Mesoscale activated states gate spiking in the awake brain

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ABSTRACT

Neuronal action potentials ('spikes') are thought to be the fundamental units of information in the brain, hence the ability to record them and to understand their genesis is crucial to our comprehension of the biological underpinnings of our thoughts, memories, and feelings.

Over the past several decades an extensive body of work has focused on the mechanisms and timescales over which neurons integrate inputs toward spike threshold. However, most of the work has been carried out in vitro or in silico, and our understanding of what underlies the generation of spike patterns in the awake brain has remained limited.

Current models emphasize either seconds-scale global states shared by most neurons in a network, or the fast input integration occurring in single neurons over the few milliseconds preceding spiking, but it’s not known whether these represent just the extremes of a continuum.

Combining a virtual reality environment with an optimized robotic system for intracellular recordings we therefore analyzed the subthreshold dynamics leading to spiking in a variety of network and behavioral states in the hippocampus, a region known to be involved in spatial navigation, learning and memory, as well as in a model neocortical region, the primary somatosensory cortex.

We discovered that the majority of spikes are in fact preceded not only by a fast, monotonic rise in voltage over a few milliseconds, consistent with fast input integration within a neuron, but also by a prolonged, gradual (tens to hundreds of ms) depolarization from baseline, which appeared to exert a gating function on subsequent inputs. Unlike the fast voltage rises, these gradual voltage rises are shared across some, but not all, neurons in the network.

We propose that the gradual rises in membrane voltage constitute a novel form of activated state, intermediate both in timescale and in what proportion of neurons participate. By gating a neuron's ability to respond to subsequent inputs, these network-mediated intermediate, or mesoscale, activated states could play a key role in phenomena such as cell ensemble formation, gain modulation and selective attention.

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1. Introduction

Although more than a century has gone by since the seminal work of Golgi and Ramon y Cahal, which marks the beginning of modern neuroscience, many of the basic principles by which neural systems operate remain obscure, especially in awake, behaving animals. In turn, this has prevented the development of rational, engineering-like strategies for the diagnosis and treatment of psychiatric and neurological disorders. To a large extent this state of affairs resulted from technological limitations, which are only now beginning to be overcome.

Neuronal action potentials ('spikes') are thought to be the fundamental units of information in the brain; however, our understanding of what drives a neuron to fire in the intact brain remains limited.

The brain displays robust ‘spontaneous activity’, which, while not associated with specific sensory or motor content (Destexhe et al., 2003; Major and Tank, 2004), nonetheless plays a major role in shaping neural and behavioral responses (Arieli et al., 1996; Fox et al., 2007; Gur et al., 1997; McGinley et al., 2015a; Zagha and McCormick, 2014).

Traditionally this spontaneous activity has been classified into highly discrete states, such as up/down, sleep/wake, active/passive in the cortex (Roš et al., 2009; Steriade et al., 1993, 2001), and theta or SWR in hippocampus (Buzsáki, 2002; Ylinen et al., 1995). More recently (McGinley et al., 2015a, 2015b; Reimer et al., 2014), however, it has been appreciated that neurons undergo continuous modulation of their activation state, though these changes are still viewed as mostly operating at the seconds (or longer) time scale, and as affecting the majority of the network.

On the other hand, in vitro, in vivo, and computational studies have shown how the last ten or so milliseconds before a spike are crucial in allowing a neuron to fire and in determining the precise timing of the spike (Azouz and Gray, 2000, 2008; Mainen and Sejnowski, 1995; Nowak et al., 1997; Pouille and Scanziani, 2001). Such dynamics appear to be highly cell-
specific, with even neighboring neurons acting to a large extent independently (Genovesio et al., 2005; Poulet and Petersen, 2008).

In between these two extremes, extracellular studies have shown that there can be rapid modulations in neural activity (e.g. spike/field coherence) during a behavioral task (Pesaran et al., 2002), and that cell ensembles can be transiently formed depending on specific behavioral performance and network history (Cohen and Newsome, 2008; Fujisawa et al., 2008). Moreover, in both cortex and hippocampus, neuronal activity has been found to be to some extent predictable by ensemble activity at the tens to hundred ms scale (Harris et al., 2003; Truccolo et al., 2010).

We hypothesized that, in addition to network-wide states, briefer activated states, specific to ensembles of neurons, would also exist. We expected that these activated states would make neurons more likely to fire in response to incoming stimuli and therefore significantly shape neural activity.

With current approaches, however, investigating such fine-grained neural dynamics, accessible only with intracellular recordings, remains something of an art form, difficult and extremely time consuming. To address this limitation, we have previously developed the Autopatcher, a robotic system that can perform intracellular recordings in vivo in a semi-automated fashion, and have more recently adapted it for use in awake animals, allowing for the first time a systematic exploration of spike generation across multiple regions and network states.

We analyzed single and dual intracellular recordings in the CA1 field of the hippocampus and in barrel cortex and discovered that, in addition to fast, monotonic rises in voltage over ~5-15 milliseconds before the spike, spikes were almost always preceded by relatively prolonged (tens to hundreds of ms), gradual membrane voltage depolarizations.

The fast monotonic rises were consistent with integration of a barrage of inputs, or with fast alterations in the timing and amplitude of excitatory and inhibitory drive (Azouz and Gray, 2008; Haider et al., 2006; Piwkowska et al., 2008; Pouille and Scanziani, 2001; Tiesinga et al., 2008). In contrast, the gradual rises appeared to provide ‘windows of opportunity’ (Haider and McCormick, 2009) for subsequent inputs to drive the cell to fire. Consistent with
this model, whereas the gradual rises appeared to be shared across at least some cells in the barrel cortex, the fast rises were cell-specific.
2. Automated whole-cell recordings in the behaving animal: the Awake Autopatcher

2.1. The importance of intracellular data

Although extracellular recordings in intact animals have been critical, over the past several decades, in shaping our understanding of how neural circuits compute in vivo during behavior, intracellular recordings, were they possible under similar conditions and with similar yield, would significantly advance our knowledge. Intracellular recordings, in fact, open a window into the inputs that the cells receives, into how these inputs are processed, and into the intrinsic properties that allow the neurons to function as they do. Moreover, the ability to precisely control the voltage and the firing pattern of a single, well characterized neuron can also be extremely useful in mechanistically proving or disproving hypotheses.

Although in vivo intracellular recordings, especially in the awake state, have so far been comparatively scarce due to their technical challenges, they have demonstrated the considerable changes in neuronal properties (Destexhe and Paré, 1999; Destexhe et al., 2003; Steriade et al., 2001) and ongoing dynamics in different network states (Polack et al., 2013; Poulet and Petersen, 2008a; Poulet et al., 2012; Tan et al., 2014; Zhou et al., 2014). They have, moreover, been instrumental in furthering our understanding of network phenomena as different as, for example, place cells (Harvey et al., 2009), grid cells (Domnisoru et al., 2013), sharp-wave ripples (English et al., 2014), and neuronal sequence generation in songbirds (Long et al., 2010). The ability to control the membrane voltage has unearthed unexpected behaviors in place cells (Epsztein et al., 2011a), while the ability to precisely control individual neurons, leaving the rest of the network unperturbed, has allowed Li and coworkers to show that a single neurons can, surprisingly, control cortical state (Li et al., 2009).

All but a handful of such studies have been recordings of single neurons, and simultaneous dual patch recording in an awake animal is probably close to the practical limit for
conventional strategies for intracellular recordings in vivo. If we want to be able to scale up our ability to intracellularly access neurons by one to two orders of magnitude, a fundamentally new approach is required. Our development of an automated patch-clamping robot, and its refinement for use in awake behaving animals represent important steps in this direction.

2.2. Whole-cell patch clamping: basic concept and previous attempts at automation

Whole-cell patch clamp electrophysiology (Hamill et al.) (Fig 1), in which a glass pipette is used to gain electrical access to the inside of a cell, enabling electrical recording under precise control of the cell’s intracellular state, has become the gold standard technique for the high-fidelity recording of electrical activity in neurons and other excitable cells. It offers an extremely high signal-to-noise ratio, and current can be delivered through the pipette to drive the neuron or to characterize its electrophysiological properties. This technique also allows the infusion of chemicals such as cell-filling dyes that enable post-hoc anatomical visualization of the recorded neuron, as well as the extraction of cell contents for transcriptomic analysis. Although initially used in cell cultures and tissue slices, it has more recently been successfully employed in intact animals, both anesthetized and awake. The main limitation of this technique, however, has always been the need for highly skilled and trained manpower, making it impractical for high-throughput screens or large-scale recordings in vivo.
Over the past two decades we have witnessed a number of attempts to create highly parallel versions of patch clamping for high-throughput drug screening, based on planar patching chip arrays. To this end people have explored the use of Si, quartz, glass (Fertig, Blick, & Behrends, 2002), teflon, PDMS (plasma treated as well as untreated) (K. Klemic, J. Klemic, & Reed, 2002; X. Li, 2006) and polyimide (Martinez, Py, Denhoff, & Martina, 2010) among other substrates, as well as a variety of geometries and fabrication procedures (Fig 2). Although quartz and glass offer the best performance (50-90% gigaseal yields in different cell-types (X. Wang & M. Li, 2003)), they are less suited to microfabrication and integration with the other components of the probes, so Si has been the most successful material, and has been reported to achieve good recordings in vitro (5-20%, higher for <1 GΩ but >100 MΩ seals) (Sordel, Garnier-Raveaud, & Sauter, 2006; X. Wang & M. Li, 2003). Coating with glass by sputtering, evaporation or spin on (Madou, 2002) and/or enhanced surface coatings (J. Xu et al., 2003) have also been explored to enhance yield.
They are all fundamentally limited, however, by their relatively poor yield and, most importantly, by being restricted to recording from cells in suspensions (excluding, e.g., neurons and any ex-vivo tissue preparation).

2.3. Non-patching strategies for intracellular recording

Whole-cell patching, however, is not the only method used to obtain intracellular electrical recordings from neurons. In fact the first intracellular recordings were performed with sharp pulled glass electrodes filled with a conducting solution (GRAHAM & GERARD, 1946) and such electrodes have been used extensively in vitro and in vivo, though their use has been progressively replaced by whole-cell patch recording, which offer lower access resistance, are more stable, and can be performed under visual guidance.
The relative simplicity of this approach, compared to whole-cell patching, however, has made it an attractive strategy to pursue when trying to scale up intracellular recordings in vivo. One of the earliest examples built on the fabrication strategy of the Utah array to fabricate arrays of sharp Si needles (Hanein et al., 2003).

Fabrication difficulties and issues with recording ability and quality led to the abandonment of the project. However, the overall strategy of gaining access to the intracellular environment by piercing the membrane with sub-um devices has been somewhat more successfully taken up in recent years by several labs, driven by advances in microfabrication (Fu et al., 2014a; Xie et al., 2012) (Fig. 3).

Ensuring acceptable cell health and recording quality in cells other than cardiomyocites, however, remains challenging, as is adapting fundamentally 2D fabrication methods to a 3D problem for in vivo use. Although the nano-FET devices developed by the Lieber group (Duan et al., 2011; Fu et al., 2014a; Tian et al., 2010) have demonstrated significant promise and are theoretically capable of high-quality recording, it remains to be seen if (and when) they can be successfully employed for multiple in vivo recordings. In addition, the complex fabrication procedure puts them well beyond the reach of most neurophysiology laboratories.

![Figure 3 Pt nanopillar array (Xie et al., 2012)](image)
2.4. The development of the Autopatcher

Despite their promise, high quality neuronal recordings with nanoscale microfabricated electrode arrays remain unproven. In light of the widespread use and excellent capabilities of whole-cell patch clamping *in vivo* we chose this technique when considering strategies for large scale recordings in behaving animals.

The initial designs centered on large fixed arrays of microscale glass or fused silica capillaries, to be actuated simultaneously. In principle fixed arrays present several advantages compared to arrays of individually moveable electrodes, in terms of ease of fabrication, use, and suitability for use in freely moving animals. However, their practical feasibility depends crucially on yield, which is inherently lower than for mobile, independently controlled electrodes. It is therefore useful to estimate what range of values it could take. If we assume that an electrode is only able to record from cell bodies, and assume an (optimistic) 100% success rate of intracellular recording from any directly accessible soma per electrode, the percentage of volume occupied by soma could serve as a proxy for the yield.

The density of cell bodies varies extensively depending on the structure being recorded from, and on the exact location within it, so that, for example, different cortical layers have very different densities, as do hippocampal layers. Taking 5% as the average value is probably reasonable, since the various electrodes would likely end up in different parts of a given structure. Another way to estimate the yield of an intracellular electrode array is to consider how many ‘steps’ it takes while proceeding blind to ‘hit’ a cell, either with sharps or patch electrodes. Again, a figure of ~5% seems reasonable (personal experience, personal communication from Miles Whittington, Fiona Lebeau, Suhasa Kodandaramaiah). If the aim is to record from 100-1000 neurons we therefore need on the order of at least 2,000-20,000 recording points.

Moreover, trying to seal and break in in all channels simultaneously by applying the same pressures throughout the array would be highly problematic because, for example, some neurons would already have achieved whole-cell configurations while others would still be in
the gigaseal stage, so that further break-in attempts couldn’t take place without killing the neurons already accessed.

Independent pressure control would therefore be highly beneficial. This could still be compatible with a fixed array, though each capillary would now have its own pressure control. However, independent pressure control over 2000-20000 channels is a non-trivial problem. Moreover, for a given minimum electrode diameter and desired yield, the amount of damage caused by a fixed, compared to an independently moveable, array would still be 20 times higher, a significant consideration when trying to record as many neurons as possible in as intact a network as possible.

We therefore decided to switch to a new design, with independently moveable patch electrodes. Since a large number of such electrodes would have to be controlled simultaneously, the patching steps would have to be automated. The stereotypy of the procedure, which follows a rigorous sequence of steps following the reaching of specific parameters (e.g. a threshold impedance increase during neuron hunting, a sufficiently high seal during the gigaseal stage, etc.) made it, in principle, highly amenable to automation.

Especially in light of the fact that the initial goal was to have the devices implanted in freely moving animals, reducing complexity and bulk of the actuation was paramount, with cost also being a constraint considering the large number of channels, so a number of possibilities were investigated. Among the ones that saw initial proof of principle testing was the idea of using gravity and the positive pressure applied to the fluid inside the pipette while searching for neurons as a mean of propelling the pipette forward. A clamp, actuated, for example, by a shape-memory alloy spring, would then lock the pipette in place once a neuron was found (see Fig. 4). This would have resulted in a massively simplified design compared to devices incorporating more conventional actuation mechanisms. Initial testing (in air, and, in one case, in agarose), indicated that the pipette could be made to move in a fairly controllable fashion in this way (though with some constraints on speed and pressure applied) and although early shape-memory alloy-based /PDMS clamps were rather crude, a fast and stable version could likely have been developed for it.
However, as a testbed for developing the patch algorithm and for initial applications it was eventually decided to postpone work on the actuation problem, and design a system around headfixed, anesthetized animals instead.

The patching algorithm, developed like the hardware described in Kodandaramaiah et al. (Kodandaramaiah et al., 2012) by Dr. Kodandaramaiah, was based on established protocols for blind patching *in vivo* (Margrie et al.), though it ultimately differed from them in several respects.

During the process of whole-cell patch clamping *in vivo* (Kodandaramaiah et al., 2012), the craniotomy is superfused with sterile 0.9% saline to keep the brain moist until the moment of pipette insertion. At the beginning of the experiment, excess saline is removed to improve visualization of the brain surface and the pipette is lowered until it touches the brain surface and then retracted it back by 20-30 μm. Its resistance is then evaluated (the normal range being 4-9 MΩ), while applying “high positive pressure” (between 800-1000mbar). If the pipette is of acceptable resistance, it is lowered to a target region within the brain (the stage labeled “regional pipette localization” in Fig. 5) while maintaining the same pressure. Upon reaching the region of interest the pressure is then lowered to “low positive pressure” (20-30mbar) and, if the resistance has not changed significantly (less than 300kΩ), indicating that the tip is intact, clean and unclogged, the “neuron hunting” stage begins. Throughout the process, a voltage square wave is applied to the pipette (e.g., 10 Hz, 10 mV alternating with 0
mV relative to the pipette holding voltage), and the current is measured, in order to calculate the resistance. During this stage the pipette is lowered in 2μm steps and the presence of a neuron is indicated by a monotonic increase in resistance over 3 steps greater than a set threshold (250kΩ). The program then stops the pipette and attempts to seal onto the neuron ("gigaseal formation" stage). The program releases the positive pressure, then applies gentle suction (-20 to -25 mbar) and gradually ramps the holding voltage down to -70 mV. Once a sufficiently strong seal has formed (greater than 500MΩ, ideally greater than 1GΩ) the user then switches the program to "break in" mode, where either pulses of negative pressure (-200-250mbar) or zapping (brief current pulses) are used to gain access to the intracellular compartment.

![Pipette Brain Regional Neuron Gigaseal Break-in pipette hunting formation localization](image)

**Figure 5** Autopatching steps (Kodandaramaiah et al., 2012) (2012) © Nature Publishing Group

Overall, the robot is very simple: it comprises a programmable linear motor, a bank of pneumatic valves, associated control electronics to move the motor and actuate the valves, and signal processing electronics to support acquisition of pipette resistance measurements (and other electrical signals) for adaptive control of the motor (Fig. 6).
The autopatcher program, in both its original and its modified versions, is written in LabVIEW (National Instruments). Overall, the system is designed to be compatible with most standard electrophysiology equipment and to be relatively low-cost, to minimize the barriers to entry for laboratories interested in adopting this technology.

2.5. Adaptation of the Autopatcher to recording from deep regions in awake, behaving animals: the Awake Autopatcher.

Although the autopatcher was developed in anesthetized animals, we ultimately wanted to be able to use it to record from awake, behaving animals. In particular, our interest in neural rhythms led us to consider recording from the hippocampus of awake, headfixed mice, navigating a virtual reality environment while running on a spherical treadmill (see Chapter 3 for details on the VR environment).

The main problem of trying to record intracellularly from awake animals is constituted by brain motion. Therefore, most of our efforts and improvements over the original autopatcher recording setup were aimed either at reducing brain motion or at dealing with its effects.

2.5.1. Minimizing brain motion

The first step toward minimizing brain motion is ensuring that the skull is as rigidly fixed in position as possible: whereas for anesthetized animal a thin delrin headplate mounted on an acrylic holder had proved more than adequate, for awake recordings we switched to a
different headplate and headplate holder design, and to a different way of attaching the headplate to the skull.

The headplates, though similar in design (Fig. 7), were now of 1/8”-thick stainless steel and had three holes that could be used to increase the area of contact between the headplate and the bonding agent, which was now dental cement, instead of dental acrylic. Moreover, the entire exposed area of the skull in the headplate window was covered in clear dental cement, forming a single solid body, with only a small craniotomy hole, drilled on the day of the experiment.

![Figure 7 Headplate CAD](image)

We quickly discovered that it was essential to make the animal motion as smooth and jerk-free as possible: to this end we developed a training protocol extending over 7-10 days, progressively habituating the animal to the recording environment, including sounds and lights. It was also helpful to replace the original motors, characterized by an unpleasant buzzing noise, with silent ones.

Finding an optimal position for the animal head not only increased animal comfort and hence performance and smoothness of motion, but also affected how much of the body’s motion was transmitted to the brain. Fixing the head just in front and 27mm above the apex of the ball proved effective.

Another major change compared to patching in anesthetized animals involved the size of the craniotomy: whereas before 1-1.5mm craniotomies were perfectly acceptable, we found that
brain motion was very significantly reduced if we kept the craniotomy diameter to less than 500µm, typically ~300µm.

To allow the use of such small craniotomies while still reaching fairly deep into the brain (1.1-1.4mm) we used extremely long-shanked patch pipettes (Fig. 8), designed to preserve optimal tip size and impedance while keeping the last 1mm or so below 100µm in diameter. An additional advantage of the long-shanked pipettes was that they seemed to cause less tissue displacement as they moved.

One problem we had in fact encountered with standard patch pipettes was tissue displacement and subsequent relaxation, which caused the cells to move with respect to the pipette tip. In addition to using long-tapered pipettes we reduced this problem by taking significantly slower steps compared to the original autopatcher algorithm (2 µm steps at 1µm/s, compared to 3µm/s) and by pausing for 10-15” upon reaching the target region but before beginning neuron hunting, to give the tissue time to relax back fully.

Finally, to further reduce brain motion, we explored the use of agar gels to cover the craniotomy (0.5-4%). However, they did not prove effective and led to an increase in pipette clogging, so we didn’t adopt it as part of our standard procedure.

2.5.2. Minimizing the effects of brain motion
Despite these precautions, brain motion could not be fully eliminated, so steps were taken to reduce its effect on yield.

One problem caused by brain motion is the increased variability in impedance readings that make detecting a neuron more difficult. To deal with this problem we increased the averaging to smooth out high-frequency changes in impedance, and also optimized the criteria for neuron detection. The original algorithm called for a monotonic 250 kΩ increase over three steps; after testing a number of other values (150-500 kΩ) and from our observations during patching we eventually settled on a program that used two alternative triggering conditions: a monotonic increase of 300 kΩ over three steps or 400 kΩ over five steps, with an increase of at least 50 kΩ per step.

It had also been suggested (Christina Demnisoru, personal communication) that one way to deal with brain motion is, upon detection of a neuron, to step back (e.g. by 10μm), then slowly re-approach the neuron. If it still satisfies the criteria for neuron detection it is then patched, with high success rate. However, upon implementing this in our code, we didn’t see any significant improvements in yield. This might be due to the difference in region (C.D. patches in the medial entorhinal cortex, whereas our recordings were all carried out in the hippocampus) and consequent differences in the kind of motion exhibited by the tissue, as well as other variables in the patching procedure (pipettes, pressures, internal solutions, etc.)

Brain motion also affects sealing, and the stability of the seal once it has been achieved. We explored tip diameters in the 1-3μm range, but pipettes with a ~2μm tip proved optimal, as is also the case for slice patching.

If the brain is moving, that means that the neuron might not remain at an optimal position with respect to the pipette tip during sealing for long, so we sped up the relevant steps (releasing pressure, applying suction, ramping down the voltage) compared to the autopatcher optimized for anesthetized recordings.
We also added a ‘smart’ component to the sealing algorithm, based on what an experienced user can do to improve the seal, based on how the cell responds. The program determined in a closed-loop fashion whether the cell was sealing successfully, and, if not, (e.g. the resistance did not increase at more than 10 MΩ/s) intervened with additional periods of low suction, small (0.5-1μm) steps toward the cell, or additional hyperpolarization (up to -95mV), constantly monitoring and adjusting its intervention to the cell’s behavior. We also made it possible for the operator to proceed manually at various points, e.g. during sealing, if the automated system was not being successful.

Breaking-in was also improved, and the user could choose between a number of preset single and double pulses of negative pressure (-100 to -350mbar, 100 to 400ms in duration), continuous low suction (~-30mbar), or progressively ramp down suction over a set period of time to a desired value (-100 to -350mbar). This was made possible by a custom-built pressure control system (see http://autopatcher.org).

One advantage of the autopatcher architecture is that all steps are controlled from the same software and can in principle be recorded, as was implemented in the Awake Autopatcher. Such data can subsequently be mined to optimize the various parameters.

2.5.3. Recording from deep structures

Recording from structures deeper than the cortex carries a number of additional difficulties, in particular an increased risk of pipette clogging, both while reaching the region of interest and during neuron hunting. The latter might possibly arise because minute debris, which didn’t affect the resistance reading, nonetheless accumulated on the tip as it descended and ‘catch’ in the tissue in an unpredictable manner as the pipette steps forward). This is usually addressed by increasing the positive pressure used. However, this can cause significant damage to the region of interest and, during neuron hunting, just push neurons away from the pipette path. Again we explored a range of possible parameters, finally settling for 800-1000mbar for ‘high pressure’ and 25mbar for ‘low positive pressure’.
In addition, we stopped the pipette 100μm above the target area and switched to an ‘intermediate pressure’ of ~3-500mbar before reaching the target region. This significantly increased the yield in the first 50-100μm of the region of interest. Switching directly to ‘low positive pressure’ would have been suboptimal, as the pipette would then have had a much larger likelihood of getting partially clogged before it reached the cells. The highest probability of successful patch is typically achieved within 100-150μm of switching to ‘low positive pressure’.

2.6. Performance of the Awake Autopatcher

In terms of yield, the animals we recorded from typically fell into fairly distinct categories: ones that yielded no data, and all stages of the process (identifying neurons, sealing onto them, breaking in) were poor, and successful ones, which yielded 1-5 (average ~1.7) neurons that could be used for data analysis, i.e. were recorded for more than 5’, had a resting potential below -55mV, and had spikes that reached at least -10mV.

In many cases it was immediately clear whether a given animal was going to belong to one category or the other, if, e.g. there was visible brain motion, the craniotomy was larger than usual, or if the brain surface had been damaged while opening the craniotomy. Even if there were no visible problems, the resistance readings in the unsuccessful cases would often be highly unstable, indicative of tissue motion.

Moreover, it’s important to keep in mind that the pipette tip size and geometry, and the internal solution employed are absolutely critical in all forms of patching, especially in vivo, whether manual or automated, so that if there were problems with either of these, no successful recordings could be achieved.

Although, as expected, lower than in the case of anesthetized animals, the recording quality and duration was suitable to address many experimental questions(Fig.9).
Figure 9 Simultaneous intracellular (top) and extracellular (bottom) recording in CA1 while the mouse is running along a virtual track. LFP theta (filtered, in green), and gamma (filtered, in red) oscillations are clearly visible.

When recording in CA1 access resistances were 37.9±14.6 MΩ and membrane resistances were 93.3±56.9 MΩ (mean ± standard deviation) (Fig. 10A). The cells were recorded for 16.1±13.4 min. N = 61 neurons (Fig. 10B).

Figure 10 Access and membrane resistances of recorded CA1 neurons (A), and histogram of the recording durations (B). N = 61 neurons
As others have observed before, blind patching tends preferentially to yield neurons that have large soma, so that, based on firing rates, responses to applied current steps, and, in a small subset of cells, biocytin staining, we believe most of the neurons we recorded from to be pyramidal cells (n = 4/4 biocytin-filled cells)

![Biocytin-filled CA1 pyramidal neuron recorded with the Awake Autopatcher](image)

**Figure 11 Biocytin-filled CA1 pyramidal neuron recorded with the Awake Autopatcher**

### 2.7. A complementary technology: ITO electrodes for artifact-free recording and optogenetic manipulation

A major advantage of optogenetics is that, in principle, it allows simultaneous recording and stimulation (Bernstein et al., 2008), which has so far proved impractical with electrical stimulation. However, it is well known (Grätzel, 2001) (see (Grätzel 2001; Honda 2004) for review) that metal or semiconducting electrodes in a solution can generate a photocurrent upon illumination. Early experimentation confirmed that commonly used metal or silicon electrodes (Fig.12) would in fact show a light-induced artifact at both stimulus onset and offset, when using light intensities common in optogenetics experiments.
Figure 12 A. Average of ten traces recorded in saline with a 25μm NiCr microwire (impedance 1.2 MΩ), illuminated at 50mW/mm². B. Artifact size (μV) vs irradiance (mW/mm²) for PtIr, NiCr, stainless steel electrodes.

While the relatively long time constant of the effect allowed the recording of spikes from stimulated cells (Fig. 13), it prevented the successful recording of local field potentials. Given the fact that synchronous activity is normally observed in the local field potentials and in spike-field coherence this represents a substantial limitation, which we aimed to address with the introduction of “light-proof” electrodes.

Figure 13 Saline (left), and in vivo (right) recordings demonstrating the photoelectric artifact following illumination. Its slow dynamics allow it to be easily separated from the single spike responses (Han et al. 2009).

The conversion of light to electrical or chemical (redox) energy, and hence the measured photocurrent, results from light acting as an electron pump. The absorption of a photon by an atom or molecule pumps an electron from a lower orbital to a higher one, giving rise to an
electron-hole (e- h+) pair: if the electron is then pumped through a wire a current has been generated. The wavelength of light that causes such a transition is that with energy equal to or greater than the difference in energies of the two orbitals. Importantly, for metal electrodes, the difference between the work function $E_f$ of electrode metal and solvation energy of electrons $E_{solv}$ corresponds to the photoenergy required for generating photocurrent, which is much lower to what would be in vacuum.

The mechanism behind the generation of a photocurrent (photogalvanic effect) in a semiconductor is similar. When a semiconductor is placed in contact with an electrolyte, electric current initially flows across the junction until electronic equilibrium is reached, where the Fermi energy of the electrons in the solid ($E_F$) is equal to the redox potential of the electrolyte ($E_{redox}$): the transfer of electric charge produces a region on each side of the junction where the charge distribution differs from the bulk material, and this is known as the space-charge layer. Photons of energy exceeding that of the band gap generate electron–hole pairs, which are separated by the electric field present in the space-charge layer. The negative charge carriers move through the bulk of the semiconductor to the current collector and the external circuit. The positive holes are driven to the surface where they are scavenged by the reduced form of the redox relay molecule (Gratzel 2001; Honda 2004)

Because the exact details of the galvanic effect depends upon the specific surface and electrolyte chemistry and light characteristics at each electrode/solution interface, all highly variable in the case of electrode used in a physiological milieu, a filtering operation, correcting in software for the artifact is impractical. The approach we chose, instead, relies on employing a conducting material that has a large enough bandgap to be only marginally affected by visible light (since $E_{\text{photon}} = \lambda v$): among commonly used materials employed for bioengineering applications indium tin oxide (ITO) appeared the most promising candidate satisfying this requirement.

Although Michigan-style probes have gained popularity, microwire electrodes are still used and preferred for many applications: they are much cheaper, simple to use, generally induce less tissue damage, and, in the case of tetrodes, allow much better single-unit separation. Since the metals in vacuum are not much affected by light, we postulated that if we could “shield” them with a layer of ITO, which would be the only conductive material in contact
with the surrounding fluid, we should get a substantial reduction, if not a complete
elimination, of the artifact.

Initial testing with electrodes fabricated from 200μm-thick ITO-coated glass slides, cut with
a diamond scribe to obtain an acute triangle shape, and insulated with nail polish except at
the final 50-75μm showed the promise of this material, with virtually non-existent artifacts
even at high light intensities.

For practical use in neuroscience research, however, the fabrication technique had to be
scalable, cheap, simple and biocompatible. Briefly, the wires or tetrodes bundles were
placed on a frame, then a holder was cast around the wires in PMMA resist, ensuring perfect
contact. The wires were then trimmed, etched and the ITO was deposited by RF sputtering.
Sputtering was chosen because it’s a simple, scalable technique which allows very mild
conditions and doesn’t involve biologically hazardous materials.

An alternative that we did explore was to use ITO nanoparticles suspension: the electrode
would be dipped in the nanoparticle solution, the ITO layer sintered at 300-500°C, and the
process repeated until an ITO layer of the desired thickness was achieved. This method
recommended itself because of its low cost, and because it gives a corrugated surface (Fig.
14), which tends to decrease impedance, in a manner similar to platinum black.

![Figure 14 SEM microgram of a tungsten microelectrode coated by repeated dipping in an ITO
nanoparticles solution followed by sintering at 400°C](image)

The microwire electrodes thus obtained did in fact show a remarkable decrease in the
photogalvanic effect (Fig. 15), the amplitude of the artifact being reduce over 20-fold over
the uncoated wires. At the same time, the impedance remained within reasonable values (1-3 MΩ).

![Graph](image)

_Figure 15 Average of 10 recordings from NiCr electrodes in saline, before (black) and after (yellow) ITO deposition (bottom trace). The top trace indicates the times of stimulation._

However, this technique tends to be very time consuming and less reliable than the sputtering method, so it was eventually abandoned. At the same time, sintering ITO nanoparticles on top of a sputtered substrate may be a useful approach to decrease the electrode impedance, if needed.

Despite the qualities of microwire electrodes, multielectrode probes have also proved extremely useful (Buzsaki 2004; Kipke, Shain et al. 2008): by having a number of electrodes aligned at a given distance from each other, not only it’s possible to accrue a large mass of data with relatively limited tissue damage, but this configuration is also perfectly suited to analyzing layered structures (such as the neocortex or the hippocampus), and allows CSD computations.

However, conventional probes are also subject to the photoelectric effect: we therefore developed ITO-on glass probes (Fig. 16) that can be fabricated with standard photolithographic method, allow multiple-site recordings, and are light-insensitive.
Figure 16A. Schematic (left) and 20x micrographs (right) of a microfabricated ITO-on-glass multielectrode probe. B. Comparison of response to light for a conventional tungsten microelectrode (left) vs. ITO-on-glass probe (right).

The microfabricated ITO-on-glass probes were initially too thick to be used in vivo; however, in vitro testing in a slice preparation in which gamma oscillations had been pharmacologically induced showed that the electrodes could record LFPs as well as conventional multisite electrode, while being free of light-induced artifacts. The size of the tip (which was fixed in a subsequent iteration of the design, with an optional thinning step) likely prevented us from effectively recording spikes from undamaged tissue. The relatively high impedance of the electrodes (3-4MΩ), however, might also have been partially to blame. There are a number of ways of bringing down electrode impedance to values more in line with standard electrodes, including sputtering a thicker ITO layer, deposition of ITO nanoparticles followed by sintering, or growing ITO nanowires. In case the single-spikes signal quality from ITO electrodes prove consistently worse than conventional metal pads, the same fabrication strategy can be easily adapted to include metal electrodes (to be used for single-spike detection) side by side with ITO electrodes (for artifact-free LFP recordings).
Overall, we have shown that a simple modification of existing technology can make the types of electrodes currently used in neuroscience research suitable for use in conjunction with optogenetic methods to allow temporally and spatially precise modulation of the activity of defined neuronal subtypes in vivo to be combined with highly parallel electrical recordings.
3. Headfixed navigation in a virtual reality environment

While using calcium and voltage sensors to image neuronal dynamics allows simultaneous recording the activity of hundreds of identified cells, and intracellular recordings give exquisitely high-resolution information about a cell’s inputs and computations, an important practical limitation to their use is constituted by their need for a very stable (on the μm scale) preparation. This has typically limited their use to in vitro or in vivo anesthetized recordings. However, in the attempt to bring such techniques to the study of brain activity in awake, behaving animals, a number of groups have over the years developed experimental setups that allow the animals to move in a semi-naturalistic fashion, while still minimizing brain motion (e.g. Buzsáki et al., 2003). For use in mice, the most successful of these approaches, that has seen fairly widespread adoption, involves a spherical treadmill to allow relatively unrestricted movement to a headfixed animal, while providing visual feedback in the form of a virtual environment (Dombeck et al., 2007; Harvey et al., 2009) (Fig. 17). This environment has been shown to cause activity patterns remarkably similar to those observed in freely moving animals, although significant differences have also been reported (Aghajan et al., 2015; Ravassard et al., 2013).

Figure 17A. Schematic of the VR setup. B. Images projected when the mouse is at the left (top) or right (bottom) ends of the virtual maze (Harvey et al., 2009) (2009) © Nature Publishing Group
3.1. Virtual reality (VR) recording setup

In light of the role of the hippocampus in navigation and spatial learning we therefore adopted a similar configuration for our in vivo, awake hippocampal patch clamp recordings.

The recording setup closely follows the overall structure of Harvey et al. (Harvey et al., 2009). The animal (Fig. 18) was securely headfixed to two headplate holders, mounted on an air table, and was free to move on an 8” polystyrene ball sustained by an air cushion. The motion of the ball was tracked by a single optical mouse placed in line with the head of the animal. The data was sent to a desktop computer, where a custom program written in LabVIEW (National Instruments) separated and smoothed the X and Y components of motion, and fed them to a custom-written MATLAB (version 2013b, Mathworks) virtual reality software. The virtual reality software (VirMEN (Aronov and Tank, 2014)) was kindly provided by Dmitriy Aronov and David Tank ahead of publication, and modified in-house.

The software updates the image to be projected based on the reported motion, and the image is then projected via a series of mirrors onto a curved translucent acrylic sheet acting as a screen.

Figure 18 VR recording setup, side (left) and top (right) view
The virtual environment consisted of a linear track with two small enclosures at the ends where the animal could turn. Animals received a reward of sweetened condensed milk diluted 1:2 in water from a spout at each end of the track after visiting the other end (Fig. 19).

![Figure 19 Virtual linear track](image)

Figure 19 Virtual linear track

The electrode employed for patch-clamp recording was mounted on a 3-axis motorized micromanipulator (Fig.18) bearing a linear stage on which the headstage amplifier and pipette holders were mounted. This allowed precise manual positioning of the pipette over the craniotomy, before beginning the autopatching protocol. The electrode moved perpendicular to the plane of the air table. In contrast, the electrode employed for LFP/MUA recordings came in from the back, at a 45deg. angle vertically and a 60deg. angle horizontally. The electrode was mounted on a miniaturized 3-axis manual micromanipulator, with 4th-axis control ensured by a programmable linear motor compatible with the autopatching program.

### 3.2. Training protocol

Animals learned to run on the virtual linear track over approximately one week. The animals were left to recover from the surgery for one week, and habituated to handling for one to two days before behavioral training began. To learn to maneuver on the treadmill and get comfortable in the testing environment, on the first two days of training the animals were placed on the spherical treadmill with the virtual reality system off and were rewarded with
undiluted sweetened condensed milk. From the third day until the end of training (typically 5-7 days) the animals were placed on the treadmill for increasing amounts of time (30 minutes to 2 hours) running in the VR linear track. Animals were rewarded with diluted (1:2) sweetened condensed milk at the end of the linear track after traversing the length of the track.

3.3. Limitations of the current VR environment

3.3.1. Limited sensory inputs

Like all similar systems, our VR environment, while allowing a relatively realistic behavior, differed in significant ways from ‘real’ reality. Not only does the mouse lack appropriate vestibular inputs in a headfixed preparation, but modalities such as smell, sound, and touch, particularly relevant for a rodent, are absent in the virtual environment. Moreover, the virtual nature of the maze made it difficult to create truly realistic ways to handle, for example, collisions with the maze walls, and turning in the virtual maze also differ significantly from what a freely moving mouse would do.

3.3.2. Lack, or scarcity, of place fields

Despite these limitations, other groups (Harvey et al., 2009; Ravassard et al., 2013) have reported robust place cell behavior in the hippocampus, which was lacking, or at least greatly reduced, in our preparation. This was true for both intracellular recordings (n = 8 cells, technical problems prevented the analysis of the relationship between position in the maze and electrophysiological data in the rest of the recordings) and for neurons recorded extracellularly (n= 43 neurons, from 2 mice over 3 sessions). A typical spike count and firing rate vs position profile is shown below (Fig. 20). The fact that place fields were lacking in both cells recorded intra- and extracellularly suggests that the problem lied in the
experimental preparation (treadmill and VR environment) itself, rather than in subtle changes in cell properties due to patching.

Moreover, anecdotally there was no difference between cells that were patched in fully automatic mode (i.e. using the Autopatcher for cell localization, gigaseal formation, and break-in) or semi-automatic mode (using the Autopatcher to locate the cell, but then sealing and breaking in manually), nor between cells patched with different pipette solutions from different investigators and labs, making it less likely that our results depended on the idiosyncrasies of Autopatching or reagents.

The average membrane potential of the neurons we recorded was -66±5mV (n=23), in line with what was reported by Harvey et al (-67 ± 4mV). The cells showed a low propensity to fire spike bursts in response to current steps (n =12 neurons), in a similar fashion to silent, but not place, cells described by Epsztein et al(Epsztein et al., 2011a) in the freely moving rat. Except for 4/23 neurons, which had an average firing rate > 1Hz, the firing rates of the recorded neurons were much lower (0.2±0.14Hz) than that of place cells reported by Harvey et al. (overall firing rate = 2.2 ± 0.4Hz; in-field firing rate = 7.3 ± 1.4Hz; out-of-field firing rate = 1.5 ± 0.4Hz)(Harvey et al., 2009). Moreover, the spike threshold was -44±3.6mV, comparable to that of silent cells recorded in the freely moving rat (~46.2 ± 1.5mV), but
higher than that of place cells under the same recording conditions (~54.9 ± 1.5mV)(Epsztein et al., 2011b). Overall, the cells we recorded showed many characteristics similar to silent cells analyzed by other groups, and were significantly different from neurons that displayed place fields.

One possible reason for the observed lack of placefields might simply be that the maze was in effect significantly shorter than, e.g. in (Harvey et al., 2009), and a well-trained mouse could transverse it in less than 2”, compared to about 20” for the maze used by Harvey and coworkers. Although in the real world place fields expand and contract as a function of the environment’s dimensions (see e.g. (Muller and Kubie, 1987)) it is not known whether that holds true in virtual environments as well.

A related problem might have been the ratio of forward to lateral motion used to translate ball motion to mouse motion in the virtual world along the central part of the maze: since the forward component of motion was heavily favored, it’s possibly that the mice came to perceive the task more as a ‘ball spinning’ task than a navigational one, especially if the visual cues were insufficiently salient.

An alternative explanation could be that the virtual maze was in fact too wide, and the mice allowed too much latitude in turning within the maze, so that instead of a well-defined linear track they perceived it as a 2D environment, within which spatial selectivity is significantly impaired in standard VR conditions(Aghajan et al., 2015).

Since acute extracellular recordings are easily obtained from headfixed mice navigating a virtual environment it would be possible to experiment with changing several parameters of the linear track in turn. Simple fixes such as extending the track, altering the settings for the motion feedback system and adding a clear spatial component by, for example, forcing the animals to lick at a specific location in the track, would likely address this problem.

### 3.4. Possible improvements upon the current VR environment and recording setup
3.4.1. Addressing the lack of place fields

There are a number of approaches that could be taken to make sure that the VR environment more closely mimics navigation in real space and generates clear place fields. One change would just be to make the maze longer, in part so that the animals have more time to pick up on the visual cues distinguishing the different segments of the maze. Altering the scaling factors translating ball motion into motion in the virtual world to make them less forward-biased would also help, though at the cost of making the task more difficult for the animals to learn and perform. Finally, it would be possible to force the animals to stop and lick at a specific location in the maze, rather than at the ends, reinforcing the need for them to recognize specific locations.

3.4.2. Toward a more realistic virtual experience

Although sound and, especially, smell might be challenging modalities to incorporate into a virtual environment, tactile-feedback virtual reality systems have been successfully built, though typically that has been used alone, rather than in conjunction with a visual virtual environment. Moving plates arranged so as to appropriately stimulate the mouse’s whiskers would be a relatively straightforward extension of our current setup.

3.4.3. Increasing the stability of the brain

It has been speculated that the motion of the ball on the air cushion might contribute to the observed motion of the brain. Replacing the air cushion with a roller system, akin to roller bearings, might provide a more stable surface. Alternatively, mounting the ball base on an appropriately damped system might allow motion of the base without transferring it to pipette holders and, ideally, brain.
3.4.4. Multipatching

We chose the configuration of the LFP/MUA electrode actuation, in part, as a step toward developing relatively economical, very compact mountings for multi-channel patching. Incorporation within VR setup brings its own challenges, as space is limited, but a design was developed that would allow up to four independent channels, all of which have a quick pipette-change feature (Fig. 21).

Figure 21 A. Spherical treadmill with two of the proposed low-cost, quick-change electrode mounts. B. Electrode mount
4. CA1 dynamics in awake, headfixed behaving animals

The hippocampus is a subcortical structure that’s highly conserved across mammals and is, among other things, prominently involved in navigation and memory formation. It’s also one of the best studied systems displaying neural oscillations, which have been postulated to be among the fundamental operating principles of the brain. Understanding what drives its neurons to fire, especially across different network states, is therefore of considerable importance.

4.1. Targeting the CA1 stratum pyramidale in the hippocampus

Experimentally, the hippocampus presents several advantages: not only its anterodorsal aspect is relatively accessible from the cortical surface (depth of the pyramidal layer being ~1.1-1.3mm), but its layered structures also shows very clear electrophysiological signatures that can be used to precisely target electrodes. Moreover, its most characteristic network dynamics (theta and gamma oscillations, sharp-wave ripples) have been shown to be preserved (at least at a qualitative level) in headfixed animals running on a spherical treadmill.

Electrodes were positioned to advance through the whole-cell recording site craniotomy parallel to the coronal plane and perpendicular to the horizontal plane and to advance through the LFP recording site craniotomy at an angle 60 degrees posterior to the coronal plane and 45 degrees inferior to the horizontal plane. The LFP electrode was slowly advanced into the brain until clear electrophysiological signatures of the hippocampal stratum pyramidale (str. pyr.) layer were observed (~600-1000 µV theta waves while the animal was running, clearly distinguishable sharp-wave ripples during immobility, multiple spikes greater than 50 µV), then retracted 25-50 µm in order to make room for the safe positioning of the patch pipette. A similar procedure was followed to map the putative location of the str. pyr. through the craniotomy to be used for whole-cell patching.
Although the geometrical arrangement of the two electrodes was calculated to place them within 500 \( \mu \)m of each other, to further ensure that both LFP and whole-cell recordings were taken from the same region of CA1, we simultaneously recorded the LFP at both locations and calculated the cross-correlation between the two signals at the beginning of the first recording day. The pipettes were considered adequately co-localized if the cross-correlation at zero lag between the two signals was above 0.85, and if, by visually inspecting the two simultaneous traces, we could confirm that the peaks and troughs of theta and gamma oscillations and of sharp-wave ripples were aligned within \(-1\) ms of each other (Fig. 22). Further validation of the electrode locations was done post-mortem, by either biocytin filling of patched cells or dye injection (AlexaFluor 594 and AlexaFluor 488, Molecular Probes)(Fig. 23).

![Graph showing simultaneous LFP recordings and cross-correlation](image)

*Figure 22 Simultaneous LFP recordings through the two craniotomies (left), showing highly correlated (correlation > 0.95) activity (right)*
4.2. Electrophysiological signatures of network states present in the freely moving animal are conserved in the headfixed preparation

4.2.1. Theta oscillations, gamma oscillations and sharp-wave ripples

Two of the most characteristic LFP signatures of hippocampal activity are theta (6-12 Hz) oscillations, present mostly while the animal is moving, and sharp-wave ripples (SWR), which occur during periods of immobility (Buzsáki et al., 2003). Both were clearly present in all recordings (Fig. 24). An average plot of the power spectrum from all LFP recordings were running also clearly reveals the presence of theta and gamma oscillations during locomotion (Fig. 25)
4.2.2. Theta-modulated gamma oscillations

In freely moving animals theta oscillations are known to modulate the amplitude of gamma oscillations nested within them. We did observe a similar phenomenon in our recordings (Fig. 26).
The visual impression is confirmed by more rigorous analysis of cross-frequency modulation (Fig. 27).

**4.2.3. Distinct bands of gamma oscillations**
Even though gamma oscillations were historically considered as a unitary phenomenon, more recently interest has emerged in differentiating different frequency bands, in particular slow (~25-45 Hz) and fast (~50-80 Hz) gammas, which have been shown to correlate with selective synchronization between CA1 and either CA3 or mEC, to have different relations with theta phase, and to be 'chunked' by individual theta cycles (Colgin et al., 2009). We observed similar patterns in our data (Fig. 28, 29, 30).

**Figure 28** Histogram of instantaneous gamma frequency from recording from n=5 mice, clearly revealing two peaks corresponding to slow and fast gamma oscillations.

**Figure 29** Z-scored energy in the slow (left) and fast (right) gamma bands, as a function of theta phase.
Figure 30 Segregation of fast/slow gamma cycles by theta cycle. Population data (left), example analysis of a few representative theta cycles (right). The measure plotted is an indicator of fast vs. slow gamma segregation, \( \frac{N_{fastcycles} - N_{slowcycles}}{N_{fastcycles} + N_{slowcycles}} \).
5. **Mesoscale activated states in the hippocampus of awake, behaving mice**

5.1. Spiking is consistently preceded by slow ramp-ups in membrane voltage (Vm)

Initial observation of the recorded traces revealed that the vast majority of spikes did not start from baseline or from hyperpolarized voltages, rapidly reaching spike threshold, but, rather, they tended to be preceded by long (up to hundreds of ms) gradual ramps in membrane voltage.

5.1.1. **Cell and spike selection, and analysis of Vm dynamics preceding spikes**

All recorded intracellular traces were visually inspected and only traces with a stable baseline with an average membrane voltage less than -45mV and spike amplitude greater than 40mV were included. Cells recorded from CA1 were also excluded if the simultaneously recorded local field potential showed evidence of electrical artifacts (e.g., due to 60Hz line noise). We recorded 20 cells in CA1 that reached our criteria for inclusion.

To examine the ramp-up in voltage preceding spiking, we initially restricted the analysis to spikes that occurred at least 300ms after a prior spike, to ensure that the ramp from baseline to spike threshold was not obscured by prior spikes. We subsequently repeated the analysis including spikes that occurred at least 100ms after a prior spike, though we excluded cases when the cell did not return to baseline between spikes (e.g., bursting), obtaining quantitatively similar results.

5.1.2. **Spike threshold calculation**

In vivo, because the membrane voltage and conductance are constantly changing, spike threshold (meaning the voltage measured at the soma, after which a spike is inevitable) can vary by as much as 5-10mV(Azouz and Gray, 1999, 2000, 2003; Henze and Buzsáki, 2001).
As a result, in vivo spike threshold is often measured as the voltage reached at spike initiation. Spike initiation is thought to occur at the inflection point in the membrane voltage before the spike peak, when there is a sudden increase in the rate of depolarization reflecting the regenerative activation of voltage sensitive sodium channels (Bean, 2007). To identify this inflection point, we used increases in the second derivative, specifically when the second derivative reached greater than four standard deviations above the mean. Based on visual inspection (Azouz and Gray, 1999, 2000, 2003; Henze and Buzsáki, 2001) this approach appeared to be optimal to detect spike initiation. This definition of spike threshold was used for all analysis, unless otherwise noted. To confirm the robustness of our findings we also used a second, fundamentally different threshold definition: the highest membrane potential in the trace not associated with a spike similar to the approach in Fontaine et al. (Fontaine et al., 2014). This was calculated by finding the all the times when the membrane potential went above baseline for at least 20ms and returned to baseline without spiking and calculating the maximum voltage during all of those periods. Presumably, therefore, any voltage infinitesimally above this would meet the criteria for spike inevitability. We called this latter method the alternate method for determining spike threshold.

5.1.3. Determining the start of the ramp-up

The start of the ramp-up in voltage preceding spikes was identified as the point when the membrane potential went above and stayed above baseline until spike threshold (computed as described in the Spike threshold calculation section). Baseline was computed by first replacing spikes in the trace with a linear interpolation over four milliseconds before and seven milliseconds after the spike peak, to ensure that most overestimates caused by the spike could be avoided, and smoothing the resulting trace with a ten second wide gaussian filter; this smooths out any variations in measured baseline membrane potential at a timescale much longer than what we analyzed (from less than one to a few hundreds of milliseconds). This produced an average of the trace without spikes that can track slow changes in voltage. We found quantitatively similar results if we calculated the baseline as the mean of the whole trace after spikes were thus removed (data not shown). The vast majority (>99%) of the time, when the membrane potential went above baseline it peaked or reached spike threshold within 400ms, therefore we excluded these rare outliers when the membrane potential did not peak or
reach spike threshold within this period.

5.1.4. Slow ramp-ups in Vm precede spiking in CA1

These ramp-ups, measured for spikes that occurred at least 300ms after the previous spike (to standardize the analysis), lasted 30.75-111.46ms (20th-80th percentiles; median 56.4ms; Fig. 31). 11.3% of the ramp-ups in CA1 neurons lasted over 150ms, with a long tail in the distribution going up to several hundred milliseconds.

5.2. Long ramp-ups occur across network and behavioral state

We then wondered whether such dynamics were preserved across different network states, and in spike bursts, which, in CA1 pyramidal neurons, are known to be in part dependent on different mechanisms form single spikes(Grienberger et al., 2014).
5.2.1. **Ramp-up analysis in different network states**

Neural activity across the hippocampal network changes markedly when animals run or sit quietly and these changes are often referred to as different network states. These network states are clearly distinguishable by the presence or absence of local field potential (LFP) oscillations in different frequency bands. (Buzsaki, 2006; Buzsáki et al., 2003) In our experiments, mice ran on a spherical treadmill while we simultaneously whole cell patched neurons and recorded the extracellular local field potential to detect changes in network state in CA1. When animals ran, we observed large theta (6-12Hz) oscillations in CA1 as others have shown (Buzsáki, 2002; Buzsáki et al., 2003; Harvey et al., 2009; Ravassard et al., 2013). When animals sat quietly, theta oscillations were no longer visible and we recorded sharp wave ripples, high frequency oscillations of 150-250Hz that last around 50-100ms and are associated with bursts of population activity, as others have observed (Carr et al., 2011; Foster and Wilson, 2006; Ylinen et al., 1995).

To detect this periods in the LFP, the LFP was first downsampled to 2kHz and bandpass filtered between 150 and 250Hz. SWRs were detected when the envelope amplitude of the filtered trace was greater than four standard deviations above the mean for at least 15ms. The envelope amplitude was calculated by taking the absolute value of the Hilbert transform of the filtered LFP. Spikes that occurred during the sharp wave ripple and during the 250ms before and after the ripples were classified as sharp wave ripple spikes because population spiking related to sharp wave ripples (such as replay events) often extends before and after the SWR itself (Davidson et al., 2009; Dragoi and Tonegawa, 2011). To detect theta, the LFP was bandpass filtered for theta (4-12Hz), delta (1-4Hz), and beta (12-30Hz) using an FIR equiripple filter. The ratio of theta to delta and beta ('theta ratio') was computed as the theta envelope amplitude divided by the sum of the delta and beta envelope amplitudes. Theta periods were classified as such when the theta ratio was greater than one standard deviation above mean for at least one second and the ratio reached a peak of at least two standard deviations above mean. Sharp wave ripples and theta periods were visually inspected to ensure that these criteria accurately detected sharp wave ripple and theta periods, respectively.
We then examined the ramp-up to spikes that occurred during theta periods, sharp wave ripple periods, or outside these periods. Twelve cells had spikes during both sharp wave ripple periods (number of cases per cell: 1, 5, 1, 3, 2, 5, 21, 1, 6, 1, 1, 1) and non-theta, non-sharp wave ripple periods (number of cases per cell: 19, 58, 32, 25, 31, 341, 167, 230, 130, 11, 3, 17). Eight cells had spikes during both theta periods (number of cases per cell: 1, 2, 9, 13, 1, 7, 9, 5) and non-theta, non-sharp wave ripple periods (number of cases per cell: 19, 58, 12, 341, 3, 167, 230, 130).

5.2.2. **Ramp-up analysis during spike bursts**

We then determined if the long ramp-ups occurred specifically preceding multiple spikes, i.e. when the first spike was followed by another spike within 10ms, a time window consistent with burst firing (see Fig. S1C, S3C). In hippocampus, 9 cells had both single (number of cases per cell: 17, 19, 5, 285, 97, 195, 81, 12, 2) and multiple spikes (number of cases per cell: 1, 8, 15, 18, 2, 1, 5, 1, 8). We found quantitatively similar results when we considered cases with a second spike within 50ms.

5.2.3. **Ramp-ups are conserved across network states and spiking history**

Spikes were preceded by long ramp-ups regardless of whether they were isolated spikes or the first spike of a burst (Fig. 32).

*Figure 32* Ramp-ups before single spikes or spike bursts. Sample traces (left) and population data (right).
Long ramp-ups preceded spikes during periods of theta oscillations, sharp-wave ripples, or during periods when neither theta nor sharp-wave ripples were (Fig. 33). For spikes during theta oscillations, ramp-ups started 26.54-93.14ms before spike threshold (20th -80th percentiles, median 63.25ms), during sharp-wave ripples ramp-ups started 35.73-123.23ms before spike threshold (20th -80th percentiles, median 60.43ms), and during periods when neither theta nor sharp-wave ripples were detected ramp-ups started 30.62-113.01ms before spike threshold (20th -80th percentiles, median 56.20ms). Ramp-up durations did not differ significantly between these states (ranksum tests of ramp-up to SWR versus non-SWR, non-theta spikes p-values > 0.02 which were greater than Bonferroni corrected p-value of 0.0042 for N = 12 cells in CA1 that had both SWR and non-SWR, non-theta spikes; ranksum tests of ramp-up to theta versus non-SWR, non-theta spikes with p-values > 0.07 which were greater than Bonferroni corrected p-value of 0.0063 for N = 8 cells in CA1 that had both theta and non-SWR, non-theta spikes). The durations of ramp-ups did not systematically depend on variables such as baseline voltage, average firing rate, or the degree to which the membrane voltage was unimodal or bimodal. Thus, long ramp-ups occur, and exhibit similar properties, before spikes occurring across a wide variety of cellular and network states.

5.3. **Ramp-ups are composed of a gradual and a fast component.**

Long membrane voltage ramp-ups, by themselves, would not necessarily be expected to produce precisely timed spikes, since noise might cause them to cross spike threshold at
irregular times (Buračas et al., 1998; Ikegaya et al., 2004; Mainen and Sejnowski, 1995; Wehr and Zador, 2003; Yang et al., 2008a). Perhaps not surprisingly, we found that the ramp-ups almost always ended in a monotonically increasing rise in membrane voltage that crossed spike threshold (Fig. 34, top), and which was quite fast – 3.55-13.10 ms in duration (Fig. 34, bottom; 20th-80th percentile; median 6.95 ms). The preceding part of the ramp-up before this fast rise we called the “gradual rise”, which lasted almost the entire duration of the ramp-up. Only 5.35% $\pm$ 8.35 of spikes did not have a gradual rise and 3.56% $\pm$ 9.50 did not have a fast rise (mean $\pm$ standard deviation, N = 18, Fig. 35), suggesting that both were needed to effectively fire spikes. Importantly, the vast majority of fast rises started below spike threshold, showing they are in fact a distinct component of subthreshold activity, rather than simply the initial component of spikes. The gradual rises and fast rises were similar in magnitude (3.15-9.76 mV 20th-80th percentiles, median 6.33 mV for the gradual rise vs. 3.33-7.86 mV, 5.3112 mV for the fast rise).
Figure 35 Most spikes had both fast and gradual rises preceding spikes. Examples of spikes lacking either component (right)

We next examined whether gradual rises that precede spikes exhibited different properties from other long-lasting depolarizing events that do not precede spikes. We identified all of the periods where the membrane potential of a neuron rose above baseline for at least 20 ms (a duration longer than the 80th percentile of the fast rises, but shorter than the 20th percentile of the ramp-ups), decomposing each such trace into a monotonic fast rise to its peak (for depolarizations without spikes) or to spike threshold (for depolarizations with spikes), with the remaining part considered as the gradual component. Thus calculated, gradual rises in voltage preceding spikes were longer (Fig. 36, 25.60-107.02ms 20th-80th percentiles, median 51.98ms, for spike events, vs. 9.55-40.70ms, median 18.60ms for non-spike events; p < 10^−50, ranksum test, N = 1182 spike events and 33666 non-spike events) and larger (Fig. 36, 3.86-10.03 mV 20th-80th percentiles, median 6.79 mV, for spike events, vs. 1.01-4.15 mV and 2.24 mV for non-spike events; p < 10^−50, ranksum test) than the gradual rises of non-spike events.
5.3.1. Fast rise analysis

Fast, monotonic rises in membrane potential preceding spikes (number of included spikes per cell in CA1: 28, 12, 68, 38, 29, 33, 22, 366, 4, 217, 247, 147, 15, 4, 5, 1, 4, 19, number of included spikes per cell in barrel cortex: 62, 5, 55, 1, 33, 148, 183, 68, 66, 42, 1, 35, 61, 9, 61, 237, 23, 268, 3, 10, 4, 12) were calculated from the time when the slope of the membrane potential became and stayed positive until spike threshold (computed as described in the Spike threshold calculation section), meaning the membrane potential rose monotonically during that period.

To compute the slope, the despiked trace (spikes were removed and linearly interpolated over from threshold to seven milliseconds after the spike peak to fully remove the spike waveform) was first smoothed with a three millisecond wide Gaussian filter and then the

Figure 36 Gradual rises preceding spikes are larger and longer than depolarizing events not preceding spikes
change in voltage over each point in time (dV/dt) was computed. A three millisecond Gaussian was chosen for smoothing the trace before computing the slope because we found this best matched our aim of minimal smoothing that would reduce noise in the slope while accurately representing the changes in the raw trace. Note that such smoothing reduces our temporal resolution at durations of less than three milliseconds: however, we were not aiming to characterize dynamics in the membrane potential significantly faster than this.

The size and duration of the fast rise in voltage preceding spikes was measured from the start of this positive slope. To determine if the membrane potential at the start of the fast rise in voltage depolarized enough to result in spiking itself, we compared this value to the maximum membrane potential not associated with spikes. Spikes for which the fast rise in membrane potential started at or below baseline were considered to not have a gradual rise in membrane potential before threshold. Spikes that had no consistent increase in membrane potential (positive slope) before the spike were considered to have no fast rise in membrane potential before threshold.

5.4. Gradual rises predict spiking

To help assess the functional importance of the gradual component of the ramp up, we looked into whether its properties could be used to predict spiking.

5.4.1. Generalized linear models

To examine how the duration of the gradual rise contributed to the probability of spiking, we used a generalized linear model (GLM, originally developed by Nelder and Wedderburn (Nelder and Wedderburn, 1992)) to estimate the probability of spiking as a function of the duration of the gradual rise. We first generated a logistic binomial model of the probability of spiking as a function of duration of the gradual rise alone. In this model the probability of a spike, p, was the dependent variable. The probability was predicted based on the gradual rise durations of depolarizing events.
Because the distribution of the dependent variable was binomial (either there was a spike in a depolarizing event or there was not) we used a binomial link function. This model is a binomial version of regression and uses the following formula, which is the canonical linking function for a binomial dependent variable:

\[ \log(p/(1-p)) = b_0 + b_1X1 \]

where \( p \) is the probability of a spike, \( X1 \) is the duration of the gradual rise, \( b_0 \) is the intercept, \( b_1 \) is the coefficient of \( X1 \). The GLM estimates \( b_0 \) and \( b_1 \) (the residuals) and yields T values and p-values for those estimates that can be used to evaluate the probability that the duration of the gradual rise predicts the probability of a spike.

When fitting such a model, one should test if the model is driven by outliers by identifying potential outliers in the residuals, excluding them, and then rerunning the model (Fox, 2015; T, 2014). To do this, we looked for influential outliers using Cook’s distance (Fox, 2015). Initially, we found some outliers when examining Cook’s distance (10-20 points above the other points with Cook’s distances of ~0.01-0.7; Cook’s distance values closer to one are more likely to be outliers). Upon closer inspection, these were all data points with long ramp-ups (>400 ms). Generally, these very long ramp-ups are at the tail of the distribution of ramp-up times, so we were concerned about the model trying to make an estimate using these outliers. We therefore decided to exclude cases with these extra-long ramp-ups (>300ms). After excluding these cases, there were no longer clear outliers. For Cook’s distance, all values were less than 0.004 for single cell analyses and less than 0.025 for paired cell analyses, well below one. Of course, there were some points that were more influential than others. To make sure that the model was not due to a small subset of influential points, we randomly selected half of the data and estimated the regressors with each half of the data separately. The estimates were very similar in both cases.

We then aimed to estimate the probability of spiking as a function of both the duration and size of the gradual rise. To do this we fit a logistic binomial model of the probability of
spiking as a function of duration of the gradual rise, the change in voltage during the gradual rise, and the interaction between these factors using the following linking function

$$\log\left(\frac{p}{1-p}\right) = b_0 + b_1X_1 + b_2X_2 + b_3X_1*X_2$$

where $p$ is the probability of a spike, $X_1$ is the duration of the gradual rise, $X_2$ is the change in voltage during the gradual rise, $b_0$ is the intercept, $b_1$ is the coefficient of $X_1$, $b_2$ is the coefficient of $X_2$, and $b_3$ is the coefficient of the interaction between $X_1$ and $X_2$. Again we looked for influential outliers using Cook’s distance and found a few outliers (1-5 points above the other points with Cook’s distances of ~0.005-0.04). These were all data points with larger gradual rises (>~18 mV in CA1). We therefore decided to exclude cases with larger gradual rises (>16 mV in CA1). After excluding these cases, there were no longer clear outliers. For Cook’s distance, all values were less than 0.01 for single cell analyses and less than 0.025 for paired cell analyses, well below one.

5.4.2. **Both duration and amplitude of gradual rises predict spiking**

As expected, the amplitude of the gradual rise could be used to predict spiking, consistent with the idea that the gradual rise progressively depolarizes a neuron toward the spike threshold, making it easier for a subsequent input to drive spiking. Unexpectedly, however, duration alone, independent of amplitude, carried information about spiking probability, such that even a relatively small (e.g. 4 mV) gradual rise, if it lasted more than 200ms could have the same effect as a much larger (e.g. 12 mV) one (Fig. 37)
5.5. Complementarity of gradual and fast rises in spike generation

We then investigated in more detail how the two components of the slow ramp-up interacted toward spike generation.

5.5.1. Assessing the role of gradual and fast rises in spiking

To determine how the size and duration of ramp-ups in voltage related to the probability of spiking, we computed the size and duration of voltage ramp-ups above baseline that did or did not result in spikes. First we identified depolarizing events when the membrane potential went above and stayed above baseline for at least 20ms to extract periods of depolarization that were longer than most fast rises in voltage. Then we measured the size and duration of the ramp-up from the start of these depolarizing events to their peak if there was no spike during the event or to spike threshold of the first spike if there were spikes during the event. Spikes that did not fall into one of these depolarizing events (e.g. spike threshold was at or below baseline) were rare and were excluded. We then separated ramp-ups in depolarizing events into gradual rises and fast rises and measured the change in voltage and duration of each of these components. The end of the gradual rise and beginning of the fast rise in voltage was identified as when the slope became and stayed positive until the peak of the event for events without spikes or until spike threshold for events with spikes.
To determine if gradual rises that tend to precede spikes were periodic, we measured the interval between the start of gradual rises. To examine the periodicity of gradual rises that were similar to the depolarizing events preceding spikes, we only included gradual rises of depolarizing events (with or without spikes) that were longer and larger than the bottom 25% of gradual rises of depolarizing events with spikes. We repeated this analysis only including gradual rises that were longer and larger than the bottom 50% of gradual rises preceding spikes and found similar results.

We then examined how the gradual rise altered the probability that a particular size or duration fast rise in voltage resulted in a spike. To do this, we computed the proportion of events with spikes as a function of these two measures. First, we binned the data by the duration or amplitude of the gradual rise and the duration or amplitude of the fast rise in voltage. For each bin, we computed the proportion of all the depolarizing events that had spikes to determine the probability of spiking as a function of the gradual rise and fast rise amplitude and duration. We computed the proportion of events with spikes per cell and then took the average of all cells (number of depolarizing events with spikes per cell in CA1: 26, 8, 64, 38, 27, 33, 22, 320, 2, 214, 245, 138, 12, 4, 5, 1, 4, 19, number of depolarizing events without spikes per cell in CA1: 5182, 1320, 711, 1610, 292, 465, 1106, 7768, 2479, 749, 1628, 962, 4840, 162, 1247, 1622, 960, Only bins that included data from at least three cells were included.

5.5.2. Gradual and fast rises interact to drive spiking

The larger the gradual rise, the more quickly the membrane reached threshold once the fast rise began, and the fast rises were accordingly smaller (Fig. 38, top left, Pearson's linear correlation coefficient, $r = -0.51$, $p < 10^{-20}$; Fig. 38, top right, Pearson's linear correlation coefficient, $r = -0.41$, $p < 10^{-20}$; $N = 1170$ spikes from the CA1 neurons). This makes sense, since the gradual rise plus the fast rise must essentially sum to equal spike threshold, and so a large gradual rise would require a smaller fast rise to result in a spike. The duration of the gradual rise, in contrast, was a weaker determinant of the properties of the fast rise (Fig. 38, bottom left, Pearson's linear correlation coefficient, $r = 0.03$, $p = 0.261$; Fig. 38, bottom right,
Pearson’s linear correlation coefficient, $r = -0.06$, $p = 0.0579$; $N = 1170$ spikes from the CA1 neurons), hinting at independence of the mechanisms underlying the gradual and fast rises.

![Image](image.png)

**Figure 38 Slow and fast rises complement each other**

As noted above most spikes were preceded by both gradual and fast rises, and the duration of gradual rises could only predict spikes partly. We thus visualized the probability of spiking when properties of both the gradual rise and the fast rise were considered together (Fig. 39). A diagonal pattern emerged: in general, the larger or longer the gradual rise, the smaller and shorter the fast rise needed to insure a high probability of spiking. Fast rises by themselves, even when they were relatively large in amplitude, did not guarantee spiking, suggesting that this interplay between gradual and fast events helps mediate spike generation in vivo in multiple brain areas. Thus, the gradual voltage rises we identified can be considered a form of activated state for the neuron, intermediate (‘mesoscale’) in duration compared to slow network-wide states (e.g. up/down states) and the fast single-neuron dynamics immediately preceding spiking.
To determine if monotonic increases in membrane potential alone were sufficient to determine when the cell spiked, we identified monotonically increasing events. We identified events when the slope of the membrane potential became and stayed positive for at least 3 ms, to identify periods that were about the length or longer than most monotonic increases preceding spikes. Then we measured the size and duration to the peak of events without a spike or to spike threshold of events with a spike. As we did for the depolarizing events with spikes, we computed the proportion of monotonically increasing events with spikes as function of the change in voltage and duration to the peak or spike threshold. We computed the proportion of events with spikes per cell and then took the average of all cells (number of monotonically increasing events with spikes per cell in CA1: 27, 11, 67, 33, 27, 31, 21, 350, 4, 186, 242, 111, 15, 4, 5, 1, 4, 19, number of monotonically increasing events without spikes
per cell in CA1: 58237, 15923, 9547, 21258, 3446, 7607, 13524, 83341, 24399, 21473, 25848, 13814, 56417, 1766.14058, 18064, 11026, 7896). Only bins that included data from at least three cells were included.

5.5.4. **Fast rises alone are insufficient to drive spiking**

Importantly, fast rises alone, even when of significant amplitude failed to elicit spikes, confirming the potentially important role of gradual rises (Fig 40 left). In addition to analyzing naturally occurring fast rises that did and didn’t result in spikes we also took advantage of whole-cell patching’s ability to inject current into a cell to drive artificial fast rises (Fig 40 right). As for the natural fast rises the spiking probability in response to single artificial fast rises was small.

![Figure 40 Spiking probability as a function of native (left) or artificial (right) fast rises size](image)

5.6. **Mesoscale activated states gate the neuron’s response to a fixed stimulus**

One way to disambiguate the effects of gradual rises/mesoscale activated states on the neuron’s response to inputs from ongoing fluctuations in network dynamics is to deliver a repeated stimulus at random times, and assess post-hoc the probability of the current pulse resulting in a spike in the presence, or absence of a gradual rise-like depolarization (Fig 41)
In all recorded neurons ($n = 9$) the presence of gradual rises significantly increased the probability of the neuron firing in response to the stimulus, across a wide range of stimulus amplitudes (resulting in depolarizations of 5-25mV) and overall probability of firing in response to the stimulus (1.5-81%) (Fig. 42, 43)

Figure 41 Example intracellular trace (top) and stimulus (bottom)

Figure 42 Probability of spiking in response to a stimulus in the presence or absence of gradual rises. Stimuli were tuned to obtain an overall probability of spiking of 1.5-3.7% (triangle), 12-27% (squares), or 37-61% (circles)
5.7. Mesoscale activated states are neither periodic nor network-wide

We aimed to determine whether gradual rises were network-wide phenomena, perhaps related to up-down states or oscillations. We measured the intervals between the starts of gradual rises. An interval measure reveals if an event is periodic regardless of the shape of the trace. Because many of the depolarizing events without spikes were short or small, these small short deviations above baseline could dominate the measure of gradual rise interval and would not reflect the periodicity of large gradual rises that tend to lead to spikes. Therefore to examine the periodicity of gradual rises that were similar to the depolarizing events preceding spikes, we only included gradual rises of depolarizing events that were longer and larger than the bottom 25% of gradual rises of depolarizing events with spikes. We found a wide distribution of gradual rise intervals (177.04 ms - 1.50 s, 20th -80th percentiles, median 527.60 ms) and while there was a slight increase in gradual rise intervals between 50-200 ms, the majority of the intervals, 76.5%, fell outside this range (Fig. 44). We repeated this analysis only including depolarizing events with gradual rises that were longer and larger than the bottom 50% of...
gradual rises preceding spikes and found similar results (data not shown).

![Figure 44 Histogram of intervals between gradual rises onsets](image)

We also examined the local field potential triggered from the start of these gradual rises and found no clear signature in the local field potential to indicate extracellular oscillations (Fig. 45)

![Figure 45 Averaged LFP centered around the onset of gradual rises](image)

### 5.8. Conclusions

Overall, we have shown that, unexpectedly, in the hippocampus of awake behaving mice the vast majority of spikes in putative pyramidal cells is preceded by a ramp-up in voltage that’s very long compared to the membrane time constant of the cell (~50-100ms vs. 5-10ms). This
ramp-up, in turn, is consistently composed of two distinct elements: a relatively slow gradual rise, which takes up most of the duration of the ramp-up, followed by a rapid monotonic increase in membrane voltage, which drives the cell above threshold. Importantly, either element by itself had a very low probability of triggering a spike, suggesting that the gradual rises exerted a gating role in the transformation of the inputs generating the fast rise into spikes.

The same gradual rises, or mesoscale activated states, followed by fast voltage rises before a spike were observed in different network states (baseline, SWR periods, theta oscillations), and in both single spikes and the first spikes of spike bursts, suggesting a degree of generality to the finding. To confirm this, we subsequently analyzed data from another region, barrel cortex of awake mice, which yielded qualitatively similar results (see Chapter 7).

When present, theta oscillations modulated the timing and duration of gradual rises, in a manner consistent with rhythmic changes in conductance and shunting inhibition.

While our preparation differed in some regards from freely moving animals (note for example the lack, or at least scarcity, of place fields), it successfully recapitulated many of the hallmarks of hippocampal network activity in behaving animals, such as nested theta/gamma oscillations and sharp-wave ripples. This suggests that the observations regarding the role of mesoscale activated states and fast rises in spike generation are likely to hold true in more naturalistic contexts as well.
6. Mesoscale activated states and neural rhythms

We have shown that gradual rises/mesoscale activated states gate the ability of neurons to respond to incoming inputs. However, it is well known that a number of network phenomena, in particular theta and gamma oscillations also shape neuronal activity in the hippocampus. We therefore decided to look more closely at the relationship between them.

6.1. Theta oscillations modulate spiking

Although we didn't observe any place field activity in our preparation, the LFP hallmarks of activity appeared normal (see Ch. 4), leading us to expect that, in a similar fashion to what is observed in the freely moving animal, CA1 pyramidal cell spiking might be modulated by the phase of the ongoing theta LFP oscillation.

At the aggregate level, that did not appear to be the case (see Fig. 46)

![Figure 46 Spike count as a function of theta phase. A. \( n = 637 \) spikes from 17 cells in which the injected hyperpolarizing current was less than 100\( \mu \)A. The distribution is not statistically different from the uniform one (\( p > 0.05 \)). B. \( n = 327 \) spikes from 8 cells in which no current was injected. The distribution is not statistically different from the uniform one (\( p > 0.05 \)).]}

There could be a number of explanations for this discrepancy: on one hand theta modulation of spiking might be less than reported for extracellular recordings because the two techniques are biased toward recording different sets of cells, on the other we have seen that little or no place fields were present in the VR environment, which might also lead to an overall
reduction in the extent of theta modulation. Moreover, the number of spikes recorded per cell varies significantly, which might also skew the results. Finally, different cells might have different phase preferences, and it has been shown that not all CA1 pyramidal cells are theta modulated (Tukker et al., 2007): both factors would tend to average out small effects. We therefore analyzed theta-phase spike modulation on a cell-by-cell basis.

We found that a fraction of the cells (9/17) showed theta modulation of spike probability with \( p < 0.05 \) (Fig. 47), though only two had \( p \)-value lower than the Bonferroni-corrected threshold of 0.0031.

Considering the relatively short duration of our recordings (5-15’ is typical), and the low firing rate of the cells, this analysis might miss some neurons that are in fact theta modulated, but fire very few spikes during the recording. To address this issue, in a subset of cells, we
delivered trains of threshold current pulses 10ms long (Fig. 48), and sorted them post-hoc according to the phase of the ongoing theta and gamma oscillations. In 2/9 neurons the distribution was significantly different from uniform (Fig. 49)

Figure 48 Sample traces from the pulse stimulation experiment. Intracellular recording (top), and LFP (bottom). Stimulation pulses are indicated in red

Figure 49 Theta phase distribution of spikes resulting from 10ms current pulses for the two neurons in which it was significantly different from uniform (p < 0.05)
6.2. Lack of membrane voltage fluctuations tracking the LFP are indicative of shunting, rather than hyperpolarizing, inhibition.

Although when the animal was moving the LFP showed very clear theta and gamma oscillations, these were, unexpectedly, not reflected in the membrane voltage (see Fig. 50). This held true both at the population level and at the single cell level.

A number of hypotheses could account for this observation. The simplest would be that only a fraction of the total population receives inputs synchronized with the LFP oscillations, and none of the cells we recorded were among them. This, however, is unlikely on statistical grounds, since we analyzed 18 neurons, and it has been estimated that one third to one half of pyramidal cells are modulated by theta oscillations (Tukker et al., 2007). Moreover, we have shown above that some fraction of the recorded neurons do in fact appear to be in some way affected by the LFP oscillation. Two broad classes of possibilities are then either that the cells participate only transiently in the oscillation, and the resulting membrane voltage fluctuations are averaged out when looking at a whole recording, or that oscillatory modulation of neuronal activity is accomplished by changes in cell conductance and/or shunting inhibition.
To test the first hypothesis we initially analyzed energygrams computed by taking the real part of the Hilbert transform of the intracellular signal during LFP theta, looking for periods lasting multiple theta cycles (e.g. 2") that had a clear peak in the theta frequency band, but failed to find convincing examples.

To look for possibly shorter theta episodes within the intracellular trace we also compared the histograms of energy in the theta band during extracellular theta and at baseline: even if the average energy was the same in the two cases, we would expect episodes of intracellular theta to show up as a peak near the higher energy values, perhaps compensated by an increase in lower energy values. However, the two distributions were not statistically significant. Finally, we looked at the average duration of periods of high theta energy in the intracellular trace in the two conditions (baseline and LFP theta), and again failed to find a difference. Although not conclusive this argues against the idea of transient bursts of oscillatory inputs to the neuron, reflected in corresponding membrane voltage fluctuations.

A possible explanation for the observed data could instead be that oscillations affect neuronal behavior continuously via rhythmic changes in overall conductance or shunting inhibition. If this was the case, we would expect the membrane voltage variability to change as a function of the theta cycle, and that is indeed what we observe (Fig. 51).

![Figure 51 Membrane voltage variability distribution as a function of theta phase](image)

Experimentally, a DC injection of depolarizing or hyperpolarizing current would reveal the underlying shunting inhibition, if present, by driving the cell away from the chloride reversal
potential. This would have to be done over a number of cells, however, considering that only a fraction appear to be modulated by the ongoing oscillations.

### 6.3. Shunting inhibition can be the basis of gamma oscillations in a computational model

Although the large majority of computational models of oscillations assume hyperpolarizing inhibition, shunting inhibition-based models have also been described, at least for gamma oscillations (Bartos et al., 2007; Vida et al., 2006), and a simplified network composed only of excitatory (E) and inhibitory (I) neurons, with sparse random connectivity, stochastic drive to the E cells and shunting inhibition can give rise to a robust gamma rhythm while lacking theta-frequency membrane voltage fluctuations (Fig. 52).

![Graphs showing neuronal index, LFP, single cell, and single cell closeup data.](image)

*Figure 52 A weak PING model of gamma oscillations employing only shunting inhibition reproduces both the observed LFP pattern and the lack of intracellular oscillations. Model and figure courtesy of Christoph Borgers, PhD*
6.4. **Gradual rises and theta oscillations**

Gradual rises have similar characteristics during periods of LFP theta and in the absence of such oscillations (see Chapter 5.2). Within times when theta oscillations are observed, gradual rises don’t significantly differ between cells that are and cells that aren’t modulated by the ongoing oscillations, suggesting that the two can be thought of as separate phenomena, though it’s possible that they share some of the underlying mechanisms. It’s also possible that the brain use different strategies to achieve the same effect (intermediate-duration activated states) during times of LFP theta and outside of such periods.

To investigate the relationship between gradual rises and theta oscillations, we initially computed the average LFP centered around the onset of gradual rises leading to spikes during LFP theta (Fig. 53), which failed to show a clear relationship between intracellular activated states and the extracellular field.

![Figure 53 Averaged LFP centered around the onset of gradual rises during LFP theta](image)

However, LFP averages, especially if carried out over cells that might have different degrees of theta modulation and very different numbers of spikes, might fail to reveal a more subtle relation between the two. We therefore analyzed, on a cell-by-cell basis, whether a relationship existed between the phase of the LFP theta and the onset (Fig. 54) or duration of the gradual rises leading to spikes. In a subset of cells the distribution was significantly non-uniform.
The relatively small number of spikes, however, didn’t allow us to establish whether gradual rises occurred during only a narrow set of theta phases, or whether the relationship varied over time.

We therefore further analyzed the recordings from four cells which showed significant theta modulation of both spiking and gradual rise onset and extended the analysis beyond the ramp-ups that led to spikes to all ramp-ups (defined as the top 10-20% of depolarizing events in duration and/or amplitude), in order to have a much larger number of data points.

A very broad phase distribution of gradual rise onsets and peaks was observed (Fig. 56).
We confirmed that, as for the other cells, this was not due to continuous theta-locked or theta-like membrane voltage fluctuations by measuring the energy in each frequency band during baseline and during LFP theta oscillations (Fig. 57), and by plotting the oscillation peak-triggered average intracellular voltage (Fig. 58). A continuous spectrogram also failed to highlight sustained (e.g. > 1s) periods of heightened theta-frequency power in the intracellular trace during LFP theta oscillations.

Figure 57 Spectral power in the LFP (top) and in the membrane voltage (bottom) during baseline (black) or LFP theta oscillations (red) for a representative neuron displaying theta modulation of spiking and gradual rise onset.
One way to account for this observation is to postulate two fundamentally independent sets of inputs reaching the neuron, one responsible for the observed LFP and theta-frequency modulation, and one underlying the gradual rises. This would be fully consistent with what we know about theta oscillations in the hippocampus, but would add an additional element determining a neuron’s behavior, at least outside of place fields.

However, it’s very likely that some of the same neurons and neuronal classes involved in the generation of the theta rhythm are also responsible for the inputs giving rise to the activated state. Moreover, the same wide distribution of onset and peak phases could in fact be accounted for by varying amplitudes and phase relationships between sets of inputs (e.g. from MDBB or CA3 and from entorhinal cortex) that are themselves rhythmic at theta frequencies.

Further recordings of population activity and intracellular inputs would be required to distinguish between these different scenarios.

6.5. Discussion

Overall, we have shown that the gradual rises are modulated by the effects of ongoing theta oscillations, at least in a subset of cells, although it’s unclear to what extent the same sets of inputs are responsible for the two phenomena.
We have also made the case that, unexpectedly, both theta and gamma rhythms in CA1 are mediated by shunting, rather than hyperpolarizing inhibition. This is in contrast with in vitro studies (Fisahn et al., 1998; Mann et al., 2005; Swann et al., 1989; Traub et al., 2000; Whittington et al., 1995) and computational models (Buzsáki and Wang, 2012; Traub et al., 2001; Wang and Buzsáki, 1996).

Whether GABAergic inhibition in a given context is hyperpolarizing or shunting is important, because shunting vs hyperpolarizing inhibition differentially affect the input-output function of neurons (Bartos et al., 2007) and may play a role in determining how easy it is for a network to synchronize in a given frequency band (Vida et al., 2006)

Considering gamma oscillations first, the only comparable study, carried out by Penttonen and coworkers (Penttonen et al., 1998), in urethane-anesthetized rats shows results at least partially comparable. In fact they report membrane fluctuations locked to the LFP close to 0 mV at resting membrane potential, in the range of the chloride reversal potential, which become significantly larger as the cell is artificially depolarized or hyperpolarized, consistent with a mostly shunting inhibition under resting conditions. Note that the recordings were carried out with sharp electrodes, less likely than patch pipettes to alter the cell’s chloride concentration, and hence the GABA reversal potential. Although they report increased gamma-frequency power during extracellular gamma, the figure shows rather broadband increase in power across virtually all frequencies, making the claim less convincing.

For theta oscillations, our data differs from what is observed under urethane anesthesia (Kamondi et al., 1998), where both a DC hyperpolarization and theta-like intracellular oscillations were observed. Part of the discrepancy likely arises from the different conditions, i.e. awake vs. anesthetized (e.g. theta under urethane anesthesia has been shown to be similar in some respects similar to theta with EC inputs eliminated). Still, a large decrease in input resistance was observed under anesthesia during theta (though it wasn’t clear if it was phase-specific). Moreover, feedforward activation of basket cells by the commissural input evoked clear shunting inhibition (Kamondi et al., 1998), suggesting that in that case, too, inhibition may be mostly shunting.
More recently, a model that aimed to recapitulate in vivo recordings around place fields showed that shunting, but not hyperpolarizing inhibition, allowed the model to reproduce all relevant features of the in vivo data (Losonczy et al., 2010). Moreover, if inhibition is rhythmic at theta frequency and shunting it would result in increased magnitude of subthreshold oscillations as the cell is depolarized (either naturally or by the experimenter), consistent with observations in both awake and anesthetized animals (Harvey et al., 2009; Kamondi et al., 1998).

A number of experiments could further prove or disprove whether, and under what conditions, hippocampal rhythms are mediated by shunting inhibition. For example, using a DC current to hyperpolarize or depolarize the neuron could ‘unmask’ the underlying inhibitory inputs, as would recording in voltage clamp mode at a depolarized potential (e.g. 0 mV), although the latter is technically challenging in the awake animal. Ideally, perforated-patch (Martina et al., 2001) recordings would be employed to ensure that the cell’s native chloride concentration, and hence GABA reversal potential, is not affected. Sharp recordings would also be advantageous in this context, and their use in awake animals has become more established in recent years (English et al., 2014; Long et al., 2010).
7. Mesoscale activated states in the barrel cortex of awake mice

We wondered if the long ramp ups to spiking observed in CA1 neurons might be unique to the hippocampus or if they preceded spikes in neurons of other circuits as well. To address this question, we analyzed whole-cell patch clamp recordings from neurons in the barrel cortex of awake mice obtained by Dr. Kodandaramaiah.

Barrel cortex has often been used as a model cortical system in the mouse and the rat, and recordings in the region can be combined with relatively straightforward sensory stimulation.

7.1. Spiking is consistently preceded by slow ramp-ups in $V_m$, composed of a slow and a fast component

We found long ramp-ups preceding spikes (Fig. 59, 60 31.64-146.50ms, 20th-80th percentiles; median 68.93ms; N = 22 neurons from 16 mice which were awake, headfixed and immobilized), similar to those observed in CA1.

![Figure 59 Long ramp-up for a representative barrel cortex neuron. Four example traces (left), and average for all traces (right)]
Figure 60 Population data for ramp-up duration in barrel cortex neurons

These ramp-ups in barrel cortex included a gradual rise which lasted most of the ramp-up followed by a fast rise (Fig. 61A, fast rise duration: 3.80-16.99ms, 20th-80th percentile; median 8.60ms) and the fast and gradual components were of similar size (Fig. 62B, 5.36-15.71mV, 20th-80th percentiles, median, 11.63mV for the gradual rise vs. 3.27-13.41mV, 6.10mV for the fast rise).

Figure 61 Duration and amplitude of fast and gradual rise components of the ramp-up

As in CA1 most spikes were preceded by both gradual and fast rises: only 9.92% ± 13.14 of spikes did not have a gradual rise and 0.21% ± 0.97 did not have a fast rise (mean ± standard deviation, N = 22 (Fig. 4).
Furthermore depolarizing events with spikes had longer (Fig. 63, 31.23-145.83ms, median 66.95ms, for spike events, vs. 11.40-64.59ms, median 26.25ms, for non-spike events; p < 10^{-50}, ranksum test, N = 985 spike events and 14429 non-spike events) and larger gradual rises than depolarizing events without spikes (Fig. 63, 7.89-16.34 mV and 12.36 mV, spike events, vs. 1.62-9.62mV and 4.44mV, non-spike events; p < 10^{-50}, ranksum test).

Figure 63 Duration (left) and amplitude (right) for depolarizing events that included (red) or didn’t include (blue) spikes.
The gradual rises in barrel cortex were also not periodic (Fig. 64, gradual rise intervals were 270.93ms - 2.13 s, 20th -80th percentiles, median 786.63ms). These results show that long ramp ups, consisting of a gradual and fast rise, occur in multiple brain regions.

**Figure 64 Inter-gradual rise interval**

### 7.2. Simultaneous dual intracellular recordings

To determine if these gradual rises are coordinated across cells, a two-channel version of our autopatcher was used to record pairs of neurons within 500 microns of each other in the barrel cortices of awake mice.

#### 7.2.1. Dual intracellular recordings analysis

The single cell analyses included four cell pairs (two cells recorded simultaneously). To further examine simultaneous intracellular activity, we recorded from additional cell pairs for a total of seven cell pairs in barrel cortex for which both cells reached criteria for inclusion (see Ch.5.1.1). In these cells we detected depolarizing events as we did for single cells. For each depolarizing event, we calculated how correlated the membrane potentials were between the cell in which the depolarizing event was detected and the nearby neuron by computing the Pearson’s correlation coefficient between the membrane potential of the cells.
from the start of the depolarizing event until the peak of the event, if there was no spike, or until spike threshold, if there was a spike. We measured the duration and amplitude of these depolarizing events, as we did for single cells, separating them into three groups: depolarizing events with a spike in the same cell used to detect the depolarizing event, with a spike in the nearby neuron but not in the same cell, or without any spikes in either cell.

To compare ramp-ups between cells, we selected spikes from one cell in the pair (the reference spiking neuron) and compared the membrane potential during the period preceding those spikes to the membrane potential of the other cell in the pair (the nearby neuron). We then repeated these analysis selecting spikes (see Ch.5.1.1) from the other cell in the pair, which would then be deemed the spiking neuron. To determine if nearby neurons tend to start ramping up at the same time, we computed the start of the ramp-up in each cell based on when that cell’s membrane potential went and stayed above baseline until spike threshold was reached in the spiking cell of the pair.

To compare the size of the gradual rise during these ramp-ups, we decomposed the ramp-up in each cell into a gradual and fast component and computed the change in voltage during the gradual rise. We also computed the size of the fast rise in membrane potential for each cell starting from when that cell’s slope became and stayed positive until spike threshold in the spiking cell of the pair. We performed these analyses first including all spikes that met criteria (see Ch.5.1.1) and then repeated them excluding cases when both cells fired within 150ms and found qualitatively similar results. Excluding cases when the two cells fired together excludes cases when we might observe ramp-ups in both cells simply because ramp-ups precede most spikes. We repeated these analysis including only cases when the two cells fired within 150ms.

7.2.2. **Mesoscale activated states are shared across cells, fast rises are cell-specific**

In seven pairs of neurons, we noted that when one neuron spiked (denoted the “reference neuron”), its ramp-up in voltage was sometimes highly correlated with the ramp-up of the other neuron (denoted the “nearby neuron”), whether the nearby neuron spiked or not (Fig. 65A).
For example, in one neuron 47.6\% of these events exhibited a correlation greater than 0.5, whereas 12.02\% of the events exhibited a correlation of less than -0.5 (Fig. 65B). Thus, uncorrelated as well as anticorrelated events could be observed (see the other 6 neuron pairs’ correlations in Fig. 66); across all cell pairs 7.27\% of events were anti-correlated (less than -0.5).

On the whole, however, the correlations in ramp-up were significantly larger during depolarizing events that led to spiking in at least one neuron of the pair than in depolarizing events that did not lead to spikes in either neuron (Fig. 66; p = 0.00021 ranksum test; N = 6282 depolarizing events with no spikes in either neuron, 289 depolarizing events with a spike in at least one neuron), suggesting a link between the coherence of the gradual rises across the network and spike generation.

Figure 65 A. Representative traces from a pair of simultaneously recorded neurons, with exemplar gradual rises that were shared (left) or not (center) between the two cells. Averages for all spikes are plotted on the right. B Histogram of correlation values across spikes for the two cells shown in A. Left corresponds to the traces in A, top, Right to the traces in A, bottom.
Indeed, this coordination was strong enough that it was to some extent possible to predict the spiking of one neuron from the duration of the gradual rise of the other neuron. Using a generalized linear model as in Ch. 5.4.1 we found that gradual rises lasting 250 ms in one neuron were associated with the other neuron exhibiting a 29.2% chance of spiking (Fig. 67, left). Note that this ability to predict the spiking of one neuron, from the gradual rises of the other, occurred to some extent even when the latter neuron was engaged in a depolarizing event that did not contain or result in a spike. As before, small gradual rises that were of long duration were as predictive as large gradual rises (Fig. 67, right).

As noted before, depolarizing events that contained a spike were larger and longer than
depolarizing events that did not (Fig. 68, left, solid red line). Perhaps surprisingly, however, depolarizing events that did not contain a spike, but during which the other neuron did spike (Fig. 68, left, dotted red line), possessed gradual rises that were also larger and longer (29.49-161.37ms 20th-80th percentiles, median 69.35ms vs. 12.05-66.95ms, 26.9ms; p < 10^{-20}, ranksum test, smaller than Bonferroni corrected p-value of 0.017 for 3 comparisons, N = 5492 depolarizing events with no spikes in either neuron, 211 depolarizing events with no spikes in the current neuron but a spike in the nearby neuron) than depolarizing events during which neither neuron spiked (Fig. 68, left, blue line). Indeed, these gradual rises were comparable to those that did precede spikes (Fig. 68, left, red line, 38.08-170.03ms 20-80th percentiles, median 89.10ms; p = 0.0267, ranksum test, greater than the Bonferroni corrected p-value of 0.017 for 3 comparisons, N= 263 depolarizing events with a spike in the current neuron, 211 depolarizing events with no spikes in the current neuron but a spike in the nearby neuron from 7 cell pairs in barrel cortex). The amplitudes of the gradual rises followed a similar pattern (Fig. 68, right; 5.96-13.10 mV 20th-80th percentiles, median 9.73mV, dotted red line; 1.76-9.59mV, median 4.63 mV, blue line; 8.69-17.59mV, median 13.32mV, solid red line; p < 10^{-20}, ranksum test, depolarizing events with no spikes in either neuron versus depolarizing events with a spike in the current neuron; p < 10^{-20}, ranksum test, depolarizing events with no spikes in either neuron versus depolarizing events with no spikes in the current neuron but a spike in the nearby neuron; p < 10^{-10}, ranksum test, depolarizing events with a spike in the current neuron vs. depolarizing events with no spikes in the current neuron but a spike in the nearby neuron; N = 5492 depolarizing events with no spikes in either neuron, 263 depolarizing events with a spike in the current neuron, 211 depolarizing events with no spikes in the current neuron but a spike in the nearby neuron), although the gradual rise was smaller in the cell that did not fire
Figure 68 Depolarizing events duration (left), and amplitude (right)

Temporally speaking, the ramp-ups began somewhat synchronously: 16.8% of ramp-ups started within 10ms of each other, 36.0% started within 20ms of each other, and start times were correlated (Fig. 69, left $p < 10^{-10}$, Pearson's linear correlation coefficient, $r = 0.389$, $N = 249$ spikes).

However, in contrast to the loose coordination of gradual rises across multiple neurons, fast rises were cell-specific: when one neuron spiked and the other did not, the spiking neuron had a fast rise of amplitude 2.93-13.95 (20th-80th percentile; median 5.98), whereas in the non-spiking cell the change in voltage was essentially absent, 0-1.89mV (20th-80th percentiles, median 0.06mV, Fig. 69, right). Thus, unlike the shared gradual rises which appeared in multiple neurons in the network, fast rises may reflect inputs specific to individual cells or small subsets of cells in the network.

Figure 69 Gradual and fast rises across simultaneously recorded pairs of neurons
7.2.3. Evolution of cell-to-cell coordination over time

The remaining six pairs of cells (Fig. 70) exhibited similar dynamics. Of interest, the same cells could alternate between periods with strong correlation between gradual rises to periods when they weren’t correlated, or were even anti-correlated. This suggests that the subsets of cells within a network that are simultaneously brought in an ‘activated’ state by the gradual rises can vary dynamically over time, and that cells can belong to multiple such assemblies.
Figure 70 sample traces, averages, and correlation during ramp-up histograms for the remaining cell pairs.
8. Discussion

We have shown that gradual rises/network integration occur in two distinct regions, in different network and behavioral states, and appear to gate, at least partially, the ability of a neuron to respond to incoming inputs. This raises, however, a number of questions: how general is this phenomenon? What is its functional and computational effect/role? How does it mechanistically come about?

8.1. Gradual rises as activated states specific to dynamically changing ensembles of neurons within a network

The fact that we observe gradual rises in both hippocampus and barrel cortex, in a variety of network and behavioral states, raises the question of just how general a phenomenon this is. Does it hold true in the same cell across different behavioral states? Within a given region, does it hold true across different cell types? And does it hold true across all regions of the brain?

We know that this dual structure (slow gating, on the tens to hundreds of ms timescale, combined with fast inputs driving the neuron above threshold) doesn’t hold true in some cases, such as, for example, tonically firing thalamic neurons, or PV+ interneurons during gamma oscillations, firing at high frequency.

However, it might still be a fairly general pattern throughout the brain, at least in slow-firing pyramidal cells. To the general class of slow network phenomena gating a cell’s response at faster timescale also belong, for example oscillations, up/down states (Buzsáki and Draguhn, 2004; Fujisawa and Buzsáki, 2011; Haider and McCormick, 2009; Sirota and Buzsáki, 2005; Steriade et al., 1993), and the more nuanced and graded ‘activated states’ described more recently (McGinley et al., 2015a).

Unlike oscillations, however, gradual rises don’t appear to be rhythmic, and they occur at significantly faster timescales (tens to hundreds of ms) than other network states. Moreover, unlike oscillations, up/down states, and more continuous activated states, (Buzsáki et al., 2012; Saleem et al., 2010; Sirota and Buzsáki, 2005), gradual rises appear to be specific to a smaller subset of cells, as reflected by the lack of LFP signature (see Ch.5). In addition,
while we often saw correlations between gradual rises across neurons in a simultaneously recorded pair (see Ch. 7), neurons’ membrane dynamics were often not correlated – or even anti-correlated – suggesting that the ensemble of neurons engaged by any one ramp event is a fraction of the neurons in the microcircuit, and that the ensemble engaged by a particular ramp event can be dynamically selected. Gradual rises might therefore be thought of as activated states specific to dynamically changing ensembles of neurons within a network.

8.2. The functional role of mesoscale activated states

The amplitude of the gradual voltage rises was ~3-10 mV. Considering that neurons are rather sensitive to small changes in membrane voltage (Carandini and Ferster, 2000; Contreras and Palmer, 2003; Haider et al., 2007; Sanchez-Vives and McCormick, 2000), such depolarized states are expected to have a very strong effect on neural responsiveness. This is in fact consistent with our current injection experiments (see Ch. 5), which show that gradual rises might exert a gating function, determining, or at least strongly affecting, whether a neuron responds to brief incoming stimuli or not. This suggests a number of possible functional roles.

At the cell ensemble level, gradual rises could be thought of as helping pre-select which cells, among a possible pool, will be likely to participate in a particular cell ensemble (Buzsáki, 2004; Harris, 2005; Harris et al., 2003). Additionally, such transient activated states could help neurons in the ensemble fire more synchronously, enhancing their downstream effectiveness, or might facilitate interaction among the participating cells, resulting in a competitive advantage over other groups of neurons. In either case, even small transient activated states could have a powerful, network-wide effect.

Transient activated states specific to subsets of neurons in a network may also complement slower and more broadly shared network states (Arieli et al., 1996; Fox et al., 2007; Gur et al., 1997; McGinley et al., 2015a; Zagha and McCormick, 2014) in explaining some of the trial-to-trial variability observed in vivo (Shadlen and Newsome, 1998). In our experiments, when injecting a subthreshold noisy current waveform in a neuron, the cell fired with very high precision (so that the exact timing of a spike appears mostly dictated by the immediately
preceding membrane voltage trajectory and inputs), not dissimilar to neurons in culture (Mainen and Sejnowski, 1995), but with low reliability. This is almost entirely explained by slow underlying dynamics at the tens to hundreds of ms timescale, which therefore effectively gate whether the neuron will or will not fire. If it will fire, it will do so at a stereotyped time in the stimulus waveform.

Functionally, such transient activated states, specific to subsets of neurons within a network may mediate the increases in excitability associated with shifts in attention (Haider et al., 2007; Murphy and Miller, 2003). Cells that share the attended sensory dimension might participate in such activated states, resulting in increased probability of spiking, both in response and in the absence of the attended stimulus (Desimone and Duncan, 1995; Luck et al., 1997; Reynolds and Chelazzi, 2004; Reynolds et al., 2000; Sundberg et al., 2009).

More generally, gradual rises could be thought of as playing a role in gain modulation, a key operation (Salinas and Sejnowski, 2001; Salinas and Thier, 2000; Treue and Trujillo, 1999) of neural circuits: at the population level, the proportion of cells that are ‘allowed’ to fire can be tuned by controlling the amplitude of the gradual rise and the number of cells displaying this activated state.

A preliminary step in testing these hypotheses would be to confirm that gradual rises do in fact affect the probability of responding to a sensory stimulus or to a more biologically meaningful form of stimulation than somatic current injection (e.g. optogenetic stimulation of afferent neurons). It’s highly unlikely that the result would differ fundamentally, however, as the voltage recorded by the patch pipette is very close to the voltage seen by the typical site of spike initiation, the axon hillock, whereby altering the voltage via the patch pipette should affect spike probability in a manner similar to more naturalistic stimuli that integrated to the same somatic membrane voltage change.

One way to explore the possible role of gradual rises in selecting cell assemblies would be to patch two neurons in a given region, while simultaneously performing either optical imaging (Dombeck et al., 2007; Göbel et al., 2007; Grewe et al., 2010; Tian et al., 2009) or large-scale extracellular recordings (Blanche et al., 2005; Buzsáki, 2004; Csicsvari et al., 2003; Shobe et al., 2015). Assuming that the cell ensembles tend to persist over time (Grewe and Helmchen, 2009; Harris, 2005; Harris et al., 2003; Luczak et al., 2009), one would expect the
same cells that fired when there was a gradual rise in both patched neurons to (be more likely to) fire again upon such occurrences, regardless of whether the patched neurons actually spiked. Conversely, a different set of cells should fire when there is a gradual rise in one, but not both, of the neurons. It’s possible, however, that gradual rises serve only to form the initial cell assembly, and subsequent reactivation can be distinct from gradual rises, though since virtually all spikes are preceded by gradual rises this is a less likely scenario.

It could also be fruitful to investigate the possible role of gradual rises in selective attention: we would expect the frequency, and perhaps amplitude and/or duration, of gradual rises to increase in neurons coding for the attended stimulus, compared to a baseline (non-attended) condition. We would also expect gradual rises to be more frequently coherent among cells coding for the same feature, and this preference to be increased by attention.

Attention has most often been studied in non-human primates, but, despite some successes (Tan et al., 2014), it’s hard to combine such experimental preparation with whole-cell patch clamping. However, some relevant tasks might be adapted to mice and rats (Humby et al., 2001; Otazu et al., 2009; Uchida and Mainen, 2003; Yang et al., 2008b), making the study of the role of gradual rises in attention significantly more tractable.

Assessing the role of gradual rises as a general gain control mechanism would perhaps be more difficult, considering that, at present, it’s not possible to monitor the subthreshold dynamics of many cells simultaneously, though this might change with the advent of better genetically encoded voltage sensors (Ghitani et al., 2015; Gong, 2015; Knöpfel et al., 2015) and imaging methods (Prevedel et al., 2014; Quirin et al., 2016; Yang et al., 2016). However, it could still be investigated if one had a clearer grasp on the mechanistic basis of gradual rises.

A fundamentally different way in which gradual rises might affect neuronal function is by setting synaptic weight. It is know that in vitro somatic membrane voltage can affect the amplitude of postsynaptic currents (Alle and Geiger, 2006; Kole et al., 2007; Shu et al., 2006; Zhu et al., 2011), but see (Apostolides et al., 2016), and it’s tempting to speculate that something similar might be happening in vivo, at least in some cell types. Although this has typically been studied with non-naturalistic patterns of somatic membrane voltage, the
gradual rises we observe are of comparable amplitude and duration to some of the experimental conditions used in vitro (Alle and Geiger, 2006; Shu et al., 2006).

8.3. The mechanistic basis of gradual and fast rises

In principle, as is the case with other network phenomena such as oscillations (see e.g. (Colgin, 2013; Roopun et al., 2008; Whittington et al., 2011)), a wide variety of mechanisms might underlie what we observe as similar gradual rises, and the exact mechanism might be relevant to their computational role. For example, if gradual rises were associated with a massive influx or release of Ca++ that would have wide-ranging consequences for plasticity and the molecular state of the neuron. To take another example, gradual rises resulting from a progressive decrease in inhibition versus an increase in both excitation and inhibition (with the predominance of the latter), by changing overall cell conductance to different degrees, might differentially affect the integration time constant of the neurons, in turn changing the neuron input-output function and its sensitivity to the temporal synchrony of inputs (Destexhe and Paré, 1999; Destexhe et al., 2003). Overall, however, they all likely share the basic function of gating the neuron’s response to subsequent inputs.

One constraint on possible mechanisms is placed by the known connectivity pattern within the region under study: for example, the relatively sparse connectivity between pyramidal cells in CA1 makes it unlikely that that region itself dynamically creates such events, e.g. in an emergent fashion similar to up/down states (Haider et al., 2006; Shu et al., 2003). In contrast, that is certainly a plausible explanation for gradual rises observed in cortical regions.

The extent to which the gradual rises are shared across the network also places some constraints on the possible mechanisms (though with few double patch recordings it’s hard to exactly quantify that): while individual neurons can display autonomous slow dynamics e.g. due to slow NMDA or mGluR receptors, the fact that multiple neurons exhibited similar gradual voltage ramps suggests a network mechanism. One possibility is that shared external inputs to multiple neurons, e.g. from the thalamus or from other cortical regions, could supply this correlated drive.

A number of possible experimental approaches (even limiting oneself to current more or less common techniques) could be taken to try and clarify this question.
An initial step could be, while patching, injecting hyperpolarizing (or small, depolarizing) pulses to measure conductance, though care must be taken in identifying gradual rises within the trace. Some evidence from recordings in which we injected threshold depolarizing current pulses, but didn’t elicit spiking, seem to suggest that conductance is in fact elevated during gradual rises in CA1 putative pyramidal cells, but in this case, by restricting ourselves to cases when the neuron didn’t spike in response to a relatively large input, we might be biasing our selection toward gradual rises where in fact there was increased conductance, leading to a smaller depolarizing response.

Taking advantage of the fact that gradual rises are sometimes shared between two neurons one could record the two cells for a period in current clamp, to establish that they do in fact show correlated gradual rises at least part of the time, then switch one recording to voltage clamp, to tease out excitatory and inhibitory components. The fact that gradual rises are not always shared between the cells makes the analysis more difficult but is not an insurmountable problem if a sufficient number of data points is collected.

This would help begin to distinguish between decreasing inhibition, increasing excitation, combined decrease, or combined increase, with one component predominant upon the other. Although still technically challenging, the use of automated systems like the multipatcher has made the collection of such data significantly easier (Kodandaramaiah et al., in preparation).

Complementary, and easier to obtain, data would be gained from simultaneous patching and large scale extracellular recordings, perhaps combined with optogenetics to identify cell types (Kravitz et al., 2013; Zhao et al., 2011) beyond what is allowed by waveform shape alone, or a combination of patching and optical imaging of genetically encoded calcium/voltage sensors. It would help reveal whether gradual rises are in fact due to self-sustained bursts of activity within a given network, and shed light on the activity of inhibitory neurons.

The wide divergence and more limited convergence of connectivity from interneurons to pyramidal cells (Freund and Buzsáki, 1996; Markram et al., 2004; McBain and Fisahn, 2001) makes them a potentially promising substrate for gradual rises shared across dynamically changing subsets of neuron within a network A progressively diminishing drive to ‘fast’ inhibitory neurons (or progressive inhibition) could give rise to the observed gradual rises. A
complementary or alternative mechanism could be the transient inhibition of interneurons with a more asynchronous GABA release, such as SOM+ interneurons in the hippocampus (Hefft and Jonas, 2005; Hestrin and Galarreta, 2005). If that was the case, brief activation of such interneurons using optogenetics would result in gradual-like patterns in pyramidal cells. In interpreting such an experiment, however, one would have to be careful to assess whether artificially induced depolarizations not only matched the gradual rises time courses, but also their amplitude distribution, conductance levels, and were consistent with voltage-clamp recordings.

Although the exact connectivity patterns of neurons in CA1 and barrel cortex differ significantly, they do have some commonalities. In particular they share an inhibitory interneuronal circuit by which VIP+ interneurons inhibit pyramidal cell-targeting PV+ and SOM+ interneurons, with the net effect that activating VIP+ interneurons disinhibits pyramidal cells in the network ((Freund and Buzsáki, 1996; Freund and Gulyás, 1997; Markram et al., 2004; McBain and Fisahn, 2001; Pi et al., 2013)). A number of studies have already shown that this interneuron circuit is a common mechanism by which multiple neuromodulators and glutamatergic projections from higher order regions can act in the cortex (Fu et al., 2014b; Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013).

Local infusion of AMPA-R antagonists, or optogenetic silencing of pyramidal cells in a region might be expected to disrupt gradual rises if they were due to self-organized local network activity, but such an intervention is likely to be of difficult interpretation. The potential role of NMDA, in contrast, could be more cleanly assessed by including a blocker such as mk-801 in the pipette solution(Popescu et al., 2007; Reeve et al., 2012). In that case, if NMDA is indeed involved we would expect a sharp decrease in the frequency, amplitude, and duration of gradual rises, in response to, presumably, the same inputs as before, since the rest of the network would not be perturbed.

The gating function of gradual rises and its shared nature is perhaps suggestive of its origin in projections from higher-order regions, or, within a region, from separate layers. Two possible approaches to validate this view would be looking at extracellular activity in regions projecting to the target one or, using calcium or voltage sensor, directly image their axonal
projections. The latter might be particularly informative is coupled with a dye in the patch pipette, allowing to focus one’s attention on axonal fibers near the cell of interest.

Of particular interest, in addition to excitatory inputs from higher-order regions, might be cholinergic, noradrenergic, and other neuromodulatory projections. Although, again, directly imaging the axonal projections near the cell of interest would yield the cleanest data, an initial test might be carried out employing pupillometry, using pupil diameter as a proxy for arousal. Recently this approach has been used to show that a significant fraction of cortical neuron’s membrane dynamics might, at slow timescales (seconds to tens of second) be explained by continuous variations in arousal level (McGinley et al., 2015a, 2015b; Polack et al., 2013; Reimer et al., 2014). Gradual rises are significantly faster phenomena, but microdilations do show some correlation at the hundreds of milliseconds timescales. The relatively low correlation might be somewhat misleading however: although we often think of neuromodulators as indiscriminately ‘bathing’ a region, it’s possible that subsets of cells might be more or less strongly modulated at different times, so that the aggregate correlation would not be a good measure. If microdilations do in fact index fast selective modulation of subsets of neurons, we would expect to see them with every gradual rise, though the converse would not be true, since the patched neuron would not belong to all subsets of cells affected at different times.

Pharmacology and/or optogenetic could be used to confirm this possibility. Indeed, there is some intriguing evidence that Ach, but not NE, blockade reduces 5-8 Hz membrane voltage fluctuations (Polack et al., 2013) in visual cortex, which might be a result of the abolition of gradual rises. A similar effect would be obtained with optogenetic silencing, while optogenetic stimulation of the projections to the region would be expected to cause naturalistic gradual rises. A complementary pharmacological approach would be to use, e.g. reuptake inhibitors, which would be expected to shift the average of duration toward longer gradual rises.

It cannot be excluded, however, that gradual rises arise mostly through mechanisms operating within the cell. Dendrites, for example, can have complex, long lasting dynamics (Grienberger et al.; Häusser and Mel, 2003) that might be reflected at the soma as gradual
rises. This could be tested with combined intracellular recording and high-resolution calcium or voltage imaging (e.g. by injecting the Ca++ dye via the patch pipette).

Getting a better handle on a putative mechanism would allow effective interventions to causally test the functional role of gradual rises. E.g., if projections from a higher region silence SOM+ interneurons and cause gradual rises, then affecting either one would drastically affect gradual rises while leaving most other aspects of network function intact.

At the current stage of our knowledge, there are too many possible mechanisms for modeling to be fruitfully brought to bear on the problem; however, that would change once more information concerning the activity of different cell types in the network and excitation/inhibition inside the neuron emerge. Even now, however, in light of the considerable impact that gradual rises, or subnetwork-specific active microstates have on neuronal responses to input, it might be fruitful to incorporate them into existing models, if only as exogenously provided, sporadic, relatively large and slow rises in Vm.

The time scale of fast rises, in contrast (~5-10ms), similar to the membrane time constants of neurons in vivo (Altwegg-Boussac et al., 2014; Gao et al., 1999; Léger et al., 2005; Yamashita et al., 2013), suggests that they may be primarily due to synchronized barrages of excitatory inputs. Once a ‘signature’ of gradual rises at the level of synaptic currents has been obtained, it would be possible to start looking for fast rise-like events, and discern the contribution of excitation and inhibition. In fact, in addition, or in alternative to, a barrage of synchronized inputs, a sudden drop, or at least a temporal shift, in counterbalancing inhibition might also account for the observed fast voltage rise.
9. Conclusions

There is increased appreciation for the role of ongoing activity in shaping overall neural computation and response to external stimuli (Azouz and Gray, 1999; Haider and McCormick, 2009; McGinley et al., 2015a, 2015b; Niell and Stryker, 2010; Petersen et al., 2003; Poulet and Petersen, 2008b; Zagha and McCormick, 2014), but a significant obstacle to our understanding is represented by the difficulty of obtaining high-quality intracellular recordings in awake animals, capable of revealing subthreshold cellular dynamics.

To address this limitation we adapted the use of automatic in vivo patch clamp, previously developed by our groups (Kodandaramaiah et al., 2012), to perform intracellular neural recordings in the awake mouse brain, by enhancements in the algorithm, hardware, and experimental preparation.

Analysis of recordings taken in both the CA1 field of the hippocampus and barrel cortex of awake mice showed that, unexpectedly, the vast majority of spikes arose from the interaction of two fundamentally distinct components: slow (tens to hundreds of milliseconds) gradual rises in the membrane voltage, and fast (few milliseconds), monotonic depolarizations that drove the cell above spiking threshold.

We have shown that the gradual component effectively gates the ability of the neuron to respond to subsequent inputs, and dual recordings have provided evidence that such gradual rises are shared by subsets of neurons within a network, evolving over time. Such gradual rises can then perhaps be thought of as cell ensemble-specific activated states, distinct from more classical network stats such as up/down states or oscillations that affect a larger fraction of the network simultaneously, as reflected by their LFP signature (which gradual rises lack).

Fast rises, likely reflecting the integration of highly synchronized barrages of inputs, in contrast, appeared to be highly cell-specific.
This dual structure held true across different network and behavioral states, and interacted with better studied network phenomena such as hippocampal theta oscillations in determining spiking output. Future studies, taking advantage of the power of in vivo intracellular recording, could use pharmacology or optogenetics to probe the mechanisms that support these cellular and network effects.

By helping pre-select which cells may respond to subsequent inputs, gradual rises might play a role in the formation of cell ensembles (Buzsáki, 2004; Harris, 2005; Harris et al., 2003), as well as in selective attention (Desimone and Duncan, 1995; Moran and Desimone, 1985; Reynolds et al., 2000), allowing cells encoding for a particular feature of interest to respond with higher reliability.
10. References


