text below). One additional awake mouse cortical recording was provided by an external lab (see Methods) to complement the existing results (Figure 5.25).

Furthermore, in contrast to the GCaMP6 mouse experiments which were designed specifically for this chapter to have long spontaneous activity periods with periodic identical stimulus periods, cat recordings analyzed in this chapter come from “unbiased” experiments: i.e. experiments carried using using randomized stimulus order. Cat experiments were made by Nicholas V. Swindale and Martin Spacek between 2009 and 2012 (described in Swindale and Spacek, 2015; see also Methods) and come from a variety of cat anesthetic preparations (see Methods) and cortical states (Figure 48-Figure 51). Importantly, they have largely randomized visual stimulus periods which were not ideally structured for use in this Chapter (e.g. neural activity during sequential spontaneous and natural scene recordings and synchronized cortical states). As can be seen in the power spectrograms and stimulus order (Figure 48-Figure 51; discussed below) only some of the cat recordings contain synchronized states that span multiple hours and contain longer spontaneous activity periods. Accordingly, the focus in the later sections of this chapter (e.g. sections evaluating firing rate order across recording epochs and various stimulus types) was dictated in part by the nature of the available cat data (i.e. varying cortical states and stimulus periods).

The Chapter begins with a brief overview of SWS, UP-state transitions as measured using single neuron recordings and LFP, and recent work by Arthur Luczak on neuronal firing order during UP-state transitions in rat somatosensory and auditory cortex. Next, the method for clustering LFP events into LECs is described and examples from cat and mouse recordings are provided. LECs are shown to have discrete CSD profiles that can be grouped across tracks and animals. Single unit latencies during UP-state transitions are then identified using peaks in LEC-triggered histograms. Two examples of LEC-triggered dorsal cortex maps are provided using VSD imaging in mice as mesoscale correlates of UP-state transitions. The remaining sections focus on tracking latency and spiking distributions during UP-state transitions as well as tracking spike order within and outside UP-state transitions. First, it is shown that latency peaks and distributions for some neurons...
Figure 50: Power Spectrograms and Stimulus Annotations - Cat C4.
change systematically over time. Next, state space analysis is used to track latency for selected (stable) neurons in randomly selected recordings to show changes over time. In-depth analysis using 6 multi-hour recordings from 2 cats are further used to show systematic changes in peak latency and distributions (i.e. changes in firing order) over periods of a few hours. A novel, ordered synchrony, metric is developed and it is shown that spiking order is also partially preserved outside UP-state transitions. Lastly, spiking order and synchrony metrics from recent studies are applied to some datasets to reveal similar gradually changing firing order across neurons. All analysis, figures and text in this chapter were made by the author.

Background

SWS and UP/DOWN-states

Over the past several decades, single neuron patch clamping methods have enabled the tracking of somatic membrane potentials of mammalian cortical and thalamic neurons across various animal states including anesthetized, naturally sleeping and awake conditions (Sakmann and Neher, 1984; Steriade et al., 1993a). These single neuron studies have identified two states: a desynchronized state present during awake and attending periods and rapid-eye-movement sleep (REM) during which neurons fire largely independently of each other (Harris and Thiele, 2011); and a synchronized state present in slow-wave sleep (SWS), quiet waking and anesthesia where neural activity has a slow oscillation (0.2Hz-0.9Hz) with individual neurons periodically oscillating between a depolarized (spiking) state and a hyperpolarized state (non-spiking) known as UP- and DOWN-states, respectively (Steriade et al., 1993a,c; Sanchez-Vives and McCormick, 2000; McCormick and Yuste, 2006; Neske, 2016; Sanchez-Vives et al., 2017). (Note: while single neuron membrane patching studies provided novel findings, behavioural and cortical states have been studied for almost 100 years using electro-encephalography -EEG and other methods). UP- and DOWN-states are known to be present in cortex, thalamus, hippocampus, striatum and cerebellum and could be “the default activity pattern of the entire cortical mantle”. That is, all other types of cortical activity underlying stimulus processing and even complex behaviour may be instantiated in cortex as a type of UP-DOWN state transition (Neske, 2016). UP-state transitions in particular (i.e. the transition of neurons from a DOWN-state to an UP-state) have been linked to multiple functions: facilitating flexible processing of information (McCormick et al., 2004; McCormick and Yuste, 2006; Haider et al., 2006); involvement in rapid changes in functional connectivity during waking behavior (Neske, 2016); and memory replay (Wilson et al., 1994; Sirotas et al., 2003; Sirotas and Buzsaki, 2005). While

Figure 50: (continued from previous page) 3 tracks from one propofol anesthetized cat (C4) shows that the recordings come from mostly desynchronized cortical state recording periods (black trace sections) with relatively brief synchronized state periods.
Figure 51: Power spectrograms and stimulus annotations - Cat C5.
Figure 51: (continued from previous page) 4 tracks from one propofol anesthetized cat (C5) shows that the recordings come from mostly synchronized cortical state recording periods (blue trace sections).

UP-state transitions can be evoked by sensory or thalamic activation (Amzica and Steriade, 1998a; Steriade, 2001) they also occur spontaneously (Amzica and Steriade, 1995; Destexhe et al., 1999; Volgushev et al., 2006).

*UP-state circuits and genesis are still being debated*

Studies over the past two decades have identified Layer 5 (L5) in cortex as a possible initiation site for UP-states with initial evidence from current-source-density (CSD) localization (Steriade and Amzica, 1996) and additional intracellular *in vitro* studies (Sanchez-Vives and McCormick, 2000) which suggested that neither thalamic input nor upper cortical layers were involved in genesis. Additional *in vivo* studies also suggest L5 cells are involved in UP-state genesis as they appear to: lead UP-state transitions (Chauvette et al., 2010), are intrinsically resonant to lower frequencies <15Hz present in the slow oscillation (Agnon and Connors, 1989; da Silva, 1991), contain a sub-type of pyramidal cells that burst and have high spine density and wide arborization within L5. A recent optogenetic stimulation study (Beltramo et al., 2013) also found that stimulation of L5 but not layer 2/3 (L2/3) neurons caused longer and more persistent transitions to UP-states. Two mechanisms by which L5 neurons could become activated and thus initiate UP-states could be (i) persistently active pacemaker L5 neurons which could initiate the cascade themselves or (ii) spontaneous initiation due to stochastic integration of synaptic activity reaching critical thresholds. While some studies suggest L5 cells ramp slowly (i.e. stochastically) while other cells depolarize quickly during UP-states (Chauvette et al., 2010) there is some evidence that L5 cells continuously fire even during DOWN-states thus possibly re-initiating UP-states following a post refractory period (Sanchez-Vives and McCormick, 2000; Hasenstaub et al., 2007; Sakata and Harris, 2009; though see a recent *in vivo* study found no spontaneous spiking in L5 during DOWN-states, Chauvette et al., 2010). However, intrinsic pacemaker resonance properties of L5 cells are also found in L2/3 neurons (Le Bon-Jego and Yuste, 2007) with persistent sodium currents which can act like central-pattern-generators (CPGs) independently of input suggesting L2/3 could also play a central role in UP-state transitions. Additional *in vitro* studies also identified core neurons in layer 4 (L4) that contribute to UP-states either spontaneously or via thalamus (MacLean et al., 2005; Rigas and Castro-Alamancos, 2007) leading some to suggest that there are as many as three sources for UP-state transitions: a cortical L4-L5/6 circuit and independent thalamocortical (TC) and reticular nucleus (NRT) populations (Crunelli and Hughes, 2010). Recent work has also found that astrocytes can trigger slow-wave-oscillation states (Poskanzer and Yuste, 2016).
**UP-state duration**

Although neurons spike the most during the early phase of an UP-state initiation, UP-states can last from 100s of milliseconds to as much as a few seconds and it is uncertain whether cell-intrinsic or synaptic mechanisms are critical, though there is some evidence for the later: in vivo current injections (inhibitory or excitatory) do not affect UP-state duration or rhythmicity (Steriade et al., 1993a; Sanchez-Vives and McCormick, 2000); and membrane potential and inter-spike interval variances during UP-states are high suggesting persistent synaptic input. Other mechanisms involved likely include: enhanced inhibitory neuron activity, synaptic depression of excitatory synapses, or hyperpolarizing conductances becoming active. One study (Steriade et al., 1993) showed that UP-states can be extended by stimulating brainstem cholinergic projectors (muscarinic signaling).

**Thalamus, brainstem and basal forebrain contribute to UP states**

Despite slow oscillations surviving thalamic lesions (Steriade et al., 1993) and occurring in cortical slabs (Timofeev et al., 2000) and in vitro (Sanchez-Vives and McCormick, 2000) more recent studies suggest thalamus has an active role in UP-states. In fact, a number of studies have shown that TC relay cells, in particular, fire post-inhibitory rebound spike-bursts before UP-state transitions in cortex suggesting thalamus initiates (Contreras and Steriade, 1995) and possibly synchronizes UP-states across cortex (Amzica and Steriade, 1995). Additionally, sensory stimulation is known to evoke UP-states, even in anesthetized animals using drift gratings (Anderson et al., 2000; Jia et al., 2010) or whisker stimulation (Petersen et al., 2003; Hasenstaub et al., 2007) supporting the role of TC neurons. Further, UP-state frequency is decreased by severing TC axons in mouse barrel cortex slices (Rigas and Castro-Alamancos, 2007) as does pharmacological blockage of TC spiking in anesthetized and sleeping rats (David et al., 2013). And fast-oscillations occurring during UP-states (discussed below) are reduced along with multi-neuron synchronization by thalamic inactivation (Lemieux et al., 2014). Interestingly, sensory thalamic relay neurons are highly inhibited after UP-state initiation but non-sensory nuclei are not (Sheroziya and Timofeev, 2014) likely because TRN neurons only connect to sensory thalamus and avoid non-sensory nuclei (Barth et al., 2002). This leaves open the possibility that non-sensory thalamus neurons (matrix neurons; Jones, 1998) may be involved in persistence and termination of UP states.

Additionally, brainstem nuclei send cholinergic, noradrenergic and serotonergic axons to cortex and thalamus, with some findings suggesting they constitute at least half of all synaptic inputs to some thalamic nuclei (e.g. cat lateral-geniculate-nucleus, LGN; Erisir et al., 1997). Basal forebrain (nucleus bassalis, and accumbens and others) also sends cholinergic axons and it seems some basal forebrain neurons prefer either UP or DOWN-states (Detari et al., 1997; Manns et al., 2000; Menas-Segovia et al., 2008; Eschenko et al., 2012; Schweimer et al., 2011).

**The function of slow oscillations and UP-states are largely unknown**

While it is unclear how cortico-thalamic circuits give rise to slow oscillations and UP and DOWN-
state transitions, even less is known about the role of such oscillations in mammals, though a number of suggestions are offered. Slow oscillations may facilitate synaptic plasticity and in particular memory consolidation. Increased synaptic plasticity during slow oscillations has been previously suggested (Moruzzi, 1965; Steriade and Timofeev, 2003) and is supported by REM sleep and performance studies (Karni et al., 1994) and findings of replay of patterns of hippocampal place cell (Wilson et al., 1994) as well as newer studies (Waters and Helmchen, 2006; Tononi and Cirelli, 2015). Such memory consolidation mechanisms may involve large dendritic calcium influx (Yuste and Tank, 1996) triggering synaptic plasticity mechanisms (e.g. CaMKII; Soderling and Derkach, 2000). It has also been suggested that during UP-states hippocampus transfers information back to cortex: sharp wave ripples (SWR) appear in hippocampus right after UP-state initiation (Siapas and Wilson, 1998; Sirota et al., 2003) and hippocampal activity patterns are transferred to cortical networks during the UP-state while cortical plasticity is high (Sirota and Buzsaki, 2005). Additionally, cellular restoration (Vyazovskiy and Harris, 2013) may be occurring during DOWN states as it is known that death occurs in familial insomnia where SWS is impossible (Cortelli et al., 1999). Another interesting hypothesis is that during UP-states the membrane properties of neurons are modified (McCormick et al., 2003) to possibly better detect sub-threshold inputs, or function as better gain control mechanisms (McCormick et al., 2004; McCormick and Yuste, 2006; Haider and McCormick, 2009) but in a highly dependent fashion on the state of the neuron.

**UP-state transitions have LFP correlates**

In two publications entitled *Cellular substrates and laminar profile of sleep K-complex* and *Electrophysiological correlates of sleep delta waves* (Amzica and Steriade, 1997a, 1998a) it was shown that K-complexes (now called UP-state transitions) recorded in cats during natural sleep were similar to those recorded under ketamine-xylazine anesthesia. Importantly, it was shown that UP-state transitions engage large parts of cortex and that single neuron depolarizations across cortex manifest as large amplitude negative depth LFP deflections (Figure 52). Additional studies over the past several years have further shown that UP-state transitions do have single (Saleem et al., 2010) and multi-channel LFP correlates (Chauvette et al., 2010) and additional work in rat hippocampus slices has also identified that some LFP waveforms have stereotyped shapes that can be grouped together (Reichinnek et al., 2010). One particular study using LFP and intracellular recordings in naturally sleeping cats (Chauvette et al., 2010) has also shown that during UP-state transitions, large amplitude multi-laminar LFP deflections correlate with single neuron UP-state initiation (Figure 53A). Importantly, however, the extracellularly recorded LFP was shown to have a more stereotyped shape during UP-state transitions than individually patched single neurons which had higher somatic voltage variability relative to each other or to their own previous transitions - even across sequential UP-state transitions separated by 1-2secs (Figure 53B). Lastly, a recent study in anesthetized macaque hippocampus suggest that grouping of LFP by shape is possible (Ramirez-Villegas et al., 2015).
Figure 52: LFP negativities mark single neuron UP-state transitions. A. Local field potential (top) and intracellular recording (bottom) of an area 5 neuron in a naturally sleeping cat reveals strong alignment between LFP negativities and single neuron membrane depolarization (dashed red lines) during UP-state transitions even in the absence of single neuron spiking (blue lines). B. An example of a single UP-state transition (same experiment as in A) measured using depth EEG (i.e. LFP), surface EEG, single neuron clamp and nearby extracellular electrode shows the alignment of LFP to membrane voltage depolarization and nearby neurons spiking during the transition. C. Same as (B) but the patched neuron does not spike while other nearby neurons spike substantially. (A-C adapted from Amzica and Steriade, 1998a with permission).

Neuronal firing order during UP-state transitions

Despite the outstanding question of genesis, UP-states are becoming more commonly studied by researchers seeking to understand neural coding in cortex (Luczak et al., 2015; Neske, 2016; Sanchez-Vives et al., 2017). A series of recent studies have focused on identifying UP-state transitions using multi-unit-activity (MUA) and evaluating single unit activity firing order during such transitions (Figure 54; Luczak et al., 2007, 2009, 2013, 2015; Bermudez-Contreras et al., 2013). In particular, the studies have shown that high-firing rate (>2Hz) neurons in rat somatosensory and auditory cortex are activated in a similar order (on average) during UP-state transition as they are during stimulus presentation (Figure 54A,B). It has been hypothesized that firing order of such high firing-rate neurons encodes information and that further identifying order for all cells and in all
Figure 53: Multi-channel LFP is more stereotyped than single neuron potentials during UP-state transitions. A. UP-state transition \((t=0\text{sec})\) recorded using laminar, multi-channel LFP (left) and intracellular voltages from two neurons (right). B. Example of two sequential UP-state transitions recorded as LFP using multichannel extracellular electrodes (black trace) and intracellular voltages for two cells (red and blue traces) shows that LFP traces are much more similar over time than single neuron membrane voltages which have different dynamics even when separated by 1-2sec (A,B adapted from Chauvette et al., 2010 with permission).

cortical areas may help reveal the coding strategy used by all cortex (Figure 54C,D; Luczak et al., 2015). Additionally, another recent study using cross-correlation histograms (CCH) has shown that groups of neurons (up to 12, average 7) recorded simultaneously in anesthetized cat visual cortex show preferred firing sequences lasting up to \(\approx 15\text{ms}\) in duration that change in part as a function of stimulus properties (Havenith et al., 2011).

While findings of firing order are evidence for spike timing neural coding theories, these firing order studies have so far been limited to analysis of only high firing rate neurons in both UP-state transitions and CCH-based studies. The CCH studies are limited by a number of factors including that they contain complex statistical tests (e.g. averaging after multi-resampling of sparse datasets and tracking sub-millisecond firing sequence errors) and require heuristics for excluding sub-optimal stimuli. They also rely on higher-firing rate neurons for generating 1ms-bin CCHs with sufficient structure (see analysis below and Figs 5.31, 5.32). And UP-state firing order studies have largely been limited to cortical areas where neurons fire spontaneously (and during stimulus presentation) at high rates (e.g. rat somatosensory and auditory cortex). Critically, using MUA to define UP-state initiation has limitations: it is potentially circular as single unit spiking is used to define both UP-state transitions and the single unit order that follows; peaks in MUA are dependent on heuristics such as the choice of histogram bin width and MUA threshold that indicates an UP-state transition. Furthermore, MUA-based definitions of UP-state transitions used so far can only capture high firing rate neurons (>2Hz) for analysis (Luczak et al., 2007, 2009, 2013, 2015; Bermudez-Contreras et al., 2013). The latter limitation excludes most (>80%) of neurons recorded
Figure 54: Latency order results from other studies. A. UP-state transition triggered single neuron PETHs in a urethane anesthetized rat somatosensory cortex recording with white dots corresponding to approximate centre-of-mass (COM) metric used in the studies. B. Example COM (red dots) and firing distributions (grey traces) for 90 rat auditory cortex neurons recorded during spontaneous and stimulus evoked periods show relatively similar order across all conditions (note: neuronal order is based on COM order during spontaneous recording periods, i.e. UP-state transition-based order). C. Hypothesized neuron firing order packets occurring during spontaneous synchronized state activity are similar to packets during tone presentation. D. Similar hypothesis as (C) but during desynchronized (e.g. awake and attending) cortical states. (A - Adapted from Luczak et al., 2007 with permission; B - Adapted from Luczak et al., 2009 with permission; C,D - Adapted from Luczak et al., 2015 with permission).
in sensory cortex where firing rates for stimulus and spontaneous activity are generally <2Hz (e.g. for mouse sensory cortex recordings analyzed in this chapter the median firing rate was 0.26Hz with 89% of neurons spiking at rates <2Hz; in cat V1 recordings presented here the median firing rate was 0.31Hz with 86% of neurons spiking at rates <2Hz; see also Methods). Thus, a single-neuron independent and largely heuristic-free definition of UP-state transition that could enable the study of all neurons’ relative firing order in all cortical areas is not yet available.

This chapter presents an improved method for detecting UP-state transitions using LFP waveforms (rather that MUA) and applies this method to visual cortex to yield novel results. The methodology developed relies on LFP events, i.e. large amplitude LFP events 50-200ms in duration to provide more temporally precise UP-state transition times for computing and tracking spiking properties (e.g. latencies) across all cells, including low firing-rate cells. The focus here is largely on extracellular recordings in anesthetized cat and mouse cortex because under such conditions slow-oscillations dominate and there are many UP-state transitions which can be identified and analyzed.

Results

Clustering multi-laminar LFP events reveals distinct UP-state transition classes

The approach for identifying UP-state transitions from LFP is similar to single-unit spike sorting approaches: event detection is carried out on the raw LFP voltage record and clustering methods are carried out on aligned multi-channel LFP events (Figure 5.8A-C; see also Methods for a complete description). Detected multi-channel LFP events in the recordings considered here had similar extracellular waveforms across most or all channels; each event contained 1 to 2 troughs and 1 peak; the events lasted between ≈50ms to 200ms; and PTP amplitudes of the events were between ≈250µV to 500µV (Figure 55D(i), E(ii)). After aligning LFP events using a centre-of-gravity iterative RMS procedure applied on the waveform on the maximum PTP channel (see Methods), features were selected and visualized using PCA (Figure 55C(iii)). The observed distributions were clustered (Swindale and Spacek, 2014) into distinct LECs, individual LFP waveforms in each LEC were re-aligned using RMS and timestamps for each waveform was exported as the temporal location of the centre-of-gravity of each waveform (Figure 5.8C(iv); see also Methods).

LEC extracellular templates were obtained by averaging aligned waveforms in each LEC (Figure 55D(i), E(ii)). The templates were distinct from each other and the PC values did not drift substantially over time (Figure 55(ii), E(iii)) indicating that LECs are largely stable (Figure 558D: cat visual cortex - Recording ID: C5.3; Figure 1E: mouse auditory cortex - MV1).

Analysis across 15 cat visual cortex tracks yielded a total of 34 LECs (≈2.3 LECs per track; see Table 5.1) and across 20 mouse sensory cortex tracks yielded 33 LECs (≈3.1 LECs per visual cortex track; ≈1.6 LECs per barrel cortex track; and 1 LEC per auditory cortex track; see Table
Figure 55: Clustering LFP events during synchronized cortical states. A. Top: power spectrogram for a cat visual cortex recording (deepest channel); Middle: synchrony index (red curve; see Methods) falls largely above threshold (dashed line) indicating synchronized states; Bottom: single unit rasters ordered by neuron depth with extracellular polytrode (right). B. Same as (A) but from a mouse visual cortex recording. C. LFP event clustering methodology: (i) 3-second synchronized state recording from a cat visual cortex recording (bottom traces) overlayed with MUA histogram shows spiking activity coincides with large amplitude negative LFP deflections as previously reported (Amzica and Steriade, 1997b, 1998b; Chauvette et al., 2010); (ii) LFP record is high-pass filtered (4Hz cutoff) and events over threshold (4 x STD, see Methods) are identified (bold traces); (iii) LFP events are aligned and clustered into LECs (see Methods); (iv) two clusters identified using PCA. D. Templates of LECs in 1C(iv); (ii) Plots of principal components for clusters in 1C(iv) vs. time showing almost no changes over time. E. Same as F, but for a mouse visual cortex recording.
### Table 2: Cat recordings and clustering details

<table>
<thead>
<tr>
<th>Recording ID</th>
<th>Anesthetic</th>
<th>Track ID</th>
<th>Sync Rec Time / Total Rec Time</th>
<th>No. of LECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1.1</td>
<td>Iso/N20</td>
<td>1</td>
<td>0.6 / 13.4 hrs²</td>
<td>2</td>
</tr>
<tr>
<td>C1.2</td>
<td>Iso/N20</td>
<td>2</td>
<td>1.4 / 9.4 hrs²</td>
<td>2</td>
</tr>
<tr>
<td>C2.1</td>
<td>Iso/N20</td>
<td>1</td>
<td>1.7 / 11.1 hrs²</td>
<td>3</td>
</tr>
<tr>
<td>C2.2</td>
<td>Iso/N20</td>
<td>2</td>
<td>4.7 / 12.0 hrs²</td>
<td>1</td>
</tr>
<tr>
<td>C3.1</td>
<td>Iso/N20</td>
<td>1</td>
<td>1.4 / 8.4 hrs</td>
<td>1</td>
</tr>
<tr>
<td>C3.2</td>
<td>Iso/N20</td>
<td>2</td>
<td>4.2 / 8.3 hrs</td>
<td>3</td>
</tr>
<tr>
<td>C3.3</td>
<td>Iso/N20</td>
<td>3</td>
<td>3.4 / 7.4 hrs</td>
<td>2</td>
</tr>
<tr>
<td>C3.4</td>
<td>Iso/N20</td>
<td>4</td>
<td>3.0 / 5.3 hrs</td>
<td>1</td>
</tr>
<tr>
<td>C4.1</td>
<td>Propofol/Fentanyl</td>
<td>1</td>
<td>3.3 / 11.9 hrs</td>
<td>1</td>
</tr>
<tr>
<td>C4.2</td>
<td>Propofol/Fentanyl</td>
<td>2</td>
<td>0.5 / 11.5 hrs</td>
<td>2</td>
</tr>
<tr>
<td>C4.3</td>
<td>Propofol/Fentanyl</td>
<td>3</td>
<td>0.9 / 4.6 hrs</td>
<td>2</td>
</tr>
<tr>
<td>C5.1</td>
<td>Propofol/Fentanyl</td>
<td>1</td>
<td>2.5 / 2.9 hrs</td>
<td>3</td>
</tr>
<tr>
<td>C5.2</td>
<td>Propofol/Fentanyl</td>
<td>2</td>
<td>4.9 / 10.6 hrs</td>
<td>3</td>
</tr>
<tr>
<td>C5.3</td>
<td>Propofol/Fentanyl</td>
<td>3</td>
<td>3.1 / 8.5 hrs</td>
<td>4</td>
</tr>
<tr>
<td>C5.4</td>
<td>Propofol/Fentanyl</td>
<td>4</td>
<td>4.6 / 6.3 hrs²</td>
<td>4</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td></td>
<td><strong>15</strong></td>
<td><strong>34</strong></td>
<td></td>
</tr>
</tbody>
</table>

1. Synchronized recording time was defined as periods with synchrony index >0.5. 2. Synchrony index threshold of 0.3 used. 3. LECs had peak-to-peak amplitude and CSD maxima below threshold and were excluded from CSD grouping and further analysis.

**LECs have discrete laminar CSDs patterns common across tracks and animals**

CSD profiles for each LEC template were investigated to determine whether there were unique *sinks* and *sources* attributed to each template (Figure 56). CSD analysis (see Methods) revealed that while during desynchronized cortical states events with high amplitude current sinks and sources are frequent and present at multiple depths across cortex, during synchronized states current sink-source pairs were more infrequent (≈1Hz - consistent with UP-state transition frequencies of 0.2-0.9Hz) and fell into distinct and spatially localized patterns (Figure 56A). Computing CSD profiles from LECs revealed a more diverse laminar profile in comparison to extracellular templates which typically have qualitatively similar shapes (and amplitudes) across most or all channels (Figure 56B: left: LEC extracellular template; right: corresponding CSD distribution). This indicates that during synchronized cortical states, UP-state transitions engage distinct cortical circuits with spatially localized sinks (e.g. neuronal action potentials) and sources (e.g. repolarization or distal inputs) in partial support of the multiple oscillator hypothesis for UP-state genesis (Crunelli and Hughes, 2010).

Computing CSDs for all cat visual cortex and mouse sensory cortex recordings LECs (Figure 57-Figure 60) revealed that similar CSD patterns could be identified across tracks from the same...
### Table 3: Mouse recordings and clustering details

<table>
<thead>
<tr>
<th>Recording ID</th>
<th>Anesthetic</th>
<th>Track ID</th>
<th>Area</th>
<th>Sync / Total Rec Time</th>
<th>No. of LECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV1</td>
<td>Isoflurane</td>
<td>1</td>
<td>Visual</td>
<td>2.9 / 3.0hrs</td>
<td>3</td>
</tr>
<tr>
<td>MV2</td>
<td>Isoflurane</td>
<td>1</td>
<td>Visual</td>
<td>3.3 / 3.3hrs</td>
<td>4</td>
</tr>
<tr>
<td>MV3</td>
<td>Isoflurane</td>
<td>1</td>
<td>Visual</td>
<td>2.6 / 3.4hrs</td>
<td>4</td>
</tr>
<tr>
<td>MV4</td>
<td>Isoflurane</td>
<td>1</td>
<td>Visual</td>
<td>2.3 / 2.4hrs</td>
<td>3</td>
</tr>
<tr>
<td>MV5</td>
<td>Urethane</td>
<td>1</td>
<td>Visual</td>
<td>5.3 / 5.3hrs</td>
<td>3</td>
</tr>
<tr>
<td>MV6(^1,4)</td>
<td>Isoflurane</td>
<td>1</td>
<td>Visual</td>
<td>2.0 / 2.8hrs</td>
<td>2</td>
</tr>
<tr>
<td>MV7(^2,4)</td>
<td>Isoflurane</td>
<td>1</td>
<td>Visual</td>
<td>2.6 / 3.1hrs</td>
<td>3</td>
</tr>
<tr>
<td>MB1</td>
<td>Isoflurane</td>
<td>1</td>
<td>Barrel</td>
<td>2.8 / 2.9hrs</td>
<td>1</td>
</tr>
<tr>
<td>MB2.1</td>
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<td>1</td>
<td>Barrel</td>
<td>2.7 / 2.7hrs</td>
<td>2</td>
</tr>
<tr>
<td>MB2.2</td>
<td>Isoflurane</td>
<td>2</td>
<td>Barrel</td>
<td>2.3 / 2.4hrs</td>
<td>2</td>
</tr>
<tr>
<td>MB3.1</td>
<td>Isoflurane</td>
<td>1</td>
<td>Barrel</td>
<td>2.8 / 3.1hrs(^3)</td>
<td>1</td>
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<tr>
<td>MB3.2</td>
<td>Isoflurane</td>
<td>2</td>
<td>Barrel</td>
<td>1.8 / 2.0hrs(^3)</td>
<td>1</td>
</tr>
<tr>
<td>MB4</td>
<td>Urethane</td>
<td>1</td>
<td>Barrel</td>
<td>7.4 / 7.8hrs</td>
<td>1</td>
</tr>
<tr>
<td>MB5</td>
<td>Urethane</td>
<td>1</td>
<td>Barrel</td>
<td>1.5 / 2.7hrs</td>
<td>3</td>
</tr>
<tr>
<td>MA1</td>
<td>Isoflurane</td>
<td>1</td>
<td>Auditory</td>
<td>1.9 / 2.0hrs</td>
<td>1</td>
</tr>
<tr>
<td>MA2</td>
<td>Urethane</td>
<td>1</td>
<td>Auditory</td>
<td>2.0 / 3.0hrs(^3)</td>
<td>1</td>
</tr>
<tr>
<td>MA3</td>
<td>Urethane</td>
<td>1</td>
<td>Auditory</td>
<td>1.5 / 3.9hrs(^3)</td>
<td>1</td>
</tr>
<tr>
<td>MA4(^2,4)</td>
<td>Isoflurane</td>
<td>1</td>
<td>Auditory</td>
<td>0.9 / 1.2hrs</td>
<td>1</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td></td>
<td><strong>20</strong></td>
<td></td>
<td><strong>33</strong></td>
<td></td>
</tr>
</tbody>
</table>

1. Tetrode recordings did not yield laminar (i.e. relative depth) information and were not used for CSD analysis. 2. VSD recordings. 3. Synchronized state defined as synchrony index >0.3 (>0.5 used for all other recordings). 4. Wildtype mice (all other mice GCaMP6s).

animal (Figure 56C, D) or even across animals (Figure 56E). Grouping all data from cat visual cortex recordings by qualitatively similar CSD pattern showed that few CSD patterns were unique to one track only: total numbers of LECs grouped into sets of 2 or more: cat V1: 30/34; mouse visual cortex: 14/17; mouse barrel cortex: 8/11; mouse auditory cortex: 3/3; see Tables 5.3, 5.4 and Figure 57-Figure 60). This suggests that individual LECs do not reflect idiosyncratic circuits (i.e. animal specific) but engage common cortical circuits present in all cats or possibly all mammals that have cortical UP/DOWN states (note: CSD patterns were not compared across species, i.e. between cat and mouse visual cortex recordings).

Lastly, in addition to the PCA space stability (see Figure 55), the temporal stability of all LFP waveforms in each LEC group was evaluated by computing the standard deviation of the full-width-half-max (FWHM) of each LFP event on the maximum amplitude channel (Figure 56F-I). 4 LEC examples provided from a single cat V1 recording (Figure 56F) have FWHM±STD distribution means of 28±7ms, 22±4ms, 24±5ms and 18±5ms, respectively (Figure 56H). Further grouping of all LECs across all cat tracks using CSD patterns (colours in Figure 56I match coloured grouping dots in Figure 57) revealed that for the first 6 LEC groups the FWHM STD distributions within
Figure 56: CSD-based LEC grouping and temporal precision. A. CSD profiles computed during spontaneous cortical activity in cat visual cortex during a desynchronized (left) and synchronized (right) recording period in the same track. (Note: CSD colour scales are normalized to be centred on current=0 μA/mm³ with -1 representing current sinks: positive current flowing away from extracellular space, and +1 representing current sources: positive current flowing into the extracellular space). B. Example of an LEC template and its CSD correlate. C. Examples of CSDs obtained from LECs from different tracks in a propofol anesthetized cat. D. Examples of CSDs obtained from LECs from different tracks in an isoflurane anesthetized cat. E. Examples of CSDs obtained from LECs from different tracks in two different isoflurane anesthetized cats. F. LFP templates of LECs from Figure 55D. G. Method for computing the stability of extracellular LFP events in each LEC using the FWHM of the first trough for each LEC event. H. Trough FWHM ±STD of all LFP events in each LEC in (F) shows that most troughs FWHM are stable (i.e. the STD is ≈5ms; see main text also). I. STDs (hollow circles) and average of STDs (filled circles) of all LEC templates belonging to the first 6 groups of LECs (see Figure 57 for colour matching; see also Methods and main text).
Table 4: Cat visual cortex - LEC groups

<table>
<thead>
<tr>
<th>Recording ID</th>
<th>Anesthetic</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>#1  #2  #3  #4</td>
</tr>
<tr>
<td>C1.1</td>
<td>Iso/N₂O</td>
<td>X</td>
</tr>
<tr>
<td>C1.2</td>
<td>Iso/N₂O</td>
<td>X</td>
</tr>
<tr>
<td>C2.1</td>
<td>Iso/N₂O</td>
<td>X</td>
</tr>
<tr>
<td>C2.2</td>
<td>Iso/N₂O</td>
<td>X</td>
</tr>
<tr>
<td>C3.1</td>
<td>Iso/N₂O</td>
<td>X</td>
</tr>
<tr>
<td>C3.2</td>
<td>Iso/N₂O</td>
<td>X</td>
</tr>
<tr>
<td>C3.3</td>
<td>Iso/N₂O</td>
<td>X</td>
</tr>
<tr>
<td>C3.4</td>
<td>Iso/N₂O</td>
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<tr>
<td>C4.1</td>
<td>Propofol/Fentanyl</td>
<td>X</td>
</tr>
<tr>
<td>C4.3</td>
<td>Propofol/Fentanyl</td>
<td></td>
</tr>
<tr>
<td>C5.1</td>
<td>Propofol/Fentanyl</td>
<td>X</td>
</tr>
<tr>
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<td>Propofol/Fentanyl</td>
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</tr>
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<td>Propofol/Fentanyl</td>
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</tr>
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<td>Propofol/Fentanyl</td>
<td>X</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
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<td>5</td>
</tr>
</tbody>
</table>

1. These 2 LECs are similar to Class 6 but may also form a separate class.

Each group (hollow circles) and for their average (filled circles) are <10ms (Figure 56I: cat visual cortex, 23/25 LECs STD <10ms; Figure 58: mouse visual cortex 13/14 <10ms).

**Most neurons lock to LEC-defined UP-states**

While a number of previous studies have shown high amplitude LFP deflections to be the global correlates of UP-state transitions (Amzica and Steriade, 1998a; Steriade and Amzica, 1998; see Figure 52, Figure 53) the methodology described here can identify UP-state transitions with significant precision: ≈5-10ms for most recordings in cat and mouse visual cortex (Figure 56I, Figure 58) and ≈5-15ms in mouse barrel and auditory cortex (Figure 59, Figure 60). The approach thus enables the investigation of single neuron firing properties (e.g. latency and order) during UP-state transitions in visual cortex of cat and sensory cortex of mouse.

The equivalence of LECs and UP-state transitions is further shown using single unit activity in two example recordings from cat visual cortex and mouse auditory cortex (Figure 61). Peri-LEC-Event-Time-Histograms (PETH) for single neurons identified via single unit spike sorting for the two highest PTP-amplitude LECs (Figure 61A-C,E-F; see also Methods). PETHs were computed by convolving each spike for every neuron with a 5ms (cat visual cortex) or 10ms (mouse auditory cortex) STD gaussian. (Note: method adopted from Luczak et al., 2007, 2009, 2013 where 20ms wide gaussians were used, but changed to reflect LFP event FWHM results presented above; see also Methods). An example PETH from a single neuron in cat visual cortex computed for the two
Figure 57: LEC groupings - cat visual cortex. Cat visual cortex grouping of all clustered LECs (34 LECs across 15 tracks in 5 cats) based on CSD profile similarity reveals that most LECs can be grouped together with other LECs from either a different track or a different cat. The coloured dots represent LEC groups analyzed in Figure 56I.
Table 5: Mouse Sensory Cortex - LEC Groups

<table>
<thead>
<tr>
<th>Recording ID</th>
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<th>Area</th>
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¹. Grouping labels are different for different cortical areas and across cat and mouse recordings (e.g. visual cortex group #1 ≠ barrel cortex group #1).

**Mouse - Visual Cortex**

Figure 58: LEC groupings - mouse visual cortex. Mouse visual cortex grouping of all LECs (17) in 5 tracks from 5 mice. Coloured dots represent LEC groups analyzed in the panel on the right for their trough FWHM STD values.
Figure 59: LEC groupings - mouse barrel cortex. Mouse barrel cortex grouping of all LECs (11) in 4 tracks from 4 mice. Coloured dots represent LEC groups analyzed in the panel on the right for their trough FWHM STD values.

Figure 60: LEC groupings - mouse auditory cortex. Mouse auditory cortex grouping of all LECs (3) in 3 tracks from 3 mice. Coloured dot represents the LEC group analyzed in the panel on the right for trough FWHM STD values.
highest amplitude LECs (Figure 56A) reveals a very narrow spiking distribution near the defined centre of the LEC events (i.e. \(t=0\)ms). In contrast, PETH results from a single neuron in mouse auditory cortex activity during events from the highest amplitude LECs (Figure 5.14E) reveal a sharp onset but a slower decay time of 10ms to 100ms (or longer) consistent with UP-state PETHs reported previously (see Figure 54A,B and Luczak et al., 2007, 2009, 2013, 2015).

Plotting PETHs for all neurons in order of PETH peak (Figure 61B-left) or cortical depth (Figure 61B-right), reveals a strikingly narrow distribution of latencies with virtually all neurons recorded firing with an \(\approx 25\)ms window of each other and individual distributions of \(\approx 5\)ms FWHM. Neurons respond to LEC #2 across a broader period of time (\(\approx 100\)ms), but also individually
exhibit narrow latency distributions similar to those for LEC #1 (Figure 61C). The different PETH distributions between the two LECs further supports the observation that UP-state transitions fall into different types. Similarly plotting mouse auditory cortex neuron PETH distributions also reveals that most neurons peak in spiking near the UP-state transition (i.e. $t=0\text{ms}$) but continue to spike subsequently (i.e. have longer tailed distributions; Figure 61E-G) consistent with previous findings in rat auditory and barrel cortex (Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013). Lastly, the proportion of neuron spikes that occur within a $\pm 50\text{ms}$ (cat visual cortex) or $\pm 100\text{ms}$ (mouse barrel cortex) window of LEC events was investigated. In the cat visual cortex recording example provided, neurons fired from $\approx 10\text{-}90\%$ of their spikes during LEC-defined UP-state transitions. There was also evidence that LECs prefered specific classes of UP-state transitions: superficial cells fired more during LEC #1 (Figure 61D - red coloured LEC) while deeper cells preferred LEC #2 (Figure 61D - green LEC). Cells from the mouse auditory cortex example fired $\approx 10\text{-}70\%$ of their spikes during LEC defined UP-states, well above chance (dashed lines in each plot indicates chance; see Methods).

Considering other cat visual and mouse sensory cortex recordings, it was found that LECs could be used to reveal strong peaks in PETH distributions for $>90\%$ of all neurons recorded (see Figure 62 for additional examples using wider windows). PETH distributions for visual cortex cells (in both cat and mouse) had narrower (i.e. $\approx 5\text{-}10\text{ms}$ wide) PETH FWHM during LEC defined UP-state transitions, while mouse auditory and barrel cortex neurons also had strong peaks but longer tailed distributions as previously reported (Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013). These findings provide further evidence that LECs are temporally precise LFP correlates of DOWN-to-UP state transitions and are tightly linked to spiking of all neurons irrespective of firing rates or cortical area.

The finding that visual cortex neurons have narrow distributions with full-width-half-maximum
activations on the order of $\approx 5$-10ms and peaks near UP-state transitions (i.e. $t=0$ms), has not been previously made. It suggests that similar to rat somatosensory and auditory cortex (Luczak et al., 2007, 2009, 2013), visual cortex neurons fire substantially during UP-state transitions. Importantly, these findings allow for the investigation of relative firing order between visual cortex neurons.

**UP-state transitions have mesoscale correlates in mouse dorsal cortex**

UP-state transitions identified as LECs have thus far been shown to have: distinct PCA-space clusters (i.e. LECs); specific CSD patterns as well as correlating with strong single neuron spiking peaks. Additional experiments were implemented to investigate meso-scale correlates of LEC defined UP-state transitions. In particular, widefield calcium activity in GCaMP6 mice (Ai93, Ai94; see Methods) was recorded simultaneously with extracellular potentials with the goal of linking UP-state transitions with dorsal cortex neural activity. Accordingly, LEC-event triggered widefield GCaMP6 activity maps were computed (similar to spike-triggered-averaging of imaging activity; Arieli et al., 1995; Tsodyks et al., 1999; Xiao et al., 2017) to identify spatio-temporal patterns (e.g. motifs) occurring in mouse dorsal cortex during UP-state transitions. Single-hemisphere (auditory-cortex) and bi-hemispheric (visual and barrel cortex) calcium activity (30Hz) recordings were made in 23 GCaMP6s transgenic mice while simultaneously acquiring extracellular potentials from visual, barrel and auditory cortex (see also Methods). Findings revealed that LEC-triggered calcium motifs (i.e. averages of calcium signals centred on the events from each LEC) correlated with global cortical activity patterns with most patterns showing depression-to-activation dynamics centred on the specific area where the extracellular recording was made (not shown).

However, as discussed above, the GCaMP6 transgenic mice used in these recordings employed a Cre-based breeding strategy and a recent study (Steinmetz et al., 2017) specifically identified Cre-dependent GCaMP6s mice as potentially having aberrant electrical activity that is visible both in the extracellular potential recordings and widefield calcium activity. Accordingly, LEC-triggered dorsal cortex calcium activity maps will not be discussed further to avoid drawing conclusions that may arise from potentially pathological cortical conditions.

However, 2 additional imaging experiments were available where voltage-sensitive-dyes (VSDs) were used in wildtype mice along with extracellular recordings in visual cortex (1 mouse; Figure 63A) and auditory cortex (1 mouse; Figure 63B; see also Methods). In contrast with GCaMP6 imaging, VSD has the advantage of reporting both subthreshold and suprathreshold neural activity and at a higher temporal resolution (150Hz). VSD motifs triggered off LECs (i.e. UP-state transitions) in mouse visual cortex peaked in amplitude nearly simultaneously with the LEC events (typically $\pm 30$ms centred on UP-state transition times (Figure 5.16); Figure 63A(ii)) with motifs revealing a gradual cortical activation preceding $t=0$ms (i.e. $\approx 100$ms to $+100$ms) suggesting a gradual build up of membrane depolarizations prior to UP-state transition. This gradual activation is expected from previous work using single neuron patch clamping techniques (Volgushev et al.,...
Figure 63: Mesoscale VSD correlates of UP-state transitions.
Figure 63: (continued from previous page) A(i). Three LEC-triggered meso-scale VSD motifs (-0.2sec to +0.2sec) from a mouse visual cortex recording (note: arrow indicates location of electrode insertion). A(ii). Temporal dynamics for motifs in A(i) from four cortical ROIs (L_RS: left retrosplenial, L_V: left visual, L_BC: left barrel, and L_M: left motor). B. Same as A but for an auditory cortex recording. (Note: dF/F0 values during spontaneous VSD activity usually peak at 0.5% and substantially lower when averaging over multiple events; see also Methods).

2006; Chauvette et al., 2010). Additionally, VSD activity was more similar across all cortical ROIs identified (see traces in Figure 63A,B(ii)) consistent with intracellular findings of simultaneous, global depolarization of neurons (Amzica and Steriade, 1995; Destexhe et al., 1999).

Average UP-state transition latencies change over time

This chapter’s main goal is to investigate whether visual cortex neurons have preferred latencies during UP-state transitions (i.e. PETH peaks) that can be systematically tracked (i.e. using a method with a defined temporal precision) and if so, to examine how order during spontaneous activity might be related to order during stimulus evoked periods. The aim was thus to extend a number of recent findings of preserved order of high-firing rate (i.e. >2Hz) neurons in rat somatosensory and auditory cortex (Luczak et al., 2007, 2009, 2013; Bermúdez-Contreras et al., 2013) to all other sensory cortex neurons including visual cortex which is an area of active research where such findings have not been previously made. However, work in this chapter also relates to theories of cell assemblies and synfire chains (Abeles, 1982b; Abeles and Gerstein, 1988; Abeles, 1991; Abeles et al., 1993; Abeles, 1982a; Ikegaya et al., 2004; Izhikevich, 2005; Schrader et al., 2008; Grun and Rotter, 2010; Gerstein et al., 2012; Torre et al., 2016; Quaglio et al., 2017) that hypothesize that networks (or chains) of functionally connected neurons exist in cortex which can be studied by identifying groups of neurons that repeatedly spike with similar inter-spike-intervals. Despite ongoing research, findings of synfire chains at the level of single neuron spiking have been limited with some even arguing that the few positive findings may be statistically insignificant (i.e. occur at chance: Baker and Lemon, 2003; or require multi-tier statistical support: Gerstein et al., 2012). A very recent study (Russo and Durstewitz, 2017) developed a “temporal-scale independent” method of searching for synfire chains but also did not find a universal "cortical coding scheme" (i.e. single neuron-based synfire chains) in rat frontal cortex or monkey visual cortex recordings.

One possibility for why strong evidence for synfire chains has not been identified is that temporal order may be involved in sensory processing but is not stable for long enough to be detected over longer recordings (i.e. order changes over time). Accordingly, before proceeding to further determine how latencies during UP-state transitions might be preserved between spontaneous and stimulus recordings UP-state transition latencies were checked for temporal stability. Using different approaches and recording examples it is shown below that even during spontaneous recording periods most neurons’ preferred latencies (i.e. peaks in PETHs) as well as spiking distributions
Figure 64: **Average UP-state latencies change over time.** A. LEC latencies for all neurons in a mouse visual cortex recording for the first 60 minutes (left) versus the entire 300 minute (right) recording. B. LEC latencies in a cat visual cortex recording for 3 sequential recording epochs (0-30mins, 30-60mins, 60-90mins) reveal that peak-latency-times observed in the first epoch are not preserved over time (see also main text). C. Example of single neuron peak-latency-times computed using a 30 minute sliding window (1-minute increments) reveals the dependency of peak latency on time window (real data: magma colour trace; shuffled data: grey trace; see Methods). Dashed green lines at the bottom indicate cell raster and black lines indicate LEC events, i.e. UP-state transitions. Inset: distributions of average latencies at each minute computed using only odd LEC events vs even LEC events reveals low error in latencies over time (i.e. small difference to the x=y dashed line). D. Top: P-value distributions for pairwise KS tests of distributions in all possible 30 minute windows reveals that distributions are similar between neighbouring periods (i.e. data along diagonal is largely similar) but can vary substantially for periods more than ≈30-minutes apart (i.e. values off diagonal; see also main text). Bottom: Same as (Top) but for shuffled data (grey traces in C(i)) reveals no statistically significant differences are present when shuffling LEC times.
(see below) are only stable for periods of <20-30 minutes and systematically (i.e. non-randomly) drift over periods of >30-90 minutes or longer.

A recent study (Havenith et al., 2011) using a cross-correlation histograms (CCH) method previously introduced (Schneider and Nikolic, 2006) has shown that sequences of up to 12 neurons from cat visual cortex show firing order in sequences of up to \( \approx 15 \text{ms} \) in duration that are referenced to internal beta/gamma oscillations (see Figure 5.31 and discussion later in this chapter). The study also found that firing sequences change as a function of stimulus properties (though 7 of 11 recorded neurons in an example provided maintained the same relative order; see Havenith et al., 2011) and that reliability increased during beta/gamma oscillations. Importantly, the study also found that even for the same stimulus "small changes in sequence can be seen" for recordings made 3-12 hours apart. While these findings of small changes were largely dismissed by the study, they are consistent with the hypothesis that order may change over time and further findings discussed in the following sections.

Below, several approaches are employed to track UP-state transition latencies including: using sliding windows to compute latencies at every minute and track their changes (Figure 64, Figure 5.18, Figure 67); identifying examples of stable and drifting triplet histograms peaks - commonly used in synfire chain studies (see below; Figure 66, Figure 67); comparing the stability and similarity of latency times of non-overlapping recordings (e.g. sequential recording periods) for individual neurons (Figure 68-Figure 70); and evaluating the stability of multi-neuron latency-state-spaces across spontaneous and stimulus evoked recording periods (Figure 68-Figure 70). A simple qualitative test for latency stationarity is to compute latencies across different recording periods (Figure 64). As an example, a 300-minute mouse visual cortex recording is used and all neuron PETHs are computed in the first 60 minutes and recomputed for the entire 300 minute recording (Figure 64A). The distributions become broader over the 300 minute period suggesting that they are sensitive to the duration of the recording. Such latency broadening is not expected if either (i) the latencies of each neuron are stationary over time; or (ii) if spiking during UP-state transitions is randomly distributed within some broader window (e.g. 100ms-200ms). A second example is provided from a cat visual cortex recording (Figure 64B). In the cat example, LEC latencies for 64 neurons were computed during 3 non-overlapping, sequential 30-minute recording epochs and displayed in order of latencies present during the first 30-minute period. The loss of order is visible and computing (Pearson) correlation between the values representing the peak latencies of all 64 neurons (i.e. pairwise comparisons of 2 64-dimensional vectors) resulted in values of 0.4 (first vs second period) and 0.3 (first vs third period). This provides an initial hint that rather than being stationary or randomly changing, spiking distributions for some neurons gradually but systematically change over time (see Figure 70 for systematic correlation value analysis; see also Discussion).

An additional method where latencies were computed using a sliding window approach was implemented (Figure 64C). The method involved computing the latency of each neuron within a 30-minute window of a recording and sliding across the entire recording in 1-minute increments.
(see Methods). This method subsumes approaches which generally compute latencies for either the entire recording or for the first vs the second half of the recording (Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013). In previous studies latencies (or more precisely COM values - see Figure 54) were only computed once for an entire recording period or experiment. Additionally, previous studies did not have the temporal precision provided by LEC events thus prohibiting further dividing of the data into shorter periods or computing latencies for all neurons, in particular lower firing rate neurons (personal communication with A. Luczak). Using the sliding window approach for the mouse visual cortex neuron example (Figure 64C) revealed a gradual change in latency over a period of 300-minutes with most of the change occurring in approximately 100-minute period (50 to 150-minute section). Two controls were implemented. First, the neuron LEC times were shuffled (Figure 64C, grey traces). The resulting UP-state transition spiking distributions are the same for each UP-state transition, but when averaged over windows of 30 minutes (or any period of time) they will lose any inherent differences that were present in the original data that may gradually change over time (i.e. Figure 64C - grey trace values were relatively similar across the entire recording). A second control was implemented where the peak latency time in each window was computed using odd vs even spikes (i.e. selected from all spike times in that 30-minute window). The differences are plotted as vertical error bars at each point in time (see Figure 64C, magma trace vertical error bars). The odd vs. even latency error is also displayed in the inset: each dot represents the peak latency in each 30-minute window computed using odd vs. only even spikes and the distance from the X=Y line constitutes the error in milliseconds (note: this is the same controls implemented previously, e.g. Luczak et al., 2007, 2009). The result is that the vast majority of latency differences are <5ms.

Last, non-stationarity was further evaluated by comparing spiking distributions between all pair-wise 30-minute epochs using a 2-sample Kolmogorov-Smirnov (KS) test (Figure 64D). For the neuron considered, all UP-state transition spike latencies (i.e. times) during the 0-30 minute recording period were compared with spike times from the 30-60 minute recording period. Thus, if the first neuron fired 100 spikes during UP-state transitions in the first 30 minutes of the recording, the relative times (i.e. relative to UP-state transition time) were compared to the transition times in the second 30 minute period of the recording. This KS test is different from comparing peak latencies - as was done above - as entire spiking distributions are compared and not just peak latency times. The results reveal that spiking distributions also change over time: spiking distributions between neighbouring epochs (e.g. <30-minutes) are more statistically similar (Figure 5.17D: darker colours along diagonal) while distributions between recording epochs farther away are more likely to be statistically different (brightest colours indicate pval >10^-6). This provides a largely independent confirmation that spiking distributions during UP-state transitions change on the order of 30-minutes or longer.

Further computing sliding-window peak-latency-times for all neurons in a mouse visual cortex recording revealed that most neurons have stable latencies on the order of several minutes, but
that the latencies drift over periods of 30 to 60-minutes (Figure 65; note: this recording was from a GCaMP6s mouse visual cortex recordings and further analysis was not pursued, however, similar latency trends in other mouse and cat cortical recordings were observed). This computation reveals that neurons maintain relatively similar orders - even as there is an overall correlation in drift across the recording - with some neurons changing their latencies substantially more than others.

The findings that for most neurons UP-state transition latencies are stable on the scale of \(\approx 30-60\) minutes but for some neurons can change over longer periods are generally consistent with previous findings for high firing rate rat somatosensory and auditory cortex neurons which largely considered recording periods of \(\approx 1-2\) hrs but lacked the temporal precision to compute drift (Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013). Interestingly, the results presented here - that neurons can change their relative latency times - are consistent with previous work identifying neuron ensembles (i.e. simultaneous firing neurons) where cell membership within each ensemble could change over periods of many minutes (Ikegaya et al., 2004). Thus, a possible explanation for some neurons’ dropping out from participation in ensemble activity may be that they simply

Figure 65: UP-state latency drift - visual cortex neurons. Mouse visual cortex recording example of all neuron PLs over a period of \(\approx 180\)-minutes. The colours represent depth of recorded neuron (see right of inset for approximate location on probe) and the inset scatter plot represents odd vs. even spike PLs for all neurons across all time windows (Note: several neurons for which the maximum odd vs. even PL error was \(> 10\)ms were excluded as they had very low firing rates or periods during which their latency could not be adequately tracked).
Figure 66: Triplet histograms - examples. Two examples of triplet-histograms from a (wild-type) mouse visual cortex (top) and cat visual cortex (bottom) recordings reveal that some neuron triplets have preferential spiking modes during synchronized state (i.e. anesthetized) cortical recordings. (Scale normalized to peak of histogram; see Methods).

drifted too far away to be visible in simultaneous two-photon activity. Overall, the findings in this section point to a possibly ever-evolving underlying temporal neural code that can be tracked and investigated using LECs (see also Discussion). The results may also provide an explanation for why positive results from synfire chain studies have been relatively rare.

Triplet histograms during synchronized state recordings

The findings above suggest that the average single neuron latency during UP-state transitions is stable over shorter periods (e.g. 30-60 minutes) but changes over longer periods. It would thus be interesting to determine whether this change of stability could be observed across the spiking order between pairs of neurons. One other method for tracking relative spike timing is to compute triplet-histograms distributions (Abeles, 1982a; Luczak et al., 2007; Markram et al., 2015) and search for the peak in histogram activity, i.e. the spiking mode of neuron triplet. The method involves selecting three neurons and computing 2-dimensional histograms where the x-axis represents the cross-correlogram (or cumulative PETH) between the first and second neurons, and the y-axis represents the cross-correlogram between the second and third neurons (see Methods). Peaks in the 2D distributions indicate a preserved temporal spiking relationship between all the neurons.

Triplet histograms were computed for randomly selected 1-hour synchronized state recordings from cat and mouse visual cortex (Figure 66). Three higher-firing rate (>1Hz) were selected and
the 1-hour long epoch was divided into 4 - 15-minute chunks. The triplet histograms (5ms bins) revealed that some neuron triplets have strong histogram peaks (Figure 66) that are near the $\Delta t=0$ bin - which is expected as during synchronized state many neurons spike together. However, a systematic analysis of triplet histograms was not further pursued given the large amounts of data that needed to be analyzed: a 1-hour cortical recording of 60 neurons results in 216,000 triplet histograms. Importantly, results presented in the above section showing that latencies drift over time - calls into question whether strong peaks could be detected given that relative spiking order could be changing. Additionally, there are some concerns in the spike-time neural coding literature regarding the correct statistical tests to be implemented to account for randomly occurring peaks in triplet histograms (Oram et al., 1999; Baker and Lemon, 2003; Hatsopoulos et al., 2003; Gerstein et al., 2012; note: most of these studies look at sparse firing during desynchronized, i.e. awake and/or behaving states).

However, as the neuron spiking considered here comes largely UP-state transitions (i.e. during synchronized state recordings) the number of spikes in the bins are much greater than those capture during desynchronized state recordings considered in previous studies (i.e. the results here are more likely statistically significant). The triplet-histogram was also applied to additional GCaMP6 mouse visual and barrel cortex recordings where longer periods of spontaneous activity were available (Figure 67; note: data comes from Cre-Emx1-dependent GCaMP6 mice). In some of those
recordings, neurons were identified that exhibited strong peaks near \( t=0 \text{ms} \) that drifted up to 30ms over \( \approx 60 \) to 90-minutes confirming that while neurons can fire in precise temporal relationships, those relationships can change over time.

**Peak latencies change systematically over time**

The findings above suggest that most, if not all, neurons have preferred peak latencies (or PLs) near UP-state transitions and that such latencies are stable on short time scales but change over longer periods of time. If PLs are relevant to stimulus processing, then the temporal relationships between individual neuron latencies during UP-states should also be present in neuronal activity occurring during stimulus processing. Several recent studies have found this, albeit only for high firing rate (>2Hz) neurons in rat somatosensory and auditory cortex where FWHM latency distributions for some neurons could be up to 100ms wide (Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013) in contrast to visual cortex distributions where they are \( \leq 10 \text{ms} \). These studies hypothesize that high firing rate neurons fire in packets which act as gating mechanisms to prepare higher-order sensory cortex areas for the arrival of new low sensory cortex information (Luczak et al., 2015).

The findings presented here, however, suggest a more complex picture where PL-based neural code may not be stable on longer time scales. This presents a challenge to the notion that higher-order areas only have to learn PL-order of lower areas if such order changes over time. It would thus be important to investigate how any putative multi-neuron neural code based on PLs might also change over time.

Evaluating the stability of a putative multi-neuron peak latency (PL)-based neural code first requires to computation of PL stability for each neuron and then visualizing PL stability for all neurons using dimensionality reduction. A 2-hour GCaMP6s mouse visual cortex recording was randomly chosen and 9 stable neurons (i.e. high PTP-voltage amplitude and stable firing rates) were identified (Figure 68A; note: none of the cat visual cortex recordings contained spontaneous activity periods longer than about 45-minutes; see also Methods). PLs for each neuron were computed at every minute of the recording using a 30-minute sliding window approach (see above) using events from the 2 LECs with the highest amplitudes (Figure 68B: colours represent the largest (red) and second-largest (blue) amplitude LECs). The relative PL time for neurons computed against the 2 LECs reveal a similar but not identical latency order is present between the two LECs (Figure 68B). Conceptually, the relative PL order of the neurons at each point in time describes a putative firing-order neural code available for stimulus processing - in partial agreement with previous hypotheses (Luczak et al., 2015; the difference here is that all neurons’ relative spike is preserved, whereas previous work suggests only high-firing rate neurons have preserved firing order). Most neurons appear to have a stable latency order that changes minimally over many minutes (in part consistent with previous findings in rat cortex).

The next step is to convert the PLs for each neuron at each point in time into a 9-dimensional (i.e. 1 dimension per neuron) PL-based vector and visualize the trajectory of that vector over the
duration of the entire 2-hour recording (Figure 68C). The PL-space vector trajectory (using the
first two components of PCA space) revealed that the two LECs defined different PL-based neural coding spaces across time. Perhaps more importantly, the highest amplitude LEC (Figure 68B,C: LEC #1 - red colour) showed a systematic - but gradual (i.e. non-random) - change over time (Figure 68A: lighter shading indicates earlier points in recording time).

This finding suggests that any putative PL-order neural code would have to change along with these - relative spiking order - relationships and a more systematic investigation would be warranted (e.g. more neurons, recordings and animals). Although mouse sensory cortex (i.e. visual, barrel and auditory) data had been acquired specifically for this purpose, it was important to avoid the potential issues discussed above in relation to Cre-dependent GCaMP6 mice (Steinmetz et al., 2017). Accordingly, the remainder of the analysis was carried out on cat visual cortex recordings and additional non-GCaMP6 mouse recordings obtained from an external lab (see Methods).

The approach implemented for tracking changes in multi-neuron PL state-space was to repeat the analysis above (Figure 68). However, non-overlapping recording periods (spontaneous and stimulus evoked) were used to preserve the independence of the compared datasets. Additionally, as some of the comparisons were made across high-dimension distributions (e.g. 9 to 64 dimensions) more appropriate distance metrics were employed: the earth mover’s distance (EMD), also known as Wasserstein metric (https://www.encyclopediaofmath.org/index.php/Wasserstein_metric), which is a method for computing the distance between probability distributions; and hyper-angles between high-dimensional vectors. Both of these measures have been previously employed in neuroscience studies: EMD has been previously used to measure distances in concept space in studies of consciousness (Hoel et al., 2013; Oizumi et al., 2014); and hyper-angle values were used to measure the difference between firing rate space vectors in two-photon studies (Carrillo-Reid et al., 2015; Miller et al., 2014). An advantage of hyper-angle values is that they are less sensitive to changes that occur in a single dimension (e.g. a single neuron’s PLs) as compared to euclidean distances. For example, if in a 10 neuron recording it is found that 9 PL values are stable across two recording epochs, but the 10th PL changes by a large amount, the euclidean distance will be biased by such a change, whereas hyper-angles tend to be less sensitive and yield a value consistent with the fact that most neuron PLs were stable.

These approaches were initially implemented in a 3.6hr cat visual cortex recording period containing 8 recording epochs - 3 of which were spontaneous activity (Figure 69; see Methods). The 8 recording periods were concatenated and spike sorted together and the LFP events were clustered into LECs. The PLs relative to the highest amplitude LEC) of 23 stable neurons (i.e. neurons with PTP>80µV that fired throughout the entire 8-recording period) were computed in each recording epoch. The PL order for the three spontaneous recording periods was visualized using PL order displayed in the first recording period (Figure 69A: note: a fourth spontaneous recording period was present, but few neurons fired during that period and it was thus excluded from analysis). The PL display order over the three spontaneous recording epochs indicates a gradual change in
Figure 68: Peak latency-space analysis. A. Firing rate distributions for 9 neurons from a randomly selected mouse visual cortex recording (see main text; see also Methods). B. PLs for neurons in (A) reveal similar but not identical latency times relative to 2 different LECs (trace colour represents firing rate of neuron as in (A)). C. PLs visualized in a PL-space (using PCA) reveal differences between latency times across different LECs (light shading: earlier in time; dark shading: later in time). D. PL-space plot for a single LEC from a different 300 minute long recording in mouse visual cortex visualized using PCA reveals similar drifting codes over time. E. Same data as (D) but shuffled in time yields a randomized distribution.
PL for some - but not all neurons. It is important to remember that the PLs were computed from completely separate recordings yet there is substantial similarity for many PLs even across 2-3 hours of recording time. Qualitatively, this result is largely consistent with findings above that show PL drift is slow but can be larger in some neurons relative to others.

Further tracking the PLs for each neuron across the three recording epochs shows that between the last two recording epochs some neurons changed their PLs substantially (Figure 69B). Next, the ∆PL changes for each neuron were computed and plotted as histograms. Given 3 spontaneous recording periods there are three possible pair-wise comparisons to be made (Figure 69C). As expected, the mean of the ∆PL distributions increased with greater time between recording epochs (Figure 69C, dashed lines indicate mean of distributions: 1.7ms, 5ms and 6.7ms for 53, 108 and 161 minutes separation, respectively). Lastly, PLs were converted into 23-dimensional vectors and the distance between each pair of vectors was computed using a cumulative ∆PL(Figure 69D) metric: the ∆PL values for each neuron across times were summed into a single representative value. The scatter plot (and linear regression fit) also confirm that epochs that are farther apart also have larger cumulative latency differences. In addition to the cumulative ∆PL metric (Figure 69D), the EMD between PL vectors at each epoch was computed with a nearly identical result to the cumulative latency metric (Figure 69E; see Methods).

Next, a similar analysis of ∆PL changes was implemented over larger numbers of recordings to determine whether similar trends would be observed (Figure 5.23). The first step was to identify pairs of spontaneous activity recordings that were <3hrs apart (i.e. to reduce potential errors from spike sorting over longer periods of time). All cat visual cortex recordings (5 cats, 15 tracks, 410 unique recording epochs) were examined specifically for periods of (i) synchronized cortical states coupled with (ii) pairs of nearby (i.e. <200min) spontaneous recording epochs (Figure 48-Figure 50: spontaneous recordings annotated with black colour). As indicated at the beginning of this chapter, the cat visual cortex recordings were acquired in an unbiased fashion, i.e. not specifically aimed at addressing questions of synchronized state PL drift across time. Considering all available cat visual cortex recordings, 6 recording blocks in 6 tracks (i.e. multi-recording epochs) were identified in 2 cats that fit the requirements of containing both synchronized state and spontaneous activity (Figure 48 - recording IDs: C3.2, 3.3, 3.4 - isoflurane + N₂O anesthetic; Figure 51 - recording IDs: C5.2, 5.3, 5.4 - propofol + fentanyl anesthetic). Within each data block, recordings selected contained both spontaneous and stimulus evoked periods. The recordings in each data block were concatenated, UP-state transitions were clustered into LECs and single units were sorted. Additionally, stable neurons (i.e. neurons with PTP amplitudes >80µV and firing throughout the recording block) were identified (# of neurons: C3.2: 17; C3.3: 23; C3.4: 13; C5.2: 23; C5.3: 11; C5.4: 15; see also Methods). The results are visualized as scatter plots of PL similarity vs ∆time between pairs of recordings using both EMD (Figure 70A) and hyper-angle (Figure 70B,C) metrics. The data was plotted separately for propofol and isoflurane recordings. The metrics were computed for pair-wise comparisons across: (i) two spontaneous recording epochs, (ii) a spontaneous record-
Figure 69: Additional methods for tracking latency drift

A. PLs for 23 neurons across 3 spontaneous recording epochs. B. Individual neuron latencies at each epoch shown in (A). C. Distribution of Δlatencies for all neurons between pairs of spontaneous recording periods. D. Cumulative Δlatency metric capturing the sum latency changes across all neurons between recording epochs. E. Same as (D) but differences between epochs were computed using an EMD metric on PL values in each epoch (see main text; see also Methods).

The one exception was from stimulus recordings under isoflurane anesthesia where PL differences between two epochs were larger on shorter time scales and more similar on longer time scales (Figure 70A, C: isoflurane plots, orange dots and traces). This trend was also present in probability distribution comparisons (discussed below) and was likely not an artifact due to insufficient data points. The most likely explanation has to do with the dissimilarity of neighbouring stimulus recordings. In particular, specific stimulus types (e.g. a natural scene or drifting grating visual stimulus) were often repeated during the recording blocks considered, but not immediately after one another - thus resulting in stimulus epochs that were closer together being more likely to be of different types as opposed to stimulus epochs that were further apart. For example, the
Figure 70: PL similarity across spontaneous and stimulus recordings. A. EMD values of PL similarity between pairwise recordings in cat visual cortex recordings acquired under propofol (left) or isoflurane (right) anesthesia in two cats (C3.2, 3.3, 3.4 - isoflurane/N₂O; C5.2, 5.3, 5.4 - propofol/fentanyl). The colours represent the types of recording periods used for the comparison (see inset). B. Hyperangle computation involves identifying the peaks of each latency of each neuron (left), building a N-dimensional vector (where N is the number of neurons) and computing the inter-epoch hyper-angle between the N-dimensional vectors (right; note: the vectors are only visualized in 2 dimensions). C. Same as (A) but using a hyper-angle metric (see Methods).

recording block from C5.2 (see Figure 51 black and blue alternating period) had four spontaneous recording periods that were interspersed with two unique movie stimuli - but the unique movie stimuli alternated so that different movie epochs were closer in time similar movie epochs (i.e. the pattern was: movie #1, spontaneous, movie #2, spontaneous, movie #1, spontaneous, movie #2, spontaneous). However, this exception seems to have a dependency on anesthetic (i.e. present in isoflurane but not propofol based anesthesia) and should be further investigated using specifically designed experiments (i.e. longer spontaneous recordings with identical stimuli interspersed in both cat and mouse visual cortex).

Lastly, PL latency similarity over time was also computed using LEC triggered spiking distributions (as opposed to only peaks of distributions, i.e. PLs in Figure 5.24). Briefly, spiking distributions were computed for each neuron within each recording epoch considered above (i.e. all spikes occurring ±100ms from all UP-state transitions were pooled into a distribution). For example, in recording ID C5.2 which had 8 epochs (see Figure 51), for each of the 23 neurons recorded, the UP-state transition spiking distribution was computed in each of the 8 epochs. Thus, for the each
neuron, there were 8 distributions which reflected when that neuron spiked relative the UP-state transitions in each of the 8 epochs. The 8 spiking distributions could then be compared against each other using a KS test (see above; see also Methods) to determine how the distributions changed over time. This test for similarity across time is a more distribution-agnostic measure of similarity and is largely independent from the PL-order tests carried out above. For example, in contrast to the PL based test above, if some neurons do not fire substantially during some recording epochs the KS test is more likely to report statistically insignificant results - whereas peak latencies will still be assigned in each epoch.

The KS test was applied on combined isoflurane and propofol data sets (Figure 71). For spontaneous-spontaneous recording comparisons the results were as expected: neuron firing distributions became increasingly different with increasing time between the epochs being compared (Figure 71A-left: trends show p-values decrease with Δtime between epochs). This is further confirmed by computing the ratio of statistically significant values (i.e. p-value < $10^{-2}$) vs all computed values in each time bin. This computation is important because most pairwise comparisons fall above p-value of 0.1 indicating they may not be significant. Accordingly, the ratio of statistically significant p-value comparisons over the total number of comparisons provides a better measure of trends considering only statistically significant comparisons (Note: p-values were corrected using Benjamini-Hochberg; see Methods). There results show - as expected - differences appear in distributions increase with separation time between epochs (Figure 71A-right). A similar result was present in the spontaneous-to-stimulus recording comparisons (Figure 71B-right; note, the linear fit was only carried out on the first three bins due as the last bin was from recording separation of >3 hrs which were not considered here).

However, the stimulus-stimulus recording comparisons yielded the same odd-ball results result as the PL similarity test across time (see above Figure 70): single neuron spiking distributions were more likely to be different between closer stimulus recordings than those farther apart (Figure 71C-right).

Lastly, single neuron PL drift over time was compared against neuron firing rate and putative cell type (e.g. pyramidal vs. inhibitory) using extracellular template width metrics previously described (Csicsvari et al., 1998; Barthó et al., 2004; Blanche, 2005; Sirota et al., 2008; Niell and Stryker, 2008; Mizuseki et al., 2009; Sakata and Harris, 2009; Figure 72). There were no clear correlations between neuron firing rate or cell type for all three datasets considered suggesting that all neuron types are subject to varying changes in latency across time.

Overall, these results provide an additional, and partially independent, confirmation that spiking-order is present during individual recording epochs and that changes to order occur gradually over time. This further supports that during synchronized states PL order is not just a result of averaging over random or epoch-dependent neuron firing order.
Figure 71: Trends in UP-state transition distributions across spontaneous and stimulus recordings. A. Left: P-value comparisons between LEC-triggered distributions of neurons recorded during two spontaneous neighbouring periods reveals that distribution similarity decreases with increased time between the recordings. Right: percentage of pair-wise comparisons that have p-values $<0.01$ increases with time between the spontaneous recordings suggesting differences are more substantial for recordings farther apart. B. Same as (A) but for spontaneous vs stimulus recordings (note linear fit on was done using first 3 datapoints only as the last datapoint reflected $>3$ hour recording separation not considered herein). C. Same as (A) but for two stimulus periods. (Note: colour same as in Figure 70; all p values corrected for false discovery rate using Benjamini-Hochberg, see also Methods).
Figure 72: Latency drift vs. firing rate and cell type - cat visual cortex.
Figure 72: (continued from previous page) A. Single neuron latency changes (y-axis) across spontaneous recording epochs (x-axis) as a function of firing rate do not show clear trends. B. Same as (A) but as a function of cell type (right scatter plot). C,D. Same as (A,B) but comparing latency changes between one stimulus evoked and one spontaneous activity epoch. E,F. Same as (A,B) but comparing latency changes between two stimulus evoked epochs. (Note: all x-values are jittered 4 data points (mins) for better visualization).

Spiking order is present outside UP-state transitions

The work in this chapter was aimed at extending previous findings from rat somatosensory and auditory cortex where UP-state transition firing order of high firing rate neurons (>2Hz) were shown to be relatively (i.e. within ≈10-20ms) preserved over recording periods (e.g. 1-2 hours; Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013). The findings above confirm that virtually all neurons in visual cortex of cat and mouse have a preferred firing order during LEC-defined UP-state transitions but that such an order changes gradually over periods of 30-120 minutes. The analysis above has largely focused on tracking neuron firing order changes during UP-state transitions across spontaneous or stimulus evoked recording periods. However, previous work in rat somatosensory and auditory cortex had also focused on stimulus evoked spiking order outside UP-state transitions (see Figure 54B). The findings were that UP-state transition firing order of high firing rate neurons (>2Hz) was also similar to the firing order during the first ≈100ms of a stimulus presentation during both awake and anesthetized recordings (Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013). In those studies the stimuli used were whisker stimuli, natural sounds (e.g. cricket chirps) or pure tones.

It is not obvious how to extend the stimulus-evoked analysis to visual cortex of cat and mouse. One issue is that the data presented here does not contain awake cat or mouse visual cortex recordings (though see Fig 5.27 for a recording from an external lab). However, designing cat experiments where recordings are made during naturally sleep (or anesthetized) periods followed by awake periods is mostly unprecedented as getting cats to focus on visual targets over time is close to impossible (note: very recent virtual reality and eye tracking tools may enable this in the future). While mouse experiments with alternating anesthetized and awake periods have been previously made (by other researchers) that data was not available (though see below for data from another lab).

Perhaps more challenging, however, is determining how to define a temporally brief visual stimulus that would emulate the natural, but brief auditory and somatosensory stimuli used in previous studies. The temporal precision of the onset of a somatosensory or auditory stimulus was critical to determining precise neuronal firing order in such studies. A briefly flashed natural image presented during an ongoing natural scene movie or uniform (dark/grey) background is artificial and arguably has no correlate in the natural visual world. Perhaps a natural scene movie that contains long stationary periods (i.e. no visual motion) interspersed with brief motion periods may
Figure 73: Method for detection of ordered synchrony. A. Detection of synchronously occurring spikes for pairs of neurons involves detection of co-occurring spikes within a 25ms window and binning the results into two bins reflecting percentage of co-occurring spikes fired by cell #1 before cell #2 (+ bin) and percentage of co-occurring spikes fired by cell #2 before cell #1 (- bin). B. Histogram of binned order for co-occurring spikes reveals one neuron fired more often before the other neuron. Difference between bins is shown and is tested for pval<0.01 statistical significance (see main text; see also Methods). Note colour scheme indicates the percentage of co-occurring spikes fired by the first neuron relative to the second neuron.

suffice - this may emulate the behaviour of a predatory animal (such as a cat) during hunting where the visual field is largely stable except for small areas where prey are moving. But it is not certain whether this would be the correlate of a brief auditory chirp or whisker deflection or whether recording from neurons whose RF was located exactly where the motion occurs would be the equivalent of such brief auditory/barrel cortex stimuli.

As such proposed recordings were not available for this chapter, an alternative method was developed that circumvents the challenges of designing novel experiments (and carrying them out) by focusing on spiking statistics (Figure 73-Figure 76). Specifically, if firing order among neurons is preserved during both spontaneous and stimulus recording periods then such order should be detectable in quasi synchronous pair-wise firing order of neurons on time-scales relevant for stimulus processing (Figure 73; see Methods). Thus, if such an ordered synchrony hypothesis holds then neurons that spike within a 25ms window of each other (a window arguably relevant for stimulus representation) should have a biased order that is detectable over longer recordings using simple statistics (e.g. using a binomial distribution test; Figure 73). The only assumption made here is that the width of the window that is relevant to stimulus processing is 25ms. This value was taken from earlier findings showing that during an UP-state transition virtually all neurons recorded fired within a 25ms window of each other (Figure 61B), but tests using 50ms window shows similar (if not even better) results as those presented here. Applying the ordered synchrony method to a randomly chosen 60 minute awake mouse visual cortex recording split into 2 halves reveals that
Figure 74: Ordered synchrony matrix - awake mouse recording. A. Ordered synchrony matrix for 68 neurons from a 60-minute awake mouse visual cortex split into two 30-minute epochs reveals some neurons fire in relative order to other neurons (i.e. similar vertical/horizontal lines). Note: all pairwise comparison values are shown (i.e. even statistically insignificant values; see also main text). B. The stability of firing order for all pair-wise comparisons across time plotted as the % spikes fired by one cell before the other during the first 30 minute epoch (x-axis) vs the second 30 minute epoch (y-axis) reveals some firing is preserved (blue line is linear fit). (continued on next page).
many neurons have substantial bias in the firing order across the two halves (Figure 74A; note: data provided by an external lab; see Methods). Comparing the relative spiking order between the first 30 minute (Figure 74B: x-axis) and the second 30 minute (Figure 74B: y-axis) reveals a bias towards preserved order (see linear fit). The preserved bias in firing order between the two epochs is statistically significant (Figure 74C - binomial test p value $3.8 \times 10^{-25}$). Considering only the statistically significant ordered firing relationships (i.e. firing order p value is $<0.01$) reveals a perfectly preserved firing order between all such pairs of neurons ($2.3 \times 10^{-10}$; Figure 74D, E).

The presence of ordered synchrony is indirectly supported by several two-photon imaging studies which have identified synchronously activated ensembles of neurons in L2/3 of mouse visual cortex over 100-500 µm regions (Cossart et al., 2003; Miller et al., 2014; Carrillo-Reid et al., 2015; Yuste, 2015). Given the lower temporal resolution of two-photon (i.e. calcium events generally reflect bursting neural activity over hundreds of ms) synchronous ensemble activation observed in two-photon calcium recordings may in fact be a correlate of ordered firing during UP-state transitions and stimulus onset.

Most neurons exhibit ordered synchrony with at least one other neuron

The methodology described above was next applied to 2 sequential cat visual cortex recordings (one spontaneous recording followed by a natural scene movie stimulus) acquired during a synchronized cortical state (Figure 75; recording ID: C5.3). Because this was a synchronized state recording, before computing the synchrony matrix all spikes occurring during UP-state transitions were removed (i.e. spikes occurring within a ±100ms window of an UP-state transition were masked). This was done to focus on order occurring outside of UP-state transitions - as order during UP-state transitions had been previously explored at length (see previous sections).

The recording time of the spontaneous recording was ≈16 minutes and that of the following natural scene movie was ≈36 minutes (Figure 75A). Computation of the ordered synchrony values for all pairwise comparisons across the 23 recorded neurons revealed that during the spontaneous recording period 11/23 neurons had statistically significant (p value $<0.001$) biased firing order with at least one other neuron (Figure 75B-left; note: longer spontaneous activity recordings would likely yield more spikes and higher percentage of significant relationships due to nature of binomial test). During the following natural scene movie recording 21/23 neurons had a biased order (Figure 75B-right). Similar results were found across other recordings, with usually >50% of all neurons having an ordered synchrony relationship with at least one other neuron. This is a remarkable result given that only 10-30 neurons were compared at a time in the recordings, suggesting that likely all neurons
Figure 75: Ordered synchrony preserved between spontaneous and stimulus evoked recordings. A. Cat visual cortex synchronized state recording containing neighbouring spontaneous and natural scene movie recordings. B. Ordered synchrony matrix for all 23 neurons recorded reveals similar spike order across many neurons (only data points with p value <0.001 are shown). C. Ordered synchrony during the spontaneous activity recording is abolished when jittering the spikes of all neurons by 25ms (see also Methods). D. Stability of of firing order for all pair-wise comparisons during the recording block (C5.3 had 5 such pairwise epochs) for spontaneous vs. stimulus evoked periods reveals that order is substantially preserved between sequential epochs (blue line is linear fit; see also Figure 73B). E. Determination of pairwise order (irrespective of % spikes fired) across all pairwise epochs considered in (D) reveals almost perfect preservation of firing order (note only data p values <0.01 were shown and considered in D,E).
Figure 76: Similarity of ordered synchrony across spontaneous and stimulus evoked periods

A. Left: distribution of inter-epoch hyper-angles between ordered synchrony vectors of neighbouring recordings in recording presented in Figure 68 reveals a peak in the 0-30 degree bin, i.e. spiking order was similar between two neighbouring epochs. Right: same as (left) but for shuffled vectors reveals largely orthogonal values (see also Methods). B. Left: same as (A-left) but for all recordings presented in Figure 65 and Figure 66. Right: same as (A-right) but for data in (B).

in cortex will have a biased ordered relationships with many other neurons (results not shown). Jittering the spikes of all neurons by as little as 25ms abolished most or all statistically significant ordering observed in the original data (see Figure 75C). Carrying out the same analysis as above (see Figure 74), reveals that the statistically significant relationships are almost perfectly preserved (117 of 118 pairs) across the two recording epochs (Figure 75D,E).

These last tests using awake mouse and anesthetized cat cortex (see above and Figure 74D,E and Figure 75D,E) confirm that firing order is largely present and conserved over periods of 30 minutes or less and that such order is lost when the temporal structure in the original (i.e. real) data is modified (e.g. Figure 5.28C).

In the last example provided above, some of the rows and columns of the ordered synchrony matrix looked qualitatively similar across spontaneous and natural scene recordings (Figure 75B). Accordingly, an additional test of similarity between neighbouring spontaneous and stimulus evoked recording periods was implemented using multi-dimensional metrics (Figure 5.29). The goal was to evaluate the similarity of ordered synchrony of all neurons across short periods of time (i.e. ≈60-minutes or less). The hypothesis was that ordered synchrony relationships would be preserved between neighbouring recordings relative to a shuffled condition. This would provide further (UP-
state transition independent) confirmation that spiking order is present in visual cortex.

For this test two values were compared for each neuron: the N-dimensional vector representing the spiking order of each neuron relative to all other neurons in the first vs. the second recording. For example, for a 23 neuron recording, the ordered synchrony vector for a neuron was computed during a spontaneous recording and then compared against the vector for a stimulus recording. This amounts to comparing the same row from two synchrony matrices. For these comparisons a hyper-angle distance metric was implemented (see above; see also Methods).

Computing the distribution of inter-epoch hyper-angles for the example provided above (see also Figure 70) revealed that synchrony order vectors across neighbouring epochs were more likely to be similar to each other (Figure 5.29A - real data). Shuffling the vectors resulted in a distribution that peaked in the fourth bin (90-120 degrees differences) suggesting that, after shuffling, most vectors were orthogonal to each other. (Note: this result is partially explained as a random rotation of a sparse high-dimensional vector is more likely to result in a largely orthogonal new vector).

This method was next implemented on all data blocks considered for the previous section (i.e. 6 multi-recording blocks from 2 cats; Figure 70, Figure 71). The goal was to determine how any potential order during a spontaneous recording period compares during a subsequent (or preceding) stimulus recording. Thus, recording pairs considered had one spontaneous and one stimulus recording. The resulting distributions revealed that in the real data there is a strong peak in the 50° histogram bin suggesting some differences were present between epochs, but overall the distribution was different from a shuffled condition which peaked in the 90° bin (2-sample KS test pval = 2.3 x 10^-26).

**Measuring spiking order using adaptive coincidence detection**

In the last two sections of this chapter two other broad approaches for evaluating firing order are briefly discussed in relation to the work presented herein. As reviewed above, the search for time structure in neuronal activity has lead to the development of several analytical methods: searching for synfire chains using triplet histograms (e.g. Abeles and Gerstein, 1988; Abeles, 1982a), identifying neuronal firing order during UP-state transitions (e.g. Luczak et al., 2007, 2015), identification of synfire-chains using statistical methods (e.g. Quaglio et al., 2017) and temporal-scale independent methods for identifying repeating sequences (e.g. Russo and Durstewitz, 2017).

An alternative approach to detecting temporal structure in time-trains (including both neural spiking data and other naturally occurring phenomena) has been proposed which is based on adaptive coincidence detection (Kreuz et al., 2009, 2011, 2013) previously introduced as event synchronization (Quiroga et al., 2002). Adaptive coincidence detection is a method for measuring overall (normalized) synchronization between two time series - including spike train rasters. Briefly, given two spike trains, the method matches each spike from one train with at most one spike in the other train and tracks order during such coincidental firing. The method considers only pairs of spikes from two neurons that are closer to each other than to any other spike from the individual
neurons. For example, if one neuron fired one spike 10ms before a second neuron fired its spike and neither neuron fired another spike (previously or after) for at least 10ms, then those two spikes (10ms apart) would be considered coincident and their inter-spike-interval (ISI) and order would be retained for further analysis. In contrast, if one neuron fired a spike 10ms before another neuron fired two spikes 5ms apart, then all spikes would be eliminated from consideration. This is because the second neuron fired (temporarily) at a higher frequency (e.g. 200Hz) than the two neuron spike sequence (100Hz) and the second neuron is thus more related to its own spike train than to the first neuron (see below for caveats to this definition of spiking relationship). The method is heuristic- and parameter-free since the "local spike rates" provide the only parameters required for the method.

Using this "adaptive" approach, three metrics were previously developed to capture different properties of pairs of spike trains: ISI-distance, SPIKE-distance and SPIKE-synchrony (Kreuz et al., 2015). These metrics are largely aimed at temporal-scale independent valuation of spike train ISI similarity, overall spike train similarity and spike train order. Briefly, SPIKE-synchronizion captures a (symmetric and normalized) measure of synchrony across all spikes from pairwise comparisons where a coincidence in spiking is defined as above. SPIKE-distance is another normalized metric that estimates the dissimilarity between spike trains (somewhat similar to Victor-Purpura distance Victor and Purpura, 1996) with low values (i.e. close to 0) indicating very similar spike trains, and high values (i.e. closer to 1) indicating very different spike trains. Lastly, ISI-distance measures interspike interval information through the ratio of instantaneous firing rates of two spike trains.

The metrics discussed above were recently implemented in a freely available Python toolbox called SPIKY (Kreuz et al., 2015). SPIKY was implemented on three randomly selected recordings in the datasets presented above (>1 hour in length) and metrics were computed for non-overlapping recording epochs of ≈25-30 minutes (Figure 77). The goal was to qualitatively assess how the metrics captured firing order for different datasets and whether they could potentially quantify order changes over time. The results were not further analyzed (for reasons discussed below) but two important conclusions can be drawn from the qualitative plots (Figure 5.30). First, the coincidence metrics indicate that non-overlapping neighbouring epochs have largely similar values. This suggests that the metrics capture similar values over time and do not reflect random properties of neuron trains as shuffling spike times (not shown) yields matrices that are substantially different across time. Second, and more relevant to work presented here, the matrices reveal small changes over time. Such changes may reflect the same neuronal firing order changes found in previous sections.

While adaptive coincidence detection methods could be investigated further (e.g. tracking metric value changes over >30 minute periods using hyper-angle or other types of distance measures), it is unclear whether an adaptive coincidence mechanism is employed in cortex. Perhaps the greatest obstacle to further use of these methods is that many if not most spikes from pairs of neurons are
Figure 77: Local time-window metrics of neural synchrony. Adaptive coincidence metrics implemented in 3 visual cortex recordings reveal similar, but slowly changing synchrony values over time.
excluded from computation due to the strong exclusion criteria of the adaptive firing rate method (see above; see also Kreuz et al. (2009)). This would imply that many or most spikes in cortex (and other areas) do not form part of a locally implemented temporal neural code (though it is possible, for example, that locally recorded neurons could be adaptively connected to other more distant neurons that were not recorded). Such a definition of coincident firing excludes almost all spikes fired during a burst. For example, if one neuron fires one spike 10ms before a second neuron fires a burst of spikes (with the first spikes in the burst <10ms apart) then not only are the two neurons’ spikes not considered as coincident (even though they fired merely 10ms apart), but all the spikes occurring during the burst must be discarded (as they will not fulfil the required coincidence criteria). The notion that most (tonic or burst) spiking is not relevant to neural coding is not a common opinion on temporal coding, and it is very difficult if not impossible to test: it would require recording most or all neurons in an organism to determine the amount of adaptive coincidence employed in cortex. Additionally, the metrics above were also not developed specifically for application to neural data and contain other assumptions (not discussed here) as well as yielding normalized and unit-less values which are challenging to directly interpret and compare with other measures of synchronization where time units (e.g. ms) are used. Future work on adaptive coincidence detection may provide useful for analysis of neuronal spike trains with some possible changes (e.g. fixing coincidence windows, or implementing time-unit based metrics; personal communication with T. Kreuz) but given the time limitations of the current work and the outstanding interpretation issues, it was not pursued further as of the time of writing.

Measuring spiking order using pair-wise cross-correlogram order and the principle of additivity

The last approach discussed here for detecting neural firing order in cortex involves identifying firing sequences by fitting gaussians to 1ms bin cross-correlogram histograms (CCH) and converting the resulting 2-dimensional histograms to 1-dimensional linear sequences using the principle of additivity - i.e. assuming that pair-wise firing order is also present in multi-neuron sequences (Schneider and Nikolic, 2006; Nikolić, 2007; Havenith et al., 2011). Perhaps the simplest way to describe this method is to note that it is a solution to the problem of converting 2-dimensional pair-wise order matrices generated in some examples above (e.g. matrices shown in Figures 5.27, 5.28) into relative 1-dimensional firing order sequences between the recorded neurons.

The approach for generating (1-dimensional) multi-neuron firing sequence from all pair-wise order (i.e. CCH) measures has been described in detail previously (Schneider and Nikolic, 2006; Nikolić, 2007; Havenith et al., 2011) and here the steps are described briefly and applied (in simplified form) to one example from a cat visual cortex recording during a drift bar stimulus presentation. As explained below, the steps in generating neuron sequences are complex and involve tracking of errors, application of several heuristics and statistical solutions to fitting gaussian distributions to sparse data.
There are two stages involved each containing multiple steps: (i) computing gaussian fits for pair-wise CCH for all neurons and (ii) converting the CCH matrices to firing sequences (Figure 78). The first step requires removing stimulus-locked rate covariation from the neural responses usually by determining when a stimulus (e.g. a drift bar) enters and exits a neuron’s RF and excluding those periods before and after from analysis, respectively. Next, 1ms-bin CCHs are computed for all neuron pairs, but for statistical reasons (i.e. fitting gaussians) only pairs of neurons with at least 3-consecutive 1ms bins that contain >8 entries each are considered (Figure 78A). As not all stimulus types evoke substantial spiking from all neurons (e.g. a non-preferred drift grating orientation) additional heuristics must be applied to exclude non-optimal stimuli from consideration. In a previous study it was found that on average only 3.5 out of 8 possible drift grating orientations could be used to generate sufficient spiking data (Havenith et al., 2011). Last, rather than fitting a gaussian to each CCH distribution, a correction must be implemented to address the “lacking distributivity of errors” (see Havenith et al., 2011 for in depth explanation). This is achieved by randomly splitting each CCH distributions into 2 halves, fitting gaussians to each half and repeating the process 100 times on the randomly selected data. The final gaussian distribution is obtained by averaging over the 100 gaussian fits.

The second stage involves computing firing sequences from the CCH matrices derived above (Figure 78B). This is done by assigning each neuron a relative time position based on the average of its CCH peaks (i.e. relative spiking times) with other neurons. For example, if, on average, neuron A fires 5ms (i.e. has a CCH peak) before neuron B and 10ms before neuron C, it’s relative time position will be 7.5ms ((5ms+10ms)/2). Additionally, if neuron B fires on average 6ms before neuron C, its relative time position will be 0.5ms ((5ms+6ms)/2). Neuron C’s relative time position will be -8ms ((-10ms+6ms)/2). Thus the 1D spiking sequence generated from the pairwise CCHs will be - A: 7.5ms, B: 0.5ms, C: -8ms (note: sum always should =0ms). There are a number of controls and error computations that can be done to test the validity of the additivity postulate (for complete details, see Havenith et al., 2011). One type of error is called additivity error and it measures the error between the 1D spiking sequence time differences and the original CCH peaks. For the example above, this is computed in a few stages. First, time differences between 2D and 1D time sequences are computed. The time differences for neuron A are A-B: 3ms; A-C: 5.5ms; for neuron B they are B-A: 2ms; B-C: 2.5ms; and for neuron C error they are C-A:5.5ms; C-B: 2.5ms. The individual errors are squared and summed for each neuron to yield a value $Q_{Add}$. For example neuron A, $Q_{Add} = 3^2 + 5.5^2 = 39.25ms$. Lastly, the error is normalized by the number of delays computed (for rationale, see Schneider and Nikolic, 2006):

$$\sigma_{add} = \sqrt{\frac{Q_{Add}^2}{(n-2)n^2}}$$

(8)

While the errors can be substantial, previous studies concluded the average additivity errors were <1ms (Havenith et al., 2011). One important note is that in the previous study of firing
Figure 78: Computing firing order using CCHs and the principle of additivity. A. Pairwise CCHs computed for 7 neurons during a drift-bar experiment reveal the presence of firing order bias across the neurons. B. Using additivity, the pairwise CCHs are converted to a firing sequence. C. Firing sequences for the same stimulus can be different for recordings 7 hours apart. Adapted from Havenith et al., 2011 with permission.
Figure 79: Local time-window metrics of neural synchrony. A. Example CCHs with significant peaks in the ±25ms bin for neurons from a drift bar recording in cat V1 (C3.2). B. PLs for the same neurons in (A) from a neighbouring synchronized state spontaneous activity recording. C. Comparison of UP-state latency difference (x-axis) vs. CCH peak (y-axis) reveals a correlation between firing times across the two methods (blue line is linear fit and CCH peaks were normalized to sum to 0ms). D. CCH-based 1D firing sequence for 3 neurons (IDs: 2, 15, 18; x-axis) vs. UP-state transition based order (y-axis) also reveals a strong correlation between neuronal firing order using the two methods (note: CCH peaks and UP-state times were normalized to sum to 0ms; see also main text).

sequences generated from CCH order, changes in firing order were observed for the same stimulus presented 7 hours apart (Figure 78C). While those results were not pursued further at the time of the publication, such firing sequence changes are consistent with the findings of UP-state transition drift over time.

Given the findings of this chapter on UP-state generated neuron firing order for all neurons - the
The best implementation of the additivity based approach would be to compare how UP-state transition order relates to stimulus driven order determined using CCHs. The ideal experimental paradigm should thus involve a synchronous state recording period (e.g. anesthetized or SWS period) - during which UP-state based firing order can be determined - followed by a desynchronized state (e.g. awake, or NREM) period during which various stimuli can be presented and additivity based order can be computed. A desynchronized state is ideal because firing order detected during synchronized states using the CCH method may nonetheless reflect firing order during UP-state transitions. This requires a spontaneous activity recording adjacent to a drift bar or drift grating recording during a synchronized state that changes to a desynchronized state during the stimulus presentation. Measuring LEC based firing order (i.e. PLs) could then be compared with the CCH gaussian fitting approach described above. Given time and space limitations on the work presented here, this method was not fully implemented in this section (note: it will be pursued in additional proposed work for publishing the work of this chapter). However, it should be noted that in previous studies CCH approach was only implemented in anesthetized cortical recordings (without clear indication of cortical state measures) during preferred stimulus orientations as it required substantial amount of spiking for the neurons considered and it usually only identified 7 neurons (on average) per recording that could be converted into a firing sequence. In contrast, UP-state transition order identified here can provide a preferred order for the vast majority of neurons even during spontaneous activity periods when spiking is very sparse.

Across all available recordings, only one synchronized state recording example was found where a spontaneous activity recording was next to a drift bar recording (C5.4). For that pair of recordings, a simplified CCH-based firing sequence approach was implemented to determine if neuronal firing order might be similar between the two recording periods (Figure 79). First, CCHs were computed during the drift bar experiment for all pair-wise neurons (23 neurons in total) and CCHs with substantial peaks were selected (Figure 79A; total unique neurons: 11; note: stimulus ON/OFF covariation was not corrected for). Next, UP-state transitions latencies for the spontaneous activity recording were computed for all neurons (Figure 79B; note: not all neurons fired during the spontaneous activity period to generate PLs). CCH-peak based order was then compared with the UP-state ∆latency order which revealed a correlation for several neurons with a linear fit that was close, but not identical to the x=y line (Figure 79C; note: CCH peaks sum was normalized to =0ms as per Havenith et al., 2011). Lastly, 5 neurons were identified which had inter-neuron CCHs with clear peaks (IDs: 2, 11, 15, 18, 21) and their firing sequence was computed as per Havenith et al., 2011 and compared with UP-state firing order (Figure 79D; note: only 3 of the neurons had PLs that were within ±25ms to match the CCH window; see also figure text). The CCH-based firing sequence order also shows strong correlation with UP-state latency based order.

While some corrective steps were skipped in this analysis, this result does indicate some correlation between firing order across the two different methods. Overall, the additivity postulate is promising and future investigations should consider it in complement to UP-state transition based
ordering as an additional way to corroborate the presence of spontaneous and stimulus driven spike order as well as confirming order drift over multi-hour recordings.

Discussion

In this chapter it was shown that during synchronized cortical states, multi-channel LFP recordings in mouse and cat cortex contain transient large amplitude LFP events lasting ≈50-200ms that can be clustered on the basis of their multi-channel extracellular waveforms into 1 to 4 distinct classes termed LECs. These large amplitude LFP events have been previously shown to be the correlates of single neuron UP-state transitions and the methodology used provides a spiking independent definition of UP-state transition and can be applied to sparse firing cortical regions including visual cortex. The identified LECs and their CSD correlates revealed that UP-state transitions fall into classes with distinctive laminar signatures and can be grouped within and across animals. LECs were also shown to have mesoscale correlates using VSD recordings. Additionally, almost all neurons showed narrow firing peaks and distributions near UP-state transitions. While both spiking latencies and distributions were stable for most neurons on the order of <30-minutes, many neurons showed changes in their PLs (and distributions) over periods of 30-120 minutes. Lastly, it was shown using both stimulus and spontaneous recordings, that even outside of UP-state transitions spiking order is biased for many neuron pairs and that such biased order also appears to change with time. In this discussion section, the findings presented above are further explained relative to existing studies and some opinions are provided for why temporal order may be present in visual cortex but appears to be drifting over time.

**LECs are a global, single-neuron independent, temporally precise correlate of UP-State transitions**

Synchronized state DOWN-to-UP state transitions (originally termed K-complexes, Amzica and Steriade, 1998a) have been studied for a few decades with the term UP-state referring to single neurons membrane transitioning from a hyperpolarized (i.e. non-spiking) to a depolarized (i.e. often spiking) state. Additionally, the term UP-state also refers to the global correlate of UP-state transitions where the vast majority (or possibly all) cortical neurons transition to depolarized states (Neske, 2016). This ambiguity in terminology is perhaps understandable as it is generally accepted that global UP-states do cause all single neurons to depolarize but not all neurons spike on every cycle (Volgushev et al., 2006; Chauvette et al., 2010). This makes it challenging to track UP-states globally solely by patching 1-2 neurons. In fact, patching 2-4 neighbouring (e.g. 100-200µm apart) cells shows that even such closely spaced neurons single can enter the UP-state cycle at different times and in different orders (Ros et al., 2009) - consistent with the present results - with membrane potential dynamics during the transition period varying substantially in different cycles (Chauvette et al., 2010; see Figure 53). As an alternative definition of global UP-state transitions, LECs have
advantages over single neuron patch clamp recordings. First, they take into account spatially broad (i.e. 100µm to 1000µm) LFP contributions from multiple sources (Buzsáki et al., 2012) from many (or all) cortical layers. Second, they are independent of any specific single neuron activity and can thus be used as single neuron-independent event triggers. Lastly, the temporal precision of LEC events can be as low as ≈5-15ms based on the stability of the FWMH of max-channel LFP trough or the width of single neuron PETH histograms. In fact, given the stochastic nature of UP-state transitions (i.e. only some cells participate every cycle), using membrane potential dynamics even from many single patched neurons to define a global UP-state transition time is not only impossible due to current experimental limitations but must ultimately rely on averaging UP-state transition on-times for individual neurons. Additionally, single neurons can take >50ms to transition to UP-states (see membrane depolarization profiles in Figure 53) and aligning and averaging over such profiles is unlikely to be more precise than an LFP based definition. When coupled with the higher precision and stereotyped shapes of multi-channel LFP signals during UP-state transitions, LECs can provide a more precise, principled and non-circular definition of global UP-state which can be used to further investigate coding in cortex. While not addressed in our study, UP-state transitions are known to mediate hippocampus to cortex interactions (Wilson et al., 1994; Sirota et al., 2003; Sirota and Buzsaki, 2005) and such studies will also benefit from temporally precise methods for identifying UP-state transitions.

Multiple LEC types suggests multiple sources of UP-state genesis

Although it has been previously shown that UP-state transitions have LFP correlates (Saleem et al., 2010; Chauvette et al., 2010), the method presented here is novel and the findings (e.g. multiple classes of LFP events) are consistent with a growing body of work identifying stereotypy in LFP recordings: LFP recordings in rat hippocampus slices showed stereotyped shapes which could be clustered (Reichinnek et al., 2010) and LFP recordings in anesthetized macaque hippocampus also exhibited LFP shape similarity (Ramirez-Villegas et al., 2015). In fact, using only MUA activity to define UP-state transitions, a recent study found two types of UP-state transitions in ketamine/xylazine-anesthetized rats (Luczak and Bartho, 2012). The findings of multiple classes of UP-state transitions is enforced by CSD correlates which have very distinct laminar patterns - common across cortical areas and different animals - supporting the involvement of discrete cortical circuits in UP-state initiation. These findings are consistent with suggestions such as the three cardinal oscillator hypothesis that UP-states can be caused and sustained by potentially independent cortico-thalamic-cortico populations: synaptically-driven cortical populations involving neurons in L4 and L5/6, glutamatergic thalamo-cortical (TC) neurons from multiple nuclei which have intrinsic oscillatory properties and GABAergic reticular thalamic neurons which also have oscillatory properties when coupled with external input (Crunelli and Hughes, 2010). The fact that UP-state transitions are stereotyped and fall into 1 to 4 classes based on their LFP waveforms - as opposed to falling into a continuum of waveforms - also supports the multiple oscillator hypothesis.
UP-State transitions might engage discrete cortical avalanche circuits

The findings of discrete CSD patterns correlating with UP-state transitions (i.e. LECs) is unexpected as while UP-state transition extracellular templates (i.e. LFP averages) are similar in shape across multiple cortical layers they can yield CSD templates that are highly heterogeneous (e.g. see Figure 56B for comparison between LFP template and CSD). This suggests that while UP-states transitions can be initiated via broad multi-laminar inputs, once activated they recruit multiple (1-4) laminar-specific circuits as they evolve into global phenomena. LEC template troughs, in particular, are generally the largest and most dynamic (i.e. fastest changing) components of the LFP during UP-state transitions and previous work has connected spontaneous "negative LFP deflections" to neuronal avalanches even in awake monkey recordings (Petermann et al., 2009).

Cortical avalanche research originated in self-organized criticality studies which sought to characterize the dynamics of naturally occurring phenomena such as weather patterns and chemical reactions (Bak et al., 1987). Self-organizing criticality is a "property of dynamic systems that have a critical point as an attractor" (https://en.wikipedia.org/wiki/Self-organized_criticality). What this means is that such systems tune themselves and always seek what are inherently unstable points between two (or more) states. In the context of neuronal activity, cortical avalanches are generally described as sequences of synchronized neural activity bursts with both spatial extent (e.g. the amount of cortical tissue engaged) and duration (e.g. the length of time they are sustained for) being captured by power law distributions which often fall precisely at the self-organizing criticality value (i.e. $1/f^{3/2}$ distributions). Cortical avalanches and power-law distributions have been shown to occur in neuronal cultures (Beggs and Plenz, 2003, 2004), cortical slices (Jimbo and Robinson, 2000; Beggs and Plenz, 2003), anesthetized cats (Hahn et al., 2017), awake monkeys (Petermann et al., 2009; Hahn et al., 2017), human EEG (Pritchard, 1992) and other paradigms. UP-state transitions specifically have been studied as phase-transitions and self-organizing criticality in several modeling studies (Mejias et al., 2010; Millman et al., 2010; Scarpetta and de Candia, 2014).

The findings presented here suggest that UP-state transitions may not only engage distinct circuits, but that they may be a type of default activity mode that manifests at a certain spatial scale (i.e. all cortical layers). It may be the case that the criticality identified in cortical recordings in general, may also describe the multi-laminar, possibly multi-cortical area, nature of UP-state transitions. Future work that focuses on the temporal and spatial dynamics of LFP and single neuron activity during UP-states transition may provide a further link between synchronized cortical states and criticality as it is present in cortex.

Spike timing codes might be ubiquitous - but difficult to track due to drift

An ongoing debate in neural coding is how information is represented by the firing of neurons with much physiological evidence supporting that neuron firing-rates are important as they are modulated by stimulus intensity (Adrian, 1926; Hubel, 1959; Shadlen and Newsome, 1994 and
many others; but see Hemmen and Sejnowski, 2006 for outstanding questions). Yet, precise neuron firing times also encode information even to µs precision (Jeffress, 1948) with more recent findings suggesting 1-2ms spiking precision (in vitro; Mainen and Sejnowski, 1995) and 10-20ms precision in-vivo during natural scene viewing (cat V1; Fregnac and Zador, 2005). A very recent finding suggests that information is encoded even in the precise spike timing of spikes within a single burst of thalamic neurons (Mease et al., 2017). Evidence of firing order between cells, i.e. of synfire chains (Abeles, 1982b) has been limited, but the studies of high firing rate neurons in rodent auditory and somatosensory cortex - that this chapter sought to extend to visual cortex - suggest neurons fire in a generally preserved order during UP-state transitions as well as stimulus presentation (Luczak et al., 2007, 2009, 2013, 2015; Bermudez-Contreras et al., 2013).

The findings presented here suggest that it is not just high-firing rate neurons in highly active areas - but that all neurons in all mammalian sensory cortex including cat V1 and mouse visual cortex may fire in a transiently preserved order during both spontaneous and stimulus evoked periods. This may mean that neurons spike together temporarily to represent specific information at various times (see also below). Future work expanding the methodology presented here using larger datasets, including naturally sleeping animals and complex stimuli, may yield further insight into how common temporal or sequence coding is in sensory cortex.

**LTP/LTD may underly temporal code change over time**

The results presented here indicating that relative firing order changes over time should not be surprising as synaptic processes underlying learning and metabolic activity are likely to constantly engage long-term-potentiation/depression (LTP/LTD) mechanisms that have as their goal the modification of connectivity between neurons. Importantly, spike-timing-dependent plasticity (STDP; Bi and Poo, 1998; Yao et al., 2004) is highly sensitive to the order of inputs and can make use of millisecond precise spiking order. Changes in connectivity driven by underlying processes that are stimulus-independent could thus manifest as changes in spiking order.

Future experiments where simultaneous extracellular and two-photon data is acquired in chronic recordings over several (e.g. 3-5) hours, and over multiple days with possible pharmacological interventions limiting plasticity may further elucidate the relationship between latency drift and LTP/LTD. However, it is also possible that latency drift is related to sleep homeostasis mechanisms that can only be properly characterized using future technologies where large scale synaptic imaging can be captured and related to single neuron latency drift.

**The null-hypothesis should be that firing order changes over time**

Although temporal order and drift have been shown using different methods, a few additional comments are warranted regarding the possibility that PL drift could result from poorly sorted neurons. First, isolating single neurons over multiple-hours of recording continues to be a challenging task.
in experimental neuroscience. Accordingly, analysis in several sections in this chapter was limited to using only large amplitude neurons that fired relatively stably over recording periods of <200 minutes in length. While some neurons may have been incorrectly sorted, it is unlikely that most neurons across multiple datasets contained sorting errors that would lead to the findings made herein. Additionally, tracking changes in spiking distributions was challenging given that cat visual cortex datasets available were not designed with this goal in mind.

While the findings were limited to datasets of ≤30 (stable) neurons which were largely selected for their stability, future recordings designed to specifically investigate latency drift (with potentially improved hardware and acquisition paradigms) should allow for the recording of several dozens or hundreds of neurons simultaneously (e.g. neuropixels probes Jun et al., 2017b) across multiple regions within a sensory area. Given that the relatively small datasets considered here already yield trends and statistically significant differences, it is likely that larger datasets will clarify the results presented here rather than diminishing them. More importantly however, is that the null-hypothesis for temporal order between spiking neurons should not be that temporal spiking relationships - if they exist - stay the same over periods of hours or days. Rather, given STDP mechanisms that operate at the millisecond scale, the extraordinary complexity of cortical networks which appear to self-organize into scale free and small-world networks, the diverse input to single neurons (up to 7,000 inputs per mouse visual cortex neuron), the inherent plasticity mechanisms such as LTD/LTP and the requirement for learning - temporal relationships of single-neurons are unlikely to be preserved for indefinite periods of time. Such temporal relationships must be inherently malleable if they are to underpin learning and plasticity mechanisms that are required for organism survival.

Put another way - it would be much more surprising and interesting - if temporal relationships are present and they are static over periods of many hours or days. This would essentially mean that a partial Rosetta Stone for understanding cortical coding may indeed be available from such temporal relationships. This was in part hypothesized by Luczak et al., 2015 in relation to high-firing rate neurons which show more temporal stability, however, the temporal variability of PLS stability in those studies was commonly ≥10-20ms and drift analysis was not likely not feasible (Luczak et al., 2007, 2009, 2013; Bermudez-Contreras et al., 2013).

Non-stationary neural codes may reveal complex metastable attractor manifolds

In two sections of this chapter it was shown that both single neuron peak latencies and spiking distributions during UP-states change on the order of dozens of minutes (e.g. 30-120 minutes or longer). An obvious question arises, namely, if stimulus information is encoded in the relative order of neurons - but such order is constantly changing - how is it possible for downstream areas to decode this information?

The simplest answer is that cortical decoders may also change simultaneously with the firing-order dependent encoders. For example, the same underlying mechanisms that lead information
being encoded in temporal but non-stationarity relationships could also underpin decoders that take advantage of non-stationarity. In fact, this is the exact hypothesis of a couple of recent theoretical studies namely that transient representation is possible in neural network models and is supported by neuroscientific evidence (Rodny et al., 2017) and that self-reconfiguration of neural circuits can be observed as a "slow drift of network architecture and dynamics" (Kappel et al., 2017).

Nonlinear systems concepts such as metastability and self-organization have been around for a long time and where introduced to neuroscience over two decades ago (Kelso, 1995). In fact, the idea of neural code drift is consistent with the notion of a "dynamically changing attractor manifold" which has been explored by some theoreticians in network models (Friston, 1997). Previous work was aimed at modeling optimal complexity and found that neural activity "dynamics are modeled by neither an ensemble of separate attractors nor a simple low-dimensional attractor, but are consistent with the attractor surface that ensues when many separate attractors are loosely coupled together" (Friston, 1997). Importantly, the work also suggested that an attractor manifold - as might be instantiated by a temporal spiking order neural code - may only appear to vary due to limitations in measurement.

Recent studies of firing rate state-space manifolds in motor cortex (Gao and Ganguli, 2015) have suggested that recording from ever increasing number of neurons may not necessarily yield additional information as a large majority of firing-rate based variances are captured on low-dimensional manifolds using even <100 neurons. In other words, the amount of variance explained by such low-dimensional manifolds increases asymptotically with increasing number of neurons being recorded in motor cortex (and possibly other association cortical areas).

However, the work presented here suggests a somewhat different picture for low sensory cortex where an underlying neuron firing order appears to change over time even in the absence of sensory stimulus or tasks (Figure 68C-E).

Additionally, it is important to note that the results presented here were based only on very small numbers of cortical neurons (i.e. 10-30 neurons for state space analysis and <100 neurons for other analysis). A more complete picture, e.g. recording from thousands or tens of thousands of visual cortex neurons in mice (or cat) may provide a much better description of potential types of codes and underlying manifolds that could change over time.

**Future work**

Despite being present only during behavioural states such SWS, anesthesia or quiescent awake states, UP-states have become an invaluable tool in investigations of neural coding in both awake and anesthetized mammalian cortex. The main aim of this chapter was to improve and extend findings of single neuron order during UP-state transitions to the visual cortex of cat and mouse. The findings of multiple classes of UP-state transition type were confirmed across multiple cortical areas in two species, and using four different anesthetic preparations. These broad experimental datasets suggest that LECs are a robust and ubiquitous phenomenon in mammalian cortex. The insight of
UP-state neuronal firing order first referenced almost two decades ago (Steriade and Amzica, 1998) has now lead to findings of preserved firing order of high-firing rate neurons (e.g. Luczak et al., 2007) and now can be extended to all spiking - in/outside of UP-states, including desynchronized states which may have no inherent UP-state structure. Further experiments, using preferably chronic recordings from multiple animals in different cortical areas may reveal distinct cortical correlates of LECs. Specifically, habituation paradigms and recordings from awake, quiescent animals, and during SWS may further reveal more nuanced neural codes and latency behaviours. An interesting goal might be to determine whether PL order resets or cycles (e.g. based on 24-hour circadian rhythms) or whether it changes unidirectionally over time to potentially describe infinitely diverse coding strategies.
Conclusion

“One could define the central goal of neuroscience as breaking the neural code - deciphering the relationships between spatiotemporal patterns of activity across groups of neurons and the behavior of an animal or the mental state of a person.”
Yuste and Bargmann, 2017

Spontaneous neural activity, i.e. activity in the absence of stimulus, is arguably an understudied field of research in systems neuroscience which is dominated by reflexive experimental protocols involving sensory stimuli and task paradigms. This thesis characterized several aspects of spontaneous single neuron activity across multiple spatial and temporal scales. Visual cortex recordings in cats and sensory cortex and thalamic recordings in mice using both extracellular electrophysiology and optical imaging provided extensive datasets which were analyzed using elementary (e.g. averaging) and more complex (e.g. state-space) analytical methods.

Analyzing the spiking activity of dozens of single neurons recorded simultaneously requires the development and testing of spikesorting algorithms. In order to keep up with developing electrode technology, novel event (i.e. spike) detection and clustering algorithms must be developed and tested against datasets containing tens or hundreds or electrode channels and ground truth of hundreds or thousands of neurons. The limited in vitro (i.e. cortical slice) datasets available contain only a few neurons (usually 1 neuron per recording) but provide the only available real tissue recordings that can be used to evaluate spikesorting algorithm performance (Fig 3.2-6). Simulated, i.e. in silico, datasets generated using the most advanced single neuron and network models (currently available) can provide useful and realistic datasets. Sorting statistics from such datasets are very similar (i.e. statistically indistinguishable) from in vivo and in vitro data (Fig 3.19). Sorting across simulated datasets reveals that while mature sorting suites can yield similar qualitative results, there are substantial differences (e.g. 50% or more neurons identified by one operator vs another) indicating operator skill may be more important than sorting suite algorithm. Sorting simulated datasets confirms that while higher density probes provide lower error rates sorting accuracy may saturate below a certain electrode density threshold (e.g. <20µm inter-channel spacing) with further increases in channel density not as useful for unit isolation. For the data analyzed in this thesis, SpikeSorter (Swindale and Spacek, 2014; Swindale et al., 2017) provides sorting results that are quantitatively as good as other sorting software suites. But many spike sorting challenges remain including sorting lower amplitude units, correcting for drift during both acute and chronic recordings, improving sorting automation especially for hundreds of channels of data and developing better ground-truth datasets representing all brain areas and cortical states. Importantly, modeling of electrode layouts appears to suggest that narrow, single column electrodes may yield similar sorting quality while being potentially less damaging to tissue. This may be
an important conclusion which should inform ongoing efforts at increasing electrode densities in extracellular probes.

The physiological findings presented in this thesis relate largely to spontaneous neural activity in mammalian cortex and thalamus and span multiple spatial scales: from the single neuron scale (e.g. 10-15µm somata) to the entire dorsal cortex of mouse (8-10mm across). Novel electrophysiological methods enabled the recording of activity of dozens of single neurons and correlating with mesoscale neuronal imaging methods that capture bihemispheric calcium activity (e.g. using GCaMP6; Chapter 4) and membrane voltage (e.g. using VSDs; Chapters 4 and 5). These methods enable the comparison - using correlation and averaging - of single neuron spikes (which have <1ms precision) to entire regions of cortex (recorded with 6.7ms - 33ms precision). The spike-triggered-mapping method used in calcium imaging supports a strong link across spatial scales even during spontaneous activity suggesting that spontaneously active neurons generally participate in monosynaptically connected, functional networks. Subcortical neurons recorded from thalamic nuclei exhibited additional variances in their relationship to dorsal cortex suggesting they are involved in more complex, likely poly-synaptic functional networks. VSD imaging provided additional insight suggesting that the homogeneity present in the cortical neuron triggered GCaMP6 maps may not be present on faster time scales and across membrane voltage profiles reported by VSDs. This supports future large-scale investigations of single-neuron VSD maps in visual (and other cortical areas) which may reveal spatio-temporal mesoscale activity patterns that span a comprehensive representation-space of mono-synaptic connectivity between single neurons and dorsal cortex. Future spike-triggered mapping work should focus on a number of improvements in both experimental acquisition and analysis including exploring cortical states (e.g. using synchrony index Saleem et al., 2010) and other cortical and subcortical areas. It would also be interestingly to use higher spatial resolution imaging such as two-photon microscopy to compute spike-triggered-maps which may reveal specific ensembles correlating with the activity of distant neurons that could not otherwise be captured (Yuste, 2015).

Spontaneous single neuron activity also appears to have an inherent structure that may be important for understanding stimulus processing. Several previous studies found order during intrinsic neuronal activity of high firing rate neurons in auditory and somatosensory areas of rats occurring during spontaneous activity (e.g. Luczak et al., 2007, 2015). Developing a novel method to pursue this work further in visual cortex revealed a number of interesting findings including that UP-state transitions appear to fall into distinct LFP-event-classes - LECs - which suggests multiple types of UP-state transitions in cortex. Using this method, the latency of almost all (>90%) neurons can be tracked during UP-state transitions to provide a better and more complete analysis across all sensory cortex. Multiple approaches further confirmed that over periods longer than 30 minutes most neurons can change their relative UP-state latencies peaks as well as distributions. Importantly, these changes can also be observed in pair-wise synchronous firing order outside UP-states and even during desynchronized or awake states. These findings enable the investigation of
firing-order based neural coding in all areas of cortex and suggest a potentially much more complex scheme for temporal neural coding whereby a constantly changing order may represent complex information for downstream areas from primary sensory cortices.

Current experimental limitations such as only being able to record from ≈100-300 neurons simultaneously with millisecond precision will likely keep the neural coding debate active for many years to come. On the basis of association cortex recordings (e.g. monkey motor cortex) some have recently argued that "recording more neurons while repeating simple behaviours may not yield richer datasets" (Gao and Ganguli, 2015) by showing that motor cortex firing-rate based neuronal activity trajectories do not increase in complexity with ever increasing number of recorded neurons.

Based on some of the studies reviewed and many findings made herein it is clear that this cannot be the complete picture of neural coding - at least not in low sensory systems. It can be argued that until thousands (or hundreds of thousands of neurons) can be recorded simultaneously with millisecond precision the debate over neural coding will likely not be resolved. Until that time, it is be important to be guided by data analyses that are generally agnostic regarding a more complete theory of cortical coding - especially in low sensory systems. Unraveling the story of mammalian cortex function seems to be much more complex with precise neuron inter-spiking relationships likely playing significant roles.


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Bionet, Biovis and cluster simulations

Allen Institute Tools: Bionet and Biovis

The simulations described in Chapter 3 were run using tools developed by the Allen Institute, the BBP and the author.

Initial simulations carried out in 2014 (see Figs 3.8, 3.12, 3.13) were based on early versions of the Allen Institute network model, currently named Bionet, 12 biophysically detailed neurons published through various BBP projects (Hay et al., 2011, 2013; Hu et al., 2009; Norenb erg et al., 2010) and the author’s custom Fortran code for computing extracellular voltages from transmembrane currents using the line-source-approximation (LSA; Holt and Koch, 1999).

Since then Bionet (http://www.github.org/bionet) has gone through further development and now functions as a "high-level Python interravce to NEURON" that enables simulating previously constructed networks and outputs data including spikes, somatic or dendritic $V_m$ or extracellular potentials. Networks can be built using any tool as long as the resulting multigraph containing nodes (i.e. cells) and edges (i.e. connections) is specified according to Bionet’s configuration. Net-Builder (www.github/netbuilder) is an existing tool for building networks that is in use. Bionet is in the process of being submitted for publication (Gratiy et al., 2017). Many contributors have participated in the development of Bionet (including the author), but the main developer is Sergey Gratiy, Scientist-1 at the Allen Institute.

In support of the latest version of Bionet, a visualization toolbox has been developed by the author named BioVis (http://www.github.org/catubc/biovis). BioVis was developed for use in Jupyter Notebook (http://jupyter.org/) which is a web application that combines live code and visualizations in an dynamic fashion. BioVis is written in Python and relies on OpenGL (https://www.opengl.org/) based polygon primitives for visualizing up to several million components simultaneously with $>10$ frames per second.

Using the Neuroscience Gateway at the University of California, San Diego

Simulating multi-neuron networks using the NEURON engine has gone through substantial improvements over the past two decades with current Python and C-based wrappers enabling the use of parallel processing on modern multi-node/core PCs and clusters. The simulations presented in this thesis were initially run on the author’s 32-core workstation and eventually migrated to Neuroscience Gateway (NSG) at the University of California, San Diego - Supercomputer Centre (https://www.nsgportal.org/). The NSG is a free super-computer resource funded by the National Institute of Health and is aimed at providing computational neuroscientists (not just NEURON modelers) with free access and technical support to computer resources (Note: NSG requires most
Figure 80: BioVis - visualization of cortical patches. A,B. Two different rat cortex networks visualized at different levels of zoom.

users to operate through a web-based portal which was not useful for the simulations presented here which were all run from the command line). It should be additionally mentioned that Canadian resources such as Westgrid (https://www.westgrid.ca/) was explored for \(\approx 10\) months and was abandoned after \(>100\) emails with technical support did not result in being to run the simulations. An additional resource SciNet (https://www.scinethpc.ca/) at University of Toronto was used successfully for shorter runs, but longer runs were not possible due to limitations and costs imposed by SciNet on end-users without grants or funding for using the resources.

Once simulations were established at the NSG, the aim was usually to generate 1-4 minutes of extracellular voltages on varying electrode layouts ranging from 60 to as much as 7,500 channels at 20KHz sampling rates (i.e. \(50\mu s\) fixed-time step in NEURON). Such simulations would usually require between 30,000 to 40,000 core-hours of run time which at the lower cost rates (UCSD Users condo/hotel rates of \$0.015/ \$0.025/core-hour) would cost between \$500-1000 per run. Over the past 2 years, more than 1 million core-hours have been used by the author via the NSG without any charges to the Allen Institute or the author.
While BioNet solves most of the technical challenges of developing Python-based interface to NEURON for running on clusters, some issues were unique to the simulations run here. First, the simulations generated large datasets 5-15TerraBytes of data per simulation that needed to be written and parsed before downloading locally for processing. Initial changes had to be made to the BioNet .hdf5 writing modules, but those have now been adopted into the latest code. However, these large writing demands are still significant and NSG suffers from periodic shutdowns due to bugs or other technical issues in the mass storage units (Note: at the time of writing - Aug 2017 - NSG is down due to such an issue that has resulted in the 3 latest simulations being corrupted).

A last comment should be made regarding tuning the connectivity parameters of the sim-
ulations. Connecting even small networks of 500 - 1000 neurons made from 12 unique neuron morphologies requires tuning of dozens of connectivity parameters and background input rates. An extensive discussion of network tuning is beyond the scope of this thesis. However, it should be noted that - as expected - non-epileptic parameter regimes are easy to obtain but at the expense of decreasing firing rates across the network. For example, very low local connectivity weights (i.e. connection strengths between neurons) could be implemented and all neurons could be driven externally using random input. This would avoid epileptic like discharges (i.e. where neurons are over activated and essentially become impossible to spikesort) - but would decrease the simultaneous spiking dynamics present in such highly connected network models. Accordingly, a lot of time was spent - using trial and error - to develop parameter sets that balanced local connectivity strength with sufficient spiking (i.e. > 1000 spikes per unit per 4 minute simulation) to facilitate spike sorting. Resulting spiking rate distributions were gaussian centred on 6-7Hz (Fig 3.14A, 3.16A). Future work should focus on improved spiking distributions that are more similar to in vivo results including lognormal shaped firing rate distributions.
As part of the effort to develop unbiased ground truth datasets for sorting algorithm development, blind testing of sorting results across multiple types of extracellular datasets is central. Initially, test datasets (see Chapter 3) were provided to spike sorting algorithm developers by direct communication (e.g. email). Datasets were provided via hyperlinks to servers (e.g. Dropbox or Google Drive) with instructions on how to load them. Users would provide sorted unit rasters which were then tested locally against the ground truth and results were forwarded (usually by email) to endusers.

A substantial improvement on this approach - without providing endusers with the ground truth rasters - is to remotely host the raw datasets and ground truth rasters and to automate the comparison algorithms to provide endusers with real-time results. To this end, a cloud-based solution was developed using the domain name `http://www.spikesortingtest.com`.

The website was written using mainly HTML, Python and Django. Django is a Python framework that enables Python applications to be run on servers (`http://www.djangoproject.com`). As the comparison algorithms were written in Python, Django made it possible to re-use the same algorithms. However, the main sorting step - comparing spike times and channel location from the sorted unit against the ground truth cell raster - turned out to be too slow for cloud computation (e.g. would require a few minutes of run time). The web browser often timed out for non-trivial datasets and even on the server side there were issues with data being overwritten by intervening processes. While some Python-only solutions could be implemented in an ideal environment (e.g. multi-processing), there were some hardware limitations on the server side as only a single (shared) core was accessible simultaneously.

The solution implemented was to write a Fortran module to do the comparison step and F2PY, a Fortran to Python interface generator (`https://docs.scipy.org/doc/numpy-dev/f2py/`) which would allow for Fortran code to be compiled and imported into Python as a standard module (i.e. variables could be passed back and forth). This had the effect of speeding up the sorting comparison substantially with results for most datasets being provided in <20 seconds.

This cloud-based solution was implemented in early 2016 with some testing carried out throughout 2016. Additional test datasets were added from *in vitro* experiments (Neto et al., 2016) and some simulated datasets from a recent publication (Hagen et al., 2015).

There are plans for adding further simulated datasets and advertising the website to endusers to generate sufficient traffic and feedback. The tool could be used to even test electrode layouts submitted by end users.
Figure 82: Spikesortingtest.com - workflow. Enduser workflow for testing spikesorting algorithms (for server side workflow see main text).
The simulations, data processing and analysis in this thesis were carried out using Python, Fortran, Django, OpenGL and C/C++. Early analysis and simulations carried in 2014 (and earlier) relied in part on Fortran. However, since 2014, Python was largely adopted for most data processing and analysis carried out in this thesis.

To this end, numerous Python tools were written for data analysis including:

- Converting extracellular raw records to standardized formats (e.g. Intan Acquisition System format, '.rhd', to a sample format used by SpikeSorter, '.tsf')
- Loading extracellular and spiking records, and various data files for analysis
- Filtering data including bandpass filters using Butterworth, Chebyshev and wavelet methods
- Concatenating, subsampling and time compression of data (e.g. for use in Chapter 5)
- Event triggered analysis (e.g. for stimulus triggered LFP, single unit or CSD plots)
- Visualizing raw data including LFP, high-pass data and CSD
- Visualizing LEC templates using LFP or CSD
- Mean-Spike-Latency (MSL) tools used for computing LEC-event triggered spiking distributions and statistics

The Python modules written to support these functions (and many others) amounted to >15,000 lines of code. They were implemented into a single menu-driven toolbox Openneuron (https://github.com/catubc/openneuron) developed using the PyQt4 Python bindings for the original Qt graphical-user-interface (GUI) toolkit (https://riverbankcomputing.com/software/pyqt/intro). The GUI required >2,000 lines of code with individual menu items having similar format. Two examples of the menus are provided (Fig C.1,2).
Figure 83: Openneuron - data pre-processing functions. Toolbox written for concatenating multiple recordings and for compressing and subsampling data.

Figure 84: Openneuron - UP-state latency toolbox. Toolbox written for analysis of single neuron latencies during UP-state transitions.
Raspberry-Pi camera - custom software

Imaging data discussed in this thesis was most commonly acquired simultaneously with electrophysiology recordings (see Chapter 4). One of the reasons for acquiring these datasets simultaneously was to match spiking records (obtained from extracellular recordings) with imaging data. One of main challenges in matching the two records is to save and match recording time stamps of one dataset to another.

Ideally, a single acquisition system (e.g. a single acquisition board) should be used that coordinates and matches dataset timestamps acquired using different modalities such as electrophysiology (usually acquired at 25Khz) and optical imaging (usually acquired at 30Hz or 150Hz). This hardware option was not available at the time of carrying out these experiments. A second, more practical, option is to use the faster acquisition system - for example the electrophysiology system which acquired data at 25Khz - to save timestamps of the slower acquisition modality. This can be done using the Epix and of imaging approaches (see Methods) implemented here as the video acquisition card can output ON/OFF pulses for each imaging frame even at a sampling rate of 150Hz.

A third option is to simply save imaging acquisition ON and OFF times, for example the times that the blue excitation light was turned ON and OFF in GCaMP6 recordings, into the electrophysiology recordings. This was done using an additional toolbox - ClampX - which could simultaneously turn the excitation light ON/OFF and send a 3.3V pulse to the Intan electrophysiology acquisition system which was saved as an auxiliary digital input. It then becomes possible to interpolate the location of each imaging frame relative the electrophysiological record and thus match each cell spike time (recorded with <1ms precision) to a specific imaging frame (recorded with 33ms or 6.7ms precision).

Both the second and third approaches were implemented for some of the data acquired for Chapters 4 and 5. However, for GCaMP6 recordings, an additional modification was implemented to correct the imaging record for hemodynamic contributions (see Ma et al., 2016). Specifically, a multi-wavelength approach was implemented (see Chapter 4; see also Methods) which required the continuous acquisition of (short) blue reflected light in addition to green epifluorescence. However, the Epix/Dalsa imaging system was monochromatic and could not be used for the simultaneous acquisition approach.

For a number of reasons (i.e. low cost, already existing imaging system, ongoing projects at the lab) a Raspberry Pi 3.0 micro-computer (https://www.raspberrypi.org/products/raspberry-pi-3-model-b/) and a natively developed RGB camera (picamera) were implemented. However, it became apparent
to the author after simultaneously acquired imaging and electrophysiology recordings that using the interpolation based method for matching the two records was not adequate (note: the picamera also did not output frame times natively in a way to enable saving them into the electrophysiological record). After a more detailed investigation, it appeared that the picamera based imaging system randomly dropped frames, or more precisely - the acquisition system froze for periods of time, and this was partially documented on picamera forums (https://www.raspberrypi.org/forums/).

As the picamera software was written in Python, the acquisition system code was changed to save individual frame times (which were already available as attributes of one of the main picamera objects) to disk for comparison offline. The results of tracking the frame times revealed that for shorter recordings (e.g. ≈6 minutes) between 1 to 3 freezes occurred amounting to a total skipped time of 0.5 to as much as 1.5 seconds whereas longer recordings (e.g. >60 minutes) dozens of freezes occurred randomly with missing time of up to 10 seconds (Fig D.1).

Correcting the imaging record using the saved time stamps enabled the correct computation of spike triggered maps for the same neuron ever after 2 hours of recording time (Fig D.2). These results confirmed that single neuron spike trains could be matched to imaging data even after 1.5 to 2 hrs of recording. Using additional single neuron recordings the corrected imaging records also confirmed that all recorded single neurons (with >25 spikes) could generate stable and expected STMs (see also Chapter 4).
Figure 86: Computing STMs using frame tracking on the Raspberry-Pi. A. Single neuron spike-triggered motif for a right hemisphere barrel cortex neuron (top) and time-course of single pixel in left barrel cortex (bottom) from the first 30 minutes of a 2 hour recording. B. Same as (A) but using spikes only the last 30 minutes of the recording.

As discussed above, the hemodynamic correction implemented relied on simultaneous acquisition of two wavelengths (blue and green) using the picamera imaging system. However, initial studies addressing hemodynamic corrections in widefield calcium imaging relied on a strobing method, i.e. acquiring images while alternating between an excitation light (e.g. 485nm - blue light) - which captured neuronal activity as green epifluorescence, and a green light (e.g. 525nm) - which captured hemodynamic absorption only but in the precise band of the emitted epifluorescence. In this way, green light representing neuronal activity could be compared to green light representing hemodynamic absorption only.

Accordingly, an additional effort was made to implement strobing on the picamera using C/C++. While passing data structures between Python and C/C++ is currently possible through a few methods, including Python ctypes (https://docs.python.org/3/library/ctypes.html), the imple-
mented solution did not fully solve the strobbing problem. There were two compounding limitations of the picamera: the dropped/frozen frame issue (see above) and the fact that the picamera used a rolling shutter (https://en.wikipedia.org/wiki/Rolling_shutter). While the first of these issues was resolved sufficiently for non-strobing applications, frame freezes would result in additional frames that would have to be dropped during strobing (e.g. the first 1-10 frames coming out of a frame freeze). However, programming the picamera to strobe at 2 times the acquisition rate (i.e. 60Hz) posed a significant challenge and was only partially solved as of the time of writing. One of the additional issues was how to balance between excitation light time (i.e. of 1ms-15ms) and shutter exposure time (1ms-15ms) in a very consistent fashion as to limit noise in the imaging activity. Ongoing efforts will likely result in a full solution - though it is unclear whether the picamera’s simple rolling shutter system could successfully be used to balance between excitation light and shutter exposure time.