Chronobiology of Epilepsy

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A fine balance between neuronal excitation and inhibition governs the physiological state of the brain. It has been hypothesized that when this balance is lost as a result of excessive excitation or reduced inhibition, pathological states such as epilepsy emerge. Decades of investigation have shown this to be true *in vitro*. However, *in vivo* evidence of the emerging imbalance during the "latent period" between the initiation of injury and the expression of the first spontaneous behavioral seizure has not been demonstrated. Here, we provide the first demonstration of this emerging imbalance between excitation and inhibition *in vivo* by employing long term, high temporal resolution, and continuous local field recordings from microelectrode arrays implanted in an animal model of limbic epilepsy. We were able to track both the inhibitory and excitatory postsynaptic field activity during the entire latent period, from the time of injury to the occurrence of the first spontaneous epileptic seizure. During this latent period we observe a sustained increase in the firing rate of the excitatory postsynaptic field activity, paired with a subsequent decrease in the firing rate of the inhibitory postsynaptic field activity within the CA1 region of the hippocampus. Firing rates of both excitatory and inhibitory CA1 field activities followed a circadian- like rhythm, which is locked near in-phase in controls and near anti-phase during the latent period. We think that these observed changes are implicated in the occurrence of spontaneous seizure onset following injury.

Epilepsy is the propensity to have seizures and is one of the most common serious neurological conditions, affecting 0.4% to 1.0% of the world population[1]. EEG recordings usually demonstrate interictal discharges (population spikes, sharp waves) over the hippocampal formation[2]. Brief synchronous activity of a group of neurons leading to a population spike shares some mechanisms with seizure generation; spikes should, however, be recognized as a distinct phenomenon[3]. Since spikes corresponding to both excitatory and inhibitory synaptic activities are significant features of network changes during epileptogenesis[4,5,6], it is imperative to understand their evolution leading up to spontaneous seizures. If one could observe and characterize the evolution of these spikes over the entire period from the time of injury up to the onset of

epileptic seizures, it would go a long way toward testing the hypothesis that specific changes in excitatory and inhibitory synaptic balance are implicit in mechanisms of epileptogenesis. We present the first demonstration of such an evolving *in vivo* imbalance, during epileptogenesis.

After the pioneering use of microelectrodes during the 1940's[7], several groups started to record local field potentials to understand the patterns of excitation and inhibition in the hippocampus. Local field potentials represent the coordinated synaptic activity in the population of the neurons. The recorded high amplitude field potentials also referred to as "population spikes" reflect the synchronized synaptic activity of the population of neurons firing together in a local area surrounding the recording electrode. Two main factors govern the shape and sign of the population spikes from the hippocampus[8]. First, neurons in the hippocampus are organized in laminar fashion with parallel position of the apical dendrites. The cell bodies in the hippocampus are densely packed and can be easily synchronized. When these neurons receive excitatory input into their cell bodies, the result is for the current to flow into the cell bodies and produce an active current sink in the measured field potential in the extracellular space. We refer to this pattern of high amplitude population spike corresponding to the summated population EPSP as the field excitatory postsynaptic potential (fEPSP). Second, coherent extracellular current flow resulting from synchronous interneuronal firing will produce an active current source in the measured field potential in the extracellular space. Population IPSPs corresponds to the largest component of current flow through the hippocampal pyramidal cells[9]. This population spike corresponding to the summated population IPSP is referred to as field inhibitory postsynaptic potential (fIPSP).

To look for an imbalance between inhibitory and excitatory spike wave discharges, we concentrated on the CA1 region during epileptogenesis since a number of *in vitro* studies have shown that there exist global changes toward more glutamatergic and less GABAergic activity in the CA1 region, resulting in the net increase in the excitability of the CA1 network during epileptogenesis[5,10,11]. Local field potentials from CA1 were recorded and high amplitude field activity corresponding to inhibitory and excitatory postsynaptic potentials were extracted (See Methods and Supplement Figure 1). A representative example of local field potential recorded with a microwire implanted in the CA1 (Fig 1A-B, and Fig 1D) is shown in Figure 1E. In Figure 1F we show the mean shape of the fEPSP field potential activity (with standard error corresponding to 95% confidence interval) obtained from a total of about 40,000 fEPSP events detected over a period of 12 days of near continuous recordings during the latent period in a rat electrically stimulated into status epilepticus. In Figure 1G we show the mean shape of the fIPSP field potential activity (with standard error

corresponding to 95% confidence interval) obtained from a total of about 24,500 fIPSP events detected over the same time interval in the same rat.

The time evolution of the normalized fEPSP and fIPSP firing rates from the age-matched sham controls and the epileptogenic latent period, are shown in Figures 2A-D. Key points worth mentioning from Figures 2 are: (1) there exists a circadian-like (with period near 24 hour) modulation in the firing rate of the fEPSP and fIPSP field activity; (2) there is no observed drift in the firing rate of the fEPSP and fIPSP field activity in data obtained from sham control rats (Figures 2A and 2C); (3) during the latent period, there is a marked upward drift in the firing rate of fEPSP field activity and a corresponding marked downward drift in the firing rate of the fIPSP field activity (Figures 2B and 2D). (4) the circadian-like modulation of firing rates of the fEPSP and fIPSP are locked near in-phase in sham control period (Figure 2E) while during the latent period following status epilepticus the two field potentials oscillate near out-of-phase with respect to each other (Figure 2F) (5) Finally, the average number of fEPSP events per hour recorded during the latent period of epileptogenesis are significantly greater than that recorded during the sham control-period, while the average number of fIPSP events per hour are significantly less during the latent period as compared to the sham controls (Figures 2G-H).

In Figure 3 the results on the imbalance in the firing rates and the phase reversal of the circadian-like oscillations of the fEPSP and fIPSP field potentials pooled across data collected from all rats are summarized The imbalance in the firing rates is quantified by estimating the drift $D = \left\langle \dfrac{\Delta f}{\Delta t} \right\rangle$ (f: firing rate) in the firing activity of both fEPSPs and fIPSPs through a least-sqaures fit of the firing rate data to a straight line, $\Delta f = D\Delta t + c$ (See Supplement Figure 2a). From Figure 3A, we see that, while the firing rates are in balance during the sham and prestatus control periods, the imbalance (as quantified by the difference in the drift rates of fEPSPs and fIPSPs) is significantly higher during the poststatus latent period ($p \approx 0.0044$, two sample t-test). The phase relationship between the circadian like firing activity of the fEPSPs and fIPSPs is quantified through a least sqaures-fit of the detrended-modulo 24 firing rate data with a sinosidual function $f(t) = a\sin(\omega t + b)$, with $\omega = 7.2722*10^{-5}$ Hz (See Supplement Figure 2b). The phase is associated with the time T_X^M (X=fEPSP,fIPSP) of maximum value obtained by $f(t)$. The phase of fEPSP and fIPSP oscillations are shown in Figures 3B. The relative phase difference is quantified as $\Delta T = \left| T_{fEPSP}^M - T_{fIPSP}^M \right|$. We see that during the control period, the two field potentials are phase-locked with a lag of around 3 hours, however during the latent period the phase lag increases to approximately 9 hours. We postulate that due to the large

relative phase shift in the oscillations of the fEPSPs and fIPSPs during the latent period the increase in firing rate of fEPSPs is not compensated by subsequent increase in the fIPSP firing activity and as a result, an emerging state of imbalance in the firing rates occurs during the latent period, making the network within the CA1 increasingly excitable and eventually triggering the first spontaneous epileptic seizure. Finally in Figure 3C-E we show the normalized cross-correlogram (NCC) of the fIPSPs with respect to fEPSPs for the sham control, prestatus epilepticus control and the poststatus epilepticus latent periods. The NCC represents the probability of observing a fIPSP event over time interval [-T,T] around an fEPSP event. During the control periods (Figure 3C-D), the timing of occurrence of the two field potentials are highly correlated with fIPSP activity leading fEPSP activity on average. However during the poststatus epilepticus latent period (Figure 3E), we see a consistent dip in the NCC, with a the minimum of NCC occurring at positive time values, suggesting the probability of firing of fIPSP event following an fEPSP event is decreased. These results suggest that the recurrent inhibition of pyramidal cells in the CA1 decreases resulting in a net increase in the excitatory field activity during the latent period.

Based on our observations, we propose a *circadian control hypothesis*, which posits that the fine balance of synaptic activity of the fEPSPs and fIPSPs in healthy controls is under circadian influence, and are phase locked with respect to each other. Injury to the brain such as electrical stimulation inducing status epilepticus, perturbs the phase relationship relative to the circadian rhythm, resulting in an imbalance in the firing rates of the fEPSPs and fIPSPs. This imbalance in turn produces an increasingly excitable CA1 network during the latent period of epileptogenesis. There is existing evidence to suggest that epileptic seizures are modulated by circadian rhythms. Though many efforts have been put into understanding the effects of endogenous biological rhythms on seizure timing[13,14,15] very little work has been done on understanding the effects of circadian rhythm expression on seizure development. It is conceivable that permanent alterations in neuronal excitability or structural lesions associated with the development of epilepsy might alter the circadian rhythm expression. Our results provide a clue to the possible consequence of the perturbation in the circadian expression in terms of phase reversal in the circadian like activity of the fEPSPs and fIPSPs following injury.

In summary, our study is consistent with previous *in vitro* studies of temporal lobe epilepsy models which show that there is a change in the net synaptic glutamatergic and GABAergic drive in the hippocampal CA1 network during the latent period leading up to the first epileptic seizure[10,12]. More importantly, we have presented the first *in vivo* demonstration of a physiological feature that exhibits circadian rhythmicity and shifts in its relative phase of oscillations during the post-injury latency period leading up to the first

spontaneous epileptic seizure. Our results may have significant mechanistic implications for the observable changes in network excitability during epileptogenesis. An intriguing conjecture is that the quantification of the evolving imbalance between the fEPSPs and fIPSPs following status epilepticus may serve as a quantitative biomarker for epileptogenesis.

Methods Summary

Local field potentials were recorded using a chronically implanted microwires (50μm polyimide insulated tungsten) in the hippocampal CA1, CA2 and the dentate gyrus. Out of a total of five rats, three rats were electrically stimulated into status epilepticus by injecting a bi-phasic current pulse through a bipolar twist stainless-steel electrode implanted in the ventral hippoampus. Continuous EEG/video data were collected at a high sampling rate of 12KHz. In house software was used to save the recorded data in a 16-bit binary format for later processing. The schematic flow chart of the data analyzed to extract the excitatory and inhibitory field potentials is given in Supplement Figure 1. All the figures were generates using custom programs within IGOR Pro (WaveMetrics, Inc)

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Author Contribution

SST, PRC and WLD provided the conceptual foundation and wrote the manuscript.

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Figure Legend

Figure 1. **Electrode localization and characterization of fEPSP and fIPSP spike patterns.** A 3-dimensional, gradient echo MR image, acquired at 17.6 T (750 MHz) with a Bruker Avance system (Bruker NMR Instruments, Billerica, MA), is shown in A) and B). The fixed brain was excised intact, washed overnight in saline, and then imaged in fluorinated oil. The image was acquired with a recovery time of 150 ms, an echo time of 15 ms, using 2 averages and a resolution of 75 microns in each direction. Orthogonal slices are shown in A) with the tip location of right-side-microarray electrode #2 (shown as the black spot within the red circles) terminating in CA1. The complete three-dimensional image volume, shown in B), illustrates an electrode tract (shown as a black line) within the brain. The electrode tract appears visible in the MR image because iron, accumulated around the site of electrode insertion, shortens the MR transverse relaxation time. Using another rat brain treated in the same way, the presence of iron is confirmed in the histological slice in C), which was prepared using Perl strain with diamine-benzidine-tetrahydrocloride. In this slice, the iron surrounding a tract is visible as the black region in the middle of the slice. D) Shows schematic diagram of hippocampus (courtesy of Dr Bruce MacIver, Stanford University School of Medicine) and location of implanted electrode array (blue box and circles in the box). E) A sample EEG trace of 3 minutes duration from recordings obtained during the latent period in one of the electrically stimulated rats. Overlaid over the raw EEG trace in green represents the time of fEPSP field potential activity and in red represents the time of fIPSP field potential activity, detected through the clustering algorithm. F) The mean amplitude profile of the fEPSP field potential activity (with standard error representing 95% confidence level) obtained from a total of 40000 fEPSP events over the entire latent period of recordings from the same rat electrically stimulated in status-epilepticus. G) The mean amplitude profile of the fIPSP field potential activity (with standard error representing 95% confidence level) obtained from a total of 24,500 fIPSP events over the same period.

Figure 2: Circadian rhythmicity and imbalance in firing rates of fEPSPs and fIPSPs. A representative example demonstrating circadian modulation in the firing rates of the fEPSP and fIPSP field potentials. In A) and B) we show the time evolution of the normalized firing rates of the fEPSP field potentials from EEG data obtained from sham-control rat C1 and during the poststatus epilepticus latent period in rat E2. In C) and D) we similarly show the time evolution of the normalized firing rates of the fIPSP field potentials from EEG data obtained from the same two rats. The time of the first spontaneous seizure following the latent period is marked in dotted blue line. Solid red line (fEPSP) and solid green line (fIPSP) represents a least-squares fit of the firing rate data to a function of the form, $f(t)=at+b\sin(ct+d)+e$, in order to emphasize the underlying circadian like rhythmicity in the firing rates. The diurnal cycle is shown in the background with gray shaded region representing the controlled dark cycle and the white region representing the controlled day cycle. The phase relationship of the circadian modulation of the firing rates between the two field potential activities during the sham-control and the latent periods are shown in E) and F) respectively. The diurnal cycle is again represented in the background. The average number of fEPSP and fIPSP events per hour over the entire duration of the sham-control period in rat C1 and the poststatus epilepticus latent period, in rat E2 are shown in G) and H).

Figure 3: Drift and the relative phase difference and cross-correlation in firing rates of fEPSPs and fIPSPs. A) The mean amplitude of the drift in the firing rate, D, of the fEPSP field potential activity and the drift in the fIPSP field potential activity in data from all the recorded rats during the three time periods of prestatus epilepticus (Prestatus), post status epilepticus latent period (Latent) and the sham-control (Sham) are shown with the standard error representing 95% confidence level. The rightmost figure shows the rate of divergence or the imbalance in the two field potential activities, as represented by the difference in the mean

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amplitude of the drift in the firing rates is shown. The standard error in this case represents the maximum and the minimum difference between the two field potential activities. B) The phase of the circadian like oscillations of the fEPSPs and the fIPSPs represented by the time in hours T^M_X (X=fEPSP, fIPSP) when the firing activity per day reaches its maximum is shown. The standard error corresponds to the 95% confidence interval. The relative phase shift as quantified by the difference $\Delta T=|T^M_{fEPSP}-T^M_{fIPSP}|$, is shown in the rightmost figure. Normalized cross-correlogram representing the probability of observing a fIPSP event in time interval of [-60:60] minutes of a fEPSP event for the prestatus, latent and the sham control periods are shown in C), D) and E) respectively.

Figure S1. Flowchart for the extraction of the fEPSP and fIPSP field potentials. A) Sample EEG trace of one hour in duration from a single microwire electrode implanted in the CA1, obtained during the latent period of recordings from one of the rats induced into status epilepticus. B) Raster plot of the spike events pooled over a period of 24 hours of the continuous EEG recordings. The pooled spike events represent the set of spike events selected from a non-overlapping time window of one hour of the EEG data, whose amplitude exceeds a threshold of 5s, where s is the standard deviation computed for each one hour of recordings of the EEG data C) Pooled spike events are then normalized in amplitude and peak-adjusted to discount for any large amplitude fluctuations and jitter in timing of the rastered spike events before feeding them into the automated clustering algorithm[16] D) The output of the clustering procedure resulting in three separate clusters (show in black, blue and red) with the corresponding proabability-density plots of the spike patterns are shown. E) The two primary clusters representing the fEPSP (red) and the fIPSP (blue) field potentials and the corresponding spike patterns represented in the probability density plots are shown. The final two spike patterns are selected by determining the cross-correlation of the events in the third cluster (black) with the mean shape-profiles of the events in the two primary clusters. Only those events in the cluster (shown in black) whose cross-correlations is >75% are included in one of the two final primary spike clusters representing the fEPSPs and the fIPSPs.

Figure S2. Procedure to determine the drift in the firing rate and the phase of circadian-like oscillations of the firing rates of the fEPSP and fIPSP field potentials. A) A representative example demonstrating the procedure used to determine the drift D in the firing rate of fEPSP and fIPSP field activities. Shown in black dotted points, are the firing rates of the fEPSP field potential events, extracted from continuous EEG recordings during the sham-control period, determined from a moving time window of one hour with an overlap of 10 minutes. The total time duration and the firing rate magnitude are normalized to scale between [0, 1]. Solid red line represents the least-squares fit to the magnitude-time normalized firing rate data with the function of the form *f(t)=Dt+b*, where *D* represents the drift in the firing rate data. The dotted blue lines represent the 95% confidence-bound for the fitted data. B) A representative example demonstrating the procedure used to determine the phase of oscillations in the firing rates of fEPSPs and fIPSPs. Shown in black dotted points are the *modulo 24, detrended and normalized* firing rate data obtained from the fEPSP events selected over the sham-control period of continuous EEG recordings. The firing rates are determined over a moving time window of 6 hours with an overlap of 5 hours. The drift in the firing rate data is removed following the procedure described in A). The detrended (after removing the drift) firing rate data is then normalized to scale between [0,1] and rastered over a time window of 24 hour (modulo 24). Leasr squares fit of the *modulo-24, detrended and normalized* firing rate data with a function of the form *f(t)=asin(wt+b)*, with *ω=7.2722*10^-5* Hz is shown in red. The dotted blue lines represent the 95% confidence bounds on the fitted data. The phase of oscillation is then associated with the time of the maximum reached by the function *f(t)* over a time period of 24 hours.

A) Coronal   Horizontal   Sagittal

B) Coronal   Sagittal   Horizontal

C)

D) CA 2   sc   CA 1   alv
   CA 3   mf   DG   pp
   fim

E)

F)

G)

A)

Normalized spike rate (y-axis): 0.0, 0.5, 1.0

Normalized time (x-axis): 0.0, 0.5, 1.0

B)

Normalized spike rate (y-axis): 0, 1

Time (hour) (x-axis): 0, 10, 20