Transitions between neocortical seizure and non-seizure-like states and their association with presynaptic glutamate release

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**ABSTRACT**

The transition between seizure and non-seizure states in neocortical epileptic networks is governed by distinct underlying dynamical processes. Based on the gamma distribution of seizure and inter-seizure durations, over time, seizures are highly likely to self-terminate; whereas, inter-seizure durations have a low chance of transitioning back into a seizure state. Yet, the chance of a state transition could be formed by multiple overlapping, unknown synaptic mechanisms. To identify the relationship between the underlying synaptic mechanisms and the chance of seizure-state transitions, we analyzed the skewed histograms of seizure durations in human intracranial EEG and seizure-like events (SLEs) in local field potential activity from mouse neocortical slices, using an objective method for seizure state classification. While seizures and SLE durations were demonstrated to have a unimodal distribution (gamma distribution shape parameter > 1), suggesting a high likelihood of terminating, inter-SLE intervals were shown to have an asymptotic exponential distribution (gamma distribution shape parameter < 1), suggesting lower probability of cessation. Then, to test cellular mechanisms for these distributions, we studied the modulation of synaptic neurotransmission during, and between, the in vitro SLEs. Using simultaneous local field potential and whole-cell voltage clamp recordings, we found a suppression of presynaptic glutamate release at SLE termination, as demonstrated by electrically- and optogenetically-evoked excitatory postsynaptic currents (EPSCs), and focal hypertonic sucrose application. Adenosine A1 receptor blockade interfered with the suppression of this release, changing the inter-SLE shape parameter from asymptotic exponential to unimodal, altering the chance of state transition occurrence with time. These findings reveal a critical role for presynaptic glutamate release in determining the chance of neocortical seizure state transitions.

1. Introduction

Epilepsy is a dynamic disease characterized by transitions into and out of the seizure state (Falco-Walter et al., 2018; Kalitzin et al., 2019). The stability of these transitions can provide key indicators of the underlying brain networks involved (Suffczynski et al., 2006). Based on the shape of the distribution of seizure and inter-seizure event durations, the inter-seizure state is stable with time, whereas seizures self-terminate with high likelihood (Suffczynski et al., 2006). This observation is markedly consistent across patients and models (Suffczynski et al., 2006; Bauer et al., 2017; Breton et al., 2019), yet the underlying cellular networks involved in these distributions are unknown. Hence, this study focuses on the synaptic activity underlying the distribution of seizure and inter-seizure event durations.

Though multiple studies have tested the synaptic mechanisms underlying burst termination (Staley et al., 1998; Jones et al., 2007), and seizure onset and termination (de la Prida and Trevelyan, 2011; Zhang et al., 2012; Trevelyan and Schevon, 2013; De Curtis and Avoli, 2016;...
Elahian et al., 2018; González et al., 2019), the synaptic mechanisms contributing to seizure state distributions remain unclear. In recent publications, pharmacologically blocking synaptic glutamate release was recommended for the treatment of epilepsy (Wong et al., 2015; Wu et al., 2015; Lazarevic et al., 2018), and could therefore be a potential synaptic contributor to these processes. Glutamatergic neuronal currents are coherent with delta frequency oscillations, in vitro (Flint and Connors, 1996; Steriade, 2003; Doi et al., 2007; Mitrukha et al., 2014; Breton et al., 2019). The delta oscillation, and its association with high frequency oscillations (> 30 Hz), is elevated during seizures (Tao et al., 2011; Ren et al., 2011; Guirgis et al., 2013; Guirgis et al., 2014; Ibrahim et al., 2013); hence, the glutamatergic network underlying these oscillations could modulate either the seizure-like event (SLE) or the inter-SLE state distributions, or both. Hippocampal electrographic burst durations and inter-burst intervals are shown to be regulated by the depletion and recovery of presynaptic glutamate (Staley et al., 1998; Jones et al., 2007). However, the pattern of glutamate release and its influence on the transitions into and out of the seizure state in the longer and more complex SLEs, as opposed to brief epileptiform bursts, are not elucidated. Furthermore, it is unknown if the changing strength of glutamatergic synaptic transmission throughout the ictal state plays a dynamic role in, or is a passive consequence of, the seizure activity (Köhling, 2014).

Presynaptic glutamate release could be modulated during seizures through multiple different mechanisms, such as those involving adenosine A1 receptors (Etherington and Frenguelli, 2004; Fedele et al., 2006; Li et al., 2007; Wang et al., 2013; Köhling, 2014; Wang et al., 2015), pannexin-1 channels (Bialecki et al., 2020; Yeung et al., 2020; Li et al., 2007; Wang et al., 2013; Köhling, 2014); hence, the glutamatergic network underlying these oscillations could modulate either the seizure-like event (SLE) or the inter-SLE state distributions, or both. Hippocampal electrographic burst durations and inter-burst intervals are shown to be regulated by the depletion and recovery of presynaptic glutamate (Staley et al., 1998; Jones et al., 2007). However, the pattern of glutamate release and its influence on the transitions into and out of the seizure state in the longer and more complex SLEs, as opposed to brief epileptiform bursts, are not elucidated. Furthermore, it is unknown if the changing strength of glutamatergic synaptic transmission throughout the ictal state plays a dynamic role in, or is a passive consequence of, the seizure activity (Köhling, 2014).

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### Table 1

Summary of seizure details recorded from the iEEG of patients with refractory epilepsy. Engel class 1 means that the patients were seizure-free after surgery. N/A indicates that the data is not available.

| Patient # | 1 | 2 | 3 | 4 |
|-----------|---|---|---|---|
| Age       | 36| 28| 21| 26|
| Sex       | F | M | M | F |
| Engel Class| N/A| 1 | 1 | 1 |
| Number of seizures | 1 | 3 | 3 | 2 |
| Sample Rate (Hz) | 2000| 2000| 2000| 500|
| MRI Findings | Abnormal Intensity, lesion | Non-lesional | Cortical Dysplasia | Normal |
| Electrode Placement | Left frontal-temporal | Left fronto-temporal | Left Dorsolateral-frontal | Right Frontal |

2. Materials and methods

2.1. Patient description

Nine seizures were recorded from 8 × 8 intracranial electroencephalogram (iEEG) grids placed on the neocortex of four patients being investigated for intractable epilepsy (Phramongkutkla Hospital, Bangkok, Thailand; Toronto Western Hospital, Toronto, Ontario, Canada). Data from these patients were used in previous studies (Cotic et al., 2014; Guirgis et al., 2015; Breton et al., 2019). Patients and the ethics committees provided informed consent and approval, respectively. The time series used for analysis was chosen from the electrodes previously identified as the seizure onset zone by expert electroencephalographers and confirmed using cross-frequency coupling based strategies (Guirgis et al., 2015). To demonstrate how the 64-electrode iEEG grid was linked to create a differential montage of 32-channels, see Guirgis et al. (2015). The data obtained from the Canadian institution was notch-filtered at 60 Hz and its harmonics up to Nyquist, and the data obtained from Thailand was notch-filtered at 50 Hz and its harmonics. Before filtering to specific oscillations, all iEEG signals were decimated to a final sampling rate of 500 samples per second. For summary of patient details, see Table 1.

2.2. Animals

C57/B16 male and female mice aged P9 to P16 (Charles River Laboratories, RRID:SCR_003792; RRID:IMSR_JAX:000664) were humanely killed for these experiments in accordance with the Canadian Animal Care (CCAC) Guidelines and with the United States Public Health Service’s Policy on Humane Care and Use of Laboratory Animals. Thy1-ChR2-YFP mice from a C57 background were obtained from the Jackson laboratories (line 18; B6.Cg-Tg(Thy1-COP4/EYFP)18Gfng/J), then housed and bred in a specialized animal facility. Optogenetic expression of ChR2 from this rodent line is mainly in the thalamo-cortical projection neurons and pyramidal cells in layers IV/V of the neocortex (Arenkiel et al., 2007). To confirm the layer-specific expression of the Thy1-ChR2-YFP, after an optogenetic experiment, we took an image of a brain slice using a confocal microscope. All surgical procedures were approved and completed in accordance with the guidelines of the Animal Care Committee of the University Health Network.

2.3. Cortical slice preparation

Mice were anesthetized using 50 mg/kg pentobarbital. Once deeply anesthetized, identified through the pedal reflex test, the mice were decapitated and their whole brain was removed. The brain was placed in an ice cold, oxygenated sucrose solution made up of (in mM): 248 sucrose, 2 KCl, 3 MgSO4, 1 CaCl2, 26 NaHCO3, 1.25 NaH2PO4, 10 D-glucose. Then the cortex was fixed to a vertical block using a cyanoacrylate adhesive. Coronal slices (500 μm) were sectioned using a Leica 1200 V vibratome. Then, each slice was hemisectioned and transferred to artificial cerebral spinal fluid (ACSF, 95% oxygen, 5% CO2 containing (in mM): 123 NaCl, 25 NaHCO3, 10 glucose, 3.5 KCl, 1 MgSO4,
1.2NaH2PO4, 1.5CaCl2. The slices were incubated for 30 min at 32 ± 0.5 °C followed by at least 1 h at room temperature prior to the start of the experiments.

2.4. Electrophysiology

For each experiment, a slice was transferred to a submerged recording chamber and perfused with ACSF (95% oxygen, 5% CO2; 10 ± 1 mL/min; 34 ± 0.5 °C). Glass electrodes (1.5 mm, World Precision Instruments) were used for both local field potential (LFP) and whole cell recordings. The LFP recording electrodes (~2 MΩ resistance) were filled with ACSF and placed within 200 μm of the intracellular electrodes in neocortical layers II/III. An Olympus BX51 microscope (OLY-150IR camera–video monitor unit) was used with an infrared filter to visualize the neurons for whole cell patching. The whole cell recording electrodes (3-5 MΩ resistance) contained a solution of (in mM): 135 K-Gluconate, 1 MgCl2, 10NaCl, 2 Na2ATP, 0.3 NaGTP-Tris, 10 NaHEPES, 0.5 EGTA, 0.0001 CaCl2, pH 7.2–7.3. Signal acquisition and storage were performed using an amplifier (Multiclamp 700B), a digitizer (Digidata 1322A) and PClamp software (version 10.2; Axon Instruments/Molecular Devices Corporation). Whole cell recordings were done in either current clamp or voltage clamp mode. Neurons were held at −70 mV for excitatory postsynaptic current (EPSC) acquisition.

Putative pyramidal neurons were identified based on their appearance under the infrared filter and their electrophysiological features identified using a series of hyperpolarizing and depolarizing current pulses (200 ms duration) through the whole cell recording electrode. A calculation of input resistance, 163 ± 59 MΩ, spike half-width, 1.63 ± 0.55 ms, and membrane time constant, 27.87 ± 14.2 ms (n = 39 cells, average ± standard deviation) was consistent with previous results (Breton et al., 2019). We did not correct for liquid junction potential since all results central to the hypothesis were following formula:

\[ \text{MPD}_{i} = \frac{a_i(i)b_i(i)}{\sum a_i(i)b_i(i)} \]

where \( a \) is the joint probability of observing all data from time 1 to \( i \), and \( b \) is the conditional probability of all data from time \( t + 1 \) to \( T \) at each hidden state \( i \).

To make the feature set used for the classifier, the LFP recording was decimated to a final sampling rate of 200 samples per second (500 samples per second for the human iEEG). Then, four time-series were extracted from the signal [0.5 to 1-Hz, 1 to 2-Hz, 2 to 3-Hz and 30 to 80-Hz] using 10,000 order Finite Impulse Response (FIR) filters run in the forward and reverse direction (filtfilt.m, MATLAB). Each feature was z-score normalized to the first 10s of the non-SLE signal. The resulting features were squared and averaged over 1 s windows. Training was performed on one randomly chosen SLE and inter-SLE time segment in low Mg2+. Testing was performed on the entire signal using 2 s windows with 1 s overlap. The marginal posterior distributions (MPDs) of the HMM were computed using the following formula:

\[ \text{MPD}_{i} = \frac{a_i(i)b_i(i)}{\sum a_i(i)b_i(i)} \]
specific state. The classified states were named inter-SLE state (state 1) and SLE state (state 2). The MPD probability of 0.5 was chosen as the threshold for the onset and termination of the events. This is the threshold for the most likely state sequence of the 2-state HMM.

2.7.2. SLE and inter-SLE criteria

To avoid potential errors in the HMM classification, for an SLE state to be considered, the LFP signal was required to rise 3× the baseline signal during the classified SLE state, and the signal was monitored for LFP artifacts. The minimal duration was dependent on the resolution of the HMM classification. Using this approach, the shortest SLE and inter-SLE intervals were 7 s under low Mg$^{2+}$ conditions. In contrast, in DPCPX, the shortest SLE state was 2 s and inter-SLE interval was 1 s, and in PTX the shortest SLE state was 1 s and inter-SLE interval was 3 s. As a fixed-length time window may under and overestimate the contribution of the low and high frequencies used in the classifier, respectively, we checked the sensitivity of the classifier to window size (Supplementary Fig. 1). The SLE state duration remained consistent for multiple time windows chosen from a window size of 2 s onward.

For visualization purposes, time frequency spectrograms were computed by convolution of the LFP signal with a complex Morlet wavelet with bandwidth of 5-Hz and 0.8125-Hz center frequency. Then the data were z-score normalized to a 10s quiescent period prior to the onset of the seizures or SLEs.
To quantify the chance of SLE and inter-SLE state transitions, the shape parameter of a gamma distribution was computed using the `gamfit` function in MATLAB. It was represented in the figures using the `histfit` function with the 'gamma' distribution specified. The gamma distribution function has a shape parameter, a scale parameter and a normalization constant (Thomopoulos, 2017). The shape of the distribution can range from asymptotic exponential (shape parameter ≤ 1) to unimodal (shape parameter > 1). In the case of asymptotic exponential, the resulting hazard function predicts a decreased chance of a state transition with time. In the case of unimodal, the resulting hazard function predicts an increased chance of a state transition with time.

Performance of the HMM classifier was determined using receiver operator characteristic (ROC) curves, as previously done (Breton et al., 2019). The SLE onset was defined as the time where the LFP signal rose to 3× the baseline signal. Baseline for each SLE was defined as 5 s of stable quiescent LFP activity between each SLE. SLE termination was defined as a return to baseline after a final field bursting event. During the inter-SLE interval, false positive (Fp) was obtained if, for each point on the MPD of state 2, state 2 was classified as higher probability than state 1, and the opposite was considered true negative (Tn). During the SLE, true positive (Tp) was when state 2 remained in higher probability than state 1, and if the probability distribution switched, this was false negative (Fn). The threshold for defining the SLE interval was varied between 0 and 1 on S2 to obtain the points on the ROC curve.

Table 2
Summary of details for seizure-like event (SLE) models used in this study.

| Model type                          | Time to SLE onset (min) | Duration of specified intervals (median ± SEM) | N’s (# SLEs/slices/animals) |
|-------------------------------------|-------------------------|-----------------------------------------------|-----------------------------|
| Low Mg\(^{2+}\) spontaneous         | 6.8 ± 2.3               | 47 ± 11                                       | 44 ± 16                     | 60/11/8                    |
| Low Mg\(^{2+}\) with electrically evoked stimulus | 5.1 ± 1.8               | 48 ± 13                                       | 65 ± 12                     | 29/7/6                     |
| Low Mg\(^{2+}\) with optogenetically-evoked stimulus | 5.5 ± 1.5               | 32 ± 11                                       | 61 ± 26                     | 30/5/3                     |
| Low Mg + CNQX + dAPV                | –                       | 0                                             | Complete Blockade           | 0/5/3                      |
| Low Mg + picrotxin, 100uM           | –                       | 13 ± 1.3                                      | 17 ± 1.7                    | 48/5/3                     |
| Low Mg + DPCPX, 0.25uM              | –                       | 67 ± 14                                       | 8.4 ± 1.1                   | 29/7/6                     |
| Low Mg + DPCPX, 0.5uM               | –                       | 27 ± 4.7                                      | 7.5 ± 1.4                   | 40/9/5                     |
| Low Mg + DPCPX, 1uM                 | –                       | 36 ± 7.4                                      | 7.0 ± 1.0                   | 31/4/3                     |

Fig. 2. The effects of glutamate and GABA receptor blockade on SLE and inter-SLE state distributions. (A) LFP recordings of SLEs under low Mg\(^{2+}\) ACSF alone, and in low-Mg\(^{2+}\) with the addition of 20 μM CNQX + 50 μM d-APV, or 100 μM picrotxin (PTX). Each example includes the stem plot showing the MPD of the HMM of the SLE state zoomed into the 0.5–0.55 probability and the LFP recording. The grayed areas indicate the timing of addition of the pharmacological antagonists. All traces and antagonists were applied during continuous low-Mg\(^{2+}\) perfusion. The circles are residual peaks in the stem plots of the MPD of the HMMs. (B) Histograms of the durations of the SLE and inter-SLE state with their respective gamma-distribution fits as defined by the MPDs for the low Mg\(^{2+}\) + PTX conditions. (C) Summary of the shape parameter for the gamma distributions for Low Mg\(^{2+}\), low Mg\(^{2+}\) with CNQX and d-APV, and low Mg\(^{2+}\) with PTX, showing unimodal SLE durations, and asymptotic exponential inter-SLE durations for both low-Mg\(^{2+}\) and low-Mg\(^{2+}\) + PTX conditions. The N’s used to compute the shape parameter are in Table 2. Datasets are considered significantly different if their error-bars do not overlap.
Sensitivity and specificity were computed as per (Breton et al., 2019).

2.7.4. Electrophysiological analysis

Time traces with shading around the curves (Fig. 6) were constructed using the shadedErrorBar function in MATLAB using the average signal ± the standard error on the average. To quantify the excitatory current response to various stimuli, the charge transfer energy was computed using the trapezoidal rule (trapz.m). Intensity was measured as the square of the voltage signal of the SLE state divided by the total SLE state time.

2.7.5. Time constant estimation

For the post-SLE state time constants, the following equations were applied:

\[ f(t) = Ae^{-\frac{t}{\tau}} + C \]  
\[ g(t) = \frac{1}{B + e^{-mt}} \]

where \( \tau \) is the time constant of the exponential curve (Eq. 2) and \( m \) is the slope parameter of the exponential curve (Eq. 3) computed over various intervals after the SLE state. \( A, B \) and \( C \) are normalization parameters of the model, and \( t \) is time. For the sucrose evoked post-SLE rate parameter approximation, we had tested multiple regressions (exponential, log, linear, sigmoid), however, the sigmoid curve best represented the shape of the data.

2.8. Statistics

Unless otherwise indicated, all bar-plots were presented in median ± 25th and 75th percentiles. To test the null hypothesis that each dataset came from a normal distribution with mean and variance unspecified, we used a Shapiro-Wilk test. If it was normally distributed, then an unpaired \( t \)-test was applied. If it was not normally distributed, then the Wilcoxon rank-sum test was applied. To test for multiple-comparisons, we applied a Bonferroni’s post-hoc correction. The Spearman Correlation was used for analyses of two variable correlations. For the empirical cumulative distribution function, the Kolmogorov-Smirnov test (kstest2) was applied to test the null hypothesis that two signals belonged to the same distribution. All statistical tests were done using MATLAB software.

3. Results

3.1. Neocortical SLEs have unimodally distributed SLE and asymptotic exponentially distributed inter-SLE state durations

This study aimed to objectively identify the distribution of SLE and inter-SLE state durations and to determine how underlying synaptic networks modulate these distributions. First, a 2-state HMM was used to (1) ensure that the identified SLE states directly modeled the seizure activity in patient EEGs, and (2) objectively define the onset and termination of the seizures/SLEs. We qualitatively examined the time-frequency spectra for nine seizures from four patients with refractory epilepsy (Table 1, Fig. 1A). We observed that all patients had high-power delta (0.5-3 Hz) and gamma (30-80 Hz) throughout their
seizures. Then, a two-state HMM (using delta and gamma features) was trained on the seizure and inter-seizure state of one patient. We tested the model on the remainder of the patient datasets and the rodent SLEs. The closer the MPD of the HMM was to 1, the higher the probability that the tested dataset resembled the trained patient dataset. Applying this approach, we observed that the SLE state modeled the seizure state, with high probability, and the 2-state HMM accurately classified the seizure state in human iEEGs (Fig. 1A, C) and the SLE state mouse neocortical slices (Fig. 1B, E). This provided an objective measurement of the onset and termination of the SLEs.

Using the objectively classified onset and termination of the SLEs and seizures, the shape parameters of the distribution of the SLE and inter-SLE state durations were measured by fitting a gamma distribution to the histogram of the classified states (Fig. 1D, F). We found that the shape parameter of the gamma distribution of both the seizure and SLE states was > 1, implying both seizures and SLEs were unimodally distributed and followed a similar underlying dynamical process. Furthermore, the shape parameter of the inter-SLE state distribution was < 1, indicating the inter-SLE state followed an asymptotic exponential distribution. To confirm that the width of the binning of the histogram did not alter the gamma distribution, we repeated the gamma distribution parameterization for various window sizes (Supplementary fig. 2), with consistent results over multiple conditions.

Glutamatergic or GABAergic synaptic activity could modify the shape parameter of the gamma distributions of the SLE or inter-SLE state (Fig. 2). Therefore, we blocked excitatory synaptic transmission using a combination of CNQX and d-APV. Then, in a separate study, we blocked inhibitory synaptic transmission using picrotoxin (PTX). The addition of CNQX and d-APV blocked the generation of SLEs (Fig. 2A). In some cases, the MPD of the HMM showed some elevated probability; however, these events were considered model confusion, as they were not associated with large amplitude activity or SLEs in the LFP. We interpreted this as a shape parameter approaching infinity or tending to zero (Fig. 2C). In contrast, PTX reduced the duration of the SLE and inter-SLE states (Table 2). Yet, the shape parameter of the SLE and inter-SLE states did not change from previous low Mg²⁺ conditions (Fig. 2C). Since modulation of GABAergic signaling led to varied timing in seizure onset, whereas modulation of glutamatergic signaling indefinitely sustained the inter-SLE interval, these data provided evidence that synaptic glutamatergic activity plays a dominant role in the distribution of inter-SLE durations underlying the neocortical low Mg²⁺ SLE model.

The next series of experiments were used to identify the association of glutamatergic currents with the distribution of low Mg²⁺ SLE durations. Evoked excitatory synaptic transmission was measured during classified SLEs. Then, we identified changes in the size of the readily releasable pool (RRP) of glutamate during SLEs using hypertonic sucrose solution. Lastly, we modulated these changes using an adenosine A1 receptor antagonist and studied the effects of this blocker on the SLE and inter-SLE states.

3.2. Evoked excitatory synaptic transmission is reduced at SLE termination and during the post-SLE state

To measure evoked excitatory currents during low Mg²⁺ SLEs, we recorded from pyramidal neurons held in voltage clamp at −70 mV, close to the reversal potential for GABA. To avoid the effects of LFP stimulation artifacts on the classification, the onset and offset of SLEs were identified using the pre-trained 2-state HMM on the intracellular currents (Fig. 3A, for confirmation that this approach did not substantially alter the statistical distributions, see Supplementary fig. 3). Then, we subdivided these states into baseline, pre-SLE, onset, and termination. Within each of these subdivided states, we examined excitatory synaptic transmission by applying low strength, short-duration stimuli to layer V, every 10s, over the course of the SLE state (Fig. 3).

After SLE termination, the evoked EPSCs reached a minimum, as compared to the SLE state.

Yet, electrically evoking these neurons may also be synchronously activating glia, interneurons, and axons-of-passage. To mitigate this, we repeated the experiment by optogenetically stimulating layer V pyramidal neurons of Thy1::ChR2 mice and recording from layer II/III. We observed a similar loss of the optogenetically-evoked EPSCs during the SLEs. These data provide evidence that there was a reduction in excitatory synaptic neurotransmission during the SLE state.

To identify if the electrical or optogenetic stimuli directly affected the SLE or inter-SLE states, we measured the time to SLE onset, the duration of the SLE state, and the duration of the inter-SLE state (Table 2). There was a shorter average duration of the SLE state in the optogenetically-evoked EPSCs, but no difference between the groups in the inter-SLE interval or time to SLE onset. We also addressed the possibility that the evoked stimuli were altering the distributions of event durations. In the case of electrical stimulation, the SLE state shape parameter of the gamma distribution was 4.1 [2.4–7.0] (shape parameter [95% confidence interval]), whereas the inter-SLE state shape parameter of the gamma distribution was 0.67 [0.4–1.1]. Furthermore, the optogenetic stimulation resulted in a SLE shape parameter of 2.6 [1.6–4.3] and inter-SLE shape parameter of 0.62 [0.39–1.0]. These data were within the confidence intervals of the spontaneous low Mg²⁺ activity, showing they were not significantly different than the controls. This verified that the stimuli did not alter the epileptic network activity. The reduced size of the evoked EPSCs at SLE termination, as compared to the pre-SLE state, provided evidence of impaired excitatory neurotransmission at SLE termination.

3.3. The readily releasable pool (RRP) of glutamate is smaller at SLE termination

The impaired excitatory neurotransmission at the end of neocortical SLEs was potentially due to suppressed presynaptic glutamate release, as noted in epileptiform bursts in the hippocampus, in vitro (Staley et al., 1996; Jones et al., 2007). Therefore, to directly probe the presynaptic glutamate release content, we applied a hypertonic sucrose solution by focal pressure ejections (Fig. 4). A loss of the current resulting from hypertonic sucrose arises due to either compromised production of presynaptic vesicles, or no vesicles available to be released (Rosenmund and Stevens, 1996).

During SLEs, we voltage clamped pyramidal neurons at −70 mV and ejected short-duration puffs of 0.5 M hypertonic sucrose from a nearby LFP electrode (5-15 psi, 5-3 ms) (Fig. 4A). Then, to further test whether the observations were due to reduced glutamate receptor sensitivity, we activated glutamate receptors by focal puffs of glutamate (2.5 psi, 5-3 ms) (Fig. 4B). We observed that the sucrrose-evoked EPSCs were smaller at SLE termination as compared to the baseline and pre-SLE state (Fig. 4C). This pattern did not occur using focal glutamate puffs. Our results supported the hypothesis that the depression of excitatory synaptic transmission was associated with a reduction of the size of the presynaptic RRP of glutamate at SLE termination.

Because of the reduction in the size of the sucrrose-evoked EPSCs at SLE termination, we hypothesized that activity during the SLE state could influence the size of the sucrrose-evoked EPSCs (Fig. 5). The first activity examined was electrographic bursting at the end of the SLEs (Fig. 5A). To assess whether the sucrrose-evoked response was dependent on the time relative to each burst, we compared how much the sucrrose-evoked EPSCs were reduced relative to the time from the peak of each burst (as defined by the lowest negative membrane deflection) (Fig. 5B). These are small responses, as expected, at the end of the SLEs. Yet, there was no correlation between the amplitude of the sucrrose-evoked EPSCs and the end of SLE bursts. Second, to determine if the severity of the SLE state influenced the amount of suppression of the sucrrose-evoked EPSCs, we measured the intensity of the SLE state (Fig. 5C). However, there was no significant correlation observed.

As a third approach, we asked whether the recovery rate of the
sucrose-evoked EPSCs correlated with the inter-SLE state duration. To do this, we estimated the slope parameter of the sigmoid curve used to fit the sucrose-evoked EPSCs during the post SLE period (Fig. 5D, E, F). The slope parameter had a moderate negative correlation with the inter-SLE interval (as classified using the LFP) and a strong negative correlation with the inter-SLE interval (as classified using the intracellular currents). To address the possibility that a similar correlation could be made using the optogenetic-evoked EPSCs, the recovery rate of the optogenetic-evoked EPSCs was measured (Supplementary Fig. 4). In this case, the slope parameter as compared to the inter-SLE duration had a strong negative correlation (as classified using the LFP). Hence, there was a reduction in the size of the RRP of glutamate at SLE termination as compared to baseline, and the longer it took for the RRP to recover, the longer it took for another SLE to occur.

3.4. Presynaptic glutamate release probability at SLE termination is dependent on adenosine A1 receptors

Previous studies have shown that the size of the RRP of glutamate is suppressed by the activation of presynaptic adenosine A1 receptors (Köhling, 2014; Wang et al., 2015; Wang et al., 2013). Therefore, we asked if adenosine A1 receptor activation resulted in the suppression of the sucrose-evoked EPSCs at the end of the SLE state (Fig. 6). We repeated the hypertonic sucrose puff protocol in the presence of DPCPX, an adenosine A1 receptor antagonist, and observed no suppression of the sucrose-evoked EPSCs at SLE termination as compared to the sucrose-evoked EPSCs without DPCPX (Fig. 6A, B). Yet, adenosine A1 receptors are said to hyperpolarize the membrane potential of the neurons, particularly at the end of SLEs. To address this possibility, we examined the amount of current required to maintain a holding potential of −70 mV for 15 s after the end of the SLE state (Fig. 6C). As compared to before DPCPX application, DPCPX application resulted in no difference in the amount of current required to maintain the membrane at a holding potential of −70 mV. Furthermore, there was no difference in the rate at which this current recovered to steady-state after the SLE (Fig. 6D). These data demonstrated that adenosine A1 receptors were associated with the suppressed RRP of glutamate at SLE termination.
3.5. Neocortical SLEs in adenosine A1 receptor blockade have unimodally distributed SLE and inter-SLE states

To recap, this study aimed to identify the chance of SLE and inter-SLE state transitions and to determine how underlying synaptic networks modulate this chance. First, the shape parameter of the gamma distribution of time histograms was used to quantify the chance of state transitions. This showed a unimodal SLE state and an asymptotically exponentially distributed inter-SLE state, specifically modulated by the blockade of glutamatergic excitatory receptors. We saw a reduction in evoked excitatory synaptic transmission at SLE termination, as compared to baseline. There was a similar reduction in the size of the RRP of glutamate. This was dependent on adenosine A1 receptors. The shape parameter of the gamma distribution of event durations in the presence of an adenosine A1 receptor antagonist could further identify the association between adenosine A1 receptors and the chance of the inter-SLE (or SLE) state transitions.

Therefore, we computed the shape parameters of the SLE and inter-SLE intervals for different concentrations of DPCPX (Fig. 7). When the adenosine A1 receptor antagonist, DPCPX, was applied, the inter-SLE duration shape parameter shifted to > 1, with non-overlapping confidence intervals on the mean as compared to low Mg²⁺ conditions. Moreover, the SLE duration shape parameter remained > 1, with overlapping confidence intervals on the mean as compared to low Mg²⁺ conditions.

Fig. 5. Correlations between the amplitude of the hypertonic sucrose-evoked EPSCs and the SLE, and inter-SLE, state parameters. (A) Example of the bursting activity at the end of the SLE state in the LFP and excitatory currents in the whole cell patch clamp recording at −70 mV. (B) Correlations between the time from burst peak and the stimulus. Amplitude correlation rho = −0.0781, pval = 0.5977; Normalized amplitude correlation rho = 0.0848, p = 0.5667 (n = 48 sucrose evoked EPSCs, 29 SLEs, 6 slices, 4 subjects). (C) Scatter plot showing no correlation between the intensity of the SLE state and the largest change from baseline of the sucrose evoked EPSC occurring either in the last 5 s of the SLE state or first 5 s of the inter-SLE state. Intensity correlation rho = 0.0844, pval = 0.6883 (n = 26 sucrose-evoked EPSCs, 26 SLEs, 6 slices, 4 subjects). (D) Example of simultaneously recorded LFP and sucrose evoked EPSCs showing prolonged recovery of the response with long inter-SLE interval. Bars above LFP trace indicate timing of sucrose ejection. Horizontal black and white bar indicates timing of the classified SLE state. (E) Data points are the charge transfer energy (CTE) of the sucrose evoked EPSCs from one example. Time zero indicates SLE termination. Slope parameter for recovery of the sucrose evoked EPSCs back to inter-SLE steady state was modeled using a sigmoid curve (Eq. 3; Slope parameter for example = 0.178 pA/s R² = 0.74). Mean slope parameter of n = 18 SLEs, 6 slices, 5 subjects = 0.398 ± 0.25 pA/s with average R² = 0.79 ± 0.16. (F) Scatter plot showing the correlation between the estimated recovery rate of the sucrose evoked EPSCs and the inter-SLE interval (Intracellular-classified: rho = −0.7525, p-value 0.0007433; LFP-identified: rho: −0.6765, p-value: 0.0037, Spearman Correlation coefficient). Only inter-SLE intervals with at least five sucrose puffs prior to the next SLE were used for this analysis.
Mg\(^{2+}\) conditions (Fig. 7C). DPCPX did not change the duration of the SLE state, but shortened the inter-SLE state duration (Table 2), and these findings were independent of the concentration of DPCPX applied. Given the intracellular results, we concluded that suppression of presynaptic glutamate release, through an adenosine A1-dependent process, is associated primarily with the inter-SLE state distribution.

4. Discussion

The mechanisms governing the transition between seizure and non-seizure states remain elusive. This study expanded on the cellular mechanisms of these transition states by identifying a non-GABAergic mechanism of the post-seizure suppression (recently hypothesized by De Curtis et al., 2019). To quantify the chance of seizure state transitions, we presented the shape parameter of the gamma distribution under various conditions. Then, we evaluated a mechanism regulating presynaptic glutamate release and associated with changes in the distribution of event durations.

4.1. Distributions of the SLE and inter-SLE state durations

Objectively measured neocortical SLEs were found to be unimodal (shape parameter > 1), and inter-SLE intervals were found to be exponentially distributed (shape parameter < 1). These results are consistent with SLEs recorded from a mouse model of Rett syndrome, in vivo (Colic et al., 2013), and epileptiform discharges measured using a computational model of epilepsy (Grigorovsky and Bardakjian, 2018). Yet, another study demonstrated that the inter-SLE state is unimodal in whole hippocampal low Mg\(^{2+}\)-induced SLEs, \textit{in vitro} (Suffczynski et al., 2006). A further report showed transition sub-states of the neocortical SLE state, \textit{in vitro}, which could be contributing to this variation (Breton et al., 2019). Similar to previous findings (Suffczynski et al., 2006), we observed that there is an independence of the duration of the events themselves and the distribution shape parameter. However, here we showed adenosine A1 receptor antagonism changed the distributions of the inter-SLE state from asymptotic exponential to unimodal. Hence, the addition of DPCPX either alters a key SLE transition sub-state, or the inter-SLE state includes a series of independent sub-states where at least one has an increased chance of a state transition with time.

4.2. Delta rhythm-classified SLE states and glutamatergic currents

As the delta oscillation is present throughout the SLE state, our results support other studies showing that the delta rhythm is driven by local glutamatergic currents (Breton et al., 2019; Flint and Connors, 1996; Steriade, 2003; Doi et al., 2007; Mitrukhina et al., 2014). However, other groups have suggested that these rhythms are generated by reciprocal connections between the neocortex and thalamus (Steriade et al., 1993; Amzica and Steriade, 1998; Lemieux et al., 2014). In the \textit{in vitro} conditions tested here, we severed the thalamocortical projections,
and so delta in the context of low Mg\textsuperscript{2+} induced SLEs, \textit{in vitro}, is driven by local neocortical networks. Furthermore, the delta oscillation has been previously used in seizure state classification \textit{in vitro} (Breton et al., 2019) and \textit{in vivo} (Guirgis et al., 2013; Guirgis et al., 2014; Ibrahim et al., 2013), and in particular, as coupled with HFOs, is a biomarker for seizure termination (Guirgis et al., 2015). HFOs are enhanced with pro-convulsant drugs (Hughes, 2008), and they allow for the differentiation between seizure and non-seizure states in the seizure onset zone of intracranial EEGs (iEEGs) of patients with refractory epilepsy (Cotic et al., 2014). Hence, glutamate could be fundamental in driving the coupled neocortical oscillations within the seizure onset zone.

4.3. Determinants of SLE frequency

Our results show that glutamatergic neurotransmission is necessary for SLEs to occur, whereas GABAergic neurotransmission modifies both the duration of the SLE and inter-SLE state, supporting the hypothesis that seizure frequency is related to the interaction of both GABA and glutamate. GABA can either be anti- or pro-epileptic in a short time period, depending on chloride movement (Ellender et al., 2014). Previous studies have shown that the low Mg\textsuperscript{2+} model is a progressively evolving model of network activity, shifting into a late recurrent discharge phase after a prolonged period of time (Dreier and Heinemann, 1991). This shift can be abrupt, and it can be associated with chloride and the interaction of different cortical circuits (Codadu et al., 2019; Burman et al., 2019). Yet, in our low Mg\textsuperscript{2+} conditions, we did not observe this abrupt shift. Our previous study showed that the first four SLEs under low Mg\textsuperscript{2+} conditions have a stable SLE termination state, as compared to the first four SLEs under 4-aminopyridine, a second SLE model (Breton et al., 2019), supporting the stability of the model over the time course of our experiments. Furthermore, as the blockade of GABA receptors did not modify the shape parameter of the distribution of the durations of the network activity, the influence of GABAergic activity on seizure transition states was not explored further in this study.

The concentration of glutamatergic antagonists used in this study limits our ability to derive a shape parameter for the SLE and inter-SLE state. Consistent with our observations, Abdelmalik et al. (2005) showed that CNQX alone, and d-APV alone, block low Mg\textsuperscript{2+} SLEs in the neocortex. Yet, future studies could consider using non-saturating concentrations of CNQX and d-APV to identify a potential modification of the gamma distributions under partial blockade of glutamate receptors.
4.4. Presynaptic glutamate release and the inter-SLE state

Our results, in the neocortex, are consistent with previous studies of epileptiform bursts in the dis-inhibited hippocampus (Staley et al., 1998; Jones et al., 2007). Staley et al. (1998), extended by Jones et al. (2007), showed that the size of the RRP of glutamate limited epileptiform burst duration and inter-burst interval. However, in these previous studies, only bursting and not distinctive SLE states were investigated. Herein, we demonstrate the dependence of the size of the RRP of glutamate on adenosine A1 receptors in the context of full long term SLEs, a different dynamic consideration. Furthermore, though these previous results correlated burst duration with the size of the RRP of glutamate, we did not observe a correlation between the SLE duration and the size of the RRP of glutamate. Unlike the neocortex, distribution of low Mg2+ inter-SLE state durations in the hippocampus, in vitro, have a shape parameter > 1 (Suffczynski et al., 2006), further impacting the dynamic consideration. Furthermore, the number of docked glutamatergic vehicles in the neocortex may be larger than in the hippocampus, and the enhancement of the number of glutamate vesicles following acute stress is different and may depend on the type of synapse studied (Treccani et al., 2014). Hence, though suppression of presynaptic glutamate may underlie both SLE and inter-SLE distributions in the hippocampus, in the neocortex, this synaptic mechanism is confined to modulating the distribution of the inter-SLE duration.

In patients, the longer the postictal depression of network activity (post-ictal generalized EEG suppression), the greater the possibility of sudden death (Lhtao et al., 2010; Moseley and DeGiorgio, 2015). Our in vitro study limits our ability to study biomarkers of sudden death in epilepsy. Yet, we provided two lines of evidence that demonstrate there is a depression of the RRP of glutamate at the end of SLEs. (1) There was a suppression of electrophysiologically-evoked excitatory synaptic transmission at seizure termination. However, stimulation of distal networks does not measure presynaptic glutamate release directly and could be due to layer V pyramidal neuron excitability. Hence, (2) we focally stimulated layer II/III pyramidal neurons using hypertonic sucrose and glutamate. In this case, the sucrose-evoked EPSCs were reduced at SLE termination, with no concomitant suppression of the glutamate-evoked EPSCs. Though these last observations may have partially been due to residual calcium at the presynaptic terminals, altering the fusogenicity of the RRP of glutamate at the end of SLEs (Stevens and Wesseling, 1999; Garcia-Perez et al., 2008), together, they support the hypothesis of depleted presynaptic glutamate release at SLE termination. However, our findings show it is not the fact that glutamate is depleted at the end of SLEs that matters to the SLE state, it is the length of time it takes for glutamate to recover post-SLE that matters to the inter-SLE state. Hence, the post-ictal depression of network activity could be associated with a failure of the recovery of the RRP of presynaptic glutamate.

4.5. Adenosine A1 receptor mechanisms in epilepsy

Adenosine is proposed as a potent seizure termination mechanism (Lewin and Bleck, 1981; During and Spencer, 1992; Dunwiddie and Masino, 2001; Fedele et al., 2006; Fukuda et al., 2010). In neocortical tissue resected from patients with refractory epilepsy, there is an up-regulation of adenosine A1 receptors (Angelatou et al., 1993). This upregulation is suggested to be an endogenous neuroprotective mechanism against seizures (Welth et al., 2019). In human neocortical slices, activation of adenosine A1 receptors reduces the occurrence of seizure-like activity (Klaft et al., 2016). Furthermore, in low Mg2+ conditions, extracellular levels of adenosine begin to rise about midway through a ~ 30-s burst of neocortical network activity (Wall and Richardson, 2015). Hence, the rise and fall in adenosine, as previously reported, is consistent with the state-dependent depletion, then recovery, of the sucrose evoked EPSCs. Adenosine A1 receptor signaling limits the spread of epileptic activity in rodent models (Fedele et al., 2006) and reduces the frequency and duration of later bursting events of SLEs (During and Spencer, 1992; Boisson, 2008; Dale and Frenguelli, 2009). Yet, in our study, because the duration, and distribution of the duration, of the SLEs did not significantly change with DPCPX treatment, the SLE state is unlikely to be directly affected by adenosine A1 receptors. A previous study showed that the post-SLE suppression of field evoked excitatory postsynaptic potentials is eliminated using an adenosine A1 receptor antagonist (Etherington and Frenguelli, 2004). Our results extend this previous study, specifically identifying a presynaptic mechanism through which adenosine A1 receptors depress the evoked excitatory neurotransmission.

It has been shown that adenosine regulates glutamate release through either a calcium- or potassium-dependent process (Köhling, 2014). We did not see evidence that more current was required to maintain the pyramidal neurons at a holding potential of ~70 mV following DPCPX application; hence, a potassium dependent process is unlikely. However, because of the small number of pyramidal neurons tested, we cannot rule out the possibility that our manipulations resulted in less hyperpolarization of other interneurons or pyramidal cells of layers III-VI (van Aerde et al., 2013), or lead to a modulation of GABA-receptors (Ilie et al., 2012; Rombo et al., 2016). This latter point is particularly critical, as a shift to repetitive bursting in the low Mg2+ seizure model could be attributed to chloride homeostasis, as previously observed (Dreier and Heinemann, 1991; Ellender et al., 2014), and DPCPX may speed up the transition into recurrent discharges. In the entorhinal cortex, a study has shown that adenosine release acts directly on the RRP of glutamate due to the activation of Gl/o proteins and protein kinase A (Wang et al., 2013), which could be occurring at the end of the neocortical SLEs observed here in the low Mg2+ media. However, the neuroprotective effect of adenosine A1 receptors has been difficult to translate into efficient antiepileptic drugs (Chen et al., 2013), which indicates the need to identify downstream outcomes of adenosine A1 receptor signaling in seizures.

The adenosine A1 receptor blockade consistently shifted the inter-SLE distribution from asymptotic exponential to unimodal, with minimal impact on the SLE distribution. Yet, other recently identified mechanisms could also similarly modulate these distributions through modulation of presynaptic glutamate release. For example, pannexin 1 channels are said to negatively regulate presynaptic glutamate release through a mechanism involving postsynaptic metabotropic NMDA receptors (Bialecki et al., 2020). Furthermore, activation of the endocannabinoid receptor CB1 could reduce presynaptic glutamate release through its inhibition of voltage-gated calcium channels (Olmo et al., 2016). Interestingly, both pannexin 1 and CB1 could be along the same signaling cascade as adenosine A1 (Kawamura et al., 2010; Lupica et al., 2017). Further studies on the influence of these two mechanisms on SLE distributions could provide a more targeted approach to epilepsy treatment.

A recent modeling study of epileptiform activity showed that increased microglial activity leads to unimodal inter-sponstaneuos electrical discharges, without altering the spontaneous electrical discharge duration (Grigorovsksy and Bardakjian, 2018). Therefore, another potential adenosine-based mechanism could be through activated microglia, which are shown to have acute (< 5 min) effects on excitatory currents (Eyo et al., 2014). Given that adenosine A1 receptors are also present on microglia, and limit their activity under cases of brain injury (Gebicke-Haerter et al., 1996; Haselkorn et al., 2010), future studies on the acute effects of microglia in in vitro seizure models may shed light on a mechanism for post-SLE changes to the RRP of glutamate modulating inter-SLE state dynamics.

Presynaptic glutamate release remains a target of interest for future epilepsy therapies. We show adenosine A1 receptors are involved in the post-SLE state through modulation of the RRP of glutamate. Therefore, our study supports research into the critical role for presynaptic glutamate release during seizures.
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