Low-intensity electric fields induce two distinct response components in neocortical neuronal populations.

Cortical activity evoked by low-intensity electric fields

Weifeng Xu1, 3, Brian S. Wolff 2, 1, 3, and Jian-young Wu1*

1. Department of Neuroscience, Georgetown University Medical Center, Washington, DC 20057, USA
2. Georgetown University Medical center Interdepartmental Program of Neuroscience
3. These two authors contributed equally to this work.

* Correspondence: wuj@georgetown.edu

Corresponding Author: Jian-young Wu

Georgetown University
Department of Neuroscience
Basic Science Building 207A, 3900 Reservoir Road, NW, Washington, DC 20057, USA
Phone: (202) 687-1614 Fax: (202) 687-0617
Email: wuj@georgetown.edu
Abstract
Low-intensity alternating electric fields applied to the scalp are capable of modulating cortical activity and brain functions, but the underlying mechanisms remain largely unknown. Here we report two distinct components of voltage-sensitive dye signals induced by low-intensity, alternating electric fields in rodent cortical slices: a "passive component", which corresponds to membrane potential changes directly induced by the electric field; and an "active component", which is a widespread depolarization that is dependent on excitatory synaptic transmission. The passive component is stationary, with amplitude and phase accurately reflecting the cortical cytoarchitecture. In contrast, the active component is initiated from a local “hotspot” of activity and spreads to a large population as a propagating wave with rich local dynamics. The propagation of the active component may play a role in modulating large-scale cortical activity by spreading a low level of excitation from a small initiation point to a vast neuronal population.

Keywords: electric field stimulation; low-intensity field; voltage-sensitive dye imaging; propagating waves

Introduction
Transcranial alternating current stimulation (tACS) can have significant modulatory effects on cortical activities and brain functions (Kirov et al. 2009; Marshall et al. 2006; Polania et al. 2012; Reato et al. 2013). Unlike suprathreshold stimulation therapies like repetitive transcranial magnetic stimulation (rTMS), tACS involves stimulation intensities that are far below the threshold to directly induce action potentials in cortical neurons. An in vitro study showed that action potentials in cortical neurons are only evoked by electric fields greater than roughly 28 V/m (Radman et al. 2009). However, under a slow alternating field, effective modulation of cortical activity can occur at much lower field intensities (Fröhlich and McCormick 2010), about 1/10th of that necessary to directly induce action potentials. This raises a question: how can such small membrane potential changes induced by an alternating electric field integrate within neuronal populations to bring about large-scale changes in brain activity?

One possible answer is that cortical local circuits serve as an amplifier to convert small membrane potential fluctuations into large-scale population (network) activity. Cortical local circuits have a highly divergent and convergent connectivity pattern; each principle neuron receives thousands of excitatory inputs, and in turn sends excitatory outputs to thousands of postsynaptic neurons (Douglas and Martin 2004). Under a slow alternating field the probability of spontaneous firing in each neuron fluctuates with the field (Radman et al. 2007; Reato et al. 2010). Since the alternating field simultaneously affects a large population of neurons, small fluctuations in firing probability may lead to a large change in the total number of spikes in the population. This increase in spiking can in turn to lead more excitatory synaptic potentials (EPSPs) throughout the divergent network, and may ultimately have a much larger effect on membrane potential than the field itself.

This possibility may be verified experimentally, by examining how the membrane potentials of neuronal populations respond to application of a slow, alternating field. The response can be separated into two components by a pharmacological blockade of excitatory synaptic transmission. Any changes in membrane potential sensitive to the blockade are likely caused by excitatory synaptic activity, and will be referred to as the
“active component”. Changes resistant to this blockade are likely caused by direct, field-induced neuronal polarization, and will be referred to as the “passive component”.

The active and passive components should also be separated by their spatial patterns in the cortical tissue. Under low-intensity electric fields neurons passively polarize in a compartment-specific fashion, with depolarization in compartments closer to the cathode and hyperpolarization in compartments closer to the anode (Radman et al. 2009). Principal neurons have a uniformly vertical arrangement in the cortex, so the degree and direction of compartmental polarization will to a large degree depend on depth within the cortex. In contrast, the active component consists of EPSPs, which should be depolarizing at all location in the cortex.

In this report we use voltage-sensitive dye (VSD) imaging to examine the spatial and temporal patterns of population membrane potential fluctuations in mouse cortical slices under applied, low-frequency (1-4 Hz) sinusoidal electric fields. Optical recording with VSD is not affected by the volume conductance artifact of the applied field, allowing for high-sensitivity measurements of subthreshold changes in membrane potential.

We indeed found that the two components can be clearly distinguished in neocortical tissue. The properties of the passive component are highly dependent on the underlying cortical cytoarchitecture, as seen with a phase-reversal zone and a number of low-amplitude zones arranged parallel to the cortical laminae. VSD signals show that the amplitude of the active component can be about 10 times larger than the passive component, suggesting that the direct effect of the applied field on membrane potential can be dwarfed by the resulting synaptic activity. We also found that the active component is organized as propagating waves, which may play a role in spreading the field-induced activity from a local hotspot to vast regions of cortex.

Materials and methods

Animal experiments C57BL/6 mice (n = 52) of both sexes from P17 to P28 were used in the experiments. The animal protocol (preparation of acute cortical slices) was approved by the Institutional Animal Care and Use Committee of Georgetown University following the guidelines of the National Institutes of Health.

Slice preparation. After being deeply anesthetized by isoflurane or an intraperitoneal injection of ketamine (100-200 mg/kg), the animals were decapitated. The whole brain was then quickly removed and chilled in cold (0 °C) sucrose-based artificial cerebrospinal fluid (ACSF) containing (in mM): 252 sucrose, 3 KCl, 2 CaCl₂, 2 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 dextrose and bubbled by 95% O₂, 5% CO₂. Neocortical slices (400 µm thick) were cut in coronal sections with a vibratome (Leica, VT1000S) between bregma 1 to -3 mm. After sectioning, the slices were transferred into an incubation chamber with ACSF containing (in mM): 132 NaCl, 3 KCl, 2 CaCl₂, 2 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 dextrose, and saturated with 95% O₂, 5% CO₂ at 26 °C. The slices were incubated for about 90-120 minutes before staining with voltage-sensitive dye.

Voltage-sensitive dye (VSD) staining, signals, and optical imaging. An oxonol dye, NK3630 (Nippon Kankoh-Shikiso Kenkyusho Co., Ltd., Japan) was used as an indicator of transmembrane potential. The slice was stained with 5 - 10 µg/ml of the dye dissolved in ACSF for 120 minutes (26°C). During staining, the ACSF was circulated and bubbled with 95% O₂ - 5% CO₂. After staining, the slices were transferred back to the incubation chamber for at least 1 hour before each experiment.
NK3630 is in the dye family that binds to the external surface of the membrane of all cells without interrupting their normal function (for review, see Chemla and Chavane 2009). The absorption spectrum of the dye shifts linearly with the changes in the membrane potential (Ross et al. 1977). The VSD signal in this report is the change in absorption of light with a 705 nm wavelength. In all experiments, the detectable signals are a change in light intensity that is roughly 0.01% to 0.1% of the resting light intensity. Staining with this dye does not cause noticeable changes in spontaneous or evoked neuronal activity (Huang et al. 2010a; Jin et al. 2002), and stained slices maintain viability for up to 24 hours. In 705 nm recording light, NK3630 molecules do not generate fluorescence, so no noticeable phototoxicity is detected (Jin et al. 2002).

In most of our experiments, we adjusted the slice and microscope plane to make the somatosensory cortex in the center of the imaging field. The VSD signals were recorded by a 464-channel photodiode array (WuTech Instruments). The two-stage amplifier circuits in the diode array subtract the resting light intensity and amplify the small signals 100 times before digitization. This achieves a 21-bit effective dynamic range. For each channel, the VSD signals were digitized at 1,600 samples/sec. The waveform of the applied electric field was sampled and digitized concurrently with the VSD signals. Optical imaging was performed on an upright microscope (Olympus BX51 WI) with a transillumination arrangement. We imaged at two spatial resolutions: With a 5X objective (0.1 NA, Zeiss) or macroscope (0.40 NA, modified from a Navitar 25 mm F 0.95 video lens), the imaging field was about 4 mm in diameter and each recording channel (pixel) collected VSD signals from an area of cortical tissue of 150 µm in diameter; with a 20X objective (0.95 NA, Olympus), the imaging field was about 980 µm in diameter and each recording channel collected signals from a tissue area of 38 µm in diameter. With a transillumination arrangement, neurons through the whole thickness of the slice (400 µm) contribute relatively equally to the VSD signal. A tungsten filament lamp was used for illumination and a 705/10nm interference filter (Chroma) was placed in the illumination path during optical recording. During imaging experiments, the slice was continuously perfused in a submersion chamber with ACSF (same as the incubation solution) at 28°C and at a rate of more than 20 mL/min. Intermittent imaging trials were performed, with at least 5 minute intervals between each trial. The total light exposure for each slice was less than 600 sec, far below what is necessary for detectable dye bleaching or phototoxicity (Jin et al., 2003).

**Generation and application of electric fields.** A pair of parallel Ag-AgCl coils or powder Ag-AgCl half-cells were used to generate an electric field in the solution. Both kinds of electrodes provide large surface areas, reducing electrolytic effects such as bubbling. The electrodes were placed in a long (65 mm) and narrow (10 mm) chamber so that the field was evenly distributed around the slice at the center of the chamber, as indicated by measurements from paired probing electrodes. Unless otherwise indicated, the electrical field was perpendicular to the cortical laminae in the imaging field (Figure 1A). In most experiments we used AC oscillating electric fields of 1 Hz for 3 cycles to induce the active component and fields of 4 Hz for 40 cycles to examine the properties of the passive component. The sinusoidal waveforms were generated by a programmable signal generator (WaveTech 10M Hz DDS function generator model 29). The field strength at the center of the chamber was measured using two parallel electrode probes 1.5 mm apart, and calibrated to the voltage output of the signal generator (Figure 1B). The current flowing through the chamber was monitored with a 10 ohm resister in series. The measured field strength was linearly related to the voltage output from the generator (Figure 1C) and thus the intensity of field is linearly correlated to the sin-wave from the output voltage.
In some of the experiments (19 animals), we added 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, non-NMDA glutamate receptor antagonist, 20 µM), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX, non-NMDA glutamate receptor antagonist, 20 µM), or (2R)-amino-5-phosphonopentanoate (AP5, NMDA receptor antagonist, 20 µM). These drugs are used for two purposes: first, to verify that the active component is dependent on excitatory synaptic transmission, and second, to block the active component so that the passive component can be imaged in isolation.

Local field potential recordings. Local field potential recordings were performed with a pair of glass micro pipets (~200k ohm impedance). The recording electrode was inserted to cortical layers II-III, about 1 mm from the edge of the imaging field. The reference electrode was adjusted in the bath to reduce the artifact from the applied field. Local field potential signals were used for verifying the activity of the active component seen in the VSD signals (Figure 1D).

Data analysis. The optical data were analyzed using the program NeuroPlex (RedShirtImaging) and programs written in MATLAB (MathWorks). Raw optical signals were digitally filtered between 0.4 – 200 Hz. The 0.4 Hz high-pass filter was chosen to eliminate baseline drift from the optical recording, which causes a background color shift in the pseudo-color images. Filtered signals were compared with unfiltered signals to verify that the 0.4 Hz high pass does not affect amplitude measurements of the active component. When working with the amplitude of the passive component (e.g., figures 4-5) we used a 4 Hz alternating field to avoid reduction by the 0.4 Hz filter. The singular value decomposition (SVD) method (Prechtel et al. 1997) was also used to remove random noise from the signal.

A Student’s t-test was used to evaluate the statistical significance of the pharmacological effects (Figure 3). A Wilcoxon rank-sum test was used to evaluate differences in median propagation velocity for different cortical layers (Figure 7D).

Results

The VSD signals of the active component closely match local field potential electrode recordings (Figure 1D). Comparing the optical and electrical signals demonstrates that the applied electrical field does not generate any artifact in the VSD signals (Figure 1D, “optical”). In contrast, the artifact could be seen in the local field potential signals if adjustments to the reference electrode did not completely cancel out the field artifact (Figure 1D, “LFP”).

VSD signals are clearly seen in single trials in each optical detector at low field intensities (Figure 2A right traces). The VSD signals reflect the membrane potential of neuronal populations in the brain tissue, with each optical detector receiving integrated signals from a large number of cortical neurons. The amplitude of the population VSD signal is proportional to the area of stained membranes; thus, if the soma and dendrites are depolarized to the same degree, dendrites should contribute much more to the population signal than the soma. Action potentials directly contribute very little to population VSD signals; most of the signal comes instead from postsynaptic potentials (for review, see Chemla and Chavane 2009).

Following the convention of Ghai et al. (2000), we 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 the pial surface (positive and negative are labeled “P” and “N”, respectively, in Figure 2A, right). Using these terms, an
“anodal field” in the literature (Nitsche et al. 2008; Paulus 2011) will be the same
direction as a “positive field” here.

The active and passive components in VSD signals

Weak alternating electric fields induced a cortical population response with two
distinct components: the active and passive (Figure 2A, right traces, and Figure 2B). In
VSD signals the active and passive components can be distinguished in several
respects. The active component is a depolarizing VSD signal that emerges during the
positive phase of the alternating field. The waveform of the active component does not
closely resemble the waveform of the applied field (Figure 2B, left), and at a low field
intensity the active component may appear only on the first few cycles (Figure 2A
traces). The passive component, in contrast, occurs in both directions of the alternating
field and closely follows the sinusoidal waveform (Figure 2B right, also Figure 5A).

The amplitudes of the passive and active components show a striking difference;
the passive component is linearly related to the field intensity, with no apparent threshold
(Figure 2D). In contrast, the amplitude of the active component has a nonlinear
relationship to the field intensity and quickly saturates at field strengths above 15-20 V/m
(Figure 2C). When using low field intensities, the amplitude of the active component can
be 5-10 times higher than that of the passive component at the same location (Figures
2A right, 2B).

Spatially, the active component is a depolarizing signal through all cortical
laminae (Figure 2B, left). In contrast, the passive component has opposite phases in the
superficial and deep layers (Figure 2B, right). With the spatial phase reversal, a positive
field will induce a passive component that hyperpolarizes the infragranular regions but
depolarizes the subgranular regions. In a band between these superficial and deep
polarizations, the passive component is undetectable (Figure 2B, center trace). With
these spatiotemporal characteristics, under a positive field both response components
are depolarizing deep within the cortex. In more superficial regions of cortex, however,
the passive component has a hyperpolarizing influence during active component
depolarization. This summation of passive hyperpolarization and active depolarization
may explain why, in more superficial regions of cortex, the amplitude of the active
component appears to reduce slightly at higher field intensities (Figure 2C).

Manipulations with synaptic transmitter blockers further distinguish the active and
passive components. The active component is extremely sensitive to the disruption of
local excitatory circuits. It can be completely blocked by 20 µM CNQX or NBQX,
antagonists of non-NMDA ionotropic glutamate receptors (representative trials shown in
Figure 3A, aggregated data in Figure 3B). The active component can also be blocked by
the NMDA receptor antagonist, AP5 (Figure 3C, 3D). In contrast, the passive component
is resistant to excitatory synaptic blockers (CNQX, AP5, both 20 µM) and the action
potential blocker, TTX (5-10 µM). These results suggest that the passive and active
components are generated by different mechanisms. Excitatory synaptic interactions are
necessary to generate the active component, but are not involved in generating the
passive component.

Threshold and time lag for inducing the active component

The active component emerges when the positive field reaches a threshold
intensity. When the field intensity is close to this threshold, the active component varies
considerably, with some cycles failing to induce the active component (Figure 4A). We
use the occurrence rate of the active component to define the threshold. For each field
intensity we test, we run three trials, each containing three cycles of a 1 Hz sine wave.
This gives 9 chances for inducing the active component (Figure 4A). If the active
component occurs during at least two of the nine cycles, then the field intensity is considered above the threshold. While the threshold varies considerably from slice to slice, the occurrence rate generally increases quickly above 4 V/m, and then reaches 100% at 20 V/m (Figure 4B). In an attempt to more accurately estimate the threshold, we found it affected by many factors, including perfusion, temperature, slice incubation time, and the interval between imaging trials. After optimizing these conditions, we measured an average threshold of 9.6 ± 2.6 V/m (mean ± SEM) across 31 slices from 25 animals, with the lowest recorded threshold being 4 V/m. Without controlling for optimal conditions, the threshold across 61 slices increased to 12.9 ± 4.8 V/m.

The emergence of the active component often lags behind the waveform of the field, most obviously seen when the field intensity is just slightly above the threshold (Figure 4A). Plotting the 50% peak time of the active component against the waveform of the applied field (Figure 4C) reveals large phase delays. These phase delays can nearly reach \( \pi \) under low field intensities (Figure 4D), though in all 1090 cases of active component examined, none emerged during the negative field (phase delay > \( \pi \), Figure 4D). The phase delay of the active component suggests that a buildup of recurrent excitation may be needed for the active component to emerge.

The passive component is a structure-specific signal

While the passive component has a stationary pattern in space, its amplitude is dependent on the neuronal architecture in the cortex (Figure 5, 6). In order to study the passive component in greater detail, we blocked excitatory synapses with CNQX or NBQX. This prevents the active component from emerging in field strengths as high as ~50 V/m. Higher field strengths improve the signal-to-noise ratio of the passive component (Figure 5A traces) and allow us to better investigate its spatial patterns.

In all cortical slices examined (between Bregma 0 to -2.5 mm) there is a nominal amplitude and phase reversal (NA-PR) zone between superficial and deep cortical layers (Figure 5B, top image, blue band). The NA-PR zone is parallel to cortical lamina and located below layers II-III. In barrel cortex, the NA-PR zone is about 491 ± 27 µm (mean ± SEM, n = 21 slices from 13 animals) deep from the pial surface (Figure 5B, C). Phase analysis demonstrates that the phase reversal line coincides with the line of the lowest amplitude in the NA-PR zone (Figure 5C). In a planar field, the NA-PR line always follows the curvature of the cortex (Figures 5, 6), which is not dependent on the direction of the field vector (Figure 5B, 5D). When the field vector is rotated from +30 to -30 degrees oblique to the depth axis, the direction of the NA-PR does not rotate (Figure 5D), demonstrating that the NA-PR line is related to the cytoarchitecture of the cortex but not the field vector. At larger oblique angles (e.g., 60-90 degree), the amplitude of the passive component was too small to accurately measure.

Using mouse whisker barrel fields, we examined how detailed the passive component amplitude can represent the cortical cytoarchitecture (Figure 6). We used a coronal slice sectioned at Bregma -1.5 mm (400 µm thick) where the whisker barrel subfield S1BF was included between two other areas of the somatosensory cortex, S1Tr (trunk region) and S2 (secondary). Under a 20X water immersion lens, each of our optical detectors received VSD signals from an area 38 µm in diameter. With this higher resolution, the whisker barrels were clearly visible in the passive component amplitude map (Figure 6, insert). Using the whisker barrels as a reference for cortical layer IV, we know that the NA-PR zone is located between cortical layers II-III and IV. The barrel structures were contrasted out by intermittent low- and high-amplitude VSD signals. There are two additional low-amplitude zones (Figure 6 L1, L2) observed between the white matter and the NA-PR zone. Unlike the NA-PR zone, phase reversal was not seen around these two low-amplitude zones. These two zones were also visible in other
sensory regions of cortex, such as visual and auditory, but were not seen outside of sensory areas, suggesting they are related to the cytoarchitecture within the granular layer of sensory cortex.

**Spatiotemporal patterns of the active component**

The active component starts from a small region (a local “hotspot”) and expands to a larger area, as in the example shown in Figure 7. This point-start pattern was seen in 33 slices from 16 animals, when the activity pattern over a larger imaging area is visualized with lower magnification objectives (5X or 10X). Globally, the active component manifests as propagating waves of variable velocity (movie S1). Rich spatiotemporal dynamics were seen in the spreading of the active component, including the emergence of multiple hotspots, variations in the velocity and direction of propagation, and interactions between local waves (Figure 7). This suggests that the spreading of the active component involves polysynaptic excitations instead of direct, long-range axonal conductance.

Figure 7, panels A and B, shows three episodes of the active component induced during the positive phase of a 1 Hz oscillating field. In VSD signal traces, the waveform of the active component varies largely across different episodes at the same location, and at different locations during the same episode (Figure 7A traces). Pseudo-color images selected from one episode (Figure 7B a - n) further show that the active component first starts from a small region (hotspot) and quickly spreads to a larger population (arrow in frame b). A second hotspot appears later in the medial region, and also expands (arrow in frame c). These two active regions expand, fuse, and then propagate in the medial and lateral directions, across several cortical areas. Non-uniform spreading is also seen, for example when the active component moves outward horizontally from the deep layers and then vertically to the superficial layers (Figure 7B, frame e – f and f-g, movie S1). In some instances, the active component oscillates a few times at the same location without propagation (frames h – n). These patterns suggest that the active component is self-organized activity in space and time, and at each local region the level of activity can go higher or lower, independent of activity in other areas.

On a more global scale, the active component is organized as a slow propagating wave. We analyzed the large-scale (>1.5 mm) spatiotemporal dynamics of 40 episodes of the active component from 3 slices. The initiation site of the active component was in every case located about 800 um below the pial surface of the cortex (Figure 7C). We also calculated the horizontal long-range propagation velocity at three depths: one near the initiation site (depth 2), one above (depth 1), and one below (depth 3). We found no significant difference between the propagation velocities at each depth (Figure 7D), and an overall median propagation velocity of 13.1 mm/s.

The propagation of the active component further suggests that, while an applied electric field can activate a large cortical area, most of the population is not directly activated by the field. Instead, the field first induces hotspots of localized activity, and propagating waves subsequently activate a large population. The propagation may be facilitated by the external electric field, especially when the activity crosses a boundary between cortical areas; as shown in the amplitude maps (Figure 8), a weaker field near the active component threshold (4-8 V/m) induced an active component in a limited local area, while a stronger field (16–24 V/m) induced an active component that propagated through the whole imaging field.

**Discussion**

We have used voltage-sensitive dye imaging to find that low-amplitude, alternating electric fields can induce a cortical population response with two distinct
components: the passive component and the active component. The two components can be distinguished by their amplitude and spatial phase distribution. Specifically, the passive component is a signal closely related to the cortical laminar structure, with a phase reversal occurring between cortical layers III and IV. The active component requires excitatory synaptic transmission and is initiated during a positive field, starting from a small area in the deep layers and then spreading to a larger area of the cortex. The active component can be ~10 times larger than the passive component and will likely dominate the overall effects of low-intensity alternating fields. Large populations of cortical neurons may be activated by the spreading of the active component rather than being directly activated by the field, and the propagation of the active component may therefore play an important role in integrating and spreading field-induced activity.

The passive component organized according to cortical cytoarchitecture

The passive component can be seen when excitatory synapses are blocked and action potentials abolished, so it is most likely a membrane potential change directly induced by the field. In theory, all cells surrounded by a high-resistance membrane would form a dipole in a planar field (Radman et al. 2007). Within the high-resistance membrane, the polarity of an intracellular electric field would oppose the extracellular field. The net result is that membrane close to the anode becomes hyperpolarized, membrane close to the cathode becomes depolarized, and membrane in center of the cell does not polarize. The polarity and spatial pattern of the passive component fit well with this theoretical prediction.

The most obvious spatial structure in the passive component is an arrangement of low-amplitude zones. The main low-amplitude zone is located between cortical layers III and IV (Figure 6). Phase reversal in the VSD signals distinguishes this main low-amplitude zone from other low-amplitude zones. This zone is seen in all cortical areas we have examined, suggesting it is related to the basic laminar cytoarchitecture of the cortex. This result is consistent with the phase reversal found in three-layer hippocampal CA3 tissue (Akiyama et al. 2011; Bikson et al. 2004).

Three aspects of the cellular organization may account for the low-amplitude zones: 1) Long cells arranged in the columnar structure form dipoles, with proximal and distal dendrites in opposite polarities and a middle section with near zero polarization. 2) An abundance of spherically symmetrical cells are not extended in space and are less affected by the field. 3) A balanced mix of depolarized and hyperpolarized cell components results in a zero net sum in the population VSD signal. It may be that all three aspects contribute to the amplitude contrast of the passive component map.

The main source of the passive component is likely to be associated with the layer V pyramidal neurons. A subthreshold external field can polarize neurons in a compartment-specific fashion, as confirmed both in intracellular recordings (Chan et al. 1988; Delgado-Lezama et al. 1999; Jefferys 1981; Park et al. 2005; Radman et al. 2009) and voltage sensitive dye imaging (Akiyama et al. 2011; Bikson et al. 2004) in the three-layered hippocampus. The pyramidal neurons in layer V are large, with their apical dendrites extending all the way into layer I (Peters 1993). As a result, these neurons have a larger vector length under the field and are likely to have larger amplitude of polarization (Radman et al. 2009). Thus, layer V pyramidal neurons form dipoles; they contribute large and anti-phase population signals in layers I-II and in layer V, while contributing nominal signal near the border between layer III and layer IV. In addition, the signals from basal dendrites of layer II/III cells and apical dendrites of short layer VI pyramidal neurons should have opposite polarities between layers III and IV, canceling each other in the VSD signals. This may also contribute to the low signal in the main
low-amplitude band. Similarly, layer VI pyramidal neurons that do not extend their
dendrites beyond layer IV may create an additional low-amplitude zone (L2 in Figure 6).

The low-amplitude signals associated with barrel structures (Figure 6) are
another example of the association between the passive component and the cortical
cytoarchitecture. While these amplitude contrasts may be associated with the barrel
hollows or granular cells, we cannot rule out the possibility that the different cells or
different areas are stained differently by the dye, thus resulting in the contrast of the
amplitude image.

When the field vector was rotated from +30 degrees to -30 degrees oblique to the
depth axis, the direction of the NA-PR did not rotate (Figure 5D), demonstrating that the
passive component is strictly related to the cytoarchitecture of the cortex and not the
field vector. When the electric field was rotated further, resulting in an orientation parallel
to the cortical laminae, we were unable to detect any passive component in fields as
large as 50 V/m. At first glance, this may appear incompatible with a recent report of
field-direction-specific polarization of somatodendritic branches (Rahman et al. 2013).
However, our VSD signals show a population summation of membrane polarizations in
the area under each detector; due to dendritic branches extending horizontally in both
directions, contributions to the VSD signal from branches specifically polarized by a field
with a horizontal orientation would likely be canceled out by contributions from dendrites
of opposite polarization.

From the passive to the active component

When the applied field is in its positive phase, the passive component in the deep
layers of cortex is depolarizing; the active component was never induced outside of this
phase of the field (Figure 4D). Under low-intensity fields, the depolarization of the active
component is roughly ten times larger than that of the passive component (as seen in
Figure 2B in the VSD signal amplitude), suggesting the active component can have a
larger effect on cortical neuronal populations than the passive component. The active
component is extremely sensitive to the AMPA/kainate receptor antagonist CNQX
(Figure 3), suggesting that local glutamatergic excitatory synapses play a key role in
integrating the small effects of a field on individual neurons into a large population event.
The threshold for inducing the active component (Figure 4B) is far below the reported
threshold for directly evoking action potentials in pharmacologically isolated cells
(Radman et al. 2009). This suggests network mechanisms are involved, such as an
elevation of spontaneous firing rate or a modulation of spike timing distributed across
many cells (Anastassiou et al. 2010; Radman et al. 2007; Reato et al. 2010).

Alternating fields elevate the firing probability only when the field is in its positive
phase, resulting in coherent increases of activity across the network (Deans et al. 2007;
Francis et al. 2003; Park et al. 2005). The coherent increase of firing in excitatory cells
may be critical for the generation of the active component, as it can lead to temporal
summation across the highly divergent and convergent cortical network. The
spontaneous firing in a large population of neurons may also engage stochastic
resonance (Collins et al., 1995; Bezrukov, and Vodyanoy. 1995), increasing the
probability of hotspots that can generate the active component. To further investigate the
emergence of the synchronized depolarization of the active component from
asynchronized spontaneous firing, multiunit spike recording and cell attached patch
recording may be needed. Both methods can be high-pass filtered to avoid the artifact
from a sinusoidal electric field. Combined with VSD imaging, spike recordings near the
origins of the active component could elucidate the dynamic process of forming hotspots
in greater detail.
It is somewhat surprising that AP5 can also block the active component. VSD signals of the active component suggest a low level of depolarization, and NMDA receptors should contribute little to subthreshold membrane potentials. However, the mild population depolarization in a large population of neurons may be sustained by a small fraction of spiking neurons. The activation of NMDA receptors in these spiking neurons might contribute to prolonging their depolarization and increasing the total number of spikes. The delayed emergence of the active component (Figure 4D) suggests that a buildup of recurrent excitation may generate the active component; NMDA conductance might play a critical role in this buildup of excitation. The large phase lag may also explain why the active component only follows low frequency fields; in our experiments, the active component in most of the slices can follow 1 Hz or lower, but not 2 Hz or higher (data not shown).

The field intensity that can induce the active component in our quiescent cortical slices (threshold ~10 V/m) is larger than the intensity for modulating spontaneous rhythms in neuronal networks (~1 V/m or lower, Francis et al. 2003; Deans et al. 2007; Fröhlich & McCormick 2010; Ali et al. 2013; Reato et al. 2010). This may be because spontaneous rhythmic activity facilitates the excitation that generates the active component. The field intensity used in human subthreshold transcranial stimulation protocols was also much lower than ours (estimated to be 1 V/m or lower, Datta et al. 2009; Marshall et al. 2006). This may in part be due to the higher neuronal density and longer axonal length in the human cortex, as well as resonance (reviewed by Fröhlich 2014) between rhythmic human cortical activity and the tACS.

In a continuous alternating field the active component occurs only during the first few cycles (Figure 2A), suggesting the involvement of complex factors at both cellular and population levels. On the cellular level, voltage-gated calcium currents (I_T and I_L) may facilitate the active component during the early cycles of the field, but may also trigger calcium-activated potassium currents (I_AHP and I_C, McCormick 2004) that reduce its likelihood during the later cycles. On the population level, the resources for the initiation, spreading, and sustaining of the active component may be inversely related to the involvement of neurons in the previous episode of active component. If more neurons are active in an earlier cycle, then more neurons may be refractory in the later cycles, and fewer neurons will be available for generating a subsequent episode of active component. We noticed that the active component may fail to re-occur after a particularly high-amplitude active component, e.g., the last active component in Figure 2 traces. This large VSD signal indicates that more neurons are involved or that the neurons involved are more depolarized. In the former case, there may be a decreased number of neurons in the population available for subsequent propagating waves (Gao et al. 2012); in the latter case, cellular mechanisms may suppress further episodes of active component.

**Active component organized as propagating waves**

The active component starts in a small area and spreads as a propagating wave (Figure 7). It appears there is a two-step process, in which the active component first becomes self-sustained in a local area (as a "hotspot") and subsequently spreads under the support of the electric field. This is consistent with a recent computational modeling study of spontaneous slow-wave oscillations under tACS, where activity emerges in one or more local “hotspots” and propagates outward (Ali et al. 2013).

Propagation of the active component may serve as a mechanism for spreading electric field-induced activity beyond areas directly affected by the field, such as a sulcus where the field vector is not optimally aligned. The propagating waves of the active component may be similar to self-sustained waves seen in vivo; the magnitude and
propagating velocity of the activity at a distance from the initiation site may not depend on the magnitude at the initiation site. Instead, local excitability and prior wave dynamics may play major roles in determining local activity (Gao et al. 2012). Such self-sustained, locally-controlled propagating waves may cause the large variations in propagating velocity and amplitude seen in the active component (Figure 7B, frames h-n).

Propagating waves of the active component can be distinguished from other waves such as epileptic waves or waves of spreading depolarization (SD, Reiffurth et al. 2012). Epileptiform activity involves recurrent excitation of a much larger magnitude than the active component, with a VSD signal amplitude about 3 – 5 times larger (Jin et al. 2002). In addition, the intrinsic optical signal (light scattering of the cortical tissue) of the active component is about 1/10 that of an epileptic wave (Jin et al. 2002; Tsau et al. 1998) and 1/100th that of a SD (Aitken et al. 1999). The characteristics of propagation are also different; epileptiform waves in cortical slices are fast (100 - 600 mm/s, Demir et al. 1998; London et al. 1989) and robust, while the active component propagation is slower (~13.1 mm/s, Figure 7D) and shows more varied patterns of propagation. The propagating velocity of the SD waves are even slower (< 0.1 mm/s, Reiffurth et al. 2012) than the active component. The SD also has a long refractory period (several minutes), while the active component can have a series of episodes occurring at about 1 Hz (Figure 1-2).

Propagating waves of the active component are similar to waves that are widely seen in the cortex accompanying sensory and motor events (reviewed by Sato et al. 2012; Wu et al. 2008). These sensory- or motor-evoked waves bring low levels of membrane depolarization from the cortical representation site to a vast neuronal population via distributed, nonspecific synaptic interconnections. Similar propagating waves also occur spontaneously in the cortex during sleep states (Huang et al. 2010b; Massimini et al. 2004). In human experiments with tACS, interactions have been suggested between a weak external electric field and cortical sleep waves. The effects of an external oscillating field were largest during slow wave sleep, where a 0.75 Hz field was more effective than a 5 Hz field in modulating sleep oscillations and facilitating declarative memory consolidation (Marshall et al. 2006). In such an experiment, the propagating waves of the active component may be interacting with ongoing cortical waves, leading to modulation of brain activity and ultimately behavior.

The active component in this report is produced by a low-frequency, alternating electric field. A persistent DC field, like those used in standard transcranial direct current stimulation (tDCS) protocols, may have different effects on cortical activity (Nitsche et al. 2008; Zaghi et al. 2010). For instance, the effects of tDCS can last minutes to hours after exposure to the applied field. This long-term effect is likely due to plastic changes in the network caused by a sustained period of elevated cortical activity (Paulus 2011). Oscillating fields, in contrast, can trigger large but transient increases in cortical activity (the active component). They may also produce long-term changes in network activity (Reato et al. 2013), likely depending on the relationship between endogenous brain activity and the frequency (Marshall et al. 2006) and phase (Polania et al. 2012) of the applied field.

In conclusion, the cortical response to subthreshold alternating electric fields not only consists of passive neuronal polarization, but can also have a network-mediated active component that is many times larger. The active component is organized as propagating waves that spread the activity from a local hotspot to a larger population, and may serve as a mechanism for modulating widespread cortical function with a small, subthreshold field.

Author contributions:
All the authors participated in all steps of the experiments including the design, data acquisition, data analysis and interpretation as well as the drafting of the manuscript.

References


19. Fröhlich F, and McCormick DA. Endogenous electric fields may guide neocortical network


---

**Figure captions**

**Figure 1. Activation of cortical slices under a 1 Hz AC field.**

*Figure arrangement.* A mouse coronal slice containing the barrel cortex is imaged by a 464-channel diode array with a 20X objective (the imaging field is marked with a hexagon). A sinusoidal alternating field is applied by a pair of parallel Ag-AgCl electrode coils (E).

*Figure B* Top: The voltage applied to the electrodes. Middle: the waveform measured from the center of the chamber (the measuring electrode probes are 1.5 mm apart). Bottom: the current passing through the chamber, measured by a 10 ohm resistor connected to the chamber in series.

*Figure C* The calculated electric field from the probes vs. the voltage applied to the electrodes. *Figure D* The active component simultaneously recorded by a local field potential (LFP) electrode and VSD imaging (optical). The LFP signals contain a large artifact from the applied field, due to imperfect cancellation. The LFP recording electrode was placed outside the imaging area (marked in *A*), with a reference electrode (not shown) adjusted to reduce the artifact.

**Figure 2. Passive and active components.**

*Figure A* Left: Schematic drawing of the cortex and locations of the VSD signals. The imaging window is marked by a blue hexagon. S1BF: Primary somatosensory cortex, barrel field; M: medial; L: lateral; Pia: pia mater of the cortex. Three optical detectors, 1 – 3, are chosen from superficial, middle, and deep cortical layers for producing the traces shown on the right. Right: the 1Hz oscillating electric field applied to the slice (red trace, P, N: positive/negative field directions) and the VSD signals from detectors 1 – 3 (black traces).

*Figure B* Expanded view of the “active” and “passive” blocks in A. The active component is a depolarizing signal in all cortical laminae, while the passive component shows a spatial phase reversal. Note that the amplitude of the active component is ~10 times larger than that of the passive component.

*Figure C* Normalized amplitude of the active component under different field intensities. The data are aggregated from 5 animals, with each color from the same animal. The amplitude of the active component for each trial is defined as the maximum of a signal induced by the stimulation. Each data point is the amplitude averaged across 6 locations in the superficial layers and across all trials with the same stimulation in that
animal. **D)** The amplitude of the passive component under different field strengths. Data are again averaged across 6 locations and across trials. The slices are bathed in 20 µM CNQX to block the active component (five colors represent data from 5 animals).

**Figure 3. Active component can be blocked by CNQX and AP5. **A) and **B). Representative examples for CNQX and AP5. Both slices were imaged with a 20X objective. Each trace is from one imaging trial, VSD signals from one optical detector in the superficial layers. The top trace in each panel shows the applied field, with 3 cycles of 1 Hz sinusoidal waves. **C) and **D). Aggregated data for CNQX (C, 5 slices) and AP5 (D, 3 slices), respectively. The amplitude of the active component was calculated as the average of the three cycles in each trial, and normalized to the average amplitude in normal ACSF for each slice. N is the number of the total trials in that condition. *** indicates significant differences (Student’s t-test, P < 0.001).

**Figure 4. Threshold and phase delay of the active component**

**A)** Emergence of the active component at fields near the threshold. The threshold for this slice is 5 V/m. Note that at near-threshold intensity, some cycles of the stimulus fail to induce the active component. **B)** The occurrence rate of active component under different field strengths (5 animals labeled by colors and shapes). The occurrence rate is defined as the fraction of cycles during which the active component occurred. **C)** A representative trace showing the applied field and a delayed emergence of the active component. The vertical dashed lines represent the time at which the VSD signal reaches half of its maximum, with the phase of the sinusoidal field (in radians) listed below the trace. **D)** Aggregated phase delay data from 1,090 occurrences of the active component. The “phase delay” is defined as the phase of the field when the active component reaches half of its maximum value, as shown in **C**.

**Figure 5. Nominal amplitude and phase reversal zone of the passive component.**

**A)** left, A section of the barrel cortex is imaged with a 20X lens. Two imaging fields are combined to make the images in B. **Right**, VSD signals from superficial (1) and deep (2) layers. The slice was perfused in 20 µM CNQX to eliminate the active component. **B)** Amplitude (top) and phase (bottom) maps of the passive component from the imaging fields shown in A. Note that the nominal amplitude and phase reversal (NA-PR) zone is parallel to the cortical laminae and here is oblique to the electric field vector. Signals from the detectors along the broken line are used for making the spatial profiles in C. **C)** Spatial profiles of amplitude (top) and phase (bottom). Note that the phase reversal line (vertical broken line) matches the point of zero amplitude. **D)** Images of the passive component amplitude from a single cortical slice exposed to a 4 Hz oscillating field (50 V/m). The slice is bathed in NBQX to suppress the active component. When the field vector (black arrows) is rotated from 0 (left) to +30 (center) or -30(right) degrees, the nominal-amplitude-phase-reversal zone (blue) does not rotate with the electric field.

**Figure 6. Cortical structures revealed by a passive component amplitude map.** A coronal slice including the barrel cortex is imaged with a 20X lens. The image is composed of 39 imaging fields. The passive component was induced by a 48 V/m, 4 Hz oscillating field. The slice was perfused with 20 µM CNQX to eliminate the active component. **NA-PR** nominal amplitude phase reversal, **L1** low-amplitude zone 1, **L2** low amplitude zone 2. Cortical areas are marked based on a mouse brain atlas (Paxinos and Franklin 2001). **RSG**, retrosplenial granular cortex; **S1Tr** Primary somatosensory area trunk region; **S1BF**, primary somatosensory cortex, barrel field; **S2** secondary somatosensory cortex. **WM**, white mater.
Figure 7. Spatiotemporal dynamics of the active component. A, The area of imaging includes somatosensory cortex (imaged with a 4X objective): S1Tr Primary somatosensory area trunk region; S1BF Primary somatosensory cortex, barrel field; S2 secondary somatosensory cortex. WM white mater. M medial; L lateral; Pia pia mater. VSD signals from four locations, 1-4, are shown in the right traces. Three cycles of an oscillating field at 24 V/m induce three episodes of the active component. Note that trace 3 has an earlier onset time, suggesting that the active component initiated nearby. B, 14 snap shots (0.6 ms) are selected from the 16,384 total images of this recording trial. The relative time (in ms) of each snapshot is marked at the bottom-left of each image, and the time of images a, e, i, k, m, n are marked by gray lines in the traces. The colors of the images represent the normalized amplitude of the VSD signal (normalized on a linear scale to the peak on each detector, peak = red, baseline= blue). The spatiotemporal patterns of the active component from the second episode are shown in Movie S1. C. The depth distribution for the initiation sites of the active components (40 episodes, 3 slices). Three black bars mark three depths where the horizontal propagation velocity was calculated in D. D. Horizontal propagation velocity. The velocity is calculated as the distance from the initiation site to the edge of the imaging field divided by the time difference. The red line inside each blue box marks the median value, and the edges of each blue box mark the 25th and 75th percentiles. Whiskers (black bars/broken lines) extend to the most extreme data points not considered outliers (99.3% coverage) and outliers are indicated by red stars. No statistical differences exist between the median velocities of any two groups (Wilcoxon rank-sum test, p > 0.1).

Figure 8. Amplitude map of the active component. Images are made from the same slice as in Figure 7, with the active component induced by 4 different peak intensities of a 1 Hz oscillating field (4, 8, 16, 24 V/m respectively). Amplitude is defined as in figure 2C. A white broken line marks the putative boundary between S1BF and S1Tr. Note that the active component only propagates beyond this boundary under the support of a stronger (16 or 24 V/m) positive field.

Movie captions
Movie S1. Spatiotemporal patterns of the active component. The movie shows one episode of the active component (the second episode shown in Figure 7A; for the location and size of the cortical tissue, see Figure 7). The movie is slowed down to 1/32 of real time; the actual duration of the active component episode is about 0.9 seconds. The pseudo-color images were generated from the normalized VSD signal from each detector; the actual VSD signal is the change in dye absorption over the resting light intensity and the amplitude is about 10^{-3}.
Figure 1

A. Experiment arrangement

B. Voltage applied on electrodes (1 Hz)
   - Waveform
   - 8 V

   Field intensity measured by probes
   - Waveform
   - 60 V/m

   Current passing through the chamber
   - Waveform
   - 9 mA

C. Field (V/m) vs. Applied Voltage (V)

D. Field, 1Hz 20 V/m
   - LFP
   - 0.2 mV

   Optical
   - 10^{-3}
Figure 2

A

B

C

D
Figure 4

A

B

C

D

Active component occurrence rate vs. Field strength (V/m)

Active component initiation phase vs. Field strength (V/m)

Field strength (V/m)

n = 1090
Figure 6

Amplitude map

- RSG
- NA-PR zone
- L1
- L2
- WM
- S1Tr
- S1BF
- S2
- Bregma -1.5 mm

Field vector

1 mm

Whisker Barrels

20111006 slice 1

Bregma -1.5