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RESEARCH ARTICLE

The effect of amygdala low-frequency stimulation on inter-hippocampal connectivity in the pilocarpine model of epilepsy

István Mihály*#, Ádám-József Berki#, Károly Orbán-Kis, Zsolt Gáll, Réka-Barbara Bod, Tibor Szilágyi

George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Romania

Objective: The aim of our study was to investigate the effect of amygdala low-frequency stimulation on inter-hippocampal network synchronization by using the phase locking value (PLV) in order to establish new biomarkers of treatment efficacy in a temporal lobe epilepsy model. **Materials and Methods**: The lithium-pilocarpine model of epilepsy was used to induce status epilepticus in male Wistar rats. Afterward, seizures were scored based on continuous video recordings. 8 weeks after status epilepticus electrodes were implanted: a stimulating electrode in the left basolateral amygdala and bilaterally two hippocampal recording electrodes in both pilocarpine-treated and age-matched control rats (N=7). 10 Pilo and 4 control animals were stimulated daily for 10 days with 4 packages of 50 seconds 4Hz trains. Inter-hippocampal PLVs were measured offline before and after stimulation trains in delta (1-4Hz), theta (4-12Hz), gamma (30-100Hz), HFO (100-150Hz), ripple (150-250Hz), and fast ripple (250-600Hz) bands using Brainstorm software. **Results**: The PLV before the stimulation was significantly lower in epileptic animals compared to controls in the delta, theta, and gamma bands. The PVLs of epileptic animals were increased by low-frequency stimulation in delta and theta bands. The PLVs in HFO and ripple band correlated positively with the changes in seizure rate, while the PLVs in the delta, theta, and gamma correlated positively with the changes in seizure duration. **Conclusion**: Amygdala low-frequency stimulation improved the impaired synchrony between the two hippocampi in low-frequency bands. The phase locking value could be useful to evaluate the efficiency of therapeutic interventions in temporal lobe epilepsy.

Keywords: temporal lobe epilepsy, amygdala stimulation, pilocarpine, phase locking value

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Introduction

Epilepsy affects nearly 1% of the world's population and its annual incidence is 50-70 per 100000 individuals, representing a severe socio-economic burden [1]. Approximately one-third of patients continue to have seizures despite receiving anticonvulsant medication [2]. Temporal lobe epilepsy (TLE) is a severe focal epilepsy type, in which the pharmacoresistant patients can undergo surgical resection of epileptic tissue, but still, around half of them continue to exhibit seizures even after surgery [3]. Deep brain stimulation (DBS) is an emerging alternative therapeutical tool in neurological disorders, and in the last years, it was observed that DBS could reduce the seizure frequency in humans and animals too, with fewer side effects than surgical resection [4,5].

Treatment efficiency of DBS is influenced by electrode placement and stimulation parameters, including frequency, intensity, train duration, etc. In humans, most trials used high-frequency (>130 Hz) stimulation protocols [6], however in rodent epilepsy models targeting the amygdala with low-frequency stimulation (LFS, <5Hz) provided promising results in the pilocarpine, pentylenetetrazole, and amygdala kindling models as well [7–9]. Our previous results already have shown that LFS reduces the mean seizure rate and duration in the pilocarpine model of TLE [10]. However, the understanding of how the mechanisms via LFS modulates the epileptic neuronal circuits is incomplete, therefore, its improvement is limited and mostly relies on empirical observations [11]. Currently, the greatest indicator of treatment outcome is seizure frequency, although the patient-reported seizure rate is not reliable in many cases due to the consciousness-impairing seizures or because of the disease-associated memory loss [12]. Presently used electrophysiological markers, like interictal epileptiform discharges (IEDs), could be used in the follow-up too, but the connection between seizures and IEDs is inconsistent [13]. For this reason, the need for new biomarkers of treatment effectiveness is on demand.

Long-range neuronal connections are reportedly implicated both in seizure generation and propagation, and it was proposed that the analysis of synchrony between distinct areas could hold valuable information regarding the progression of epilepsy, and the rate at which focal seizures spread to generalized seizures [14–16]. Therefore, studying the large-scale operation of epileptic networks may facilitate the understanding of interictal brain activity and may contribute to the development of objective biomarkers of treatment efficacy [17].

Several studies on focal neocortical and temporal lobe epilepsy measured the functional connectivity with various methods using electroencephalography (EEG) [18], functional magnetic resonance imaging (fMRI) [19], and magnetoencephalography (MEG) [20]. Overall, these studies found increased connectivity within and nearby

* Correspondence to: István Mihály. E-mail: istvan.mihaly@umfst.ro
# These authors have equally contributed to the article.

the epileptogenic foci accompanied by impaired synchrony across long-range, distant connections [18–20]. However, there is a lack of data regarding the function of long-range neuronal interaction when brain stimulation is applied for TLE.

We hypothesize, that inter-hippocampal synchronization is impaired in the pilocarpine model of temporal lobe epilepsy, and this pathological synchronism is modulated by low-frequency stimulation of the amygdala. Measuring inter-hippocampal synchronization could serve as a marker of treatment effectiveness in the pilocarpine model of epilepsy.

Materials and Methods

All experiments were conducted in accordance with the 2010/63/EU directive of the European Parliament and the local regulations approved by the Ethics Committee for Scientific Research of the George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Târgu Mures, (ethical committee license no: 340/17 November 2017, extended by no. 54/2 April 2019). For the experiments, male Wistar rats were used; at the time of the induction of status epilepticus (SE) the rats were 6 weeks old. Housing was in standard conditions, in plexiglas cages under controlled environmental (~23 °C) and light conditions (12 h dark-light cycle), with food and water being accessible all time. The animal groups were the following: DBS-Pilo (pilocarpine-induced and electrically stimulated, n = 10), DBS-Control (electrically stimulated healthy controls, n = 4), and SHAM-Control (implanted but non-stimulated healthy controls, n = 3).

The lithium-pilocarpine model was used to induce status epilepticus as described in our previous publication [10]. Briefly, systemic injection of pilocarpine (30 mg/kg, i.p.) induces SE in rats. LiCl (3 mEq/kg i.p.) was given 16 h before to potentiate the effect of pilocarpine. Methylscopolamine (1 mg/kg) was given 20 minutes before pilocarpine to alleviate peripheral cholinergic activation. All substances used for epilepsy induction were purchased from Sigma–Aldrich (St. Luis, MO, USA). SE is characterized initially by head-nodding, clonic jerking, and jaw movements. Later generalized tonic-clonic seizures appeared, which were classified by their gravity based on the revised Racine scale, from 1-6 [21]. After 2 hours of SE diazepam (5 mg/kg, i.p.) is administered to halt the seizures. Animals were rehydrated i.p. after induction. Control animals received only lithium and i.p. saline solution, and a single dose of diazepam 2 hours after saline administration. Following SE, animals were placed in individual cages and continuously video-monitored throughout the whole study. The severity and average duration of spontaneous recurrent seizures were determined based on these recordings according to a revised Racine scale [21].

Electrode implantation protocol: Detailed settings of electrode implantation, stimulation protocol and recordings were described in our previous publication [10]. Briefly, pilocarpine treated rats (approx. 8 weeks after SE) and aged-matched controls had stereotaxic electrode implantation protocol in general anesthesia. All electrodes were implanted according to the Paxinos and Watson's atlas for rats [22], with the bregma as reference. The stimulation electrode was implanted in the left basolateral amygdala (BLA, AP -2.8 mm; ML +5 mm to bregma; DV +8.4 mm to skull surface). The hippocampal recording electrodes were placed as follows: AP: -3.6 mm, ML: ± 2.6 mm, DV: 3.6 mm. Two screw electrodes were used for epidural recordings (AP: +2mm, ML: ±2 mm) and two more were placed at the posterolateral surface of the parietal bones and used as reference and ground electrodes. Electrodes were connected to a headstage connector and fixed with dental acrylic. At the end of the experiments, correct electrode positions were verified by Nissl staining. Animals with incorrect electrode positions, as determined after the experiment, were excluded from the analysis.

Stimulation protocol and local field potential (LFP) recording: After a 10-day long post-surgical recovery, animals were placed in a plexiglas cage (40x45x50 cm) housed in a Faraday cage and were kept in the same standard conditions as before. The headstage was connected to a swivel contact, which made it possible to apply electrical stimulation and record EEG at the same time, on a freely moving rat. The electrical signal was amplified by an 8-channel preamplifier and an amplifier system (SUPERTECH Multiamp SMA-4a, Supertech, Pécs, Hungary). Signal was passed through on a 0.16 Hz low-pass filter, 8 kHz high-pass filter, and a 50 Hz Notch filter of the Multiamp amplifier system. Data acquisition was done by an A/D converter (PCI 6036E, National Instruments, Austin, TX, USA), with a sampling rate of 5 kHz.

A BioStim gate-controller and pulse pattern generator (STC-8b) connected to a Bipolar Floating End-stage (BSE-3b; both from Supertech, Hungary) were used for stimulation. The parameters of stimulation were the following: square wave, biphasic, 100 μs pulse duration, 500 μA intensity, with a regular interpulse interval at 4 Hz. A daily package consisted of a 50-second train applied 4 times, with 5-minute pauses between each train. Simultaneous EEG was recorded starting 5 minutes before the first and halted 5 minutes after the last train. All rats were stimulated for 10 days.

Phase locking value (PLV): To estimate the functional connectivity between the left and right hippocampus, the method of phase locking value measurement was used. In this approach, the frequency-specific instantaneous phase angle is calculated for two different signals and the resulting phase difference is extracted for all time-bins. PLV measures the variability of this phase difference over time [23]. The result is between 0 and 1, high PLVs indicate strong synchrony between neuronal oscillations that emerged from signals measured from distinct brain areas. According to Lachaux et al., an important advantage of the method is that ignores the amplitudes of signals therefore,

the connectivity cannot be overestimated due to the presence of amplitude covariance [23].

PLV was calculated using the Brainstorm Toolbox [24] for MATLAB (The Math Works Inc, Natick, MA, USA).

From each daily 30 minutes long LFP recording, the pre-stimulation 5-minute periods and the last 5-minute periods (post-stimulation period) were selected for PLV analysis, in the case of stimulated groups. In the case of SHAM-Control animals, the first and last 5-minute intervals were extracted and analyzed from the 30-minute recordings. Inter-hippocampal PLV was measured on the signals obtained from the left and right hippocampus.

The analyzed frequency bands were delta (1–4 Hz), theta (4–12 Hz), gamma (30–100 Hz), HFO – high frequency oscillations (100–150 Hz), ripples (150–250 Hz) and fast ripples (250–600 Hz).

Histology: At the end of the recording and stimulation protocol rats underwent deep anesthesia (Ketamine-Xylazine, 90 mg/kg + 10 mg/kg) and were transcardially perfused with ice-cold normal saline solution (0.9%, 1 min 30 s) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) solution containing 15% picric acid. Brains were further incubated in paraformaldehyde for 24 h, then 60 μm coronal sections were cut by using a Leica vibratome (Leica VT 1000S, Leica, Germany). For correct electrode positioning, cresyl-violet staining was applied and sections were checked using light microscopy.

Statistical analysis: Data are expressed as mean and standard error of the mean (SEM). Statistical analysis was performed using the GraphPad PRISM version 8. Paired t-test, unpaired t-test and Pearson correlation were used to evaluate parametric data, and Mann-Whitney test, Wilcoxon test as well as Spearman correlation were used to evaluate non-parametric data. A $P < 0.05$ indicates a significant difference.

Results

All selected animals (N value) that underwent pilocarpine administration developed spontaneous recurrent seizures based on video recordings. The control group had no epileptic seizures.

By comparing the PLVs measured in the first 5 minutes of the recordings between the control and pilocarpine groups (before DBS in this group) it was found that the DBS-Pilo group exhibited a significantly lower PLV in delta, theta, and gamma frequency bands. In HFO and fast ripple bands, PLVs were lower too, but the differences were not significant. In the ripple band, PLVs were higher, but the difference was also not significant (Figure 1).

In order to evaluate the effect of LFS on PLV, the 10-day average of pre-stimulation and post-stimulation PLVs were compared from all groups. In the DBS-Pilo group, it was observed that the PLVs increased significantly in delta and theta frequency bands after LFS. In the gamma band, there was no modification, whilst in HFO, ripple, and fast ripple bands the PLV decreased, but not significantly. After the DBS, the significantly increased PLVs in the DBS-Pilo group (in delta and theta bands) still differed significantly from those measured in controls (Figure 1). The PLVs did not increase throughout the 10 days of stimulation and there was no significant difference between the PLVs measured on first and last days of DBS (*delta*: 0.51 ± 0.04 vs. 0.42 ± 0.05; *theta*: 0.44 ± 0.04 vs. 0.42 ± 0.07; *gamma*: 0.22 ± 0.03 vs. 0.25 ± 0.01; *HFO*: 0.08 ± 0.04 vs. 0.10 ± 0.04; *ripple*: 0.12 ± 0.05 vs. 0.11 ± 0.03; *fast ripple*: 0.28 ± 0.1 vs. 0.23 ± 0.04, $p>0.05$ for each comparison).

No difference was found between the PLVs of SHAM-Control and DBS-Control groups (*delta*: 0.67 ± 0.05 vs. 0.74 ± 0.02; *theta*: 0.61 ± 0.04 vs. 0.70 ± 0.03; *gamma*: 0.15 ± 0.02 vs. 0.15 ± 0.03; *HFO*: 0.08 ± 0.02 vs. 0.10 ± 0.04; *ripple*: 0.12 ± 0.03 vs. 0.11 ± 0.04; *fast ripple*: 0.23 ± 0.04 vs. 0.27 ± 0.04; $p>0.05$ for each comparison). The LFS did not modify the PLV in DBS-Control group (*delta*: 0.74 ± 0.02 vs. 0.73 ± 0.02; *theta*: 0.70 ± 0.03 vs. 0.71 ± 0.03; *gamma*: 0.25 ± 0.01 vs. 0.27 ± 0.01; *HFO*: 0.10 ± 0.04 vs. 0.12 ± 0.04; *ripple*: 0.11 ± 0.03 vs. 0.11 ± 0.04; *fast ripple*: 0.23 ± 0.04 vs. 0.19 ± 0.02; $p>0.05$ for each comparison). Hence, the comparison between the epilep-

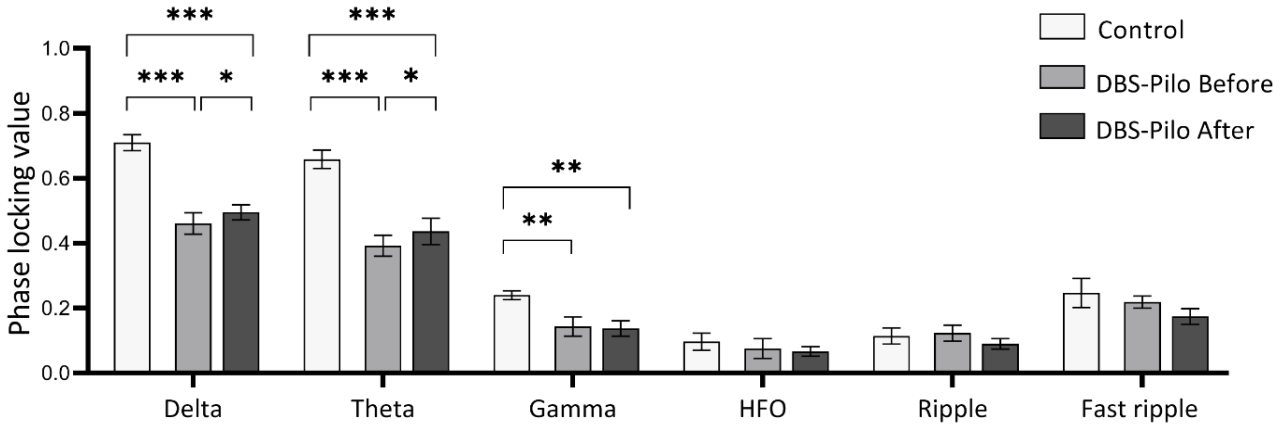

Fig. 1. Phase locking values (PLVs) in different frequency bands in the control and DBS-Pilo groups. All values are the averages of 10 days. Epileptic animals (DBS-Pilo Before) showed significantly lower PLVs than controls did in the delta, theta, and gamma frequency bands. After DBS, the PLV of the DBS-Pilo group increased significantly in delta and theta frequency bands. Data were represented as mean±SEM, * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

tic and control animals were made by combining the results of the SHAM-Control and DBS Control groups.

In the case of all animal groups, the delta and theta PLVs were approximately two times higher than the PLVs measured in the gamma, HFO, ripple, and fast ripple.

Phase locking values' relation to seizure parameters: As the DBS reduced the average seizure rate and seizure duration (30% decrease in the seizure rate and a reduction by 26.5% in seizure duration), it was feasible to test if the changes in these parameters are related to the PLV. The changes in seizure rate and seizure duration were calculated as the differences between the average seizure rate during the 10 days of DBS compared to 10 days before DBS. It was found that the pre-stimulation HFO and ripple PLVs correlated positively with the change in seizure rate (Table I, Figure 2A and B).

The changes in seizure duration correlated positively with the pre-stimulation PLVs in the delta, theta, and gamma frequency bands (Table I, Figure 2C and D).

Discussion

In the present study, we found that inter-hippocampal synchronism was decreased in distinct frequency bands in epileptic rats compared to healthy ones and that amygdala

Table I. Correlation between the power of decrease in seizure parameters and pre-stimulation PLVs

	Correlation Coefficient and P-value	Delta	Theta	Gamma	HFO	Ripple	Fast ripple
Change in seizure rate	r	-0.3713	-0.3383	0.1513	0.7182	0.7764	0.4021
	p	0.3253	0.3732	0.6982	0.0345*	0.0179*	0.2834
Change in seizure duration	r	0.7598	0.8280	0.7785	0.0969	-0.2530	-0.1036
	p	0.0287*	0.0111*	0.0295*	0.8194	0.5419	0.8071

*p<0.05

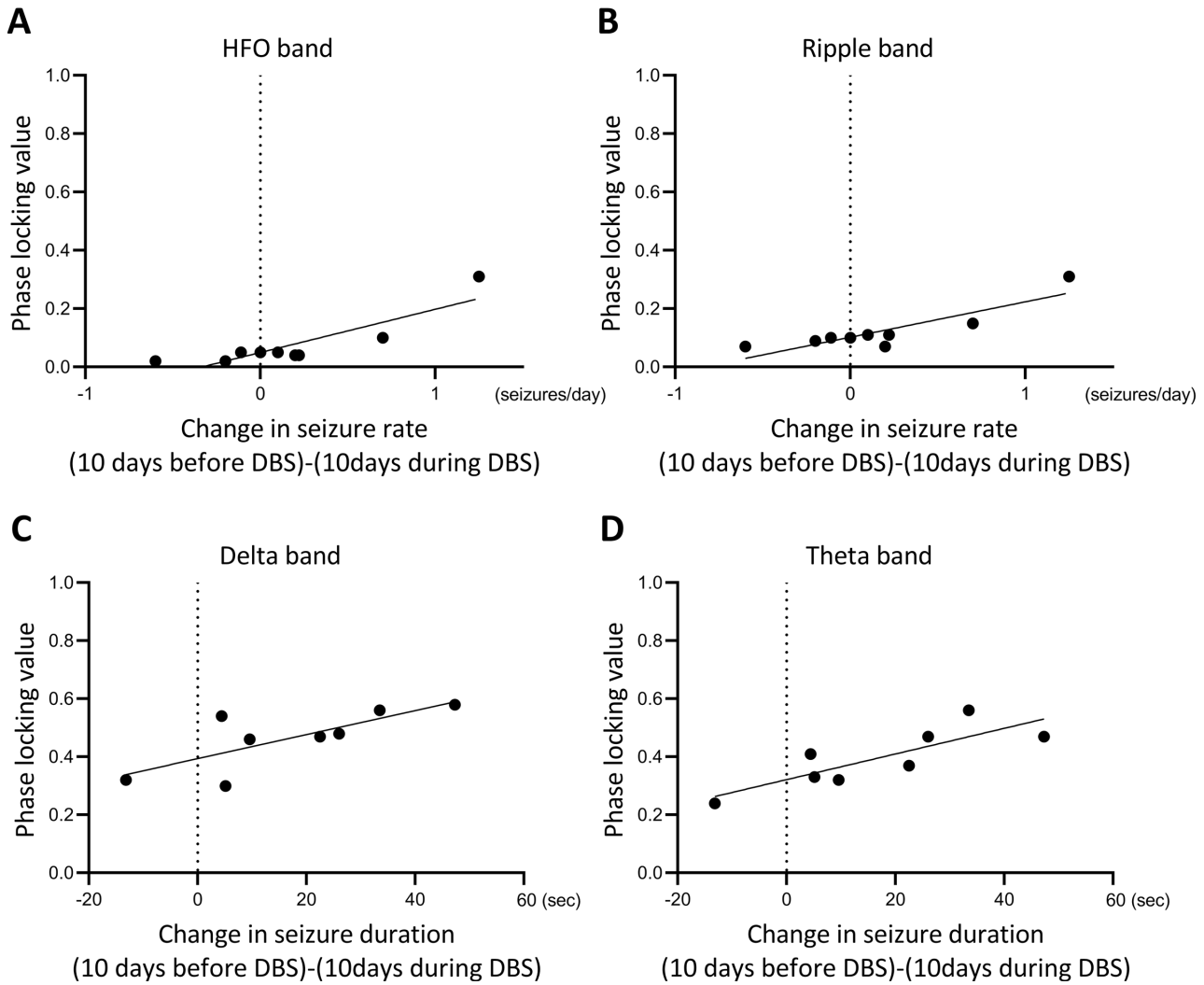

Fig. 2. (A) Correlation between average PLV in HFO band before DBS and change in average seizure rate of individual animals. Significant positive correlation was found (r=0.71, p<0.05). (B) PLV in ripple band and change in seizure rate. Significant positive correlation was found (r=0.77, p>0.05). (C) PLV in delta band and change in seizure duration. A significant positive correlation was found (r=0.75, p<0.05). (D) PLV in the theta band and change in seizure duration. Significant positive correlation was found (r=0.82, p<0.05). Positive values on the abscissa indicate a decrease in seizure rate or seizure duration while negative values indicate an increase.

LFS re-increases it. LFS does not interfere with the phase locking values of healthy rats.

The estimation of PLV could be an effective approach to explore the large-scale network synchrony between distant brain territories, in our case allowing an in-depth comprehension of inter-hippocampal synchronism both in epileptic and healthy animals. We found a considerable difference in the strength of synchronization in different animal groups. Epileptic animals exhibited substantially lower connectivity value in low and middle-frequency bands (from delta to gamma), indicating the presence of pathologically decreased network synchronizations in TLE. Contrarily, in high-frequency domains, no difference was found between the animals.

The impaired cortical connectivity in pathologically rearranged epileptic brain areas was observed also in various human studies [18,20]. In experimental temporal lobe epilepsy models it was found that epileptiform events were preceded by short-term modifications of bilateral hippocampal coherence [25]. In the kainic acid (KA) model of epilepsy, 8 weeks after KA injection decreased intrahippocampal synchrony (between CA1 and Dentate Gyrus) was found in the theta band (4-12 Hz) which was related to episodic-like memory deficits [26]. In the same model, when KA was injected unilaterally in the hippocampus, similar to our results, decreased inter-hippocampal synchronism was found in the 2-32 Hz frequency domain already during the latent phase of the model, and it was independent of recurrent seizures. The theta oscillations were reduced ipsilateral to the injection site during the latent phase, and bilaterally during the chronic phase [27]. Meier et al. noticed decreased connectivity at higher frequencies (100-400 Hz) appearing ~10 s before epileptiform events in KA-induced epilepsy at least 6 weeks after KA injection [25]. We extend these observations, as in pilocarpine model of epilepsy animals had altered connectivity during interictal periods in gamma band (30-100 Hz) too approximately 3 months after SE, in the chronic phase of the disease.

In human patients with mesial TLE, the EEG and fMRI connectivity measurements also revealed decreased limbic connectivity relative to the healthy control group [18,28]. Moreover, recently Elahain et al. suggested developing PLV-based algorithms that may help to accurately delineate and characterize the seizure onset zones using the EEG of epileptic patients [29].

It is noteworthy that in each group (Control and DBS-Pilo) the PLV showed higher values in case of slow oscillations (delta and theta) in comparison with high-frequency oscillations, which further highlights the fact that functional connection across large networks is established by the extensive presence of lower frequencies at high power densities, whilst fast oscillations are generated transiently within local operating units [30,31].

It was proposed that the readjustment of hippocampal rhythms could exert an antiepileptic effect in TLE models [32,33]. Here we demonstrate that unilateral amygdala LFS enhances the inter-hippocampal connectivity in chronically epileptic rats. The improvement of phase synchrony value was most outstanding in the theta band which acquires importance as the power of theta oscillations reportedly were increased during different anticonvulsant treatments for TLE in animal models [33–36]. Moreover, it was reported recently, that increasing the relative theta power by external stimuli could have anticonvulsant effects in rats [10,32] and could be used as a marker for the effectiveness of the anticonvulsant intervention. However, we should remark that in our case the PLVs were not increased to the values measured in controls.

In a recent EEG study carried out on patients with focal epilepsy, it was found that eslicarbazepine acetate (an anticonvulsant drug) reverted the pathologically modified global connectivity after at least 3 months in all analyzed frequencies (2-60 Hz) while the seizure rate diminished by more than 50%[18]. In our case, the connectivity enhancement was present immediately after the four 50 s long stimulation trains but no gradual improvement was noticed across the 10 days, therefore, we propose that amygdala LFS with 4 Hz for 4x50 second/day has a short-term neuromodulator effect.

The inter-hippocampal PLVs' relation to seizure parameters is still poorly understood. We found that LFS reduced more the average seizure duration in animals exhibiting higher inter-hippocampal synchrony in low and middle-frequency bands (<100 Hz) measured before the stimulation. As well, the seizure rate decrease was more pronounced in animals presenting higher synchrony in HFO and ripple bands during the aforementioned period. These observations suggest, that the PLVs in the delta, theta, gamma, HFO, and ripple frequency bands may be used to assess the pathomechanism of TLE and that the efficacy of LFS in this pathology may be estimated with interictal inter-hippocampal synchrony values measured before the stimulation. In a magnetoencephalography based study including patients with TLE and focal epilepsy, it was reported that the degree of the decrease in global mean connectivity in the alpha band (8-12 Hz) was related to the duration of the disease (in years) and the average number of seizures with loss of consciousness [20]. Moreover, it was reported that with the progress of the illness as the cumulative number of seizures increases, the long-range connectome suffers a continuous decline [37,38] and the degree of functional connectivity impairment positively correlates with episodic memory dysfunction observed in human TLE patients [39]. Altogether, these findings suggest that synchrony could play a decisive role both in the evolution and evaluation of the disease.

We analyzed frequencies up to 600 Hz to detect possible frequency domains with higher susceptibility for alterations in PLV. Both the difference between the two groups and the change in synchrony values between the two hippocampi were present only at low and middle-frequency

bands. This could suggest, that synchronism between distant brain structures like the two hippocampi in our case, may rely mainly on lower frequencies, but further studies should be done to definitely clarify this issue.

Conclusion

The analysis of phase locking value revealed decreased synchrony between the two hippocampi in the delta, theta, and gamma bands in the pilocarpine epilepsy model. Amygdala LFS improved the value of connectivity between the two hippocampi in the low-frequency bands (delta and theta), which could be an explanation for the antiepileptic effect of LFS. Our results suggest, that measuring the PLV can be used in a complementary way to evaluate patients with temporal lobe epilepsy and to assess the efficacy of antiepileptic treatments.

List of abbreviations

BLA- basolateral amygdala
DBS - deep brain stimulation
EEG – electroencephalography
fMRI - functional magnetic resonance imaging
HFO – high frequency oscillation
IEDs - interictal epileptiform discharges
KA – kainic acid
LFP – local field potential
LFS - low-frequency stimulation
MEG – magnetoencephalography
PLV - phase locking value
SE – status epilepticus
TLE – temporal lobe epilepsy

Author contributions

IM: (Conceptualization; Methodology; Investigation; Data collection; Data analysis and interpretation; Project administration; Resources; Writing - original draft; Writing - review and editing)
Á-JB: (Conceptualization; Methodology; Investigation; Data collection; Data analysis and interpretation; Visualization; Writing - original draft)
KO-K: (Validation; Data analysis and interpretation; Supervision; Writing - review and editing)
ZsG: (Data analysis and interpretation; Supervision; Writing - review and editing)
R-BB: (Conceptualization; Methodology; Investigation; Data collection)
TSz: (Methodology; Validation; Data analysis and interpretation; Supervision; Writing - review and editing)

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Conflict of interest

None to declare.

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