Seizures As Imbalanced Up States: Excitatory And Inhibitory Conductances During Seizure-Like Events.

ABBREVIATED TITLE: Excitation/Inhibition Balance During Seizures.

AUTHORS (last name, first name):
Žiburkus, Jokūbas¹, Cressman², John R., and Schiff, Steven J.³

¹. University of Houston, Dept. of Biology and Biochemistry, Houston, TX.
². George Mason University, Krasnow Institute for Advanced Studies and Dept. of Physics and Astronomy, Fairfax, VA.
³. The Pennsylvania State University, Center for Neural Engineering, Depts. of Engineering Science and Mechanics, Neurosurgery and Physics, University Park, PA.

CORRESPONDING AUTHOR: Jokūbas Žiburkus, PhD;
4800 Calhoun Rd. SR2 Rm 349
Houston, TX 77204-5001
E-mail: jziburkus@uh.edu

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ABSTRACT

Precisely timed and dynamically balanced excitatory (E) and inhibitory (I) conductances underlie the basis of neural network activity. Normal E/I balance is often shifted in epilepsy resulting in neuronal network hyperexcitability and recurrent seizures. However, dynamics of the actual excitatory and inhibitory synaptic conductances ($g_e$ and $g_i$, respectively) during seizures remain unknown. To study the dynamics of E and I network balance, we calculated $g_e$ and $g_i$ during the initiation, body, and termination of seizure-like events (SLEs) in the rat hippocampus in vitro. Repetitive emergent SLEs in 4-aminopyridine (100μM) and reduced extracellular magnesium (0.6mM) were recorded in the identified CA1 pyramidal cells (PC) and oriens-lacunosum moleculare (O-LM) interneurons. Calculated $g_e/g_i$ ratio dynamics showed that the initiation stage of the SLEs was dominated by inhibition in the PCs and was more balanced in the O-LM cells. During the body of the SLEs, the balance shifted toward excitation, with $g_e$ and $g_i$ peaking in both cell types at nearly the same time. In the termination phase, PCs were again dominated by inhibition, while O-LM cells experienced persistent excitatory synaptic barrage. In this way, increased excitability of interneurons may play roles in both seizure initiation (Ziburkus et al. 2006) and in their termination. Overall, SLE stages can be characterized in PC and O-LM cells by dynamically distinct changes in the balance of $g_e$ and $g_i$; where a temporal sequence of imbalance shifts with the changing firing patterns of the cellular subtypes comprising the hyperexcitable microcircuits.

KEYWORDS: excitatory/inhibitory balance, synaptic conductance, seizure, interneurons, microcircuits.
INTRODUCTION

Animal behavior emerges from precise and finely tuned inhibitory and excitatory neuronal activation patterns (Buzsaki 2006; Klausberger et al. 2003; Klausberger and Somogyi 2008; Shen et al. 2011; Turrigiano and Nelson 2000; Yizhar et al. 2011). In normal neuronal activity in vitro and in vivo, such as recurrent cortical UP states, $g_e$ and $g_i$ may be exquisitely balanced (Borg-Graham et al. 1998; Haider et al. 2006; Shu et al. 2003). During initiation of scratch-like network behavior in turtle spinal motor neurons and interneurons, $g_i$ and $g_e$ peak in phase with the total membrane conductance (Berg et al. 2007). When transitioning between awake and sleep states, the $g_e$ and $g_i$ in neocortical cells in vivo show complex interactions and inhibition can transiently dominate during these cycles (Rudolph et al. 2007). Given that normal network states depend upon appropriately balanced E and I cell interplay, it is important to characterize the nature of the $g_e/g_i$ balance in pathological network activity as a function of identified E and I cell subtypes.

In hyper-excitable epileptic tissue, seizures are historically thought to stem from decreased $g_i$ and increased $g_e$. The growing body of experimental and theoretical evidence suggests that interneuron activity is preserved in human (Cohen et al. 2002; Huberfeld et al. 2011; Truccolo et al. 2011) and animal (Avoli et al. 1996a; Higashima et al. 1996; Lopantsev and Avoli 1998; Velazquez and Carlen 1999) epileptic tissue. Furthermore, seizure synchronization patterns in vitro and in vivo are complex and, similarly to other behavioral states (Buzsaki 2006), appear related to precise E and I cell subtype-specific, interactions (Fujiwara-Tsukamoto et al. 2010; Gutkin et al. 2001; Netoff and Schiff 2002; Truccolo et al. 2011; Ullah et al. 2009; Ziburkus et al. 2006). E-I cell interplay during SLEs and seizure EEG data from human patients (Schiff et al. 2005) support a natural partitioning of seizures into initiation, body, and
termination stages. Despite the importance of $g_e$ and $g_i$ dynamics in network disorders such as epilepsy, ratios of $g_e/g_i$ during seizures have not been studied. Furthermore, to date, all of the studies on $g_e$ and $g_i$ dynamics, with the exception of putative spinal interneurons in turtles (Berg et al. 2007), have been limited to explorations of putative neocortical pyramidal cells. This leaves a void of information on spontaneous $g_e/g_i$ balance and dynamics during seizures in distinct cell subtypes.

To investigate dynamics of $g_e$ and $g_i$ before, during, and after the SLEs in identified excitatory and inhibitory hippocampal cell subtypes, we used a pharmacological seizure model and recorded synaptic conductances in PC and O-LM cells in transverse hippocampal slices, in area CA1. $g_e$ and $g_i$ calculations were derived from multiple recurrent SLEs recorded in the same cell voltage-clamped at different holding potentials. Our data suggests divergence in network input dynamics between E and I cells, whereby different dynamical ratios of $g_e/g_i$ were observed during distinct SLE stages. Collectively, these findings demonstrate a novel dynamical pattern of excitatory and inhibitory conductances underlying the temporal and spatial evolution of SLEs.

METHODS

Animals. Experiments were performed on Sprague Dawley rats (P18-P30) with approval from the Institutional Animal Care and Use Committees of George Mason University, Pennsylvania State University, and the University of Houston.

Electrophysiology. Postnatal day 18-30 rats were anesthetized with diethyl-ether, decapitated, brains removed, hippocampi isolated, and transverse 350µm sections cut in cold
dissection buffer (in mM: 2.6 KCl, 1.23 NaH₂PO₄, 24 NaHCO₃, 0.1 CaCl₂, 2 MgCl₂, 205 sucrose, 20 glucose) using a vibratome.

Slices were incubated for an hour in artificial cerebrospinal fluid (ACSF; pH 7.3, 30°C, in mM: 130 NaCl, 0.6 MgSO₄, 3.5 KCl, 1.2 CaCl₂, 10 glucose, 2.5 NaH₂PO₄, 24 NaHCO₃) aerated with 95%O₂-5%CO₂, transferred to a submersion recording chamber (Warner Instruments) and perfused (1ml/min, 34°C). Borosilicate glass micropipettes (3-6 MΩ) for voltage clamp recordings contained (in mM): 120 cesium gluconate, 5 N-(2,6-dimethylphenyl carbamoylmethyl) triethylammonium bromide (QX-314), 2 KCl, 0.5 EGTA, 20 HEPES, 10 phosphocreatine, 0.3 NaGTP, 2 NaCl, 4 MgATP, and 0.3% Neurobiotin (pH 7.25, 295mOsm). A few recordings were also performed in current clamp, using similar intracellular solution, except cesium gluconate was replaced by potassium gluconate, and QX-314 blocker was excluded from the pipette (Ziburkus et al., 2006; Hazra et al., 2012). Extracellular recordings were performed with glass micropipettes (1-3 MΩ) filled with 0.9% NaCl.

Alignment of the seizure start times was based on an identifiable feature of the extracellular recording - the fast positive extracellular shift (FPES, 1-5mV, Fig. 1 in (Ziburkus et al. 2006)). O-LM interneurons were targeted because of their importance in normal (Gillies et al. 2002; Klausberger and Somogyi 2008; Pike et al. 2000; Somogyi and Klausberger 2005) and pathological hippocampal rhythms (Aradi and Maccaferri 2004; Ziburkus et al. 2006), and their vulnerability in epilepsy (Oliva et al. 2002; Sanon et al. 2005; Santhakumar and Soltesz 2004). E cells in pyramidal and I cells in oriens layers were visually preselected using differential infrared contrast microscope (Zeiss) with a mounted zoom-tube. Extracellular electrodes were placed in the upper stratum (st.) pyramidal near the patched cells. Recordings were performed using
Axon MCC 700A and 700B amplifiers (Molecular Devices), filtered (4KHz whole-cell, 1KHz extracellular), and digitized at 10KHz (Digidata and Pclamp7, Molecular Devices).

SLEs were induced pharmacologically by applying 100 µM of 4-AP (Sigma) in decreased magnesium (0.6mM [Mg$$^{2+}$$]$_o$) ACSF. 4-AP increases inhibitory and excitatory neuron signaling by partial blockade of repolarizing A-type potassium channels causing broadening of action potentials and membrane depolarization (Avoli et al. 1996b; Avoli et al. 1988; Somjen 2004; Ziburkus et al. 2006). The decreased Mg$$^{2+}$ in the ACSF partially removes n-methyl-d-aspartate (NMDA) receptor blockade and reduces divalent charge screening (Huberfeld et al. 2011), increasing the duration and intensity of 4-AP in vitro seizure-like events. Both pharmacological models or in conjunction have been used to mimic temporal lobe epilepsy and pharmaco-resistance, often observed in the intractable forms of epilepsy (Albus et al. 2008; D'Antuono et al. 2010; Loscher 2011; Wahab et al. 2010).

To determine excitatory synaptic reversal potential ($E_e$) in this convulsant solution, we blocked inhibition using picrotoxin (PTX, 50µM). In a few instances, a bipolar stimulating electrode (200µm outer diameter, WPI) was placed either on the Schaefer collateral pathway (n=5) in the st. radiatum or on the alveus/oriens excitatory projections to CA1 pyramidal cells (n=4) and interneurons (n=5), respectively. In all cases, while in the presence of PTX, spontaneous burst firing occurred and electrical stimulation was unnecessary. Likewise, a stimulation-evoked inhibitory synaptic reversal potential ($E_i$) was measured in the presence of NMDA (R-2-amino-5-phosphonopentanoate, APV, 25µM) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor (6-cyano-7-nitroquinoxaline-2,3-dione, CNQX, 10µM) antagonists. $E_i$ for pyramidal cells was measured by stimulating st. pyramidale/oriens (n=5) or pyramidale/radiatum (n=5) borders. $E_i$ for the horizontal oriens
interneurons was measured by stimulating oriens (n=6) or oriens/pyramidale border (n=5). These varied sites of stimulation were chosen based on the known anatomical locations of the bulk inhibitory and excitatory projections to these cell subtypes (Freund and Buzsaki 1996; Pouille and Scanziani 2004). All of the drugs were bath applied.

To track the temporal evolution of inhibitory and excitatory conductances during the seizures, E and I cells were voltage clamped at a range of holding potentials (−80 to +40mV). Cells used for final analysis had to meet the criteria of undergoing at least 6 seizures at different positive and negative holding potentials while maintaining a stable recording and an input resistance higher than 100MΩ. To test for stability of the patch and cell, after each holding potential (−80 to +40mV) we checked that when the cells were held at −60 to −80mV during inter-seizure intervals, the change of bias current did not exceed 150pA of the original holding current value. We randomly alternated between positive and negative holding potentials for each subsequent ictal event. The pre-ictal SLE state in 4-AP is commonly associated with increases in the frequency of firing and bursting in the individual cells and networks, monitored by whole-cell and extracellular DC recordings. Increases in frequency of firing and bursting monitored electrically and through the audio speaker were used as the cues by for experimenter to anticipate the impending SLE and change the holding potential with compensated capacitance. If a seizure did not occur, the cells were returned to the negative holding potential (−60 to −80mV) and changes in bias holding current were monitored. Liquid junction potential value (LJPV) using this whole cell solution was measured as +13mV. All of the holding potentials and reversals are reported with the adjusted LJPV value.

Data analysis and dynamic conductance measures. Data was analyzed using Clampfit and Matlab. To analyze interictal burst (IB) reversals, the minimal amplitude (root mean square –
RMS) of spontaneous activity observed at a given holding potential was considered as a synaptic potential reversal value. To determine the IB reversal values we used two voltage clamp protocols: continuous holding square wave-like current injections and slowly depolarizing (15 sec.; -80mV to +40mV) ramp current injections. To find the reversal potential within the ramps, we calculated the smallest RMS value of cell current deflections within 1000 millisecond (ms) bins and matched these nominal current measurements to the corresponding membrane voltage value. Since the two experimental methods using square wave and ramp current injections did not show significantly different results, we report IB reversal values grouped from both types of measures and according to their respective cell subtypes.

To record synaptic current reversal during prolonged SLEs we held the cells using continuous voltage-clamp at a range of membrane potentials (-80 to +40mV). To calculate excitatory and inhibitory conductances a similar analysis method to that previously used in the in vivo (Borg-Graham et al. 1998; Rudolph et al. 2007) and in vitro (Haider et al. 2006; Shu et al. 2003) experiments. In order to calculate the average total reversal potential ($E_T$) over the course of a seizure we binned the data every 100ms. Within the time bins, all of the seizure episode currents were measured across all of the holding potentials for each cell. In order to isolate the synaptic currents, we first calculated the portion of the current produced by the inherent leakiness of the cells. To account for this leak current, we calculated the total amount of current (at a given membrane potential) during the 1-second period prior to the ictal discharges. We visually selected a second of data that did not contain large bursts. We then subtracted this ambient leak value from the subsequent bursts and ictal event currents. The current values at each holding potential were fit to a line. The intercept with the abscissa reveals the total synaptic reversal potential ($E_T$) and the slope gives the total membrane conductance ($g_T$). The $g_T$ is the
sum of the excitatory and inhibitory conductances ($g_e$ and $g_i$). The total reversal membrane potential $E_T$ is a potential value that would balance the total synaptic drive. That is, it is the potential at which excitation and inhibition are balanced. Depolarized $E_T$ indicates domination by the excitatory, inward current, and the hyperpolarized $E_T$ values indicate a shift toward outward inhibitory current. $E_T$ and $g_T$ values are required to calculate the individual excitatory and inhibitory conductances by using time dependent Ohm’s law. By inserting the measured excitatory and inhibitory reversal potentials ($E_e$ and $E_i$) we then calculated the individual conductances, $g_e$ and $g_i$. Our calculations were done using the following set of equations (1-4).

Where $V$ is the voltage clamp holding potential, and $I_T$ is the total recorded current:

\[ V = I_T(t)/g_T(t) + E_T(t) \]  

(1)

\[ g_T(t) = g_i(t) + g_e(t) \]  

(2)

\[ g_i(t) = \frac{g_T(t)(E_e - E_T(t))}{E_e - E_i} \]  

(3)

\[ g_e(t) = \frac{g_T(t)(E_i - E_T(t))}{E_i - E_e} \]  

(4)

Conductance values for all of the pyramidal cells and the interneurons were averaged across the E and I cells respectively, and are presented here with the standard error of the mean.

**Statistical Analysis.** Running ANOVA analysis was performed on the individual $g_e$ and $g_i$ and on $g_e/g_i$ ratios throughout the evolution of the SLEs (Figure 6). Values at confidence level of 95% or higher were considered as significant. To determine time course of the the $g_e/g_i$ ratio
differed significantly during the process of SLE, we normalized ge and gi individually (Figure 6 C-D).

**Immunohistochemistry and neuronal reconstructions.** The cells that exhibited morphological features of pyramidal cells and were immunohistochemically negative for parvalbumin were identified as pyramidal (Fig. 1). The cells that contained horizontally projecting dendrites, axonal terminals extending toward the apical pyramidal dendrites to the radiatum-lacunosum-moleculare border, and were positive for somatostatin were identified as O-LM cells (see summary table in Figure 1).

During the recordings, cells were filled with neurobiotin. Neurobiotin filled cells were fixed for 24 to 76 hours in 4% paraformaldehyde in 0.1M phosphate buffer (PB). The neurobiotin filled cells were visualized using 7-amino-4-methylcoumarin-3-acetic acid (AMCA excitation/emission)-conjugated streptavidin (1:1000, Vector Laboratories, Burlingame, CA, USA). Somatostatin and parvalbumin immunoreactivity was tested using a mixture of two primary antibodies raised in rabbit or mouse (Maccaferri et al. 2000). For somatostatin (SOM) we used the following: mouse monoclonal anti-somatostatin at 1: 1000. For parvalbumin (PV): polyclonal rabbit anti-parvalbumin at 1: 1000. Parvalbumin staining was used to make sure that recordings were indeed done from pyramidal CA1 cells. Somatostatin stain in conjunction with subsequent neuronal reconstructions was used to identify O-LM interneurons. Secondary antibodies were conjugated with fluorophores (anti-PV was conjugated with fluorescein isothiocyanate, FITC and anti-SOM with Tetramethyl-rhodamine isothiocyanate, TRITC). Following the staining, slices were wet mounted using aqueous medium and analyzed using fluorescent (Zeiss 2FS) and confocal (Olympus BX) microscopes equipped with sensitive cameras (Cooke Sensicam or Hamamatsu Orca). Using a 40x lens we identified neurobiotin
positive cells that were also positive for somatostatin and parvalbumin. Following fluorescent antibody staining and analysis, the slices were demounted, washed, and re-stained with streptavidin. Briefly, using the Vector stain ABC Elite kit (Vector Laboratories) we incubated the slices with 0.1% Triton X overnight and then used standard 3',3'-diaminobenzidine histochemical methods. Axonal visualization was improved using a PB solution containing nickel ammonium sulfate. The recorded cell morphologies were then reconstructed using Neurolucida (Microbrightfield, Figure 1).

RESULTS

Epileptiform activity recordings in the CA1 pyramidal cells and OLM interneurons.

Single and dual whole-cell and simultaneous extracellular recordings were performed in the identified hippocampus CA1 area O-LM and PCs. O-LM cells were targeted due to their high susceptibility to damage by seizures (Oliva et al. 2002; Sanon et al. 2005; Santhakumar and Soltesz 2004) and their involvement in seizure initiation in the in vitro 4-AP model (Ziburkus et al. 2006). O-LM cells were first selected visually based on their somatic location in the oriens, bipolar horizontally extending dendritic trees, with the occasionally detectable axon branching toward radiatum and lacunosum moleculare off one of these horizontal dendrites. Exact subtypes of the recorded cells were confirmed by post hoc morphological reconstructions and dual immunohistochemical staining using distinct cellular markers (Figure 1). Some cells were only partially filled with neurobiotin, thus allowing us to trace only portions of the axons. To confirm
that basket cells were not recorded when targeting pyramidal layer neurons, we used antibody
directed against parvalbumin positive cells. Post-hoc all of the pyramidal layer cells used in the
analysis were identified as the PCs. Somatostatin positively stained 85% of all of the putative O-
LM cells.

To analyze inward and outward synaptic currents and their reversals during short interictal
bursts and prolonged seizures, we employed a well-studied in vitro seizure model of 4-AP and
lowered [Mg^{2+}] (0.6mM; (Isaev et al. 2005; Khosravani et al. 2003; Khosravani et al. 2005;
Ziburkus et al. 2006)). Whole-cell recordings were performed in current and voltage-clamp
modes. The internal recording solution for voltage-clamp contained blockers for potassium and
fast sodium conductances (Cs^{2+} and QX-314) and neurobiotin. This experimental paradigm
allowed us to isolate inhibitory and excitatory synaptic currents (Figure 2). Since the active
membrane properties of the cells were rapidly blocked by QX-314, we could not reliably employ
electrical characteristics to aid in distinguishing between the recorded cell subtypes (Gupta et al.
2000). In addition to blockade of the voltage-gated sodium channels, QX-314 also blocks G-
protein gated potassium conductances, such as GABAb (Alreja and Aghajanian 1994; Otis et al.
1993; Slesinger 2001). Thus potassium conductances in these experiments were blocked by both
cesium and QX-314 in the pipette solution.”

**Interictal burst reversals in the CA1 pyramidal cells and OLM interneurons.**

We first studied the reversal potentials of the interictal bursts (IB). IBs analyzed were the
initial bursts that preceded SLE activity, or the bursts from the slices that never formed SLEs
(Hazra et al., 2012). Two modes of voltage-clamping were used to record burst reversals:
continuous clamp at different holding potentials (from -80 to +40mV) or slow (120mV/15 sec)
ramp current injections (from -80 to +40mV; Fig. 2). To calculate the IB reversal, we found the potential at which the current fluctuations were minimal. Values calculated using depolarizing square pulses (Fig. 2A-C) or ramps were not significantly different and are here shown grouped together (Figure 2D). IBs in the two cell types reversed at significantly different membrane potentials (Figure 2D). In PCs $E_{\text{Total}}$ was -27.6mV (±7.2mV, St. Dev., n=13) and in O-LM cells $E_{\text{Total}}$ was more depolarized and averaged at -7.7mV (±9.6mV; n=21). The burst reversal potential showed such disparity even when dual whole-cell recordings were performed simultaneously in the two cell subtypes in the same slice. Comparatively more depolarized reversal potential values in O-LM cells support previous findings of more pronounced excitability of oriens interneurons prior to PC firing during interictal bursts and SLEs (Aradi and Maccaferri 2004; Ziburkus et al. 2006).

Measuring $E_e$ and $E_i$ in hyperexcitable media.

To calculate the dominant conductances during the prolonged duration SLEs we first needed to determine the excitatory and inhibitory reversal potentials ($E_e$ and $E_i$ respectively) in the presence of 4-AP and lowered [Mg$^{2+}$]$_o$ (Figure 3). Prior to application of 4-AP, equivalent stimulation of the Schaffer collaterals in normal ACSF solution in the presence of either the inhibitory or the excitatory blockers showed the $E_e$ reversed at 0mV (±6.7mV) and $E_i$ at -73mV (±5.9mV), (corrected for LJPV, n=5 in each condition). To measure $E_e$ in the presence of 4-AP, the inhibition was blocked by addition of picrotoxin (PTX) to the bath. In 4-AP and PTX, we observed spontaneous long duration excitatory bursts which reversed at highly positive membrane potentials ($E_e$=+23.5mV, Figure 3). In turn, to measure $E_i$ in the presence of 4-AP, all of the excitatory synaptic transmission was blocked by APV and CNQX and inhibitory currents
were electrically evoked. Inhibition reversed at the value of $E_i = -60\text{mV}$. This showed that in the absence of intrinsic voltage-gated properties of the cells and 4-AP, excitatory and inhibitory synaptic currents reversal values are shifted toward more positive membrane potentials.

**When excitation equals inhibition: $E_{total}$**

After determining the $E_e$ and $E_i$ values, we needed to determine the total synaptic current reversal potential values – $E_T$. During the repetitive spontaneous SLEs, PC and O-LM cells were temporarily voltage clamped at a range of negative to positive holding potentials (-80 to +30, Methods). Figure 4 (A-B) illustrates simultaneous dual whole-cell recordings in current and voltage clamp modes and the extracellular recording of the SLEs. The start of the body of SLEs was always determined by the fast positive excitatory shift (FPES) in the extracellular signal (Figure 4A, B). Qualitative observations of seizures in interneurons and PCs indicated that there is a dynamic conductance interplay associated with the initiation, body, and termination phases of the seizures.

An O-LM interneuron example with fourteen seizures recorded at different holding potentials is shown in Figure 4C. It illustrates that outward currents corresponding to inhibition, revealed at positive holding potentials, were more prominent before and after the ictal discharges; and inward currents, corresponding to excitation revealed at negative holding potentials, dominated during the body of the ictal discharges (Figs. 4A-C). When several seizures were acquired in the same cell at different holding potentials, it allowed us to measure dominant synaptic currents and produce linear fits of the average current-voltage (I-V plots) dynamics at every second before, during, and after the body of the SLEs. To isolate the synaptic inputs, leak currents were subtracted from the total current values. Figure 4D shows the calculated and fitted values of the
leak and synaptic currents before the start of the body of seizure for this illustrative interneuron. Leak currents increased as the holding potential was set at increasingly depolarizing potential values (Fig. 4D).

To measure \( g_e \) and \( g_i \) ratios, we selected only the data from O-LM and PC subtypes that were confirmed immunohistochemically and morphologically. Furthermore, the final conductance analysis was performed only on the data from the cells that showed sufficient number of seizures successfully recorded to incorporate at least six different holding potentials (see Methods section). For the final \( g_e/g_i \) calculations, leak currents were subtracted throughout the entire extent of the seizures as is shown in Figure 4D for the start of the SLE.

In order to calculate \( g_e \) and \( g_i \) we needed to determine the total synaptic conductance values \( g_T \) and the total synaptic reversal values \( E_T \) (equations 1 and 2). Holding cells at an array of potentials allowed us to calculate the average current-voltage (I-V) relationships in the pyramidal and O-LM cells throughout the entire extent of the seizure period (Figure 5, A and B). Average I-V plots for PC and OLM cells are shown in Figure 5 panels A and B. Each inset in the plots show the average SLE I-V plots for all of the O-LM and PCs at 1 sec intervals (calculated at an array of the holding potentials). The average SLE I-V plots were fitted linearly and the total synaptic conductance values \( g_T \) were obtained from the slope of these curves. The calculated slope values were steeper in O-LM cells as compared to the PCs.

Individual representative and average total synaptic reversal potential (\( E_T \)) values are shown in Figure 5 (C -E). Similarly to the ictal-like events reversed at more positive potentials in O-LM interneurons (Figure 5D, F) as compared to the PCs (Figure 5C, E).
To calculate $g_e$ and $g_i$ we also needed to determine the driving force, defined by the difference in the excitatory $E_e$ and inhibitory $E_i$ (Figure 3) and total synaptic conductance reversal potential $E_T$ (Figure 5; Equations 3 and 4). In both cell subtypes, the $E_T$ shifted to more positive values during the body of the seizures. In O-LM interneurons this excitatory shift persisted into the termination part of the seizures. In PCs, $E_T$ returned to pre-seizure levels 15 seconds after the start of the ictal event (FPES).

$g_e$ and $g_i$ interplay during SLEs.

Calculated average conductance values showed that prior to the initiation of the SLEs, inhibitory conductances dominate PCs (Figure 6A and C). In O-LM cells, inhibitory synaptic currents were more balanced in amplitude with the excitatory ones. During the start of the ictal events, $g_i$ and $g_e$ peak during the FPES with $g_e$ dominating both cell types (Figure 6A-D). Excitation remains dominant in OLM cells (Figure 6 B and D) while inhibition dominates PCs during the termination stage (Figure 6A and C). This strikingly increased excitation in the O-LM cells during the ictal discharge (Figure 6B and D) could be linked to the failure of the ‘inhibitory veto’ (Trevelyan et al. 2006), causing interneurons to enter a temporary depolarization block reported earlier (Ziburkus et al. 2006). The prolonged, yet gradually equalizing $g_e/g_i$ ratios during the termination phase in the O-LM cells could also be the only remaining mechanism of excitation-driven inhibitory termination of the SLEs (Fujiwara-Tsukamoto et al. 2004).

Statistical comparison of the $g_i$ and $g_e$ changes in the two cell subtypes highlighted the differences in the synaptic dynamics (Figure 6 E and F). Conductance balance in the PCs during SLE was significantly different at the initiation and termination of the SLEs, as the neurons were dominated sequentially by excitation and then inhibition (Figure 6E). In comparison, in O-LM
cells conductance balance showed a significant increase in excitation that lasted well into the body and termination stage of the SLEs (Figure 6F). This suggests, that during epileptogenic-like hyper-excitability the nearby located interneurons and PCs tune into distinct network processes, some dominated by inhibition, others by excitation.

DISCUSSION
To our knowledge we report the first comparative whole-cell voltage clamp study in excitatory and inhibitory cells during SLEs. The E/I balance in pyramidal and O-LM cell subtypes differed dynamically during the progression of the SLEs. At the onset of the SLEs, PCs were dominated by inhibitory inputs, but O-LMs received relatively balanced E and I inputs. Inhibition again dominated during the termination of SLEs in the PCs. In O-LM cells, excitation dominated the termination phase of the SLEs suggesting that continuous excitation of the inhibitory circuits persist, and may be an important mechanism to terminate the seizures. These dynamic changes in E and I conductances also support the notion of earlier work which suggested a tripartite division of seizures into distinct initiation, body, and termination stages (Schiff et al. 2005; Timofeev and Steriade 2004; Ziburkus et al. 2006). Present measurements provide a new perspective into the underpinnings of these sequential dynamical SLE stages. Conductance increases during pathological activity.

Large conductance increases during epileptiform events was previously observed only in the model of ‘paroxysmal depolarizing shifts’ (PDS) (Rutecki et al. 1987). When shorter duration PDSs were voltage-clamped in the presence of K-channel blockade (tetraethylammonium – TEA) in putative PCs, the authors found that initiating and terminating synaptic currents reversed at more negative potentials compared to the peak of the PDS. This suggested that the time points
preceding and following the PDSs contained a larger component of inhibitory synaptic input. Although in TEA, $E_e$ was measured, $E_i$ was not and synaptic conductance calculations were omitted (Rutecki et al. 1987).

E/I imbalance is often assumed to underlie seizures in epilepsy (McCormick and Contreras 2001); nonetheless, dynamics of $g_e$ and $g_i$ balance during more prolonged seizures have not been studied until now. Recently, the E/I balance theory also extended into the studies of the social behavior dysfunctions, such as autism and schizophrenia (Gogolla et al. 2009; Hashimoto et al. 2003; Kehrer et al. 2008; Markram and Markram 2010; Rubenstein and Merzenich 2003; Vattikuti and Chow 2010; Yizhar et al. 2011). As dynamical instabilities, seizure and SLE initiation may represent near maximal E and I conductances. It is likely, that the relation between the effects of altered E/I balance in seizures, and that of cognitive disorders and social dysfunction, will likely rely on different microcircuit patterns of activity.

**UP state vs SLE.**

Perhaps the best dynamically characterized normal recurrent neuronal network events are UP states in neocortical PCs recorded in vitro and in vivo (Borg-Graham et al. 1998; Haider et al. 2006; Shu et al. 2003). During UP states, the inhibitory and excitatory conductances in PCs in ferret neocortical slice preparations were shown to be balanced suggesting little temporal excitatory and inhibitory interplay (Shu et al. 2003). When the dynamic total reversal potential values were calculated from the *in vivo* studies in anesthetized ferrets, the initiation and termination phases of the UP states were shown to be dominated by excitation (Haider et al., 2006)). Alternatively, Rudolph and colleagues have shown that there can be a variety of E/I conductance changes during UP states in awake and naturally sleeping cats (Rudolph et al. 2007). Nonetheless, the differences in synaptic input dynamics between inhibitory and
excitatory cells using dynamic instead of the fixed $E_i$ and $E_e$ reversal potential values remain unknown. To date, all of the studies of E/I balance in UP states have used either experimentally or theoretically derived fixed $E_i$ and $E_e$ values when calculating synaptic conductances.

In the current study, we used similar techniques that were previously employed in vivo and in vitro to study UP state activity (Berg et al. 2007; Borg-Graham et al. 1998; Haider et al. 2006; Hasenstaub et al. 2005; Rudolph et al. 2007; Shu et al. 2003). To date, our understanding of the dynamical changes in the E/I conductance balance is limited to the analysis of the UP states. We used a common seizure model and calculated conductances in two subtypes of cells using whole-cell patch clamp during SLEs. We showed that there is a considerable disparity in the way CA1 O-LM interneurons and PCs receive network inputs as a function of distinct seizure stages. Our findings may be related to diverse activity patterns in local neuronal subsets observed in vivo in cats and in epileptic human patients (Rudolph et al. 2007; Truccolo et al. 2011).

Using dynamical total reversal potential values we show that during SLEs, similarly to UP states, $g_e$ and $g_i$ peak at the start of the seizures. Nevertheless, the SLEs studied here demonstrate a unique temporal interplay of $g_e$ an $g_i$, consistent with the temporal spiking activity interplay previously found in the same neuronal subtypes (Ziburkus et al. 2006). How UP states, or seizures, might dynamically function in disorders of E/I balance such as autism, schizophrenia, or models of other diseases remains to be investigated. Furthermore, additional studies of the excitatory and inhibitory conductances in other seizure and epilepsy models will be necessary to determine if the dynamics observed in 4-AP are present in other chronic or posttraumatic epilepsy models. In general, the complex interactions between and among the excitatory and inhibitory networks make it difficult to map the results of this experiment to other models. However, the temporal interplay and the maximal excitatory synaptic input barrage during the
ictal-like events observed here are likely to be a shared feature in many types of seizures in which E and I cell activity is preserved. Remarkably, the E/I interplay and heterogeneity in epileptiform cell signaling observed in 4-AP has now been demonstrated in several seizure models, and even in the epileptic human brain recordings; although we cannot be sure that the conductance measurements are the same, we do know that the interplay pattern is being found in many differing models, and may have broad applicability in helping to explain the instabilities in seizures (Cymerblit-Sabba and Schiller 2010; Derchansky et al. 2008; Fujiwara-Tsukamoto et al. 2010; Huberfeld et al. 2011; Lasztoczi et al. 2009; Trevelyan and Schevon 2012; Truccolo et al. 2011).

From the methodology perspective, dynamic total reversal potential provides a good approximation of the conductance changes (Hasenstaub et al. 2005). Nevertheless, we are cautious when interpreting the data based on dynamic total but static individual inhibitory (E_i) and excitatory (E_e) potential reversal values. In our experiments, seizures arise under the global influence of partial potassium conductance blockade of 4-AP. This compound elevates the resting membrane potentials towards depolarization, in both excitatory and inhibitory cells, and generates patterns of activity that share many similarities with in vivo experimental and clinical seizures. Furthermore, blockade of glutamatergic or GABAergic receptors may influence E and I reversal potentials through changes in steady state levels of ions. Although such pharmacological manipulations are used as a standard in such studies, we are cautious when assuming that the E_e and E_i values determined using synaptic receptor blockers are stable during heightened states of network excitability.

Nevertheless, beyond this pharmacological manipulation of normal hippocampus, changes in ionic driving forces as a function of ongoing activity has been noted in a variety of in vivo
models of epilepsy. In the pilocarpine model of epilepsy, $E_i$ can be shifted by upwards by up to 9 millivolts (Benini et al. 2011). In neonatal excitatory and inhibitory neurons of the hippocampus, chloride reversal was recently reported to be at an average of -50mV value, about 20mV above the value reported and calculated in mature neurons in vitro (Dzhala et al. 2010). On the other hand, Khirug et al report that even a single seizure can redistribute chloride transporters and lower the $E_{\text{GABA}}$ into more negative values in the CA1 neurons (Khirug et al. 2010). These findings all suggest that increasing excitability through partial depolarization of neurons may generate seizures, and perhaps the precise level of depolarization required to observe seizures may be less important than the functional balance between excitation and inhibition achieved.

UP states, interictal bursts, and especially prolonged duration SLEs allow for significant changes in the extra and intracellular ionic compositions (Cressman et al. 2009; Ullah et al. 2009), which must alter the individual $E_e$ and $E_i$ values. Apart from the intracellular chloride loading, increases in the intracellular sodium and extracellular potassium $[K^+]_o$ concentration often take place during epileptogenic or heightened neuronal activity (Somjen 2004). During seizures, $[K^+]_o$ can change by 3-4 times of its basal concentration, yet even small fluctuations in $[K^+]_o$ raise $K^+$ reversal potential exerting significant effects on neuronal excitability. For example, our ongoing computational investigations suggest that increases in extracellular potassium concentrations from 4mM to 12mM causes over 10 millivolt shift in the reversal potential of glutamatergic AMPA receptors (Cressman et al. 2009). Nonetheless, the ultimate and comprehensive representations of the E/I dynamics will require individual ionic driving forces underlying $E_e$ and $E_i$ to be simultaneously and dynamically monitored, a challenge presently beyond our technical capabilities.
Furthermore, extracellular calcium concentration during the ictal events in 4-AP can also drop dramatically. In previous studies, extracellular calcium concentration in brain slices was shown to drop from 1.8 to ~1.3mM (Avoli et al. 1996b) during ictal activity. In cultured cell preparations, similar reduction in calcium resulted in nearly fourfold reduction in the amplitude of excitatory postsynaptic potentials (Rettig et al. 1997). Our experiments were performed in 1.2 mM calcium concentration, a low baseline level, and show that the excitatory synaptic activity increases during the interictal bursts and ictal-like events; with a selective and temporal loss of OLM interneuron spiking activity (Hazra et al. 2012; Ziburkus et al. 2006). In the absence of sufficient extracellular calcium, synaptic transmission may be reduced and electrical coupling may play a role in the dynamics we see. However if the cell were attached ohmically to a set of voltage source that changed in a stereotypical way during a seizure then the currents at lower holding potentials would be affected in similar pattern to ones held at higher potentials. This is not seen in these experiments as the time course of excitatory and inhibitory currents do not reflect such common patterns. Finally, one recent report suggests that gap junctions may not play as significant of a role as was previously thought in generating epileptiform activity in the 4-AP model (Connors 2012).

*E and I cells ‘tuning into’ different network inputs.*

The increased variability and depolarized values of the total reversal potential during interictal burst and SLEs in the O-LM cells suggest that interneurons of the same subtype may receive or tune into different inhibitory and excitatory synaptic inputs. Increased excitation of the O-LM cells is also consistent with our previous reports using current clamp recordings in the same seizure model which showed O-LM cells discharging spikes at higher rates before seizure
initiation, followed by the prolonged depolarization block during the body of the SLEs, during which PCs fired at high rates (Schiff S 2008; Ziburkus et al. 2006).

Under conditions in which voltage-gated conductances are not blocked, the balance of E and I inputs depends upon their relative somatodendritic location (Pouille and Scanziani 2004). Computational modeling and experiments show that space clamp non-uniformity is substantial for voltage-gated conductances even in the proximal branching dendrites of non-spherical cells (Armstrong and Gilly 1992). Inclusion of voltage-gated conductance blockers (QX-314 and Cs\(^{2+}\)) in our experimental paradigm allowed us to use our recorded cells as passive antennas tuned into the hyperexcitable network (Bar-Yehuda and Korngreen 2008; Fleidervish and Gutnick 1996). In this way, both the inhibitory and the excitatory conductances in the two cell subtypes were unaffected by the active voltage-gated conductances (Bar-Yehuda and Korngreen 2008). Attenuation in the strength of the synaptic inputs would then depend only on the passive membrane properties and the overall axial resistance. Given that O-LM cells are electrotonically more compact than PCs, this could cause overestimation of the total reversal potential values in PCs. Previous *in vivo* and *in vitro* measurements of conductances employed sharp electrode recording techniques in putative PCs (Berg et al. 2007; Borg-Graham et al. 1998; Haider et al. 2006; Hasenstaub et al. 2005; McCormick 2003). Although this type of recording does not affect the intracellular environment to the same extent as does the patch pipette, it is at a disadvantage in its space-clamping ability. Nevertheless, it remains to be seen whether epileptic conductance dynamics observed here in distinct inhibitory and excitatory cell subtypes follow the same trends *in vivo* and in other *in vitro* seizure models.

*The role of early inhibitory network recruitment and its clinical significance.*
During SLEs, inhibition dominated PCs except for a restricted period of runaway excitation at seizure initiation. In many normal brain processes, such as UP states or other neuronal oscillations (Buzsaki 2006), inhibition plays a precise role in either balancing or shaping the excitatory responses. For example, during both the conscious and unconscious states, neocortical neurons can be dominated by inhibition (Rudolph et al. 2007). Other experiments in neocortical slices using cell-attached recordings and calcium imaging showed that an inhibitory ‘veto’ precedes the breakthrough excitation of epileptiform discharges (Trevelyan et al. 2006; Trevelyan et al. 2007). These experiments suggest that inhibitory inputs play important roles in the early defense and potential forecasting of epileptic activity.

When interpreting EEG recordings of seizures clinically, one has to consider that underlying variations in the signal may stem from interplay of inhibitory and excitatory networks and their conductances (Schiff et al. 2005; Truccolo et al. 2011). In an experimental setting, focally generated theta rhythms can be synchronized by local inhibitory inputs (Glasgow and Chapman 2007). In epilepsy, potentially overcompensating increases in inhibition may also signal the start of the seizure (Truccolo et al. 2011). The pronounced early inhibitory component in PCs (Figure 6A) in the current study suggest that network inputs may be controlling these cells at first but then failing and allowing runaway excitation to peak at the start of the SLEs. Likewise, increased excitation of the inhibitory cells following the peak of the conductances sustains the overall inhibitory network activity and promotes the inhibitory seizure ‘terminating veto’. It seems likely that these novel network mechanisms act concurrently with others, such as changes in ionic conductances (Cressman et al. 2009; Ullah et al. 2009), contributing to a transient pathological failure in neuronal activity control. We are hesitant to over-simplify seizure dynamics, and instead suggest a multifaceted mechanism of pathology that spans failures in individual ion
concentrations, intrinsic cellular properties and network dynamics. Greater understanding of intrinsic cellular and network failures during seizures will help us construct more accurate dynamical models to be applied for improved seizure understanding, forecasting, and cell subtype-specific control strategies.


Figure legends:

Figure 1. Examples of O-LM interneurons and excitatory pyramidal cells and summary of the recordings. A. Neurolucida reconstructions of O-LM (left) and pyramidal (right) cells filled with neurobiotin (NB). Somas and dendrites are in black and axons in white. s.l.m. – stratum lacunosum moleculare; s.p. – s. pyramidale; s.r. – s. radiatum; s.o. – stratum oriens. B. Examples of the recorded neurobiotin-filled cells (NB) cross-stained with parvalbumin (PV, green) or somatostatin (SOM). Top photomicrograph illustrates NB-positive cell in the pyramidal layer surrounded by PV-positive pyramidal layer interneurons. Bottom: SOM and NB positive O-LM interneuron. C. Table summarizing electrophysiological studies and cell identification criteria.
Figure 2. Interictal burst reversals in O-LM and pyramidal cells. A. Concurrent whole-cell and extracellular recording traces of spontaneous excitatory potentials in a pyramidal cell held at -70mV in a current clamp mode. B. Excitatory postsynaptic currents recorded in the pyramidal cell held at -70mV using voltage-clamp. C. Inhibitory currents recorded in the pyramidal cells at +20mV. D. Interictal burst reversal potential values were significantly different between the two types of cells: Pyramidal (-27.6 ± 7.2mV, n=13) and O-LM (-7.7 ± 9.6mV, n=21) cells (p<0.0001, unpaired t-test).
Figure 3. Inward (excitatory) and outward (inhibitory) current reversals. A. Spontaneous excitatory inward current bursts in the presence of picrotoxin (PTX) at an array of holding potentials (representative traces from CA1 pyramidal cell). B. Confirmation that these were glutamatergic excitatory potentials was performed by addition of AMPA and NMDA receptor blockers (CNQX, 10μM and APV, 25μM) and holding the cell at -70mV in current clamp mode. Bottom traces: five and ten minutes following addition of the blockers. C. Inhibitory burst reversals in the presence of APV and CNQX (representative traces from CA1 pyramidal cell). D. Blockade of inhibitory currents by PTX (50μM) at 0mV holding potential in voltage clamp mode 5 and 10 minutes following addition of the blocker. E. Average $E_e$ and $E_i$ reversal potentials in 4-AP. ($E_e = +23.5\pm6.9mV$ and $E_i = -60\pm9.7mV$).
Figure 4. In vitro seizure recordings in current and voltage clamp modes. A. Traces of repetitive seizures recorded simultaneously in the current (pyramidal, top trace), voltage clamp (O-LM interneuron, middle trace), and extracellular recordings (bottom). PC is in current clamp at -63mV and O-LM interneuron is held at -70mV in voltage-clamp. Cesium gluconate was substituted with potassium gluconate and QX-314 was omitted from PC recording solution. B. Same cell pair as in A, but O-LM interneuron is voltage clamped at -+40mV to record outward currents. All of the seizure ictal event start times were aligned to the fast positive extracellular potential shift (FPES, Ziburkus et al 2006a). C. Example of fourteen repetitive seizure traces recorded at different holding potentials (-80mV to +35mV) in the same O-LM interneuron as in A and B. Note the qualitative differences in the dominant currents before, during, and after the termination of the ictal events. D. Calculated synaptic and leak currents for the traces in C shown at a single time window for one second preceding the FPES.
Figure 5. Total synaptic current reversals during SLEs. A and B. Average continuous current-voltage (I-V) curves in the pyramidal (E, n=7) and O-LM (F, n=13) cells. Each blue trace represents the average I-V values derived from a number of holding potentials at one second intervals (insets show an expanded I-V curve and its linear fit from near the start of the SLEs). The slope values are the calculated average total conductances. C and D. Total reversal potential plots from the representative set of pyramidal (left, n=7) and O-LM (right, n=5) cells. Each line/color corresponds to averaged measurements in one cell. Seizure start time (FPES) at 0 seconds is indicated with the dashed line. E and F. Average plots of the total synaptic reversal potentials E_{total} or E_{T} for the pyramidal (C, n=7) and O-LM (D, n=13) cells.
Figure 6. Dynamics of $g_e$ and $g_i$ in PC and O-LM cells during SLEs. A and B. Average relative $g_e$ and $g_i$ values during SLEs in the PC (A, n=7) and O-LM (B, n=13) cells. Inhibition dominated PCs. Excitation and inhibition was more balanced in the O-LM cells prior to the ictal events, but excitation dominated the post-ictal phase in the O-LM cells. C and D. Normalized $g_e$ and $g_i$ values during SLEs in the PC and O-LM cells from A and B. Multiple running ANOVA analysis was performed on the conductance data with a confidence level of 95% or higher. The asterisks in panels A through D denote whether the values at seizure onset is significantly different from any other time point across the seizure. E and F. Multiple running ANOVA analysis on the average $g_e/g_i$ ratios for PC (E) and O-LM (F) cells. Asterisks indicate the ratio of the conductances in the given bin that were significantly different ($p<0.05$) from the index point (filled square at seizure start in E, and before the FPES in F). Conductance ratio for PC cells showed difference at 6 different time points: 2 before and 4 after the seizure. In the O-LM cells, only 3 time points at the start of the ictal events were significantly different. Dashed lines indicate the start time of the SLEs.
FIG. 1

A

s.l.m.

s.r.

s.p.

s.o.

B

PV

NB

SOM and NB

10μm

C

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FIG. 2

A. EPSP

B. EPSC

C. IPSC

D. Histogram of total potentials with N=21 and N=13.
FIG 5.

A. PYRAMIDAL

B. O-LM

C. E_{total} (mV) vs Time (sec)

D. E_{total} (mV) vs Time (sec)

E. E_{total} (mV) vs Time (sec)

F. E_{total} (mV) vs Time (sec)
FIG 6.

A. PYRAMIDAL

Conductances (nS/mm²)

Time (sec)

B. O-LM

Normalized Conductances

Time (sec)

C. PYRAMIDAL

D. O-LM

E. Bin number

F. Bin number