ELECTRIC FIELDS AND SLOW CORTICAL ACTIVITY

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Brian S. Wolff, B.S.

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Brian S. Wolff, B.S.

Thesis Advisor: Jian-Young Wu, Ph.D.

ABSTRACT

Electrical activity in the brain gives rise to endogenous electric fields, which are the cause of many signals recorded from the nervous system. Electric fields can also be applied from external sources to modulate the excitability of neuronal populations in both research and clinical settings. I use a brain slice model to investigate the effects of oscillating electric fields on cortical networks. A combined approach of voltage-sensitive dye and two-photon calcium imaging techniques provides a detailed view of the spatiotemporal patterns of network activity and its modulation by electric fields. I find that electric fields induce very small polarizations that can generate much larger population events. I find that low extracellular calcium levels facilitate slow oscillatory activity by increasing sodium conductance, and that this confers an increased sensitivity of the network to electric fields. This suggests that electric fields and extracellular calcium levels may act in concert to synchronize cortical networks. And finally, I provide evidence for a novel theory that electric fields influence and reinforce the activation of neuronal ensembles during Up states. Together these results suggest a significant new hypothesis, that the generation of electric fields is an important mechanism in the network activation for consolidating memories during sleep.
The research and writing of this thesis is dedicated to everyone who helped along the way.

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Chapter I: Introduction

Electric Fields and Neuronal Activity

**Endogenous Electric Fields.** Neuronal activity in the brain is, fundamentally, the movement of charged particles across cellular and subcellular compartments separated from each other by thin membranes. The charges inside and outside of neurons give rise to electric fields that can be measured in a variety of ways, most commonly by nearby electrodes. When these electrodes are only a short distance from the neuronal activity that generates the fields, they can record very fast signals from single neurons ("spikes") as well as much slower signals from populations of neurons ("local field potentials", or LFP). As electrodes become farther removed from the neuronal activity, only the low-frequency signals from large populations are recorded. This is the case with the recordings in an electroencephalogram (EEG), where electrodes are placed on the scalp and are able to detect signals only from highly synchronous changes in membrane potential across large populations of cortical neurons. Well-known examples of such signals are the spindles and delta waves that characterize the distinct stages of sleep.

Extracellular or EEG electrodes can pick up such large signals because the cytoarchitecture and functional organization of the cerebral cortex are particularly well-suited to generating large fields. The most numerous and typically largest cells in the cortex are pyramidal cells, which have large apical dendrites forming tufts at the outer surface of the cortex. The dendritic tufts connect to soma deep within the cortex via long, thick dendritic cables running perpendicular to the cortical surface. Electrophysiological recordings have shown that when active, pyramidal neurons can
generate current sources and sinks that are separated by long distances across the depth of the cortex, forming long dipoles (Mitzdorf 1985). This large degree of separation between sources and sinks, between relatively positive and negative regions, can give rise to large electric fields. The functional organization of cortex into columns, where nearby pyramidal neurons tend to be synchronously active, allows the populations of parallel-aligned pyramidal neurons to simultaneously generate overlapping electric fields. Together, these fields sum up into the very large LFP signals recorded by nearby electrodes, or even more distant electrodes outside the skull with EEG. In contrast, cells with a high degree of symmetry will be unable to form dipoles, and thus generate only very small fields. Thus, brain regions that have a columnar organization, like the cortex or hippocampus, can generate higher-amplitude LFPs than brain regions that lack this organization.

**Applied Electric Fields.** Long after the discovery of EEG and the local field potential, it was found that the process can work in reverse, that fields generated outside of the skull can influence electrical activity in the cortex. The earliest research focused on treating psychiatric disorders in human subjects (Redfearn et al. 1964, Costain et al. 1964, Herjanic et al. 1967) through the use of transcranial direct current stimulation (tDCS). This technique involves placing a small electrical stimulator next to the scalp above a cortical region of interest, which generates an electric field across the surface of the cortex. By measuring motor-evoked potentials in the presence and absence of tDCS over the motor cortex, investigators have directly shown that the scalp currents influence the excitability of cortical motor neurons (Priori et al. 1998, Nitsche & Paulus 2001). Specifically, an anodal or cathodal stimulator will induce current in
opposite directions, resulting in an electric field that can have a net excitatory or inhibitory effect, respectively. Recently, promising studies have used tDCS to targeting the motor cortex to facilitate motor learning and memory (Reis et al. 2008), as well as recovery from neuronal injury or stroke (Williams et al. 2009).

Much of what is known about how fields interact with neuronal activity has been learned through experiments with brain slices, where fields can be of predictable and uniform intensity across populations of cells. By applying such fields in the presence of synaptic blockers, it has been shown that electric fields affect neurons in the same way that neurons generate fields: individual neurons or neuronal compartments oriented parallel to the field vector polarize (Chan et al. 1988, Bikson et al. 2004, Radman et al. 2009). Similarly, neurons with high symmetry or a perpendicular orientation are relatively unaffected (see Figure 1 for a schematic of this orientation dependence). And much as regions with large numbers of parallel-oriented neurons are able to generate large electric fields, they are also particularly susceptible to modulatory effects of applied electric fields.

Pyramidal neurons of the cortex are highly sensitive to the effects of electric fields, which can affect spike rates by bringing a soma closer to or further from spiking threshold. In short, where currents directed from the apical dendrite toward the soma bring the soma closer to spiking threshold, and currents in the opposite direction bring the soma further from spiking threshold. Thus, applied electric fields can facilitate or inhibit neuronal activity in hippocampal (Ghai et al. 2000, Bikson et al. 2004, Gluckman et al. 1996), cerebellar (Chan & Nicholson 1986), and cortical (Fröhlich & McCormick 2010) networks. Further, oscillating electric fields can show a periodic effect on

Experiments in vitro have shown measurable effects on neuronal populations from electric fields as small as 1 V/m (Ghai et al. 2000, Francis et al. 2003), and estimates of the magnitude of the electric fields used during conventional tDCS in vivo are even lower (Datta et al. 2009). For comparison, endogenous cortical electric fields can be up to 4 V/m during slow wave activity and up to 20 V/m during epileptiform events (Jefferys 1995). The implications of this are that endogenous fields may play a role in synchronizing neuronal activity, even during non-pathological events (Fröhlich & McCormick 2010, Anastassiou et al. 2011). This process, where neuronal populations activated in synchrony influence the excitability of nearby neurons, is termed ephaptic transmission. These small fields, whether endogenous or exogenous, will induce a roughly 0.1 – 0.3 mV change in the somatic membrane potential of a pyramidal neuron for every V/m of field intensity (Deans et al. 2007, Radman et al. 2009). And though this change is small, the network effects in the cortex can be large. This is likely because cortical neurons are so tightly packed in parallel orientations, allowing a field across even a small region to affect a very large number of cells. The effect may then be amplified by the high degree cortical synaptic connectivity, where a small change in excitability of cortical pyramidal cells is amplified by the large number of their synaptic outputs.

**Cortical Up and Down States**

EEG recordings during sleep show characteristic oscillatory patterns, and sleep is categorized into different stages based on these patterns. Though the amplitude and
frequencies of these patterns of oscillation may vary across species, they are remarkably conserved at least among terrestrial mammals (McCormick & Bal 1997). In 1993, a series of papers from the lab of Mircea Steriade identified a novel oscillation in the cortex of anesthetized cats, and later generalized to other sleeping or anesthetized mammalian species (Steriade et al. 1993a-c). They named it the "slow oscillation" due to its low frequency, which is below 1 Hz. The slow oscillation is formed by the synchronous switching of cortical neurons between two stable resting potentials, one more depolarized (the “Up” state), and one more hyperpolarized (the “Down” state). Neurons are much more likely to fire action potentials during the depolarized Up state, and the slow oscillation can therefore regulate the timing of other cortical activity patterns, such as spindles and sharp waves. Other sleep-associated cortical EEG patterns, such as K-complexes (Amzica & Steriade 1997, 1998) and possibly also delta waves (Sirota & Buszaki 2005) are aspects of the slow oscillation, though delta waves can also be considered a distinct thalamically-generated oscillation (Steriade 1993b, Steriade & Timofeev 2003). In addition to sleep, the slow oscillation can be seen under certain types of anesthesia, and even during quiet wakefulness (Petersen et al. 2003).

Spatiotemporal Dynamics of the Up State. The cortical Up state can propagate as a wave across broad regions of cortex, with both the transition to the Up state and to the Down state being highly synchronized. High-density EEG recordings in humans have estimated the propagation speed to be very fast, between 1 and 7 m/s (Massimini et al. 2004). The high speed of propagation suggests that long-range connectivity is synchronizing distant regions, since polysynaptic propagation through local networks is much slower (Amzica & Steriade 1995). In contrast, voltage-sensitive
dye imaging in anesthetized rodents (Petersen et al. 2003, Xu et al. 2007) and cortical slices (Sanchez-Vives & McCormick 2000, Wester & Contreras 2012) has estimated the speed of Up state propagation to be slower, from 10 – 100 mm/s. This slower propagation is likely mediated by local networks.

Using electrode arrays to measure spiking activity across cortical depth in vitro, it was found that a propagating Up state first activates layer V neurons, and subsequently recruits neurons in layer VI, and then neurons in layers I – IV (Sanchez-Vives & McCormick 2000). This sequence was later confirmed using a vertical electrode array in vivo (Sakata & Harris 2009), though in vivo many Up states may not involve any spiking in layers II/III (Kerr et al. 2005, Sakata & Harris 2009). A recent in vitro study further demonstrated with local TTX injections that the subgranular layers V – VI are necessary for horizontal propagation of the Up state, while the infragranular layers are not (Wester & Contreras 2012).

In cortical networks, the sustained firing during the Up state includes both excitatory and inhibitory neuronal activity. Both kinds of activity are present throughout the duration of the Up state, though more spiking activity is recorded earlier in the Up state, and importantly, both are required. This can be demonstrated with pharmacological blockers. Blockade of either NMDA or non-NMDA ionotropic glutamate receptors abolishes transitions into the Up state, leaving the cortex in a perpetual Down state (Sanchez-Vives & McCormick 2000, Shu et al. 2003). However, it has been suggested that NMDA receptors may play more of a facilitory role than a necessary one (Cunningham et al. 2006, Steriade & Timofeev 2003). Blockade of GABA_A receptors also eliminates Up states, resulting instead in epileptiform activity.
The epileptiform bursts are clearly distinct from Up states, due to their high-amplitude and short-duration local field potential, and bursts of tonic activity in individual cells (Sanchez-Vives & McCormick 2000).

**Extracellular Calcium and Up States.** Much of our understanding of Up and Down States comes from a model of cortical Up states in brain slices. Up states in brain slices are usually recorded in a lower concentration of calcium and magnesium ions (~ 1 mM) relative to the “standard” 2 mM concentration (Sanchez-Vives & McCormick 2000, Shu et al. 2003, Cunningham et al. 2006, Fanselow & Connors 2010, Wester & Contreras 2012, Tahvildari et al. 2012). It has been reported that no Up states occur in 2 mM solution (Sanchez-Vives 2000), though there are studies from one group that regularly records Up states in a 2 mM solution (Cossart et al. 2003, Ikegaya et al. 2004, MacLean et al. 2005, Watson et al. 2008, Poskanser & Yuste 2011).

Nonetheless, the 1 mM concentrations are closer to the physiological concentrations *in situ* (Sanchez-Vives & McCormick 2000, Massimini et al. 2000) and are used in most studies to facilitate *in vitro* Up/Down state transitions. But why are low calcium and magnesium concentrations important? To understand why, I must first discuss some basic electrophysiology.

The neuronal resting membrane potential is determined by the concentrations of various ions on either side of it. It can be dynamically adjusted by changing the permeability of the membrane to specific ions. Neuronal membranes at rest are generally most permeable to potassium ions, as constitutively open channels allow them to leak through the membrane. These potassium leak currents drive the membrane potential towards potassium’s reversal potential (approximately -90 mV).
Under normal conditions, the neuronal resting potential is slightly depolarized relative to this value (-50 to -80 mV) due mostly to leak sodium currents. Sodium can pass through some nonspecific monovalent cation channels, such as the HCN channels associated with the hyperpolarization-activated current, $I_h$ (Robinson & Siegelbaum, 2003). There are also two major sodium-specific leak currents. One is the persistent sodium leak current, $I_{NaP}$, which shows some voltage dependence (Crill 1996). Another is a voltage-independent background sodium leak current, $I_{L-Na}$, mediated by NALCN channels (Ren 2011).

An increase in extracellular concentrations of sodium or potassium cations has an excitatory effect on neurons. This can be explained by changes in the electrochemical gradients, and calculated with reasonable accuracy using the Nernst equation. However, an increase of extracellular calcium has an inhibitory effect on neurons. This is not due to their electrochemical gradient; calcium ions have little direct effect on membrane potential due to the low concentrations both inside and outside of the cell. Instead, calcium ions can affect membrane potential by gating sodium and potassium currents, though both intracellular and extracellular mechanisms.

Intracellular calcium notably can activate hyperpolarizing potassium currents through a number of different channels and on a variety of time scales. For example, a slow hyperpolarizing current, $I_{AHP}$, plays a large role in terminating burst firing in neurons, and is activated by the intracellular calcium that accumulates after each action potential. This current may also play a role across longer time scales, however, including the termination of cortical Up states (Compte et al. 2003, Sanchez-Vives et al.)
If a decrease in extracellular calcium decreases calcium entry into cells, this is one possible mechanism for calcium’s effect on neuronal excitability.

Extracellular calcium can reduce conductance through a number of different channels, including voltage-gated sodium channels, which not only allow sodium influx during action potentials but also the persistent sodium current during only slightly elevated membrane potentials (Su et al. 2001), and NALCN channels, which mediate roughly 70% of the basal sodium leak current (Lu et al. 2007, Lu et al. 2010). Both types of channels are sensitive to calcium, showing substantial changes in conductance with small changes in extracellular calcium concentration. The persistent sodium current may play a function of “gain control” in layer V cortical neurons (Astmann et al. 2006), and at least partially determines their spontaneous firing rate (Mao et al. 2001). In addition, the NALCN channel is heavily implicated in neuronal population rhythms. Mutations in one of its subunits can disrupt pacemaker activity in neuronal systems, resulting in disrupted breathing in mice (Lu et al. 2007) and both inverted circadian locomotor patterns and abnormal rhythmic motor activity in flies (Nash et al. 2002).

**Subcortical Slow Synchronization.** The cortex sends extensive long-range projections to many subcortical regions, and the cortical slow oscillation can recruit other brain areas into synchronous oscillation via these connections. Up and Down state transitions can be observed in the striatal spiny neurons in vivo, and the transitions are dependent on cortical inputs (Kasanetz et al. 2006). Both the hippocampus (Hahn et al. 2006, Battaglia et al. 2004) and amygdala (Crane et al. 2009) also show periodic increases in excitability synchronized with cortical Up states, both of which are dependent on synaptic activity that is likely of cortical origin. The slow oscillatory
changes in hippocampal excitability can then propagate further through the limbic system, including the nucleus accumbens (O'Donnell 1995). Thus, the cortically-generated slow oscillation can influence the timing of activity across much of the brain.

This is most clearly true for the thalamus. Slow oscillation in the cortex recruits thalamic neurons (both relay and reticular neurons) into a synchronous slow oscillation in vivo (Steriade et al. 1993c). This thalamic oscillation can be induced in isolated thalamic slices by pharmacologically mimicking cortical excitation of metabotropic glutamate receptors (Hughes et al. 2002). Of course, excitatory thalamic neurons send considerable feedback to the cortex, and this feedback affects the oscillation in the cortex. In particular, Up or Down state transitions in the cortex can be triggered by electrical stimulation of thalamocortical neurons in vitro (MacLean et al. 2005, Rigas & Castro-Alamancos 2007, 2009) and in vivo (Steriade 1993b), as well as by sensory stimulation, which presumably excites the cortex via thalamic afferents (Petersen et al. 2003). In fact, it has been suggested that while the Up and Down state transitions can be generated in isolated cortex, the thalamus plays a fundamental role in regulating the timing and frequency of Up/Down state transitions (Steriade 1993b, Contreras & Steriade 1995, Crunelli & Hughes 2010). This is supported by evidence that deafferented cortical slabs show a reduced frequency of Up state transitions (Timofeev et al. 2000) and that the presence of intact thalamocortical connections in brain slices increases cortical Up state frequency (Wester & Contreras 2012). This can alternatively be viewed as the thalamus being an additional source of the recurrent excitation that generates Up states in cortical slices (Sanchez-Vives & McCormick 2000), rather than being the result of fundamentally different properties of the thalamus.
**Slow Oscillation and Memory.** There is much evidence for a role of slow-wave sleep in facilitating memory consolidation (Diekelman & Born 2010), with a relationship between the amplitude of the slow waves and performance on memory tasks (Huber et al. 2004, Mölle et al. 2004). Further, specific patterns of neuronal activations associated with a particular behavior during wakefulness are replayed during the Up states of slow wave sleep in both the cortex and hippocampus (Ji & Wilson 2007, Euston et al. 2007). These patterns suggest that the slow oscillation may facilitate memory consolidation by grouping neurons into organized ensembles that fire during the Up state (Cossart et al. 2003). Its role in memory may also be through coordination of higher-frequency oscillations such as sleep spindles (Gais et al. 2002). However, the precise role of the slow oscillation itself in memory consolidation is difficult to assess, in part due to high correlations between slow oscillation, spindle activity, and even the overall levels of cortical and subcortical activity.

**Electric Fields and Slow Cortical Activity**

The study of how electric fields are influenced by slow cortical activity is not without precedent. In published literature there are two approaches taken to studying this. The first is a series of studies in human subjects, in which slow, oscillating fields were applied to human subjects. The second is an in vitro study in ferret cortical slices, where the timing of spontaneous Up states was modulated by sinusoidal AC fields. The research in this thesis builds on the results of these studies, so they will be discussed in detail here.

**Applied Fields in Humans.** In a study published in 2006, Marshall et al. (2006) applied transcranial fields in human subjects during sleep in human subjects. The field-
generating electrodes were placed on the skin above the frontal cortex and oscillating DC currents were run through the electrodes at a frequency matching the cortical slow oscillation (~0.75 Hz). The subjects slept while exposed to the electrical stimulation, and compared to controls (sham stimulation), they spent more time in deep sleep and had increased scores on a subsequent test of declarative memory.

The authors provide evidence that the 0.75 Hz oscillating field enhanced the cortical slow oscillation, as immediately after terminating the applied field subjects showed an increased power at this frequency in EEG recordings. In addition, they found that the applied fields increased the occurrence of spindle oscillations (8 – 15 Hz) during sleep. Faster fields (5 Hz) had no effect on these measures, nor on performance in the test of declarative memory, so the effect was dependent on field frequency. Thus, the authors conclude the slow oscillation has a causal role in declarative memory consolidation, and it can be enhanced by applied electric fields.

In a follow-up study (Kirov et al. 2009), the authors applied the same fields during wakefulness rather than sleep. During wakefulness, the fields did amplify low-frequency oscillation, but did not affect declarative memory consolidation, demonstrating that the effects of applied fields depend on brain state. Interestingly, the applied fields did facilitate declarative memory encoding, likely due to an increase in higher-power oscillation in the theta (4 – 8 Hz) and beta (15 – 25 Hz) ranges.

**Applied Fields in Rodents.** An advantage of in vitro experiments is that they allow a more detailed exploration of mechanisms. Fröhlich & McCormick (2010) applied oscillating AC electric fields to slices of ferret cortex to explore the role of fields in modulating slow activity. Importantly, they applied fields at a very low frequency
(0.075–0.375 Hz) and of a strength similar to those occurring endogenously (2 – 4 V/m). They measured multiunit spiking in slices showing spontaneous oscillation between Up and Down states, and concluded that fields of endogenous magnitude could affect the timing of Up state onset. The overall effect of this was to increase the slow oscillation frequency through a reduction of Down state duration. This means there was an increased number of Up states over time, and an increased overall level of activity. This is evidence not only that exogenous electric fields can modulate Up state timing, but also that endogenous fields may act as a feedback mechanism promoting the Up state, and consequently, overall levels of activity.

**Cortical Ensemble Activation**

As technology improves, so do our methods of measuring activity in many neurons simultaneously. Multielectrode arrays and calcium imaging can record the spiking activity of tens or even hundreds of neurons simultaneously, revealing coordinated neuronal activity patterns. Such groups of neurons that encode information or perform computation through their coordinated activity are referred to as neuronal ensembles. Populations of neurons can encode information through ensemble activation in a number of ways. One method is termed “population coding”. An example of this is in the primary motor cortex, where single neurons may encode a specific trajectory for limb movements, but the overall output to the muscles is determined by a vector sum of these encoded trajectories (Georgopoulou {et al.} 1986).

Similar active ensembles are observed in sensory cortex. Primary visual cortex (V1) is arranged in a retinotopic fashion, with neurons in different regions of V1 responding to visual stimuli from different portions of the visual field. Additionally, cells
can be tuned to different features of an image within that location of the visual field, such as orientation or motion (Hubel & Wiesel 1962). Information about the entire image is thus represented as an ensemble of cells, distributed across a large network (Pasupathy & Connor 2002). Individual cells of the ensemble, tuned to separate parts of the image, can then feed forward into higher levels of processing on the entire image (Brincat & Connor 2006).

**Development of Sensory Ensembles.** Before sensory inputs first arrive during cortical development, spontaneous cortical neuronal activity is dense, meaning the majority of neurons are recruited into synchronous activation. Thus, there is an absence of specific ensemble activation in sensory cortex during early development. Rodents develop hearing in the second postnatal week (~P10-P12), and auditory cortical connectivity undergoes rapid refinement at this time (Oswald et al. 2007). Mice receive visual inputs for the first time at eye-opening, which takes place at approximately P12-P13. Before eye-opening, spontaneous visual cortical activity is dense (Adelsberger et al. 2005), and after eye-opening spontaneous activity patterns become sparser (Rochefort et al. 2009).

From birth through the first postnatal week, dense spontaneous activity is first mediated by electrical synapses and intrinsic ion conductances (Allene et al. 2008), but is subsequently replaced by large, low-frequency network depolarizations mediated by NMDA receptors, called the cortical early network oscillations (cENO). The GABA_A receptor may also contribute to these oscillations (Garaschuk et al. 2000), as increased chloride conductance through GABA_A receptors is depolarizing in immature neurons. However, the GABA_A conductance may instead contribute to a separate set of cortical
depolarizations that are more typically associated with the hippocampus, called giant depolarizing potentials (Allene et al. 2008). Regardless, recent evidence has demonstrated a progression from the very slow early network oscillations (less than 0.05 Hz) to the mature slow oscillation (~0.5 Hz). During this progression, the waves not only become more frequent, but also sparser (Rochefort et al. 2009). Thus, the large depolarizations that recruit nearly all neurons in the network are replaced by Up states that activate particular neuronal ensembles. And importantly, in the absence of visual input, this sparsification process is delayed (Rochefort et al. 2009).

**Up State Ensembles.** Ensemble activation has been observed in the spontaneous activity in slow-wave cycles during sleep (Kerr et al. 2003, Luczak 2006). Both calcium imaging and multielectrode array methods have shown that patterns of spontaneous ensemble activation are stable over the time course of minutes (Kerr 2003) to tens of minutes (L uczak et al. 2007, Han et al. 2008). Intriguingly, the ensembles activated during sleep appear to be replays of the same ensemble activity occurring during waking behavior, with simultaneous replay in both hippocampal and cortical populations (Ji & Wilson 2007, Euston et al. 2007). This spontaneous replay occurs during sleep (Kenet et al. 2003, Ji & Wilson 2007, Euston et al. 2007) but not during wakefulness (Xu et al. 2012). Since long-term or remote memory is more dependent on cortex than on hippocampus (McGaugh 2000), and since sleep has demonstrable effects on long-term memory consolidation (Stickgold 2005, Marshall et al. 2006), this has led to the hypothesis that the neuronal ensembles are consolidating memory in the cortex during sleep.
Spontaneous ensemble activity is preserved surprisingly well in vitro, showing that it can emerge as a property of cortical networks. In slices of mouse visual cortex, a small fraction of the large pyramidal neurons in layer V fire spontaneously in the absence of glutamatergic transmission, due in large part to persistent sodium currents (Na_p) and hyperpolarization-activated cation currents (I_h) (Mao et al. 2001). These intrinsically active neurons may be the origin of spontaneous cortical activity (Chagnac-Amitai & Connors 1989), consistent with observations that spontaneous cortical activity typically emerges in layer V (Sanchez-Vives & McCormick 2000, Sakata & Harris 2009). Regardless, temporally correlated firing emerges in cortical networks, which requires fast glutamatergic transmission and is mostly associated with Up states. The patterns of ensemble activation in vitro resemble attractor dynamics, with multiple recurrent activation patterns across overlapping populations of neurons (Cossart et al. 2003). Highly similar patterns can stably repeat over 10s of minutes (Ikegaya et al. 2004), similar to reports in vivo (Luczak et al. 2007, Han et al. 2008).

**Voltage and Calcium Imaging**

The experiments described in this dissertation use two different methods to measure physiological signals in the cortex. These methods use fluorescent dyes to measure either voltage changes or calcium signals in neurons or neuronal populations. Because voltage-sensitive dye imaging and calcium imaging are direct measurements of membrane voltage and intracellular calcium concentration, respectively, they do not pick up artifacts from extracellular fields. This is very important, as the exogenous fields applied throughout the experiments in this dissertation would contribute very large signals to any conventional electrode recordings. This problem is addressed in many
published studies by the use of multielectrode recordings and signal subtraction algorithms; however, the imaging techniques used here provide a more elegant (and accurate) approach to recording neuronal activity.

**Voltage Sensitive Dye Imaging.** A voltage-sensitive dye (VSD) is a lipophilic molecule that can passively absorb into membranes, with fluorescent properties that depend on the voltage across the membrane. The dyes can theoretically absorb into any membrane, including the plasma membranes of neurons and glia as well as intracellular membranes. Under normal circumstances, however, there is little fluctuation of membrane potential in glia or organelles, so while they may contribute significantly to the baseline fluorescence, the measured VSD signals (change in fluorescence over time) should be of neuronal origin.

Unlike traditional electrode recordings, VSD signals come only from cell membranes. Thus, VSD recordings are not affected by extracellular volume conductance across the tissue. VSD signals are also unaffected by artifacts from an applied field, as they measure the voltage across the thickness of the membrane. Consider an applied field that is 1 V/m. Across a membrane that is ~3 nm thick, this applied field is 3 nV, which is orders of magnitude smaller than normal millivolt fluctuations in membrane potential. However, the polarization of cortical pyramidal neurons by a 1 V/m field has been measured to lead to a ~0.3 mV change in membrane potential (Radman et al. 2009). Across the 3 nm thick membrane, this polarization results in a 100,000 V/m field, many orders of magnitude greater than the applied field. Thus, the changes in dye fluorescence or absorption correspond to neuronal membrane potential changes and not to changes in the applied field.
By measuring the changes in fluorescent properties, VSD imaging allows the monitoring of activity simultaneously across large populations of neurons. VSD imaging has very high spatial and temporal resolution, as dyes can change their fluorescent properties fast enough to follow action potentials (Antic et al. 1999). However, the resolution is in practice limited by the low magnitude of fluorescence changes, which under typical conditions is three to five orders of magnitude smaller than the baseline fluorescence.

The VSD experiments described in this dissertation have high temporal resolution, but spatial resolution is sacrificed to allow a high signal-to-noise ratio. I use the absorption dye NK3630 and bright-field imaging with a 464-photodiode array, where each photodiode measures changes in fluorescence absorption from a region of cortex 100 – 150 μm in diameter. Thus, each detector will be recording aggregate absorption changes across thousands of neurons at a time, including the cell bodies of some neurons and the processes of others. Because of this, individual spikes or postsynaptic potentials are not seen in the VSD signals; rather, the signals correspond to changes in membrane potential occurring simultaneously across large populations of neurons. To put it most simply, VSD reports synchrony.

It is only with VSD imaging that I can characterize the two components of the cortical response to electric fields, which we term the passive and active components. The passive component is passive polarization of cells or cellular compartments, which should scale linearly with the intensity of the field. Thus, for a sinusoidal oscillating field, the VSD signal should appear as a simple sinusoid. In contrast, the active component is changes in membrane potential caused by synaptic activity, and should scale
nonlinearly with the intensity of the field. So for a sinusoidal field, the active component VSD signals will have a steeper rise and decay.

**Two-Photon Calcium Imaging.** Calcium imaging is conceptually similar to VSD imaging, and is similarly unaffected by artifacts of the applied electric field. Rather than being bound to cellular membranes, however, calcium indicators diffuse freely in the cytosol and can bind to free calcium ions. Their fluorescent properties depend on calcium binding state, and changes in fluorescence thus correspond to changes in intracellular calcium concentration. I use AM (acetoxymethyl ester) dyes, which are activated by intracellular esterases, and thus extracellular calcium concentration changes do not contribute to changes in fluorescence.

Under normal physiological conditions, action potentials cause a large depolarizing spike that back-propagates through the soma and dendrites. This depolarizing spike opens voltage-gated calcium channels across the neuronal membrane, resulting in a very large elevation of somatic calcium concentration. This increase in somatic calcium is what is measured by the fluorescent calcium indicators. Regulators of calcium concentrations within neurons are very complex, but subthreshold changes in membrane potential that are unassociated with spikes should not appreciably affect calcium conductance through the membrane (Mao et al. 2001), and therefore should not be detectable with calcium indicators. Thus, calcium signals have the same all-or-none response characteristics as action potentials themselves, and calcium signals are taken to be a measure of neuronal spiking activity. In contrast, voltage-sensitive dyes report any changes in membrane potential, including fluctuations that never reach spiking threshold.
The calcium imaging experiments described in this dissertation use two-photon imaging. Two-photon microscopy differs from conventional bright-field microscopy in that a longer wavelength of light is used, and the fluorophore is excited only when multiple low-energy photons reach it simultaneously. For example, the experiments in this dissertation used Oregon Green as a fluorophore, which shows peak absorption at 488 nm. With two-photon imaging, the fluorophore is excited by light with a wavelength of 800 nm. This has three major advantages. First, the higher-wavelength photons will be scattered less by the tissue being imaged, allowing imaging deeper within the tissue. Second, since fluorophores only emit photons when simultaneously excited by multiple photons, there is less excitation outside the plane of focus, leading to a more favorable signal-to-noise ratio. Finally, the lower-energy photons and lower levels of overall light reduce bleaching of the dye and phototoxicity.

Two-photon calcium imaging allows very high spatial-resolution images, and can be used to look at very small subcellular compartments such as dendritic spines. In this proposal, however, it is used to look at large networks with cellular resolution. But while the spatial resolution is much better than with VSD imaging, the temporal resolution is far worse. This is for two reasons. First, the microscope uses a scanner to acquire the images, where each pixel in the x-y plane is imaged sequentially, limiting image acquisition to the speed at which the scanner can move. Second, and more importantly, the calcium dyes themselves are slow. While VSD changes signals very rapidly according to voltage changes across the membrane, calcium indicators take time to bind to and dissociate from calcium ions. Further, calcium indicators can slow
down the calcium dynamics they measure, for it is only dissociated calcium ions that can be cleared from the cytosol.

**List of Abbreviations**

AC field – alternating current (the field changes direction between positive and negative)

DC field – direct current field (the field vector does not change direction)

Ca.Mg – calcium and magnesium ions

cENO – cortical early network oscillation

CSD – calcium-sensitive dye (equivalent to “calcium indicator”)

EEG – electroencephalogram

$I_{\text{AHP}}$ – after-hyperpolarization current

$I_h$ – hyperpolarization-activated cation current

$I_{L-Na}$ – voltage-independent sodium leak current

$I_{\text{NaP}}$ – persistent sodium current

LFP – local field potential

NALCN – nonselective sodium leak channel, also known as VGCNL1

tDCS – transcranial direct current stimulation

V1 – primary visual cortex

VSD – voltage-sensitive dye
Figure 1. Schematic of neurons in an electric field. Applying voltage across distant electrodes creates an electric field between them. The field direction is defined as the direction of induced current flow, where positive charge flows toward the negatively-charged cathode. In this schematic, the parallel electrodes generate a field that induces current flowing from left to right.

(A) A hypothetical pyramidal neuron, with the apical dendritic tuft on the left and cell soma (blue) on the right. Under the influence of the electric field, a dipole is induced in the pyramidal neuron. Here, the cell soma is depolarized relative to the apical dendritic tuft. Action potentials are more likely to initiate in a depolarized soma, and thus the field has an excitatory effect.

(B) When the orientation of the field with respect to the neuron is reversed, the dipole is reversed; thus, the soma is hyperpolarized relative to the dendritic tuft. In this case the electric field has an inhibitory effect.

(C) When neurons are oriented perpendicular to the field, the soma is unaffected by any induced dipoles. Thus, this cell is not appreciably modulated by the field.

(D) When neurons are symmetrical around the soma, as is the case with most interneurons, the soma is unaffected by any induced dipoles. Thus, this cell is not appreciably modulated by the field.
Chapter II: Central Hypotheses

Central Hypothesis for Chapter IV: Very small neuronal polarizations can generate very large population events.

Single-unit recordings show only a 0.1 – 0.3 mV change in the somatic membrane potential of pyramidal neurons per V/m of electric field (Deans et al. 2007, Radman et al. 2009). Transcranial stimulation techniques in human subjects apply fields at or below 1 V/m (Datta et al. 2009), which will directly induce a change in membrane potential that is far too small to evoke action potentials in isolated neurons. In fact, field strength required to directly evoke action potentials in pharmacologically isolated pyramidal neurons is more than an order of magnitude larger, between 28 and 79 V/m (Radman et al. 2009). It is not well-understood how the small change in membrane potential directly induced by an electric field can have such a large effect on the cortex, one that in human subjects can even alter behavior. I use an in vitro brain slice preparation to test the hypothesis that small changes in spiking probability at the single-unit level can translate into large-scale changes in network activity.

More specifically, I predict that the cortical response to electric fields can be divided into two components: the “passive component” and “active component”. The passive component is the changes in membrane potential caused by the field-induced polarization of neurons. The active component is the change in membrane potential caused by synaptic activity. I use voltage-sensitive dye to show that these two components are separable and have distinct spatiotemporal properties. Additional experiments with calcium imaging suggest that layer V neurons are the primary drivers of the active component.
**Novelty and Significance.** Electric fields applied across neuronal populations will cause large signal fluctuations in electrode recordings, which can be much larger than the neuronal signals themselves. Because of this, studies of electric field effects have generally focused on high-frequency spiking activity in the presence of static or very low-frequency applied fields. In the experiments presented in chapter IV of this dissertation, I avoid this problem by using fluorescent imaging instead of electrical recordings. In particular, voltage-sensitive dye imaging has allowed me to record changes in membrane potential occurring across cortical brain slices, offering an unprecedented look at small, subthreshold changes in membrane potential across large populations of cortical neurons.

Most if not all published studies have focused on steady-state fields (Bikson et al. 2004, Radman et al. 2009, Akiyama et al. 2011) or on the modulation of spike timing by time-varying fields (Francis et al. 2003, Deans et al. 2007, Fröhlich & McCormick 2010). Single-trial VSD imaging of neuronal activity provides a view of how low-frequency fields affect low-frequency changes in membrane potential. I combine this approach with the use of fluorescent calcium indicators to examine the spiking behavior of neurons under oscillating fields.

Transcranial stimulation techniques in humans have been used to facilitate motor learning and memory (Reis et al. 2008), as well as facilitate recovery from neuronal injury or stroke (Williams et al. 2009). There is promising evidence that they can be used to facilitate declarative memory encoding and consolidation (Kirov et al. 2009, Marshall et al 2006), suggesting further benefits possible with this technique. However, targeted design of therapeutic protocols will require an understanding of how large-
scale changes are caused by applying low-intensity fields across the cortex. The experiments in chapter IV are a significant step in acquiring such an understanding.

In addition, these experiments provide an opportunity to compare voltage and calcium imaging techniques, and to my knowledge is the first study to look at brain rhythms with a combination of both approaches. I use different electric field stimuli to compare similar activation patterns, and then view membrane dynamics with VSD imaging and spiking behavior with calcium imaging. In both cases, this reveals limitations in what can be observed with a single imaging technique, and even show some differences that are hard to reconcile. Understanding the limitations of these two techniques is important for any investigator who uses them, and will be valuable in interpreting the data they generate.

**Central Hypothesis for Chapter V**: Low extracellular calcium levels facilitate slow oscillatory activity by increasing sodium conductance.

Development of an *in vitro* model of cortical Up states has helped improve our understanding of mechanisms behind their initiation and propagation. In order to see regular spontaneous Up states in brain slices, the concentration of calcium and magnesium ions in solution must be kept low, usually between 0.8 and 1.2 mM (Sanchez-Vives & McCormick 2000, Fanselow & Connors 2010). However, the reasons for this have not been explored. I use primarily calcium imaging to explore spontaneous neuronal activity with varying concentrations of extracellular calcium and magnesium ions in order to better understand their respective roles. Additionally, the use of pharmacological blockers allows assessment of the role of sodium channels.
**Novelty and Significance.** The model of spontaneous cortical Up-state-like activity in the presence of low extracellular calcium and magnesium ion concentrations is not novel. However, it is not established what role calcium specifically plays, and even whether it significantly contributes to the effect. There are many possible mechanisms by which these ions may affect spontaneous cortical Up states, but a controlled in vitro investigation is lacking in the literature. Further, calcium-modulated sodium currents contribute to many kinds of rhythmic activity in the brain, such as breathing rate and circadian rhythms (Ren 2011), but their involvement in spontaneous Up state generation has not been directly demonstrated.

There is evidence that extracellular calcium concentrations fluctuate *in vivo* during slow-wave sleep (Massimini & Amzica 2001), as the cortex shifts between Up and Down states. The proposed reason is that the increased neuronal firing during an Up state results in rapid movement of calcium from extracellular to intracellular environment, resulting in reduced extracellular calcium concentrations. Confirmation of this hypothesis *in vitro* would suggest that this reduction in extracellular calcium caused by the Up state may actually stabilize the Up state. Acting as positive feedback, the fluctuating calcium levels may enhance synchrony during slow wave sleep.

**Central Hypothesis for Chapter VI:** Networks with slow oscillation are much more sensitive to electric fields.

Slow oscillating fields have been used in human experiments to improve the consolidation (Marshall et al. 2006) or encoding (Kirov et al. 2009) of memory. These effects are state-dependent; in other words, they depend on the presence of slow wave sleep. The frequency of Up states *in vitro* can be reversibly increased by decreasing
the extracellular concentration of calcium and magnesium ions. I manipulate the frequency of Up states by changing these extracellular ion concentrations and test the dependence of field effects on synchronized activity in the slice.

**Novelty and Significance.** There has been no study of how electric field effects depend on spontaneous Up-state activity in the slice. There is only one published *in vitro* study on the modulation of Up states by electric fields (Fröhlich & McCormick 2010), and my use of *in vitro* imaging allows me to describe this phenomenon further. For example, VSD imaging allows exploration of whether applied fields affect the spatiotemporal properties of Up states.

Testing this hypothesis may help explain results observed *in vivo*, that the effect of applied oscillating fields can depend on brain state (Marshall et al. 2006, Kirov et al. 2009). Additionally, the applied fields in my experiments are of comparable magnitude to endogenous the electric fields that occur *in vivo* during the slow oscillation, suggesting that effects of the applied fields used in this experiment may generalize to the effects of endogenous fields. This would lend support to the idea suggested by Fröhlich & McCormick (2010), that endogenous electric fields may enhance slow oscillation synchrony. Finally, these and most other *in vivo* protocols for transcranial stimulation involve the use of DC fields, whereas most *in vitro* studies have used AC fields. Here I test both AC and DC fields to investigate whether this property of the field is important in determining its effects. The results of these tests have important implications in designing both *in vitro* and *in vivo* stimulation protocols.
Central Hypothesis 1 for Chapter VII: Developmental sparsification is due to changes in local cortical networks.

It has been shown that spontaneous cortical activity becomes sparse in rat visual cortex during the second week of development, when the eyes first open (Rochefort et al. 2009). However, the mechanisms behind this change are unknown. Here, I use an in vitro visual cortical slice preparation to isolate local cortical networks and determine whether the sparsification is cortically mediated, as opposed to being mediated by changes earlier in the visual processing stream. Additionally, the in vitro preparation will allow access to deeper layers of cortex, for which the sparsification process has not been examined.

Novelty and Significance. This phenomenon has been demonstrated in vivo, but never in vitro. In addition to isolating cortical networks, the in vitro preparation allows for controlled application of the GABA\textsubscript{A} blocker, bicuculline. This allows investigation of the role of local inhibition in this phenomenon.

Sparsification is a developmental phenomenon occurring not just in visual cortex, but in auditory cortex as well (Rochefort et al. 2009). In both cases, it occurs shortly after meaningful sensory input first reaches the cortex. Exploring the mechanisms behind its occurrence may aid our understanding not only of the developmental phenomenon, but also how sensory information is encoded by the nervous system.

Central Hypothesis 2 for Chapter VII: Electric fields influence ensemble activation during Up states.

Using two-photon calcium imaging, I investigate whether the same spontaneous ensembles are consistently reactivated during Up states evoked by applied electric
fields. Further, this allows us to see if repeated electric field exposure activates the same ensembles of neurons, or whether the ensembles are as varied as those occurring spontaneously.

**Novelty and Significance.** Slow oscillating fields have been used in human experiments to improve the consolidation (Marshall et al. 2006) or encoding (Kirov et al. 2009) of memory. The mechanisms are unknown. It has been theorized that memory is stored across large ensembles of neurons in the hippocampus and cortex. In fact, Up states consists of active ensembles of neurons both in vitro (Cossart et al. 2003) and in vivo (Kerr et al. 2005), but it has never been addressed how these ensembles are influenced by electric fields. Confirmation of this experimental hypothesis would be the first evidence that electric fields can reinforce the activation of particular neuronal ensembles. The repeated activation of ensembles may explain how electric fields can enhance aspects of memory, an idea that to my knowledge is completely novel in the literature. Testing this hypothesis in vitro allows assessment of the plausibility of this mechanism.
Chapter III: Methods

All presented data were acquired using voltage sensitive dye imaging and two-photon calcium imaging. The standard techniques are described below, and only deviations from this standard procedure will be noted in the next chapters. For a schematic of the two imaging setups, see figure 2.

Brain Slice Preparation

For all experiments, C57BL/6 mice of either sex (p15 – p22 unless otherwise specified) were anesthetized with ketamine or a mixture of ketamine and xylazine, and quickly decapitated. The head was submerged in iced, oxygenated artificial cerebrospinal fluid (ACSF) that has the sodium replaced with sucrose (containing in mM: 226 sucrose, 2.5 KCl, 1 CaCl₂, 4 Mg₂SO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 20 dextrose, and sometimes 0.5 ascorbate). The brain was quickly removed from the skull and a vertical cut was made by hand through the anterior cerebellum. The posterior surface of the forebrain was glued to a block, and 400 μm thick coronal slices were cut on a vibratome stage (Leica VT1000s) and transferred to a holding chamber with “normal” ACSF (in mM: 126 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 20 dextrose; saturated with 95% O₂, 5% CO₂). Importantly, slices were perfused steadily in this incubation chamber and maintained at 27 – 28°C.

Field Generation

Oscillating fields were generated by applying voltage across two parallel coils of electrodes. The electrode coils generated electric fields with an intensity that was uniform along the length of the electrode. While imaging, slices were located between the electrodes, with the imaged region of cortex running parallel to the electrodes (see
Following the convention of Ghai et al. (2000) and Fröhlich & McCormick (2010), I refer to a “positive” field as when the electric current flows from the pial surface into the deeper cortex, and a “negative” field as one in which current flows outward toward the pial surface. Using these terms, an “anodal field” in the tDCS literature (e.g. Nitsche & Paulus 2001) will be the same direction as a “positive field” here. The intensity of an oscillating field is always expressed as the peak-to-peak value.

**VSD Imaging**

**Staining.** Slices incubated for 2 – 6 hours prior to staining. For staining, each slice was bathed in 50 ml normal ACSF with 0.02 mg/ml of voltage-sensitive dye NK3630 (Nippon Kankoh-Shikiso Kenkyusho Co., Ltd., Japan) for 2 hours at 27 – 28°C. The stained slices were transferred back to the incubation chamber for 1 – 2 more hours, and then to the experiment chamber for imaging.

**Imaging.** Imaging was performed with a 464-channel photodiode array (WuTech Instruments) on an upright microscope (Olympus BX51 WI) with a transillumination arrangement (see Jin et al., 2002). Unless otherwise specified, imaging was done with a 4X objective. Oscillating electric fields were generated by a programmable signal generator (WaveTech). The waveform of the electric field was sampled concurrently with the VSD signals at 1600 samples per second in all channels. Data were viewed online using NeuroPlex software (RedShirt Imaging).

Slices were rapidly perfused at 27 – 28°C with “recording” ACSF, which was the same as “normal” ACSF except with 3.5 mM KCl and varying concentrations of CaCl₂ and MgSO₄. Imaging was done in discreet trials in order to minimize bleaching and
phototoxicity. Perfusion was stopped during each imaging trial in order to reduce noise in the recordings. One trial was run every three minutes.

**Data Analysis.** Offline analysis was performed by custom scripts written in MATLAB (Mathworks). First, the location of the cortex in the imaging window was marked according to differences in baseline fluorescence across detectors. For each cortical detector, the signal was converted to $\Delta F/F$ values by dividing all samples by the resting light intensity (the signal when the transillumination light is turned on minus when it is turned off). Traces were subsequently filtered between 0.5 and 55 Hz.

Passive component amplitudes were calculated at the frequency of the applied field using FFT. Passive component latency was calculated by taking the cross-correlation between the field waveform and the VSD signal at various time lags, and taking the lag for which correlation was maximal.

Baseline-to-peak amplitude (also referred to as “active component amplitude”) was calculated as the maximum value during the peak minus the minimum value occurring within a short time window before the peak. Total depolarization values (e.g. figure 14B) for each event were calculated by taking the area between the VSD trace and a linear interpolant created between the hand-marked onset and offset of activity; event durations were the timespan between the hand-marked onset and offset.

To calculate the detector at which an active component or Up state initiated, data was first smoothed spatially in a 1-detector radius. A time window was selected by hand that encompassed the entire event onset, and the onset time for each detector was defined as the first sample at which the signal reached 50% of the maximum signal. The detector for which this occurred earliest was defined as the initiation location.
Calcium Imaging

Staining. Slices were incubated for 2 – 3 hours and then transferred into the imaging chamber, where the slice was rapidly perfused at 27 – 28°C with “recording” ACSF, which was the same as “normal” ACSF except with 3.5 mM KCl and variable concentrations of CaCl$_2$ and MgSO$_4$. Staining procedures were adapted from the methods in Garaschuk et al. 2006. In brief, the slice was viewed through an upright microscope with a 20X objective, and the calcium dye (Oregon Green BAPTA-1, AM) was pressure-injected into the slice for 20 – 30 seconds through a glass micropipette (2-5 MΩ resistance). Two to six injections were made into the cortex, with three well-placed injections required to fully stain the depth of the cortex (figure 7A shows the extent of staining). The fluorescence signal peaked 20 – 30 minutes post-injection, which is when recording started.

Imaging. Two-photon imaging was performed with a commercially purchased LaVision Biotec system with a pulse laser at an 800 nm wavelength. The scanner was mounted on an upright microscope fitted with a 20X (unless otherwise specified) water-immersion objective. Imaging frame rates were 3 – 4 Hz. Packaged software (LaVision Biotec) was used to acquire and store the images, and for online viewing of the data.

Data Analysis. Offline analysis was performed by custom scripts written in MATLAB (Mathworks). Cells in each image were marked in automated fashion, and (often substantial) corrections were made by hand. Additional corrections for motion over time were performed by correlating a binary “mask” image with the raw image in each frame. Traces were generated for each cell and converted to $\Delta F/F$ values by dividing by the first decile of all samples in the trace. Synchronous events or Up states
were identified by changes in the average fluorescence across the entire image. Long recordings (>60 seconds) were high-pass filtered above 0.02 Hz to remove drift.

Calcium transients were identified based on a measure which I call a “z-score”, though this is a misnomer, as I am defining it as the number of standard deviations from the first decile, rather than the number of standard deviations from the mean. I used the decile instead of the mean because the signal baseline and signal mean were substantially different for cells showing frequent calcium transients. Traces were converted from $\Delta F/F$ values to these z-scores by dividing each sample by its “expected standard deviation”. I used this expected standard deviation to avoid very frequent transients being marked as noise by my automated procedure. To calculate this expected standard deviation, the standard deviations of all samples for each cell were calculated and regressed against the baseline fluorescence of the cell (in the absence of calcium transients, the relationship between baseline fluorescence and the signal standard deviation was linear). A “calcium transient” was marked when the z-score was greater than 3 for two or more consecutive frames. The z-score traces were also used to generate the “raster” plots used in chapter V (for example, see figure 9). The procedure was verified by hand for randomly selected cells, and in all cases transients were reliably marked with >95% accuracy for 20X images, and 100% accuracy for 60X images.
Figure 2. The stimulations used during voltage and calcium imaging procedures are similar. In both, a slice is placed between two silver-chloride electrodes. An oscillating current is run through the electrodes to generate an oscillating electric field across the tissue, with a direction parallel to the cortical depth axis in the center of the imaging region. Unless otherwise indicated, slices are exposed to five cycles of a 1 Hz AC field. In the VSD setup, applied fields were sinusoidal, whereas in the calcium setup, applied fields were square-wave.

The imaging techniques themselves are very different. The voltage sensitive dye imaging procedure in these experiments shows voltage changes across large cell populations (thousands of cells per detector). Low magnification is used in order to record from a large portion of the slice. The spatial resolution is very low, but the temporal resolution is very high.

The calcium imaging procedure shows calcium changes in the soma of individual cells. Most images were obtained with a 20X objective, which allowed imaging of 100-200 cells at a time. Somatic calcium signals in neurons are highly correlated with action potentials. Two photon imaging was used, allowing for a high spatial resolution, but low temporal resolution.
Chapter IV: Electric Fields in the Cortex

Central Hypothesis

Very small neuronal polarizations can generate very large population events. Accordingly, the cortical response to electric fields can be divided into two components: the “passive component” (small polarizations) and the “active component” (large population events).

Results

Passive and Active Components

VSD signals were clearly seen in single trials. The blue traces in figure 3 show sample VSD signals from somatosensory cortex exposed to a 16 V/m AC field, oscillating at 1 Hz. The signals in this example show both passive and active components visible in the same trace, with the active component (black arrows) appearing only during the first two cycles of the applied field. It is not shaped like the field stimulus, but is instead a large depolarization occurring during the positive phase of the applied field. It is also much larger than the passive component, which is clearly visible only during the last three cycles of the applied field, when there is no active component. The passive component, in contrast to the active, is shaped like the stimulus waveform (here, a sinusoid), and its direction depends on depth within the cortex. When the field is positive, the passive component is hyperpolarizing in the superficial layers of cortex and depolarizing in the deeper layers. When the field is negative, the polarity of the passive component is opposite.

The passive and active components are generated by different mechanisms. To explore this, I blocked fast glutamatergic transmission with the AMPA/kainate receptor
antagonist CNQX. 20 µM CNQX completely blocks the active component (figure 3, red traces during the first two cycles of oscillation), but does not affect the passive component (red traces during the last three cycles). Identical results can be seen using 1 µM TTX to block the sodium channels necessary for action potentials. Since the passive component does not require synaptic transmission, we can interpret the passive component to be the membrane potential changes from neuronal polarization induced by the field. The active component, in contrast, is depolarization induced by excitatory glutamatergic transmission.

**Spatiotemporal Properties of Population Activity**

The passive and active components have many other distinguishing characteristics. By measuring responses to electric fields in the presence of CNQX or NBQX, I can see the passive component with high signal-to-noise without any averaging. The passive component is stationary, showing very consistent spatiotemporal properties. The phase relationship between the passive component and the applied field shows that the cortex is divided into two clear regions: one superficial region that is always hyperpolarized by a positive field, and one deep region that is always depolarized by a positive field (figure 4, top-right). The passive component does not propagate; it retains a consistent phase relationship everywhere it is detectable, with one abrupt phase reversal. There are three distinct bands of peak amplitude (figure 4, top-center), one near the superficial surface of cortex which is always hyperpolarized by a positive field, and two in a deeper region of cortex, which are always depolarized by a positive field. Between the superficial and deep regions, there is a region with no detectable passive component (also apparent in the middle pair of traces in figure 3).
This region is at the precise location of phase reversal between the superficial and deep regions. It runs directly parallel to the cortical base and surface within somatosensory or visual cortex, and does not change if the slice is moved or rotated. Thus, the location of this zone depends on properties of the cortex, and not on the angle of the field vector, nor proximity to either electrode.

The active component, in contrast, manifests as propagating waves of depolarization across large cortical areas. It starts with small “seeds” of activity (figure 5A-D, frame 0) and propagates outward as a wave. The seed is typically located near where the cortical depth axis is parallel with the applied field vector, and is almost always in or near layer V, though its precise location can vary. In all observed cases, the active component has spread across the entire depth of cortex, though the extent and speed of lateral propagation has varied considerably. Pseudocolor maps are shown in figure 4 for the first cycle of active component during three separate trials (Figure 5A-C). In all cases, the active component initiates near layer V (black arrows in the 0 ms frames), and depolarization quickly spreads both upward towards layers I-III and horizontally in both directions. Similar to reported propagation of Up states in vitro (Wester & Contreras 2012), the horizontal spread is first to the deep layers, and subsequently to the more superficial layers, rather than being a sequential activation of columns.

When the field is applied for several consecutive cycles, the active component may be evoked by many or all of them. Fields of higher intensity tend to evoke activity on more cycles, however there is considerable variance even when using the same stimulus on the same cortical slice. For example, figure 5 shows the same 1 Hz, 14
V/m field applied across the same slice, with each trial occurring three minutes after the previous one. A 14 V/m field would consistently generate the active component on the first cycle of the field, but responses to the 2\textsuperscript{nd} – 4\textsuperscript{th} cycles were inconsistent, and the fifth cycle never generated a response. The wave properties of the active component evoked on the first cycle of the applied field tends to be the same in each trial (figure 5A-C), with activity generated at a seed and spreading outward. Subsequent cycles of active component often have more irregular spatiotemporal properties; in figure 4D, the active component spreads horizontally as a wave but generates a second peak ~500 ms later. This second peak may be a reflection of the initial wave travelling laterally through the auditory cortex (up in the image), since it only propagates medially.

In all recordings, the active component peaks propagated laterally across the imaging field in at least one direction. There were examples of waves failing to propagate along one of the two horizontal directions in two of the five slices tested, but this occurred in less than 10% of the evoked waves. I calculated the velocity of the waves by averaging signals across cortical depth and determining the onset times at each distance from the wave initiation site (onset was defined as when the signal reached 50% of maximum). This method is not very precise, but should be accurate over many samples. The median propagation velocity recorded was 13 mm/sec with 94% of calculated values being less than 100 mm/sec (figure 5E). This fits well within ranges observed for spontaneous wave propagation both \textit{in vitro} (Sanchez-Vives & McCormick 2000, Wester & Contreras 2012) and \textit{in vivo} (Petersen et al. 2003, Xu et al. 2007).
Properties of the Field

Changing the properties of the applied field affects the active and passive components differently. I first applied fields of varying intensity and frequency in the presence of 20 \( \mu \text{M} \) CNQX to see how this affects the passive component. Consistent with earlier studies of polarization by electric fields (Deans et al. 2007, Anastassiou et al. 2011), the passive component amplitude showed a linear relationship with the field intensity (figure 6A). This was true regardless of the frequency of the applied field. The passive component amplitude decreases exponentially with increases in frequency of the applied field, again regardless of field intensity (figure 6B).

The active component, rather than showing a linear relationship with the stimulus intensity, shows characteristics of an all-or-none response. The active component is induced only if the field reaches a threshold positive value, which ranged from 5 \(- 10 \) V/m across the 10 slices tested (with a mean of +7.4 V/m, which occurs during the positive phase of 14.8 V/m peak-to-peak AC fields). Note that this active component threshold is lower than the threshold for evoking action potentials in individual cells, for which the lowest reported value is 28 V/m (Radman et al. 2009). Increases in field intensity above the active component threshold have little effect on the active component amplitude (figure 6C). The active component is also more sensitive to low-frequency fields (figure 6D).

Cellular Activity

To investigate cellular activity, I used two-photon imaging to record intracellular calcium levels in individual neurons within a small cortical population. Slices were placed in a recording chamber and calcium dye (Oregon Green BAPTA-1, AM) was
pressure-injected into cortical tissue to stain a small region of visual cortex (figure 7A). This AM dye will bind only to intracellular calcium. I used a two-photon scanning microscope with a 20X objective, allowing continuous monitoring of 100 – 200 cells at a time. Calcium transients in single neurons are discreet events, and correlate very highly with the firing of action potentials (Mao et al. 2001, Kerr et al. 2003). Slices were exposed to 1 Hz AC electric fields as in the VSD imaging experiments, except the fields were square-wave instead of sinusoidal. The region of cortex chosen for staining was aligned such that the apical dendrites of pyramidal neurons were parallel to the applied field vector. However, earlier experiments with VSD suggest that the active component is initiated near this location, but not always at this location. Thus, I cannot determine whether the active component is initiating within the imaging window, or whether it is being initiated elsewhere and propagating through the imaging window. Unsurprisingly, no equivalent of the passive component was visible in calcium signals, as membrane depolarizations that do not result in action potentials should not produce a detectable somatic calcium transient (Mao et al. 2001).

We recorded from seven slices exposed to 1 Hz AC fields, with each imaging window containing primarily layer V neurons. In each case, the fraction of neurons firing during at least one cycle of each applied field was linearly related to the intensity of the stimulus (figure 7B). The fraction of neurons showing calcium transients can also be referred to as the “density” of activity; these terms will be used interchangeably. The density of activity was proportional to the threshold stimulus for that particular slice and not the absolute increase in field strength. Thus, if the threshold for evoking activity was 14 V/m, then twice as many cells would be activated by a 28 V/m field; if the threshold
was 24 V/m, then twice as many cells would be activated by a 48 V/m field. There was an eventual plateau; even fields approaching 40 – 50 V/m would not induce calcium transients in more than half of the neurons. In each slice I recorded from, a substantial fraction of cells (20 – 40%) would never fire during an applied field. To confirm that these cells were capable of showing calcium transients, in two slices I added 1 – 10 μM bicuculline at the end of the experiment, and in both cases more than 96% of cells would show calcium transients during the resulting epileptiform activity (figure 10 in chapter V provides an example).

We found that the threshold for evoking calcium transients in pyramidal neurons was between 14 and 24 V/m. This range is slightly higher than the active component thresholds determined using VSD (10 – 20 V/m), but is still below the reported field intensities for directly evoking action potentials in an isolated pyramidal neuron (28 – 79 V/m). It is therefore exceedingly likely that much of the neuronal firing is driven by the same mechanisms as the active component described in the VSD recordings. That is, the synchronous activation of large groups of neurons is driven by network mechanisms, even if a few very sensitive neurons may be directly activated by the field.

Layer V pyramidal cells have been reported to be the most sensitive to electric fields (Radman et al. 2009), and my calcium imaging data confirms this. For three slices, I recorded across a large extent of the cortical depth in order to compare cells in layer V to cells outside of layer V. I first identified layer V pyramidal cells in the images by their size and shape. Next, all cells in the image were put into one of two groups: those appearing across the same range of depths as the identified pyramidal neurons of layer V, and those appearing either more superficial or more deep than any identified
layer V pyramidal cells. This approach to categorizing layer V cells may produce both false positives and false negatives, but should be sufficiently accurate to identify broad trends. Regardless of field intensity, there was always a higher fraction of these identified layer V cells firing at least once during the five cycles of applied field (figures 7C & 6D).

The temporal patterns of calcium transients within layer V cells were also different. I would often observe calcium transients in the same layer V cells during most or all cycles of applied field oscillation, even for fields at threshold (figure 7E, top of figure 7F). Outside of layer V, this never occurred for fields at threshold. As field intensity was increased, the fraction of layer V cells firing during multiple cycles increased rapidly. Cells outside of layer V, however, increased only moderately between 100 and 150% of the threshold field, followed by a substantial increase between 150 and 175% (figure 7E, middle and bottom of figure 7F).

**Discussion**

The principle finding in this chapter is that the cortical response to oscillating electric fields has two components: a passive component, which is polarization directly induced by the field; also an active component, which is depolarization caused by synaptic activity. VSD recordings show that they have very distinct spatiotemporal properties, and are differently affected by the frequency and intensity of the field. Calcium imaging data suggest that the active component begins in layer V.

The experiments in this chapter are the first exploration of electric field effects in the cortex using VSD or calcium imaging, which allow a novel view of how the activity changes over space and time. An important finding is that the active component can be
5–10 times larger than the passive component, and will dominate overall measurements under a low-intensity, oscillating electric field. The active component starts from a small seed and propagates outward as a wave. Through this propagation, the active component may play a role in spreading the effects of a local field to a much larger population of neurons, including those that may not be directly polarized by the field.

The Passive Component

The passive component takes the shape of the applied field and is unaffected by pharmacological glutamate blockers or TTX, and is likely the direct polarization of neurons under the field. The largest contribution to the signal is most likely from layer V pyramidal neurons, with the soma and basal dendrites in layers IV-VI acting as one half of a dipole, and the apical dendrite and tuft in layers I-III acting as the other half. This fits in well with current theory (Jefferys 1995, Buzsaki et al. 2012) and with prior studies showing somatic polarization in cortical (Radman et al. 2009) and hippocampal (Bikson et al. 2004, Deans et al. 2007) neurons by applied electric fields. It also comports with my calcium imaging data that show more calcium transients in layer V neurons under weak applied fields (figure 7C-E), suggesting that layer V somata are most strongly affected.

The most prominent feature of the passive component is a stationary zone, near the border between layers III and IV, which features undetectable amplitude and an abrupt phase reversal. This can be explained by a simple polarization of layer V pyramidal neurons, where the zero-amplitude region is located at the center of the dipole, but there may be other factors contributing. Several other factors may
contribute. First, it may be that cells in this zone project primarily horizontally or symmetrically (e.g. stellate cells) and thus do not polarize to a significant degree. Second, VSD signal is an average across all membranes beneath a detector, meaning a flat signal can be caused by a lack of any changes or by a balance of positive and negative changes. Thus, the measured passive component may be influenced by polarization of pyramidal and non-pyramidal neurons in more superficial layers, which have ends of their induced dipole at different depths. They are not as vertically-elongated as many of the layer V neurons, due to their proximity to the tufts in layer I, but should still be polarized by fields to a lesser degree; thus, they may influence the location of the detected phase-reversal. They may also generate the second peak in the deep layers, as seen in the amplitude map in figure 3.

It is worth noting that the exponential frequency-amplitude relationship in figure 6B addresses an inconsistency in the literature; Deans et al. (2007) reported an exponential decline in amplitude with increased frequency, whereas Anastassiou et al. (2011) reported a linear decline. This may be explained by differences in recording techniques. Like Deans et al., I am measuring amplitude using VSD, which exclusively reports potential changes across membranes, most of which should be from dendrites and influenced by local transmembrane currents. Anastassiou et al. used intracellular (somatic) and extracellular electrodes and recorded the potential between these two electrodes. The extracellular electrode may primarily be measuring the bulk flow of extracellular current. This extracellular current would be almost entirely determined by (and linearly related to) the applied field, since it is much larger than any endogenous fields that can be generated in the absence of highly synchronized neuronal activity. I
believe this is a more compelling explanation than the insufficient number and mobility of electrodes that Anastassiou et al. ascribed to the Deans et al. study.

**From Passive to Active Component**

The active component can only be induced when the deep layers are depolarized by the passive component (in more than 20 slices examined). VSD imaging revealed that the active component was always first initiated in or near cortical layer V, and calcium imaging showed that layer V neurons were most likely to fire. However, the field intensity that can induce the active component is below the reported threshold for directly evoking action potentials in pharmacologically isolated cells (Radman et al. 2009). Prior studies have shown that even extremely small fields can modulate spike timing of hippocampal neurons (Francis et al. 2003, Reato et al. 2010), and many layer V neuron show high levels of spontaneous activity (Le Bon Jego & Yuste 2007). Thus, it may be that the active component is initiated through modulation of spike timing in layer V pyramidal cells by the passive component. Consistent with this, I found that the active component is highly sensitive to TTX and glutamatergic antagonists, suggesting that synaptic activity plays a key role in integrating the small effects of a field on individual neurons into a large population event.

Interestingly, the polarization caused by applied fields resembles the profile of endogenous fields measured in vivo. Current source density analysis has shown that cortical slow waves consist of superficial and deep regions of cortex acting as alternating current sources and sinks (Buzsaki et al. 1988, Fröhlich & McCormick 2010). Thus, current flows into the deeper layers of cortex when neuronal activity is high (e.g. Up states) and out of the deeper layers when activity is low (e.g. Down states). The
passive component here sets up a similar dipole, which may facilitate the synchronous firing in the active component.

**The Active Component**

The active component involves the recruitment of large populations of neurons into propagating waves. Propagating waves occur in vivo in response to sensory stimuli, and can spread depolarization from one brain region to distant brain regions. For example, a visual stimulus can evoke activity in primary visual cortex, which then propagates into secondary visual or association cortices (Takagaki et al. 2008). By the same mechanism, the propagating waves of the active component that I recorded with VSD can turn small changes in local membrane potential into a large event involving large cortical regions. This may explain how relatively local application of weak applied fields *in vivo* can result in measurable changes at the behavioral level.

In a continuous oscillating field the active component always appears on the first cycle, but does not always occur on every cycle (e.g. figure 5 traces). This suggests the involvement of complex factors at both cellular and population levels. On the cellular level, there may be a buildup of inhibitory/shunting factors after several occurrences of active component. For example, the repeated calcium transients observed primarily in layer V pyramidal neurons (figure 7E) may eventually hyperpolarize the cell via the slow calcium-activated potassium current ($I_{AHP}$, Sanchez-Vives & McCormick 2000). On the population level, propagating waves can be sustained by recurrent excitation (Sanchez-Vives & McCormick 2000), which may require recruiting new populations of neurons into subsequent waves. This may be particularly important outside of layer V, as these cells rarely fire on multiple cycles (figure 7E). After being recruited into the active component
during one cycle of the field, these cells may be refractory during subsequent cycles, eventually leading to a loss of the necessary recurrent excitation to sustain propagating waves.

**VSD vs. Calcium Signals**

We used VSD imaging and two-photon calcium imaging to record the activity induced by electric fields, and found that the active component showed characteristics of an all-or-none response. This means an applied field above threshold will not evoke more activity than a field at threshold. With calcium imaging, however, I found that the fraction of cells spiking under the field linearly increased well past the threshold for activating minimal activity. So why will increasing the field intensity above threshold increase the calcium signals but not the voltage signals? There are two main possibilities.

The first possible explanation is that an increase in excitatory synaptic transmission is balanced by an increase in inhibitory transmission. Calcium signals are measuring somatic calcium transients, which highly correlate with action potentials of single neurons. The VSD signals, on the other hand, are mostly the result of postsynaptic potentials, averaged over large regions of space. So the question then becomes, how do more action potentials not lead to more postsynaptic depolarization? When very low-intensity fields activate a minimal fraction of neurons, the observed calcium transients are mostly in excitatory layer V pyramidal cells (top two images in figure 6F). As field intensity increases, more of the smaller cells become activated, some of which will almost certainly be inhibitory. It may be that as more cells are activated with higher field intensities that the increased inhibition will balance out the
increased excitation, leading to very similar overall depolarization across the populations measured with VSD.

An alternative explanation is that I am misinterpreting the active component threshold in my calcium data. It could be that the active component threshold is much higher in the calcium imaging setup, and that it is not until the point when the calcium response saturates (e.g. at 1.5 times the threshold in figure 6C-E) that we can observe the “active component”. This would mean that the numbers of neurons responding at lower field intensities are not sufficient to generate a signal large enough to see in VSD imaging. It would also mean that the active component threshold in the calcium imaging setup is much higher than in the VSD setup. This could possibly be due to poor slice health or to inadequate perfusion in the recording chamber, though there were attempts to take these variables into account. It could also be due to the dyes themselves affecting physiology, though these effects should be minimal.

This alternative explanation may be unlikely, as synchronous cortical activity is visible in VSD even when it involves a small fraction of neurons firing. In my calcium imaging experiments, fields below the intensity at which the response saturates still induce activity in up to 25% of the neurons within the imaging window. This is considerably denser than the 10% activation seen in most Up states in vivo (Kerr et al. 2003, Rochefort et al. 2009), which are clearly visible with VSD imaging (Petersen et al. 2003, Xu et al. 2007). It is also well within the range of densities of Up states seen in vitro in my later experiments (chapter V figure 8) and by others (MacLean et al. 2005), which are also clearly visible in vitro with VSD (Wester & Contreras 2012, chapter V figure 7).
Figure 3. The response to a sinusoidal AC electric field shows distinct active and passive components. Each trace is from a single trial, averaged across 8 detectors centered at the point marked on the slice (top image), filtered between 0.5 and 30 Hz. Regardless of depth, the active component (arrows, blue traces) appears as large depolarizations occurring shortly after the field (black trace) reaches its peak (dotted lines). Blocking fast glutamatergic transmission with CNQX abolishes the active component, leaving only the passive component (red traces). The passive component is the same shape as the stimulus waveform, appearing sinusoidal here. The passive component direction depends on cortical depth: in the more superficial area (detector 1) it is in opposite phase relative to the more deep area (detector 3). Between these two areas is a smaller area where the passive component does not appear (detector 2).
Figure 4. The passive component has a laminar pattern. (Left) A picture of a cortical slice, with a box drawn around the region being analyzed. (Top-Center) Amplitudes of the passive component are calculated for each detector by FFT. The grayscale map shows the spatial arrangement of normalized passive component amplitude across this region. The amplitude of the passive component depends on depth within the cortex, and is mostly uniform across short horizontal distances. (Bottom-Center) Amplitudes are plotted vs. depth for five slices of somatosensory cortex. The region with no detectable amplitude is on average 500 um below the surface of the cortex, roughly corresponding to the border between cortical layers III and IV. Averaging across five slices of somatosensory cortex, the polarization per unit stimulus peaks at roughly $3 \times 10^{-5}$ for every V/m of stimulus, and reaches zero near a depth of 500 um below the cortical surface. (Top-Right) The phase relationship between the field and passive component waveforms was calculated for each detector using cross-correlation. The location of passive component phase reversal corresponds with the region with near-zero amplitude shown on the grayscale map, here between 4 and 5 detectors deep. (Bottom-Right) Plotting the latency for each trial across the same five slices shows that the phase reversal is at different depths, likely depending region of somatosensory cortex. However, it always shows an abrupt change from roughly phase-aligned (0 ms latency) to antiphase (500 ms latency). Data is from 56 trials total.
Figure 5. The active component is a propagating wave originating from a local seed of activity. The traces at the top are of the applied field (gray trace, 14 V/m) and three VSD signals (black traces). Each trace is the average across six detectors spanning the depth of the cortex (black mark on the top-right image). The traces are from consecutive trials from one slice of visual cortex (top-right picture). Pseudocolor images are generated for frames 64 ms apart during the events labeled A-D in the traces. Data is spatially smoothed with a 1-detector radius. Hot colors correspond to higher ΔF/F (depolarization), and cool to lower. The applied field vector is along the left-right axis. (A – C) The first peak of each trial follows a characteristic pattern of initiation (arrow) and propagation. (D) Later peaks often show different spatiotemporal properties from the stereotyped first peak. In this example, it initiates from the same location (arrow on frame 0), but has a second peak (arrow on frame 512). (E) Horizontal propagation velocity is calculated based on the time it takes the wave to propagate from one set of detectors to those horizontally adjacent.
Figure 6. The cortical response to an oscillating electric field depends on the field frequency and intensity. (A) The amplitude of the passive component is linearly related to the intensity of the applied field, regardless of the frequency of the field. (B) The amplitude of the passive component declines exponentially with increasing field frequency, regardless of the field amplitude. Data is averaged across four slices. (C) The active component of all four slices shows characteristics of an all-or-none response, where a large enough field intensity will trigger a response that quickly saturates. (D) The active component is more sensitive to low-frequency fields, failing to initiate as the frequency increases (30 V/m fields).
Figure 7. The cellular response depends on electric field intensity. Two-photon calcium imaging was performed on a small region of cortex, often spanning the entire depth of cortex, but always including layer V. Cells that were clearly astrocytes were excluded from analysis based on morphology and higher baseline fluorescence. A cell was considered “active” when its level of fluorescence was three standard deviations greater than baseline for multiple consecutive frames. (A) Fluorescent image of stained region of visual cortex. One column is stained, and imaging with a 20X objective allows detection of calcium spikes for 100-200 neurons at a time, as shown in part F. (B) The fraction of cells in the image that show calcium transients at least once during the five-second field application increases linearly with field intensity until reaching ~175% of threshold. These data are from 7 slices, with thresholds ranging from 12 – 24 V/m, with a mean of 17 V/m.

Continued on next page. (C) Cells in the pyramidal layer V (red) are most sensitive to the field, and evoked calcium transients within this layer are denser than in other layers (blue) at all field intensities. (D) Data from part C normalized (with non-normalized values on the y-axes). (E) Same as the plot in part D, except cells are considered “active” when they fire during most (three or more) cycles of oscillation rather than just one. (F) Sample two-photon images of cells from one slice stained with a calcium indicator. Active cells are marked in color, depending on whether they are inside (red) or outside (blue) of the pyramidal layer. Outlined cells fire during at least one cycle of oscillation, and filled cells fire during three or more cycles of oscillation.
Chapter V: Extracellular Calcium and Up States

Central Hypothesis

Low extracellular calcium levels facilitate slow oscillatory activity by increasing sodium conductance.

Results

Imaging Spontaneous Activity. First, I established that the lower concentration of extracellular calcium and magnesium ([Ca.Mg]_e) increased the frequency of spontaneous cortical activity. I used two-photon calcium imaging of mouse visual cortical slices to record intracellular somatic calcium signals. Small regions of cortex were pressure-injected with Oregon Green BAPTA-1, AM dye, which reports only intracellular calcium. Because of this, in each image the cell soma appears brighter than the background, which includes cell processes and extracellular space. figure 8A shows the average spontaneous changes in fluorescence across all pixels of a 235 x 363 \( \mu \text{m} \) imaging window centered on layer V of visual cortex. The images include neurons, neuropil, and astrocytes; however, astrocytes are a small minority of cells and contribute very little to overall fluorescence changes. In addition, the signals in this trace are high-pass filtered above 0.2 Hz, which should remove contributions by the very slow calcium signals observed in the soma of astrocytes (Poskanser & Yuste 2011). The averaged trace in figure 8A shows one high-amplitude peak of synchronous calcium increases (closed arrow) over 5.5 minutes of recording in 2 mM [Ca.Mg]_e, as well as several smaller peaks of spontaneous activity (open arrow). After switching recording solutions to 1 mM [Ca.Mg]_e, the frequency of spontaneous activity increases, including both high-amplitude and low-amplitude peaks.
We used VSD imaging, as described in the previous chapter, to image spontaneous activity as well. To avoid bleaching/phototoxicity, my VSD recordings are only 10 – 20 seconds long with 2 – 3 minutes between each recording trial. Nonetheless, by chance I would often record spontaneous activity during the recordings. I found that the peaks of synchronous activity resembled cortical Up states, including sustained depolarization for more than 500 ms across large populations of cells (figure 8B). This was the case for the spontaneous activity observed in both 1 mM and 2 mM [Ca.Mg]_o, though due to the sparse sampling of my recordings, only five spontaneous Up-state-like events were recorded in 2 mM [Ca.Mg]_o, and the sample may not be entirely representative. Consistent with reports in the literature, I found that spontaneous activity was heavily reliant on sufficient oxygenation. Thus, the perfusion of extracellular solution is considerably faster in the recordings in this and subsequent chapters when compared to the VSD recordings in chapter IV.

In calcium recordings in 1 mM [Ca.Mg]_o, the average fluorescence changes as shown in figure 8A are not only more frequent, but are also higher-amplitude. To investigate the fraction of cells contributing to each spontaneous event, I used a semi-automated process to mark individual cells in the image, typically recording from 100 – 200 cells at a time with a 20X objective (see example image in figure 8). Fluorescence changes within the boundaries of the marked cells were used to generate traces, and a cell was considered “active” when the fluorescence increased more than three standard deviations above the baseline for two consecutive frames (3 – 4 Hz sampling rate). The amplitude of the change in the mean fluorescence across the entire image (figure 9, blue traces) corresponds with the fraction of cells active (density) during the
spontaneous events (figure 9, raster plots). I found that spontaneous activity in 1 mM [Ca.Mg]_e was on average denser than spontaneous activity in 2 mM [Ca.Mg]_e; however, there was considerable overlap between the distributions (figure 9, box plot).

Spontaneous events in 1 mM [Ca.Mg]_e did not lack sparser activity; rather, spontaneous events in 2 mM [Ca.Mg]_e lacked denser activity. In the box plot in figure 9, the distribution of density across all events appears unimodal, suggesting spontaneous events in 1 mM [Ca.Mg]_e do not fall into separate categories of “sparser” and “denser” events, with only the former regularly occurring in 2 mM [Ca.Mg]_e.

**Inhibition.** Cortical Up states require a balance of recurrent excitation and inhibition (Shu et al. 2003). As expected, the spontaneous events in 1 mM [Ca.Mg]_e were completely abolished by bath application of the AMPA/kainate receptor antagonists CNQX or NBQX (no spontaneous events across 5 – 10 minutes of recording from each of 6 slices). The spontaneous activity patterns included an important role for inhibition. I compared the spontaneous activity in 1 mM [Ca.Mg]_e before and after adding the GABA_A antagonist bicuculline (1 μM) to the bath. The sparse spontaneous events (~15% of cells in each event) quickly became very dense, recruiting nearly every neuron into each burst of activity (figure 10A). The calcium signals in each neuron also became much larger (compare the brightness of the individual cell events in figure 10A).

VSD imaging showed the effects of a GABA_A antagonist were consistent with those in the literature for Up states (Sanchez-Vives & McCormick 2000, Mann et al. 2009, Sanchez-Vives et al. 2010): 1 μM bicuculline increased the amplitude of spontaneous events and shortened the duration (figure 10B-C).
**Calcium affects Sodium Channels.** Next, I tested whether calcium ions specifically contribute to this change in spontaneous activity by repeating the previous experiment, but holding \([\text{Mg}^{2+}]_e\) at 1 mM. Spontaneous events were infrequent when \([\text{Ca}^{2+}]_e\) was 2 mM, but became far more frequent under 1 mM \([\text{Ca}^{2+}]_e\) (figure 11, traces). This effect was reversed by bath application of 10 \(\mu\text{M}\) gadolinium chloride, a blocker of sodium currents through voltage-dependent and leak channels. This restored spontaneous event frequency to the approximate level seen with 2 mM \([\text{Ca}^{2+}]_e\).

Spontaneous events were infrequent with either 2 mM \([\text{Ca}^{2+}]_e\) or 10 \(\mu\text{M}\) GdCl₃, but those that did occur under showed a reduced in density compared to 1 mM \([\text{Ca}^{2+}]_e\) (figure 11, raster plots).

We questioned whether this effect on spontaneous activity could be explained by a simple elevation of resting potential across the network. As the Nernst equation predicts, increasing extracellular potassium ion concentrations depolarizes neurons (Somjen 1979). I found that an increase in extracellular potassium chloride (\([\text{KCl}]_e\)) from 3 to 5 or 8 mM did not increase the frequency of spontaneous synchronous events (figure 12A). This is despite the increased the number of spontaneous calcium transients that occurred throughout the entirety of the recording (figure 12B). For comparison, I found that when reducing the \([\text{Ca.Mg}]_e\), there were no changes of comparable magnitude in the rate of spontaneous spikes that occurred outside of the large spontaneous events (i.e. during a Down state). As with the calcium and gadolinium experiments, here there were too few observed spontaneous events (7 total) to make a definitive statement about density. Nonetheless, the spontaneous events that did occur under higher \([\text{KCl}]_e\) did not show an increase in density (figure 12D).
Finally, to examine the possibility that extracellular calcium was influencing neuronal activity through intracellular mechanisms, I used a 60X objective to get signals with a better signal-to-noise ratio for each neuron, though at the cost of capturing only 20 – 30 cells per image (figure 13A). I found that the number of cells showing calcium transients during each event was reduced by increasing $[\text{Ca}^{2+}]_e$, but that there was no significant change in the amplitude of the individual calcium transients (figure 13B-C).

This is in contrast to the effect of bicuculline, which led to a dramatic increase in intracellular calcium concentrations (figure 10A).

**Discussion**

The principle findings are that reducing the calcium concentration alone promotes spontaneous synchronous cortical activity. Blocking sodium channels with gadolinium demonstrates that this increase in spontaneous activity may depend on calcium-modulated sodium currents.

**Up States in vitro.** I used low extracellular concentrations of calcium and magnesium ions to reproduce an in vitro model of spontaneous Up states in slices of mouse visual cortex. While I do not have intracellular recordings that demonstrate the bistable membrane potential that defines Up states (Steriade 1993a), the spontaneous cortical activity in my recordings demonstrates many properties consistent with Up states. They show sustained depolarization for more than 500 ms, their regularity depends on low $[\text{Ca.Mg}]_e$ (Sanchez-Vives & McCormick 2000, Fanselow & Connors 2010, Wester & Contreras 2012), they occur at a frequency consistent with other in vitro recordings in mouse sensory cortex (Wester & Contreras 2012), they are sparse (Kerr et al. 2003, Rochefort et al. 2009, Cossart et al. 2003, MacLean et al. 2005, Watson et
al. 2008), and they show similar sensitivities to disruptions in glutamatergic (Sanchez-Vives & McCormick 2000, Cunningham et al. 2006) and GABAergic (Sanchez-Vives & McCormick 2000, Mann et al. 2009, Sanchez-Vives et al. 2010) transmission. Thus, though this model of Up states may not be an entirely faithful reproduction of what we call Up states in an organism, the spontaneous activity shares many characteristics in common with the in vitro Up state literature, and “Up” states may be the most appropriate categorization. Further, Up state activity has much in common with spontaneous ensemble activation in the absence of an Up state (Cossart et al. 2003, Ikegaya et al. 2004), so the properties of spontaneous activity described in this chapter likely apply to Up states, and I will refer to them as Up states in this discussion.

The frequency of spontaneous activity in the VSD and calcium imaging setups was similar (figure 7C), leading me to believe that the spontaneous events in the calcium imaging data are the same kind of spontaneous events that are seen with VSD. The frequency of spontaneous events in 2 mM solution was higher in the calcium imaging setup than in the voltage imaging setup, however, which may be due to a misclassification of smaller-amplitude calcium activity (open arrow in figure 7A) as an “Up state”. Instead, these smaller-amplitude calcium signals may be synchronous activations of groups of neurons independent of Up/Down state fluctuations, termed “cortical flashes” by Cossart et al. 2003. In this and other in vitro papers from that lab (Mao et al. 2001, Ikegaya et al. 2004), they found that such synchronized firing in the cortex usually, but not always, occurs during an Up state.

Decreasing [Ca.Mg]₀ to 1 mM increased the average number of neurons recruited into each Up state (figure 9). However, it did not change the amount of
calcium that enters a cell during its participation in a spontaneous event (figure 13). Intracellular calcium signals show a linear relationship between calcium signal amplitude and the number of action potentials fired (Mao et al. 2001). The lack of change in intracellular calcium suggests that the properties of neuronal spiking during spontaneous events is unaffected by the change in [Ca.Mg]e. This is in stark contrast to a blockade of GABA\textsubscript{A} receptors, which dramatically increases the levels of intracellular calcium in nearly all cells during spontaneous events (figure 9). This fits well with earlier studies, showing a transition from slow sustained spiking during Up states to high-frequency bursting when GABA\textsubscript{A} is blocked (Sanchez-Vives & McCormick 2000, Mann et al. 2009, Sanchez-Vives et al. 2010).

**Extracellular Calcium.** I found that a reduction in extracellular calcium ions alone could account for a large increase in the frequency of spontaneous synchronized activity (“events”) in the cortex, and that this effect was reversed by 10 mM Gd\textsuperscript{3+}, a blocker of sodium channels. This is not due to a simple nonselective depolarization across the network. When depolarizing neuronal membranes by increasing the concentration of extracellular potassium ions, I found the anticipated increase in overall spontaneous spike rates, but I did not see an increase the frequency of spontaneous Up states. Extracellular calcium is likely exerting an effect on voltage-gated sodium channels, which can mediate a persistent sodium current under non-spiking conditions. Extracellular calcium can also reduce permeability through the NALCN sodium leak channel (Lu et al. 2007). The NALCN channel is widely expressed across the mammalian nervous system, and has been strongly implicated in rhythmic network
behavior, including respiratory and movement-related rhythms (Ren 2011). It may have a role in slow cortical rhythms as well.

Gadolinium may also act by blocking kainate receptors (Huettner et al. 1998) in addition to voltage-gated and leak sodium channels, and this could account for the reduced frequency of spontaneous events. Changes in kainate receptor function, however, are not likely to be mediating most of the effects in seen in these experiments for several reasons. First, a literature search turned up no evidence that extracellular calcium modulates kainate receptor conductance in the way that gadolinium does, yet the effect on Up state frequency seems the same. Second, the in vitro Up states should depend critically on kainate receptor function, as the AMPA/kainate antagonist NBQX completely abolishes Up states in my experiments, and a study in entorhinal cortical slices suggests that the effect of NBQX is mediated primarily through kainate receptors (Cunningham et al. 2006). Nonetheless, I have not ruled out that changes in kainate receptor function due to extracellular calcium or gadolinium concentration increases contribute to the results we see here.

**Significance.** There is evidence that this calcium regulation of Up states is physiological. During slow wave sleep, extracellular calcium oscillates in phase with the slow oscillation (Massimini et al. 2000). Here, I propose a model by which extracellular calcium dynamics act as a positive feedback mechanism during Up state generation. First, through either organized or stochastic processes, a small population of neurons reaches sufficient levels of depolarization to fire synchronous action potentials. With the firing of action potentials, large quantities of calcium enter the cells, and the local extracellular calcium concentration is reduced. This reduction in extracellular calcium
increases conductance through sodium channels, increasing levels of depolarization throughout the network. This depolarization facilitates the recurrent excitation necessary to drive Up states (Sanchez-Vives & McCormick 2000, Shu et al. 2003), increasing the probability of a network-wide Up state. This model can be tested with a wider array of techniques in cortical slice preparations, and could potentially help our understanding of cortical rhythmic behavior in general.
Figure 8. The frequency of spontaneous events is affected by calcium and magnesium ion concentrations. (A) A sample of calcium signals in visual cortex averaged across a population of 125 cells and the surrounding neuropil (235 x 363 μm imaging window) shows small groups of cells spiking irregularly (open arrow), and larger Up-state-like events occurring only rarely (closed arrow). After increasing [Ca.Mg]_e to 1 mM, the large events occur much more frequently. (B) Example of VSD signals in visual cortex, showing spontaneous depolarizations lasting 500 or more milliseconds in 1 mM solution (bottom trace). In 2 mM solution (top trace), spontaneous activity usually appears to be shorter in duration and smaller in amplitude; however, this sample may not be representative, since spontaneous activity was only seen five times during the short-duration VSD recordings. (C) Under both the calcium imaging and VSD imaging setups (6 slices for calcium, 12 slices for voltage), spontaneous events were seen at similar frequencies in 1 mM and 2 mM [Ca.Mg]_e.
\[ \Delta F/F = 0.02 \quad \text{20 sec} \]
Figure 9. Spontaneous events are denser in lower [Ca.Mg]_e concentrations. The black raster plots at the top each represent a two-minute calcium recording from 126 cells displayed in the bottom-left image. The top color plots contain one row of pixels for each cell in the imaging window, with brighter colors corresponding to higher calcium signals at each time point. Any calcium signals less than three standard deviations from baseline are set to zero (black). “Calcium transients” were defined as when the signal exceeded this three-standard-deviations threshold for multiple consecutive frames. Blue traces beneath each plot are the mean calcium signals across the entire imaging window, including cells and neuropil. Under both 2 mM (left) and 1 mM (right) [Ca.Mg]_e solution, calcium signals have a clear temporal structure, with larger-amplitude signals corresponding to denser activity. The box-whisker plot on the bottom-right shows the distribution of event density with each solution: there are much denser events when using 1 mM [Ca.Mg]_e solution, and though the distributions are significantly different (p < 0.003), they overlap considerably. Box plot data are from two slices, 68 events in 1 mM [Ca.Mg]_e solution, 23 events in 2 mM Ca.Mg solution.
Figure 10. The GABA<sub>A</sub> blocker bicuculline (1 μM) induces epileptiform activity that is different from the spontaneous activity in 1 mM [Ca.Mg]<sub>e</sub> solution. (A) Calcium signals from 158 neurons and surrounding neuropil as bicuculline is added to the bath (1 mM [Ca.Mg]<sub>e</sub> solution). The amplitude of the average calcium signal (blue trace) increases by a factor of 7 – 8, and nearly all neurons are recruited into each event. (B) VSD recordings of spontaneous activity under control conditions and with 1 μM bicuculline. (C) The VSD recordings are consistent with reports in the literature: the duration of network depolarization is decreased by the GABA<sub>A</sub> antagonist (p < 10<sup>-5</sup>), and the baseline-to-peak amplitude of depolarization is increased (p < 10<sup>-4</sup>).
Figure 11. Changing the calcium concentration alone affects spontaneous event frequency. 
$[\text{Mg}^{2+}]_e$ is maintained at 1 mM throughout. Spontaneous event frequency increases when extracellular calcium is decreased from 2 mM (event in left raster) to 1 mM (events in central raster). This increase in spontaneous event frequency is reversed when the sodium channel blocker GdCl$_3$ is added to the bath, suggesting that extracellular calcium may affect spontaneous activity by blocking this leak sodium channel.

Event frequency data are from two slices (different animals); raw event counts are in the following table.

<table>
<thead>
<tr>
<th>[Ca/Mg]</th>
<th>Slice 1</th>
<th>Slice 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/2 mM</td>
<td>4 10</td>
<td>0 7</td>
</tr>
<tr>
<td>2/1 mM</td>
<td>3 5</td>
<td>3 5</td>
</tr>
<tr>
<td>1/1 mM</td>
<td>17 10</td>
<td>25 11</td>
</tr>
<tr>
<td>GdCl$_3$</td>
<td>5 6</td>
<td>6 7</td>
</tr>
</tbody>
</table>
Figure 12. Higher extracellular KCl concentrations increase spontaneous spike rates but do not increase the frequency of spontaneous Up-state-like events. (A) Increasing $[\text{KCl}]_e$ from its normal concentration of 3 mM to elevated concentrations of 5 mM or 8 mM fail to increase the frequency of spontaneous events. (B) The rate of spontaneous calcium transient occurrence, averaged across all cells in the imaging window, increased with KCl concentration (lower-left plot). (C) The rate of spontaneous calcium transients occurring outside of large network events shows little if any increase when the $[\text{Ca.Mg}]_e$ is reduced. (D) The density of the few observed spontaneous events did not increase under elevated $[\text{KCl}]_e$.

Event frequency data are from two slices (different animals); raw event counts are in the following table:

<table>
<thead>
<tr>
<th>[KCl]</th>
<th>Slice 1</th>
<th>Slice 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>3 mM</td>
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<td>7</td>
</tr>
<tr>
<td>5 mM</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>8 mM</td>
<td>2</td>
<td>14</td>
</tr>
</tbody>
</table>
Figure 13. Intracellular somatic calcium transients are unaffected by the change in [Ca.Mg]e concentration.  (B) The black traces on the right are from the numbered cells in the image in (A), and the blue trace is the average fluorescence change across all pixels (cells and neuropil).  (C) After increasing [Ca.Mg]e concentration from 1 to 2 mM, population events become sparser (right; one slice, 14 events), but the amplitude of the calcium transients does not change (left; 23 cells, 160 transients).
Chapter VI: Electric Fields and Up States

Central Hypothesis

Networks with slow oscillation are much more sensitive to electric fields.

Results

AC Fields Trigger Up States

We determined the threshold AC field for each slice by applying electric fields of varying intensity across the cortex and using VSD imaging to record population activity. The data in this chapter are cumulative across slices of visual and somatosensory cortex, as there were no detectable differences between how the two responded to applied electric fields. As in chapter IV, I found that this threshold was on average roughly 15 V/m in 2 mM [Ca.Mg]₀ solution. And as in chapter V, I found that changing the extracellular solution to 1 mM [Ca.Mg]₀ results in regular spontaneous cortical activity matching many characteristics of Up states. Immediately after each spontaneous event, there is a “refractory” period during which the slice will not initiate population activity, shown to be roughly 3 – 6 seconds long in a study by Sanchez-Vives & McCormick (2000). This spontaneous activity could interfere with my determination of the threshold, as fields above threshold would not evoke activity during this refractory period. To avoid the influence of this refractory period on my analysis, I discarded trials in which spontaneous activity was observed in the 5 – 10 seconds after imaging started but before the field was applied. The threshold was also defined as the field intensity necessary to evoke activity in at least 3 of 4 trials, as unseen spontaneous activity had a small probability of making the cortical tissue refractory to stimulation. Nonetheless, the
threshold was almost always very clear, with activity either consistently evoked, or consistently not evoked.

Slices in 1 mM [Ca.Mg]₀ were much more sensitive to applied fields. figure 14A shows sample VSD traces from a visual cortical slice exposed to threshold electric fields. In 2 mM [Ca.Mg]₀, activity is consistently evoked by the field on multiple cycles, with a threshold of 14 V/m. When the extracellular solution was changed to 1 mM [Ca.Mg]₀, in most experiments a 4 V/m stimulus would consistently evoke activity in the cortex. Importantly, oscillating 4 V/m fields are at the upper range of electric field intensities measured in vivo during slow oscillatory activity in neocortex (Fröhlich & McCormick 2010). The total amount of depolarization during the five-second threshold field was unaffected by the [Ca.Mg]₀ (figure 14B). To determine this, I used the applied fields to evoke activity under both concentrations and quantified the area between the active trace and the estimated baseline (a linear interpolant between the onset and offset of activity). This measure of total depolarization was unaffected by [Ca.Mg]₀ (40 trials in 5 slices, p > 0.6).

We next used VSD imaging in 1 mM [Ca.Mg]₀ to determine where in the slice the depolarization begins. Unlike other in vitro reports which saw activity initiating in layer V during cortically-generated Up states (Sanchez-Vives & McCormick 2000) or layer IV during thalamically-generated Up states (Wester & Contreras 2012), I found that the onset of spontaneous cortical activity can be located anywhere across the depth of the cortex (figure 13C, top histogram). The location of the onset of cortical activity was significantly affected by applied fields (p = 0.003 from a two-sample Kolmogorov-
Smirnov test), as applied fields skewed the onset distribution towards the deep layers (figure 13C, bottom histogram).

The discrepancy between the location of depolarization onsets in my VSD recordings and the electrode recordings by Sanchez-Vives et al. (2000) may be explained by the techniques used. Since VSD measures primarily postsynaptic potentials, we may see an onset in the superficial layers even when layer V neurons are the first to fire action potentials. And unlike another VSD study (Wester & Contreras 2012), my coronal slices should not contain thalamocortical connectivity, and thus the onset will not be restricted to layer IV. An alternative explanation, however, may be that this is a result of imprecision in my analysis. VSD recordings contain low-frequency noise that is the result of very subtle motions in the imaging sample, which tend to be larger toward the edges of the sample, which in this case is the cortical surface. This noise may increase the probability of detecting depolarization as initiating in the superficial layers, though this change in probability should be the same for evoked and spontaneous activity. In this case, my results would indicate that though the spontaneous activity may be initiating in layer V, more of the early depolarization would be occurring in the deep layers in the evoked condition relative to spontaneous.

Field Intensity Affects the Response

Increasing the intensity of the applied field does not show a clear effect on individual Up states (figure 15A). Similar to the effects on cortical activity I reported in chapter IV (figure 6C), “suprathreshold” fields (2 – 3 times the magnitude of the threshold field), did not show a significant effect on the amplitude of depolarization when compared with spontaneous or threshold-evoked events (figure 15B, left, p > 0.28).
Similar to reports in ferret cortical slices (Fröhlich & McCormick 2010), the applied field intensity also has no significant effect on Up state duration (figure 15B, center, p > 0.58). However, calculating the total depolarization (area under curve for the entire 5 seconds for evoked events, or for 5 seconds after the onset of a spontaneous event) showed significantly more depolarization under high-intensity fields than for threshold (figure 14B, right, p < 0.001) or spontaneous (p < 0.02) events, mostly due to more cycles of depolarization occurring under higher intensity fields. There was no difference between the amount of depolarization during threshold-evoked and spontaneous events (p > 0.50); at threshold, only the probability and timing of Up states was affected by the field.

When the applied field intensity was increased, this increased the probability of evoking Up states on each successive cycle of the applied field, and increased the synchrony between the applied field and the VSD signals. To examine this further, I determined the location of the onset of activity, and averaged the signal from this detector across all trials for which either a threshold or suprathreshold field was applied (67 trials, figure 15C). When doing so, the amplitude of the VSD signal increases when applying a higher-intensity field. The increase in this case was roughly proportional to the stimulus, with a 2.5-fold increase in the VSD signal amplitude resulting from a 2.5-fold increase in the average stimulus. Cross-correlation of the stimulus and VSD waveforms also shows that under lower-intensity fields, the VSD signal lags farther behind the field (figure 15D).
DC Fields

So far, the described experiments have been with AC fields, which oscillate between a positive value and an equivalent negative value. I established that there is an increased sensitivity of slices in the low \([Ca.Mg]_e\) solution. Interestingly, threshold fields in 2 mM \([Ca.Mg]_e\) consistently evoked activity on the first cycle of oscillation, but threshold fields in 1 mM solution consistently evoked activity not until the second cycle of oscillation (figures 14A, 15A&C). To explore the reasons for this, I applied DC fields (fields that oscillate between a positive or negative value and zero) to the cortical slices in the same manner as AC fields.

Slices in 1 mM \([Ca.Mg]_e\) solution show regular spontaneous Up state activity. I found that with 1 mM \([Ca.Mg]_e\), the threshold field was the same peak-to-peak intensity regardless of its polarity, and that Up states were initiated after the first negative-to-positive transition. This is illustrated in the left-side traces of figure 16. A positive DC field begins at the trough of the waveform, and activity begins halfway through the first cycle as the field completes its first trough-to-peak transition (figure 16B, left). An applied negative DC field begins at its peak, and activity thus begins after one complete cycle, when the field reached its first peak (zero) after passing its trough (figure 16C, left). An AC field starts between its peak and its trough, and thus activity does not start until after 1.25 cycles, when the field first reaches its peak after passing its trough (figure 16A, left).

In 2 mM \([Ca.Mg]_e\), there is little spontaneous Up state activity, and the cortex responds quite differently to DC fields. Instead of requiring a positive transition, cortical activity is initiated when the field reaches a sufficiently positive value, as shown in the
right-side traces of figure 16. Thus, for a slice with a 16 V/m AC threshold, activity will be evoked on the first cycle (figure 16A, right). For this same slice, a positive DC field of 8 V/m will be sufficient to evoke activity, since both applied fields reach a maximum of +8 V/m (figure 16B, right). Under 2 mM [Ca.Mg]_e conditions, negative DC fields of a higher intensity (48 V/m) will not evoke any activity (figure 16C, right).

Of the six slices tested in 2 mM [Ca.Mg]_e conditions, one actually did respond to negative DC fields. For this slice, the threshold intensity for negative DC fields (24 V/m) was still much larger than the AC (12 V/m) and positive DC (5 V/m) thresholds. The activity was initiated as the field reached its positive peak (zero V/m), as is normally seen when using 1 mM [Ca.Mg]_e solution (figure 17, bottom-right). See figure 16D for a summary of threshold data across all slices tested, and figure 17 for a summary of when activity initiates with fields of different AC or DC polarity.

**Relation to Passive Component**

In chapter IV, I used VSD to show that the cortical response to electric fields has a passive component, which is the change in membrane potential directly induced by an applied field. The active component, which is the change in membrane potential that arises from synaptic activity, appeared in 2 mM [Ca.Mg]_e solution when the field intensity was high enough. We can interpret this to mean that the active component initiates when the superficial layers are sufficiently depolarized by the passive component. This interpretation does not fit well with what happens when using DC fields in 1 mM [Ca.Mg]_e solution, so I next investigated whether [Ca.Mg]_e affects the passive component.
We used NBQX to block fast glutamatergic transmission and isolate the active component, as described in chapter IV. In both 1 mM and 2 mM [Ca.Mg]e, the passive component behaved the same, establishing antiphase oscillating regions in the more superficial and deeper regions of cortex (figure 18A). DC fields appeared to result in a DC waveform, in which a positive DC field would result in the deeper layers alternating between baseline and a more depolarized state, while the more superficial layers alternate between baseline and a more hyperpolarized state. However VSD imaging includes substantial baseline drift, so this observation is a subjective one and may not be accurate. However, it is consistent with intracellular recordings from pyramidal neurons, which show a uniform DC polarization when applying uniform DC fields (Deans et al. 2007, Radman et al. 2009).

When using fields at the threshold for evoking activity in 1 mM [Ca.Mg]e (~4 V/m), the passive component is too small to measure without averaging. Instead of directly measuring the amplitude of the passive component at these intensities, I instead measured the relationship between field intensity and passive component amplitude. Reducing [Ca.Mg]e from 2 mM to 1 mM had no effect on this relationship (figure 18B, top plots). In both 1 mM and 2 mM solution, the relationship was the same regardless of whether the field was AC, positive DC, or negative DC (figure 18B, bottom plots).

Finally, since negative DC fields should only hyperpolarize the deep layers, I investigated whether the activity evoked by negative DC fields in 1 mM [Ca.Mg]e initiates within the deeper layers of cortex. I found that it does; there was no significant difference in distributions of the depths at which activity initiates for positive and negative DC fields (figure 19A, p > 0.89 from a two-sample Kolmogorov-Smirnov test).
The polarity of the DC field also had no significant effect on the total depolarization during each field (figure 19B, $p > 0.64$) nor the relative amounts of depolarization in the superficial vs. deeper layers (figure 19C, $p > 0.94$) that are oppositely polarized by the passive component.

**Discussion**

The main finding in this chapter is that reducing the $[\text{Ca.Mg}]_o$ not only increases Up state frequency, but also dramatically increases the sensitivity of cortex to 1 Hz oscillating electric fields.

**Electric Fields Evoke Up States.** The evoked Up states appear similar to the emergence of the active component in chapter IV. The first cycles of the field do not evoke Up states in a smooth probabilistic fashion, but instead do so at a discreet threshold; fields with a sufficiently high intensity will consistently evoke Up states, while lower-intensity fields will consistently not evoke Up states.

We found that increases in the intensity of the applied field above threshold do not affect the characteristics of each individual Up state; the baseline-to-peak amplitude of depolarization is unchanged, as is the Up state duration. Similar to an earlier report (Fröhlich & McCormick 2010), I found that rapid Up and down state transitions could be evoked at the frequency of the applied field, even when it was faster than the rate at which they spontaneously occur. Higher intensity fields would do this more reliably, and generate more total depolarization over a 5-second exposure.

**Extracellular Calcium and Magnesium.** One result I found surprising is that while in 2 mM $[\text{Ca.Mg}]_o$, the total amount of depolarization in response to threshold
fields looks identical to the response in 1 mM [Ca.Mg]_e. The depolarization observed in 2 mM [Ca.Mg]_e in response to applied electric fields appears to be resulting from an Up state, which is seen much less frequently in 2 mM solution (Sanchez-Vives & McCormick (2000) report no Up states in 2 mM [Ca.Mg]_e, but Cossart et al. (2003) and other studies by Rafael Yuste’s group do report them, albeit at lower or unspecified frequencies). This suggests that increased [Ca.Mg]_e does not affect how Up states are sustained or terminated, but does strongly suppress their initiation. In this experiment, I provide evidence that electric fields can overcome this suppression and initiate regular Up state transitions in 2 mM solution in vitro.

Applied electric fields and reduced [Ca.Mg]_e may have a synergistic effect on Up state transitions. Lowering [Ca.Mg]_e can induce Up states in the absence of fields. Applying higher-intensity electric fields (~15 V/m) can induce Up states even in high [Ca.Mg]_e. But with the reduction [Ca.Mg]_e, even very small applied fields (~4 V/m) can induce Up states. And there is evidence that both of these mechanisms are physiological, as in vivo experiments have shown that both extracellular calcium (Massimini et al. 2000) and endogenous electric fields (Jefferys 1995, Fröhlich & McCormick 2010) fluctuate rhythmically along with the cortical slow oscillation. Both of these mechanisms may play a physiological role in generating the recurrent excitation necessary to push an entire network into the sustained depolarization of an Up state.

**AC and DC fields.** Another result I found surprising is that a DC shift of the applied field waveform between DC+, AC, and DC- affects how the cortex responds to the field when in 2 mM [Ca.Mg]_e but not in 1 mM. This difference is most notable in the case of negative DC fields, which trigger Up states in 1 mM [Ca.Mg]_e at a very low
amplitude (~4 V/m), but in 5 of 6 cases would not trigger Up states at all in 2 mM [Ca.Mg]e. The Up states evoked by negative fields in 1 mM [Ca.Mg]e were initiated near the field maxima (0 V/m), and were as far as I could tell completely identical to those initiated by positive DC fields. I do not know the mechanism, but a number of possibilities present themselves.

First, and simplest, there may be more spontaneous spiking present in the 1 mM condition. Even very low intensity fields have a large effect of neuronal spike timing (Francis 2003), and a higher spontaneous spike rate would allow a larger degree of modulation of the spontaneous spike rate. It would also reduce the effect on spike timing necessary to generate enough recurrent excitation to trigger an Up state. This simple explanation does not fit well with the experiments in chapter V, which showed little change in Down state spike rate when [Ca.Mg]e was reduced to 1 (figure 11C).

However, as discussed in chapter V, calcium imaging is much better at detecting bursts of spiking than it is at single action potentials, so it is possible that an increase in lower-frequency, non-burst spiking with low [Ca.Mg]e was simply not detected in the chapter V experiments.

A second explanation is that there may be cellular changes that allow for a “rebound” effect from the release of hyperpolarization only in 1 mM [Ca.Mg]e. The most obvious candidate for mediating such an effect would be the I_h current, which is a nonspecific cation current activated by hyperpolarization. However, this current should not be affected by extracellular calcium or magnesium ions, and any contribution to the rebound effect should also be present in 2 mM [Ca.Mg]e concentrations. Leak sodium currents can be strongly affected by extracellular calcium concentrations, most
prominently through the NALCN channel (Lu et al. 2010) but also possibly through
currents may add an AC component to the passive DC polarization. However,
intracellular recordings in DC fields have not shown such an AC component (Deans et

Network effects may also facilitate a “rebound” excitation. Spontaneous active
cells have been recorded in layer V during down states in both 1 mM (Sanchez-Vives &
McCormick 2000) and 2 mM (Mao et al. 2001) [Ca.Mg]e. These cells will be heavily
modulated by applied fields, while inhibitory interneurons will be very weakly modulated
(Radman et al. 2009). Thus, a negative field may reduce the rate of spontaneous
spiking in these pyramidal cells, reducing the fraction of cells in a post-action-potential
refractory period, and also reducing feedback inhibition. Both may facilitate a rebound
burst of synchronous firing after the release of pyramidal hyperpolarization by the
oscillating field. In addition, second-messenger systems play a role in the in vitro slow
oscillation, for example through GABA_B receptors (Mann et al. 2009) and purine
signaling (Poskanser & Yuste 2011), which can affect membrane conductance on a
more delayed time scale (but well within the 1-second period of applied field oscillation
used here).
**Significance.** The experiments in this chapter are the first evidence that electric field effects in the cortex are state-dependent. In conditions of spontaneous slow oscillation, very small fields can have large effects on voltages changes in cortical slices, and negative fields can evoke activity. In conditions without spontaneous slow synchrony, these small fields do not have an effect, and negative fields do not evoke activity. This is despite the fact that the passive polarization (passive component) is the same in both conditions. Thus, the effect of applied fields depends on the active state of the network. This may explain a portion of the results of Marshall et al. (2006), where slow oscillating fields helped consolidate memory only when applied during slow-wave sleep. It may be that outside of slow-wave sleep, the slow applied fields still polarize the cortex, but they do not have a large effect on coordinated synaptic activity. Finally, my data also strongly suggest that the conventional understanding, where anodal (positive) fields are excitatory and cathodal (negative) fields are inhibitory, does not necessarily apply when using oscillating fields.
Figure 14. Electric fields can initiate Up-state-like events in cortical slices. (A) VSD signals from a visual cortical slice exposed to threshold fields in different [Ca/Mg] concentrations. When using 2 mM solution, the field intensity must be 3 – 5 times higher in order to evoke network activity. (B) Average total depolarization depolarization (area under curve) evoked during the 5-second threshold field exposure in different [Ca/Mg] concentrations. Though the threshold is much lower for evoking network activity in 1 mM solution, the total during the field is the same. Data is from 5 slices, 40 trials. (C) The image of a somatosensory cortical slice is marked with the initiation spot of each spontaneous or evoked Up state in 1 mM [Ca/Mg] solution. On the right are histograms pooling data across 100 events in 6 slices. Spontaneous events can initiate in the superficial or deep layers of cortex, but field-evoked events more consistently initiate in the deeper layers of cortex (two-sample Kolmogorov-Smirnov test p=0.003). Data are from 6 slices, 100 events.
Figure 15. The electric field intensity affects voltage changes across the network. (A) VSD signals from a visual cortical slice in 1 mM [Ca.Mg]e exposed to a threshold (4 V/m) and a suprathreshold (14 V/m) field. Both evoke similar events, but there are more of them during the suprathreshold field. (B) There is no significant difference in the individual sustained depolarizations occurring spontaneously or evoked by threshold or suprathreshold fields, both in terms of duration (p>0.26 for all comparisons) and baseline-to-peak amplitude (p>0.58 for all pairwise comparisons). The amount of total depolarization evoked by fields above threshold is significantly larger than that for spontaneous (p=0.02) or threshold-evoked (p=0.007) events. Data is from 13 slices, 118 trials. (C) Average VSD traces across 67 trials from 13 slices. There is a much more robust effect from fields 2 – 3 times the threshold (bottom trace) than from those at threshold (top trace). (D) Cross-correlation between the stimulus waveform and the averaged waveforms in part C. The correlogram shows that suprathreshold fields evoke activity that follows the field more closely (at a lower latency) than activity evoked by threshold fields.
Figure 16. The effects of DC fields depend on [Ca/Mg]e. In 1 mM solution, activity depends on the changes in the electric field. In 2 mM solution, activity depends on the absolute intensity of the electric field. (A) AC fields in 1 mM solution (left) evoke responses on the second cycle of applied field oscillation, whereas in 2 mM solution (right), activity is evoked on the first cycle. (B) DC+ fields evoke activity on the first cycle regardless of [Ca/Mg]e. (C) When applying negative DC fields in 1 mM solution (left), activity is evoked between the first and second cycle, when the applied field first reaches its peak of zero. Negative fields in 2 mM solution (right) fail to evoke activity, even with very high field intensities. (D) Left: Average thresholds for three field types (AC, DC+, and DC-) with intensities quantified as the peak-to-peak distance in the stimulus waveform. Right: the same data, but with thresholds quantified as baseline-to-peak. Activity is evoked in 1 mM solution when the distance between the highest and lowest values (peak-to-peak distance) of the applied field is sufficiently large. Activity is evoked in 2 mM solution when the applied field reaches a sufficiently positive value. 2 mM DC- data is not plotted because no activity could be evoked by such fields in 5 of 6 slices. 1 mM data is across 10 slices, 2 mM AC data is across 8 slices, and 2 mM DC data is across 6 slices.
Figure 17. Histograms of the first event onset time in response to threshold applied fields in 1 mM [Ca/Mg]_e solution. The dashed line is the stimulus waveform, and the solid lines are the VSD signals averaged across trials (13 slices, 176 trials total). (Top-left) Activity most often starts after the peak of the second cycle when AC fields are at threshold. (Top-right) When AC fields are sufficiently above threshold, activity usually begins instead very close to the peak of the first cycle. (Bottom-left) Positive fields nearly always evoke activity at the peak of the first cycle, and activity during the second cycle is unusual. (Bottom-right). Negative fields evoke activity that begins at the end of the first cycle, after the field has reached its highest positive value. Data is from the same slices as figure 16.
Figure 18. Changing \([\text{Ca/Mg}]_e\) does not alter the passive component. (A) Sample traces with 20 μM NBQX: a 16 V/m AC field induces identical passive components in 1 mM (black traces) and 2 mM (gray traces) \([\text{Ca/Mg}]_e\) solutions. (B) Top traces: Slices are exposed to 20 cycles of an applied field in either 1 or 2 mM \([\text{Ca/Mg}]_e\) plus 20 μM NBQX. The amplitude of the passive component is calculated by FFT. Both 1 mM and 2 mM data are normalized to the expected amplitude in 2 mM solution, based on a linear regression of all calculated amplitudes for all stimulus intensities in 2 mM solution. The data show that the passive component is unaffected by changes in \([\text{Ca/Mg}]_e\), regardless of stimulus intensity. The data are from two slices, 36 trials. Bottom traces: Passive component amplitudes are determined as in part B, except instead of curves for each extracellular solution, there are curves for AC and positive and negative DC fields. Data is normalized to expected amplitudes for AC fields. The data show that the passive component amplitude is only dependent on the peak-to-peak amplitude of the applied field, and is not affected by DC polarity. The data are from 2 slices, 51 trials.
Figure 19. Positive and negative fields both evoke activity that begins in the deeper layers of cortex. (A) Left: initiation locations of events evoked by positive and negative fields in one slice of visual cortex. Right: histograms of the depth at which activity initiates across seven slices (the distributions are not significantly different, p>0.85). (B) The total amount of depolarization (area under curve) induced by positive and negative fields does not differ between AC or DC fields at threshold (p>0.64 for all comparisons). Data is from 4 slices, 50 trials. (C) Depth ratio is the total depolarization in the more superficial half of cortex divided by the total depolarization in the deeper half of cortex. DC polarity does not affect this ratio (p>0.94 for all comparisons). All cumulative data (everything except the image in A) is from 4 slices, 50 trials total.
Chapter VII: Up State Ensembles

Central Hypotheses

Developmental sparsification of ensemble activation is due to changes in local cortical networks. Electric fields influence this ensemble activation.

Results

Sparsification

To investigate the sparsification of visual cortex, I imaged calcium transients in visual cortical neurons. Images were taken with a 60X objective, allowing simultaneous recording from 20 – 40 neurons (figure 20A). The extracellular solution consisted of 2 mM [Ca]_e and 1 mM [Mg]_e. Unlike in the experiments in chapter V, not all slices showed spontaneous synchronous events, likely due to differences in slicing procedures (these slices were cut at room temperature). In those slices that did not show spontaneous activity (8 of 12), I evoked activity with low-amplitude electric fields as described in chapters IV and VI. The spontaneous and evoked events are all grouped together in the data shown, as spontaneous and evoked events did not seem in any way distinguishable.

We cut slices from animals ranging from P10 to P18. For each slice, I would record calcium signals in a small group of 20 – 40 neurons across several synchronous events, and then move the imaging window to a new group of neurons. Astrocytes were eliminated from analysis based on their morphology and brighter staining. I subsequently used SR-101 in two slices to specifically label astrocytes, and confirmed that they were being correctly identified by visual inspection. The imaging window was recorded as being either in layers II/III or in layers V/VI.
We found that the sparsification of spontaneous activity was preserved in vitro. Consistent with the in vivo results from Rochefort et al. (2009), synchronous population events in slices taken from mice before eye-opening (P10 – P12) involved a very large fraction of neurons (figure 19B), while events after eye-opening (P14 – P18) involved fewer neurons (figure 19C). This was true whether the events were spontaneous or evoked by electric fields. Comparing images from layers II/III to images from layers V/VI showed that the sparsification occurred not only in the superficial layers II/III, but also in the deeper layers V/VI (figures 20D & E).

The reversal potential for chloride ions undergoes a large shift in development, and as a consequence GABAergic transmission goes from being excitatory in early development to inhibitory. The shift is not sudden, but is instead gradual throughout the first postnatal month in mice (Luhmann & Prince 1991). In addition to changes in the effect of GABA$_A$ receptors, inhibitory synaptogenesis is also taking place at the time of eye-opening (de Felipe et al. 1997). I hypothesize that these changes in GABAergic neurotransmission are responsible for the sparsification in the visual cortex. I added the GABA$_A$ antagonist bicuculline (5 µM) to slices taken before and after eye-opening. In slices taken before eye-opening, bicuculline had no effect on the density of spontaneous or evoked activity (figure 20F, left), though it did increase the frequency of spontaneous activity. In slices taken after eye-opening, the sparse activity became dense, appearing in all ways similar to the synchronous activity seen in slices before eye-opening (figure 20F, right).
Electric Field Effects

The next set of experiments were all mouse visual cortical slices with 1 mM [Ca.Mg]$_e$, with all slices taken after eye-opening (P15 – 18). I used two-photon calcium imaging and a 20X objective to record calcium signals from 100 – 200 cells simultaneously. In each slice tested, I recorded from the same group of throughout the entire experiment; this allowed me to monitor which cells were active in the Up states occurring over ~90 minutes of recording time.

Three slices were recorded from in total. In each, a region of cortex was pressure-injected with AM calcium dyes, and the extracellular solution was then switched from 2 mM to 1 mM [Ca.Mg]$_e$. I first recorded spontaneous events for 8 – 10 minutes. After this, I recorded events evoked by 5-seconds of applied 1 Hz square-wave fields (at or near threshold), applied every 30 – 60 seconds until a comparable number of Up states were observed under both conditions. In one of the three slices, this was carried out twice. A total of 114 spontaneous and 106 evoked Up states were included in the final analyses. Each group of active cells in each Up state is considered an “ensemble”.

During recording, it was clear there were some cells particularly likely to fire during each application of oscillating field (figure 21A – interstimulus recording time is truncated for illustrative purposes). These cells were sometimes part of spontaneous ensembles as well, though not as frequently as evoked (figure 21B). Up state events were marked in each recording, and for each cell in all three slices, I quantified the fraction of spontaneous as well as field-evoked events it participated in. Each cell is plotted in figure 21D. There was a weak correlation between spontaneous and evoked
firing; cells that were part of spontaneous ensemble activation were more likely to also be a part of evoked ensemble activation. From this plot, I conclude that *in vitro* Up states frequently consist of the same ensembles of neurons, whether spontaneous or evoked.

We next wanted to determine whether the ensembles were more consistent when occurring spontaneously, or more consistent when they were evoked by electric fields. To determine this, I performed in-group comparisons on the same data. I correlated half of the spontaneous events with the other half, and half of the evoked trials with the other half. I placed alternating events into each group, as schematized in figure 2 (left). Evoked events showed a much higher correlation within group (r = 0.84) than when compared to spontaneous (figure 21D, r = 0.50). Spontaneous events also showed a higher within-group correlation (r = 0.61), though the difference was small.

The correlation between evoked and spontaneous ensembles was lower than either in-group comparison. A simple explanation for this could be the passage of time, as due to the experimental design, all spontaneous recordings preceded all evoked recordings, and thus more time passed between the average inter-group comparisons than between the average in-group comparison. Additionally, other studies have described spontaneous ensemble activation as being stable over 10s of minutes (Ikegaya et al. 2004, Luczak et al. 2007, Han et al. 2008). To investigate how time changes affect the correlations measured earlier, I split the within-group correlations differently. This time, the first half of all events would go into group one, and the second
half would go into group two (figure 22A, right). By doing this, I increased the average amount of time between Up states being compared.

We found that this had little effect on spontaneous correlations (figure 22D), but the higher correlations under the evoked conditions ($r = 0.84$, figure 22C) were reduced to a level even lower than the spontaneous condition ($r = 0.46$, figure 22E). Thus, evoked ensembles are only highly correlated when the time between Up states is low. This was not only true for the data set as a whole, but for each individual slice (figure 22F). This data suggests that relative to spontaneously active ensembles, electric fields can preferentially activate certain ensembles of neurons, but this ensemble “preference” is only stable for a short time.

**Discussion**

The first main finding of these experiments is that the developmental sparsification, in which dense waves of activity are replaced by sparse ensemble activation, is a phenomenon of local circuits spanning the depth of cortex that can be reversed by blocking GABA$_A$ receptors. The second main finding in this chapter is that during Up states, applied fields can lead to less diverse patterns of ensemble activation than those that occur spontaneously.

**Sparsification.** Developmental sparsification, in which dense waves of activity are replaced by sparse ensemble activation, is a phenomenon of local circuits spanning the depth of cortex. It is reversed in the visual cortex by blocking GABA$_A$ receptors. Perhaps more surprisingly, the blockade of GABA$_A$ receptors in visual cortex does not affect the density of the waves of activity before eye-opening.
GABAergic synapses are present as early as P4, though they increase in number throughout the first postnatal month (de Filipe et al. 2007). Blocking GABA_A does not decrease the density of waves in the visual cortex just before eye-opening, though it does increase the frequency of spontaneous events. This suggests that the GABAergic system has a complex role in this developmental stage, suppressing the initiation of cortical activity but not affecting the number of neurons recruited into an event once it has been initiated.

During the few days after eye-opening spontaneous events become sparse, and this sparsification process is reversed by blocking GABA_A receptors. This suggests that the maturation of local inhibitory circuits is behind the sparsification process. This is likely due to a combination of increased GABAergic synaptic density and the changes in chloride reversal potential that turn GABA from an excitatory neurotransmitter to an inhibitory neurotransmitter.

**Electric Fields.** During Up states, applied fields can lead to more similar ensemble activations than would occur spontaneously. Electric fields polarize neurons to a degree dependent on the length of the neuron along the field vector (Radman et al. 2009). Thus, the effect we see may be due to the field only increasing the firing probability for a particular subset of neurons: the large pyramidal neurons that are optimally aligned with the field vector. These cells, and by extension their postsynaptic targets, would be more likely to participate in the ensemble activation during each Up state.

The similarity of evoked ensembles appears to only be stable for a short time, as the high correlation disappears when comparing the earlier half of evoked trials to the
later half. There may be number of reasons for that. Most trivially, it could be due to
changes in the positioning or health of the slice over time. When comparing calcium
images at the beginning and end of each experiment, there is a small of the drifting of
the slice in the direction of perfusion (the largest was \( \sim 12 \, \mu m \)). This drift is parallel to
the field vector, and there does not appear to be any rotational movement, so it is
unlikely to change which cells are most influenced by the field. Nonetheless, I cannot
rule out the possibility that the small movement of the slice relative to the field-
generating electrodes can account for the changes we see. Slice health should be
stable over time, as we see spontaneous Up states for the duration of calcium staining
(90 – 120 minutes) and for far longer in the VSD setup (more than 3 hours). Further,
dead cells typically show a large increase in fluorescence due to elevated intracellular
calcium levels, which was not seen here.

Aside from these more trivial explanations, a potential cause of the short duration
of stable evoked ensemble activation is synaptic plasticity, or a gradual change in
synaptic strengths. Inhibitory neurons participate in and fire during Up states (Fanselow
& Connors 2010), and inhibitory neuron plasticity is thought to play a role in the
irregularity of cortical activation states (Vogels et al. 2011). Even through Hebbian
mechanisms, inhibitory plasticity may account for the instability of evoked ensemble
activation.

Another possibility is a change in spontaneous activity levels. There is
spontaneous activity in some pyramidal neurons (Mao et al. 2001), which may be the
initial seed of excitation that drives a network into a spontaneous Up state (Chagnac-
Amitai & Connors 1989). There is also spontaneous Down state activity in a subtype of
inhibitory neurons (Fanselow & Connors 2010). Spontaneous activity can change over the time scale of 10s of minutes, with many causes including second-messenger signaling or metabolic changes. This change in spontaneous activity could influence the excitability of neurons across the network, in affect changing the conformation of the most stable attractor states (Cossart et al. 2003).

**Significance.** The intensities of the fields used in these experiments are close to normal physiological levels (~6-12 V/m, which is only slightly higher than the 2-4 V/m fields measured during the slow oscillation in vivo by Fröhlich & McCormick 2010). This suggests that endogenous electric fields may play a role in stabilizing attractor dynamics, reinforcing the activation and reactivation of particular neuronal ensembles during the slow oscillation. Electric fields may play a role in the replay of waking ensembles during slow-wave sleep (Ji & Wilson 2007, Euston et al. 2007), which may be part of the process of memory consolidation during sleep. Additionally, it may help explain the results of the Born et al. (2006) study, where the application of slow oscillating fields across the cortex of human subjects improved their scores on tests of declarative memory. These applied fields may be reinforcing the endogenous fields during Up states, resulting in “deeper” attractor basins within the dynamic network, and facilitating the ensemble reactivations that may be important in memory consolidation.
**Figure 20 (Previous Page).** Sparsification is a phenomenon with local cortical mechanisms. (A) Two-photon image of cells labeled with a calcium dye in a slice taken from mouse visual cortex at P11, before eye-opening. Imaging is with a 60X objective. (B) Calcium traces from the cells labeled in A. Averaging across all pixels shows three spontaneous population events within the imaged region (blue trace). Each population event corresponds with a large fraction of cells being activated, with activity from individual cells is shown in the black traces. (C) Calcium traces from a P16 visual cortical slice. Averaging across all pixels shows four spontaneous population events in the imaged region (blue trace). Each population event corresponds with a small fraction of cells being activated (black traces). (D) The density of cortical activity in the visual cortex gradually decreases after eye-opening. (E) Developmental sparsification occurs deep in the cortex as well as in the more superficial layers (p < 10^{-5}). (F) 5 μM bicuculline has no effect on the density of events before eye-opening, but reverses sparsification in older animals (p < 10^{-5}).
Figure 21. Spontaneously ensemble activation is weakly correlated with field-evoked ensembles. (A) Sample traces from six cells (black traces) responding to three separate trials of a 1 Hz, 9 V/m electric field (blue trace). Often, the same cells are active in successive trials. (B) The same cells (black traces) are only sporadically active in spontaneous events (can be seen in the green trace, which is the mean signal across the whole image). (C) Image showing the location of the cells. (D) For each cell over the course of an experiment, the fraction of all spontaneous events it participated in was plotted against the fraction of all electric-field-evoked events it participated in. Figure D data is from three slices, 131 spontaneous events and 106 evoked events.
Figure 22 (Previous Page). Electric fields evoke more regular ensembles than those that emerging spontaneously. Data is from three experiments done in 1 mM Ca/Mg, each gathered within a 90 minute period. (A) In the remaining figures the same data as shown in figure 20D will be similarly plotted, but split into two groups via two methods. Method 1 controls for changes over time: each event is numbered sequentially, and all odd-numbered events go into group 1 and all even-numbered events go into group 2. Method 2 does not control for changes over time: the first half of all events go into group 1 and the second half go into group 2. (B) Spontaneous ensembles correlate with other spontaneous ensembles to a higher degree than they do with evoked ensembles (figure 20D), though the difference is small ($r = .65$ vs .50). (C) Evoked ensembles correlate with other evoked ensembles to a much higher degree than with spontaneous assemblies ($r = .84$ vs .50). This suggests that evoked events recruit the same cells more consistently than spontaneous events do. (D) When grouping spontaneous events into early vs. late in the experiment, the correlation is reduced slightly ($r = .57$ vs .65). (E) When grouping evoked events in to early vs. late in the experiment, the correlation is reduced dramatically ($r = .46$ vs .84). (F) Tables show the breakdown across all three slices. With method 1 (left table), each slice shows much higher correlation among evoked assemblies than among spontaneous assemblies. With method 2 (right), there is no consistent difference across slices.
Chapter VIII: Discussion

The experiments described in previous chapters were based around a series of central hypotheses outlined in chapter II. All hypotheses deal with a common question: how is recurrent excitation driven in cortical networks? In chapter IV, I show that very small polarizations from applied electric fields can generate very large population events. As pyramidal neurons form long dipoles across the cortex, they can become more likely to fire. This small effect on firing probability is amplified by the large number of neurons affected, and the even larger number of excitatory synaptic outputs.

In chapter V, I show that a reduction of extracellular calcium ions can similarly drive recurrent excitation, likely by increasing sodium conductance. Again, this is a small effect on the firing probability of single neurons, but a large effect on synchronous population events driven by recurrent excitation.

The results of chapter VI combine these concepts, showing that networks with slow oscillation are much more sensitive to the effects of electric fields. When low concentrations of calcium and magnesium facilitate recurrent excitation, a very small “push” by an electric field is necessary to push the recurrent excitation past the threshold for a large population event.

Discussion of these chapters in isolation, however, misses the larger picture. This final chapter discusses the broader relationships between the central hypotheses and their experimental results. Specifically, I address how the concept of passive and active components relates to changes in extracellular calcium ion concentration. Next, I discuss implications for how endogenous electric fields and physiological fluctuations of extracellular calcium may interact in an organism. And finally, I discuss how this relates
to the ensemble activations studied in chapter VII, with proposals for further experiments to expand on this more preliminary work.

**Passive and Active Components and Extracellular Calcium**

The principal finding in chapter IV is that the cortical response to oscillating electric fields has two components: a passive component, which is polarization directly induced by the field, and an active component, which is depolarization caused by synaptic activity. The passive component is small and stationary, and being the subthreshold polarization of neurons under a field, it has received considerable study (Bikson et al. 2004, Deans et al. 2007, Radman et al. 2009). The novelty in my experiments in chapter IV is a more complete description of its spatial patterns in the cortex and across large populations. But the main contribution these experiments make to our knowledge is the relationship between the passive and active components.

The active component is presumably generated by the polarizations that make up the passive component. Since the activity appears to emerge in or near layer V (figure 5), and since the large pyramidal neurons appear to be most sensitive to the field (figure 7), the active component is likely driven by the modulations of spontaneous spike timing in the pyramidal neurons of layer V. The depolarizations of the active component are much larger than those induced by the passive component, and can spread across a much larger area as a propagating wave. This discrepancy in size becomes magnified considerably when, as shown in chapter V, the extracellular calcium and magnesium concentrations are reduced from the “normal” 2 mM concentration to the more physiological concentrations of 1 mM. In these conditions, fields of physiological
intensity (< 4 V/m) induce passive polarization that is not detectable in VSD signals, yet evoke Up-state depolarizations that are orders of magnitude larger.

The experiments in chapter IV show an interesting disconnect between the characteristics of the passive component and the active component, which now appears to be an Up state. In “normal” extracellular solution, sufficiently depolarizing the deep layers of cortex appears to give rise to recurrent excitation that drives the active component. However, after reducing extracellular calcium and magnesium to 1 mM, and only after doing this, the active component can be driven by negative DC fields that should only hyperpolarize the deep layers. These experiments suggest that the reduction of extracellular calcium and magnesium ions enables the recurrent excitation to be driven by a release of hyperpolarization. But as far as I can tell, the passive component induced by these fields is unaffected by the change in extracellular solution. The passive component is very resistant to manipulation; changes in extracellular ion concentrations or addition of pharmacological agents fails to affect its characteristics, both in my experiments and others (Akiyama et al. 2011).

**Further Experiments.** A series of experiments could help us understand the “rebound” excitation. One question is whether there are single-cell mechanisms behind this. Intracellular recordings under an electric field, though technically complicated, have been performed in brain slices (Deans et al. 2007, Radman et al. 2009). With such a setup, I could explore whether passive hyperpolarization can activate cation currents via I_h or some other intracellular mechanism. These mechanisms may not be visible in VSD imaging due to the limited spatial resolution, and would not be visible in calcium imaging because they involve subthreshold changes. Importantly, I could then
test whether such cation currents are affected by extracellular calcium’s modulation of sodium channels.

Additionally, network mechanisms could be most simply investigated by applying negative DC polarizations of varying length. For example, if 5 ms negative field followed by a normal 500 ms positive field evokes activity, then I know the hyperpolarization is less important than the total change in applied field. Alternatively, perhaps low calcium and magnesium confer excitatory properties to negative fields. In this case, a sufficiently long exposure to a single DC negative field could evoke activity.

For a more comprehensive understand this phenomenon, I can also explore the role of magnesium. While calcium has a demonstrable effect on the permeability of the neuronal membrane to sodium, the role of extracellular magnesium is to this point ignored in our discussions. It mediates the voltage-gated property of the NMDA receptor, which is important and possibly even necessary for Up states (Sanchez-Vives & McCormick 2000, Cunningham & Whittington 2006). In chapter V, I showed that reducing extracellular calcium facilitates Up states, but it may be that extracellular magnesium plays a vital role in how the cortex is affected by a DC shift in the applied oscillating field.

**Extracellular Calcium and Electric Fields in Normal Brain Function**

An exciting theory that has received a revived interest of late is that naturally occurring electric fields contribute positive feedback in generating recurrent excitation in the cortex (Fröhlich & McCormick 2010, Anastassiou et al. 2011). In chapter V, I suggest that extracellular calcium may play a similar role: synchronous activity triggers widespread calcium influx, depleting the extracellular environment of calcium ions. This
in turn increases sodium conductance across neuronal membranes, driving nearby neurons closer to firing threshold and facilitating recurrent excitation.

But what is more intriguing is the evidence in chapter VI suggesting that extracellular calcium and electric fields may have a synergistic role in driving recurrent excitation. Up states can be driven by relatively high-intensity fields when extracellular calcium concentrations are elevated, but when extracellular calcium levels are reduced, neurons become much more sensitive to the fields (figure 14A). Similarly, the same intensity electric field will be able to generate more depolarization in the cortex when calcium concentrations are reduced. In the intact animal, slow wave sleep oscillations produce calcium fluctuations (Massimini & Amzica 2001) and electric field fluctuations (Fröhlich & McCormick 2010) that are both synchronous with the slow oscillation. Thus, electric fields and fluctuating extracellular calcium may be acting in concert to amplify the recurrent excitation that drives synchrony in the cortex.

If this is true, evidence suggests this would primarily apply to slow-wave activity, and not to higher-frequency oscillation. Experiments in chapter IV show a dramatic drop in the effects of applied fields (1/f) at higher frequencies, and the endogenous fields generated by oscillatory behavior also decline with frequency (again 1/f, Buzsaki et al. 2012). In addition, calcium dynamics are slow, and extracellular calcium concentrations may not follow faster oscillation to a significant degree. However, slow waves during sleep play an important role in synchronizing higher-frequency oscillations both inside and outside of the cortex, and thus any effects on the slow oscillation can have widespread effects throughout the brain.


**Recurrent and Specific Excitation**

The data in chapters IV and VI suggest that low-intensity electric fields can drive recurrent excitation in the cortex, and that under the right conditions this can trigger an Up state. The experiments in chapter VII suggest that low-intensity electric fields can also affect which neurons participate in the Up states, increasing the similarity of ensemble activations within a short time window. As previously discussed, this may explain how the applied fields used by Marshall et al. (2006) and Kirov et al. (2009) affected memory.

The fields used in the experiments were of a magnitude comparable to the endogenous fields occurring during slow wave sleep. Thus, it may be the case that endogenous fields have a role not only in promoting synchrony, as suggested by Fröhlich & McCormick (2010), but also in reinforcing repeated patterns of neuronal activation. There is an abundance of evidence demonstrating a link between the slow oscillation and memory (Diekelmann & Born 2010), and this may be mediated by the replay of events during the slow waves (Ji & Wilson 2007, Euston et al. 2007). The replay of events likely involves the repeated firing of specific ensembles. Thus, it may be that endogenous fields are an important mechanism in stabilizing specific ensemble activations during sleep, and in doing so may play an important role in memory.

**Further experiments.** The data supporting this hypothesis are still very preliminary. Further *in vitro* experiments could elaborate on the higher correlation among electric-field evoked Up states, and help assess the practicality of moving *in vivo*. Notably, for this phenomenon to be relevant to an intact organism, it would need
to be at least somewhat robust to the position of the cortex relative to the field. This can be easily tested with slight adjustments of electrode positioning in the imaging setup.

It would also be important to investigate the source of the higher correlations in evoked Up state ensembles and their transient stability. The deep-layer pyramidal neurons are a likely source of both. There is spontaneous activity in some pyramidal neurons (Mao et al. 2001), which may be the initial seed that drives a network into a spontaneous Up state (Chagnac-Amitai & Connors 1989). This spontaneous activity may not be stable long-term, and changes in this activity may affect which cells are recruited into each Up state. By monitoring both spontaneous single-cell firing along with synchronous events, we can see whether the stability of spontaneous pyramidal firing and the stability of similar Up state ensembles follow a similar time course.

In addition, deep-layer pyramidal cells should be most sensitive to effects of the applied field, so an important test would be to divide the analysis into two groups, pyramidal cells and other. This will allow me to see if the correlated activations are due only to the reactivation of similar groups of pyramidal neurons, rather than due to a correlated activation of their postsynaptic targets. Only in the latter case would in vivo imaging experiments be practical, since in vivo imaging experiments are limited in practice to the superficial layers of cortex. In the former case, if the correlations were driven exclusively by pyramidal cells, the experiments could still be conducted in vivo by measuring pyramidal cell spiking with multielectrode arrays, though this would be more difficult and less rewarding.

Overall, understanding these correlations may prove difficult, but could be of very significant impact in understanding how memories are formed and maintained.
References


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