iScience, Volume *23*

Supplemental Information

Delta-Frequency Augmentation and Synchronization in Seizure Discharges and Telencephalic Transmission

Ping Chou, Guan-Hsun Wang, Shu-Wei Hsueh, Ya-Chin Yang, and Chung-Chin Kuo

Transparent Methods

Animals

All experiments including the care and use of animals were conducted in strict compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA, and the protocols approved by the Institutional Animal Care and Use Committee of National Taiwan University College of Medicine (Approval Number: 20130443 and 20160391), and that of Chang Gung University (Approval Number: CGU106-081, CGU106-206, CGU107-277, and CGU108-027), Taiwan. All animals were maintained in a vivarium with controlled 12-hour dark/light cycle, and ad libitum access to food and water. Wistar rats and wild-type C57BL/6 mice were purchased from BioLASCO Taiwan Co., Ltd., Taiwan. Thy1-ChR2-eYFP (line 18, #007612) transgenic mice, which express Channelrhodopsin-2 at glutamatergic pyramidal neurons within the basolateral amygdala (Jasnow *et al.*, 2013) were purchased from Jackson Laboratory.

Implantation of electrodes and optic fibers

During stereotaxic surgery, male Wistar rats (6-8 weeks, 250-300 g) were anesthetized by intraperitoneal infusion of 1 ml/kg of normal saline mixture solution containing 50 mg/ml Zoletil 50 (Virbac, France) and 10 mg/ml Xylazine (Sigma Aldrich, USA), and then mounted on a stereotaxic frame (Narishige, Japan). Each rat was implanted with bipolar insulated tungsten electrodes (0.002 inch in diameter, <0.5 mm apart, California Fine Wire Company, USA) into desired brain regions bilaterally following the Paxinos and Watson Brain Atlas for electrophysiology recordings. The kindling stimulation site, however, was always in the left basolateral amygdala (BLA in rats, posterior 3 mm, lateral 5 mm, and deep 8.8 mm relative to bregma), where an

additional pair of electrodes was implanted for stimulation. Two stainless steel screws were wound with silver wires (A-M System, USA) and tighten on the skull above cerebellum as well as on the nasal bone as reference and ground electrodes, respectively. Finally, the electrodes were welded on connectors (Omnetics Connector Corporation) and the scalp wound was covered with dental cement. Following similar procedures, optical fibers (200 μm, 0.37 NA, Newdoon Inc., China) glued with electrodes were implanted into the brain (BLA in mice, posterior 1.5 mm, lateral 3 mm, and deep 4.8 mm relative to bregma) of male Thy1-ChR2-eYFP transgenic mice (6-8 weeks, 20-30 g) for optogenetic stimulation. After surgery, animals were allowed to rest for 1~2 weeks and orally administered with ibuprofen (Sigma Aldrich, USA) in drinking water for 3 days before experiments. After recording, animals were sacrificed with intraperitoneal injection of overdose Zoletil 50. A 5-mA constant current was then passed via the electrodes for 20 sec to cause electrolytic lesions that mark the position of the electrodes. Animals were then perfused with 0.9 % normal saline and 4 % formaldehyde via the ascending aorta. The brain was removed and stored at 4 ˚C in 4 % formaldehyde at least overnight for postfixation, and then stored in 25 % sucrose solution for at least 3 days for cryoprotection. Afterwards the brains were sectioned by a cryotome (CM1950, Leica, Germany) at 30 μm thickness, and stained with cresyl violet acetate (Nissl staining, Sigma Aldrich, USA) to verify the positions of the electrodes. Only the data from the rats with verified correct location of all electrodes are used for further analysis.

In-vivo kindling procedures

At first, a session of 50 Hz biphasic square pulses (1 ms each phase, ±50 μA in amplitude, 10 sec per session) with a stimulus generator (STG4002, Multichannel

Systems, USA) was applied to the left BLA. If there are no afterdischarges (i.e. epileptiform discharges lasting for at least 5 sec) induced after the stimulation session, the procedures were repeated with a current amplitude increment of ±10 μA every 2 min to a maximum of ±300 μA. The current amplitude inducing the first appearance of afterdischarges is defined as the afterdischarges threshold (ADT). Those rats which did not have an ADT ≤ ±300 μA (~2%, 4 out of 24 rats) were not used for further experiments. We then applied sessions of 50 Hz biphasic square pulses (1 ms each phase, 10 sec per session) at ADT (mean ADT = 130 ± 27 μA for 20 rats) for rapid kindling stimulation. The stimulation sessions were repeated every 20 min, 10 times a day for up to 4 days (Morales *et al.*, 2014), and the 20-min period right before the first stimulation in each day was taken as baseline. Behavioral seizure scores were evaluated in every session according to modified Racine's stages (Racine, 1972): stage 0) normal behavior; stage 0.5) epileptiform discharges (afterdischarges), absence-like, freezing behavior; stage 1) facial clonus and mouth clonus; stage 2) head nodding; stage 3) unilateral forelimb clonus; stage 4) bilateral forelimb clonus with rearing; stage 5) rearing and falling; stage 6) wild running and jumping. When an animal progressed into stage-5 or higher seizures for 3 sessions, it was considered as successfully kindled and the kindling protocols were concluded. As a working definition, we also take seizures without vivid convulsions (Racine stages 0.5-2) as low-stage, and those with vivid convulsion or associated violent behaviors (Racine stage 3 and above) as high-stage. All of the 20 included rats (rats that had an ADT ≤ ±300 μA) were successfully kindled. But only the data from 7 rats, which have verified correct positions for all electrodes, were used for further analysis.

In-vivo photostimulation

The blue light for optogenetic stimulation was generated by a 473 nm diode-pumped solid-state (DPSS) laser (Newdoon Inc., China). The stimulation parameters for optical stimulations were controlled by a touch panel and verified with sample recordings. A 50 Hz (1 ms/pulse) light stimulation for 10 sec was applied via an optic fiber to the left BLA to mimic electrical kindling stimulation. The light power was typically changed between 2 to 50 mW (measured at the optic fiber tip) with a step increment as small as 2 mW for ADT measurement. All of the 9 mice show an ADT ≤ 50 mW. We then applied sessions of 50 Hz biphasic square light pulses (1 ms each phase, 10 sec per session) at ADT (mean ADT = 5.6 ± 0.3 mW for 9 mice) for optokindling stimulation. All of the 9 included mice (mice that had an ADT ≤ 50 mW) were successfully kindled. But only the data from 2 mice, which have verified correct positions for all electrodes, were used for further analysis.

In-vivo electrophysiological recording

We recorded in-vivo electrophysiological signals which were band-passed from 0.1 to 3000 Hz with a 40 dB/decade cut-off rate filter and a 60 Hz notch amplified with 1400 gain through an analog amplifier (Model 3600, A-M System, USA), and then digitized with a sampling rate of 25 kHz (DataWave Technologies, USA). To separated local field potentials (LFPs) and multi-unit spikes (MUs), signals were passed through a Butterworth 2^{nd} order infinite impulse response (IIR) filter with 6 dB/oct roll-off to get LFPs (100 Hz low-pass) and MUs (300 Hz high-pass) simultaneously during, or through a finite impulse response (FIR) filter after, the recordings. A 3-axis accelerometer (Analog Devices, USA) was anchored to the headstage (Plexon, USA), measured the acceleration of gravity in tilt-sensing as a motion sensor. The mechanical signals of acceleration of gravity were then converted

to voltage signal and input into amplifier with the same filter and amplification setting.

Signal analysis

LFPs signals were down-sampled to 500 Hz, and characterized in the frequency domain by power spectrum density (PSD) function in Welch's method with Hamming window (with a window length of 1024 points and half of the data overlapped, giving the resolution of 0.488 Hz in each signals; pCLAMP 10, MDS Analytical Technologies, USA). For time-frequency analysis, the first 10-sec segment was taken for analysis and then shifted by 1 sec for the next 10-sec segment until the completion of the full-length signal. Coherence analysis between each two signals was processed under function *mscohere* in Matlab (Mathworks, Natick, USA), defined by squared cross-spectrum between the two signals, and divided by the auto-spectrum of each signal. The value of coherence is between 0 to 1, where 1 indicates that one signal can be linearly transformed into the other one in the frequency domain, and 0 means completely lack of linear relationship. For phase analysis, we calculated instantaneous phase of FIR filter with 21^{st} order Hamming window filtered delta (1-5 Hz) band of LFPs via Hilbert transform (Neuroexplorer, Nex Technologies, USA). If two signals were synchronized, then they have linear correlations in statistics (r = 1 for positive correlation and = -1 for negative correlation). The coefficient of determination (R^2) denotes the square of r, which ranges from 0 to 1 and indicates how well the real values of one signal could fit with the other one with a linear prediction.

To evaluate the synchrony between the MU activities in bilateral BLAs, we took the interspike distance ("SPIKE-distance") in the time axis as the key estimator (Kreuz *et al.*, 2013). The analysis is done by a graphical user interface in Matlab, SPIKY, which

is provided online (Kreuz *et al.*, 2015). SPIKE-distance is derived from the distance between two neighbor spikes in the first train and the closest one in the second train for each spike, and then applied to calculate the "dissimilarity" score (S), which signals the desynchrony of firing patterns rather than single event synchronization. The value of S is between zero and one, and is addressed to measure the differences of timing between two spike trains instantaneously. Zero indicates that the two spike trains are identical or fully synchronized (i.e. no SPIKE-distance differences between the two spikes trains), and one indicates a fully desynchronized condition. We then denote 1 minus S as "similarity", which serves as a more straightforward indicator of the degree of synchronization between the two spike trains. To sorting the single units, the spikes in MUs were detected under a threshold detection algorithm. Specifically, MUs were calculated for average and standard deviation for negative peaks. Three-sigma rule were then applied to extract spikes (i.e. those >3 SD were detected and taken as spikes) (SciWorks 10.0, DataWave Technologies, USA). The extracted spikes were subsequently calculated under principle component analysis (PCA) algorithm, and generated waveform boundary clusters on a PC1vs. PC2 plane. Any waveform that falls below the upper waveform boundary and above the lower waveform boundary will be considered as part of the cluster. The criteria of waveform boundary clustering are as follows: waveform deviation≦3 SD, inter-cluster deviation ≦3 SD, and mean spike number per cluster≧3. To display the LFPs-SUs coupling during afterdischarges, we set the timestamp of each single unit spike as the zero time points, and the oscillating waves in LFP recordings in the flanking 1-sec (0.5 sec on each side) period are realigned and then averaged to get spike-triggered average (STA) waveforms (Neuroexplorer, Nex Technologies, USA). We also calculated

probabilities of each sorted single unit firing in a given phase of the delta cycle. For this purpose, the original data obtained with digitization at 25 kHz were band-filtered into the delta band (1-5 Hz) were computed for their instantaneous phase via Hilbert transform, with each zero phase timestamp taken as the start of a periodic fluctuation. We then documented the spike timestamps at the instantaneous phase of each cycle (Neuroexplorer, Nex Technologies, USA), and plotted spike probabilities as vectors in different degrees. Vectors in each epoch were calculated based on their mean magnitude and direction to get the mean resultant vector in the custom Matlab codes. The magnitude of the mean resultant vector is defined as phase-locking value (PLVs), and the direction of the mean vector is given in the figures in degree. Only those units with a spike count $\geqq 5$ in each epoch were addressed by this analysis.

Brain slice preparation

Wild-type C57BL/6 of both sexes (aged p20-28) were decapitated under isoflurane anesthesia to obtain the brain which was placed into ice-cold oxygenated choline-based cutting solution (containing [in mM] 87 NaCl, 37.5 choline chloride, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, and 0.5 $CaCl_2$). Coronal slices containing BLA (Niittykoski *et al.*, 2004; Sosulina *et al.*, 2010) (270 μm in thickness) were cut on a vibratome (VT1200S, Leica, Germany), and oxygenated in the cutting solution for 20 minutes at 30°C and then in saline (containing [in mM] 125 NaCl, 26 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, and 2 $CaCl_2$) for 20 minutes at 30°C before electrophysiological experiments.

Electrophysiological recording and electrical stimulation in brain slices

The slice was then placed in the recording chamber perfused with oxygenated (95%

O_2/5% CO_2) saline by a peristaltic pump (Gilson Medical Electric, USA) at a stable rate approximately ~5 ml/min. A ×4 objective was used to visualize BLA, and individual neurons were seen with a ×60 water immersion objective on an upright microscope (BX51WI, Olympus, Japan) and were recorded in whole-cell current-clamp mode at room temperature with 3-6 MΩ borosilicate pipettes containing K^+-based solution (in mM, 116 $KMeSO_4$, 6 KCl, 2 NaCl, 20 HEPES, 0.5 EGTA, 4 MgATP, 0.3 NaGTP, 10 $NaPO_4$ creatine, and pH 7.25 adjusted with KOH). A micropipette puller (DMZ-Zeitz-Puller, Germany) was used to make the recording electrodes of borosilicate capillaries (1.65-mm outer diameter, 1.28-mm inner diameter, Harvard Apparatus, USA). Kindling-like stimulation (Goddard *et al.*, 1969; Tuunanen *et al.*, 1997) applied in BLA (1-s train of 0.4-ms monophasic rectangular pulse at frequency of 60 Hz at an intensity of 400 μA, World Precision Instruments A356) was performed by using a paired electrode filled with 0.9% NaCl. The stimulating electrode was placed within the BLA tissue and the grounding electrode placed right above but not touching the surface of BLA slice. We always waited for 4 minutes to ensure full recovery (most neurons can fully recover within 2 minutes after stimulation) between each stimulation session. The signals were amplified with a Multiclamp 700B or axopatch 200B amplifier (MDS Analytical Technologies, USA), sampled at 20 kHz, filtered at 5 kHz, and digitized at 10-20 kHz with a Digidata-1440 analog/digital interface along with pCLAMP software (MDS Analytical Technologies, USA). Usually a current-clamp recording with good quality would last at least 60 min. In pair-recordings, the two neurons are always no more than 300 μm apart.

Identification of BLA pyramidal neurons and interneurons

Neurons in BLA can be separated into two groups, namely pyramidal neurons, and

non-pyramidal or interneurons (Sah *et al.*, 2003). After membrane breakthrough, a depolarizing step current of sufficient magnitude (Mahanty and Sah, 1998) is injected at membrane potential of -60 mV to elicit cellular discharges. Interneurons have smaller diameter and little or no spike frequency adaptation whereas pyramidal neurons possess longer diameter and exhibit prominent spike frequency adaptation in response to current injection (Washburn and Moises, 1992; Rainnie *et al.*, 1993; Mahanty and Sah, 1998; Sah *et al.*, 2003; Spampanato *et al.*, 2011). In this study, we would like to focus on parvalbumin-containing (PV+) INs because they may critically involve in network synchrony in BLA as well as in other structures (Muller et al., 2005; Woodruff and Sah, 2007a; Woodruff and Sah, 2007b; Spampanato et al., 2011; Tremblay et al., 2016). Although some reports have showed that the PV+ INs may have heterogeneous electrophysiological properties, the majority of PV+ INs exhibits no spike frequency adaptation in response to depolarizing step current injection (Rainnie et al., 2006; Woodruff and Sah, 2007a). There are even studies with PV+ INs never showing spike adaptation injected with depolarizing current (Woodruff and Sah, 2007b; Chu et al., 2012). On the other hand, it is generally accepted that PNs show spike adaptation with depolarizing pulses (Sah et al., 2003). We thus limited the sampled INs to those showing no spike frequency adaptation to decrease the likelihood of erroneous inclusion of PNs. We also visualized the morphology of pyramidal neurons and interneurons with immunofluorescent confocal imaging performed on the same acute brain slices used for electrophysiological recordings following protocols developed by McLeod and colleagues (Mcleod et al., 2017). With confocal microscopy (LSM780, Zeiss, Germany), pyramidal neurons and interneurons were identified by immunopositivity of Ca^{2+}/calmodulin-dependent protein kinase II antibody (CaMKII, 1:1000; Biorbyt, UK) and parvalbumin (PV,

1:1000; Synaptic Systems), as well as specific fluorescence in glutamatergic and GABAergic neurons in Thy1-ChR2-eYFP and Gad2-Cre;Ai32 mice , respectively. Ai32 (#024109), Gad2-Cre (#010802) and Thy1-ChR2-eYFP (#007612) transgenic mice were from Jackson Laboratory. In addition, we also used recording electrodes to fill BLA neurons with high concentration (0.4%, for faster labeling) of Lucifer Yellow CH, lithium Salt (Thermo Fisher Scientific, USA) in K^+-based solution, and identified their morphology on a fluorescence microscope. As a working definition, the smaller (longest diameter$\leqq$10 μm) neurons of non-pyramidal morphology that showed little spike frequency adaptation were then identified as interneurons, while larger neurons (longest diameter >12 μm) of pyramidal morphology that exhibit spike frequency adaptation were identified as pyramidal neurons.

In-vitro data analysis

All brain slice recording data were analyzed with pCLAMP 10 (MDS Analytical Technologies, USA), SigmaPlot 12 (Systat Software Inc., USA) and Excel 2013 (Microsoft, USA) software. The definition of burst is modified from Rainnie's group (Rainnie et al., 1993) to count the number of burst discharges in slice recording data. Postsynaptic events having at least one spike on top of rapidly summating high-frequency EPSPs were defined as burst discharges (Rainnie et al., 1993). Only EPSPs and IPSPs with amplitude greater than 3 mV were included in analysis. When two cells were recorded simultaneously, any two postsynaptic events (burst discharges, EPSPs or IPSPs, each from different neurons in a pair-recording) recorded in the two cells, respectively, having a difference in the onset time <50 ms are defined as synchronized events. For the pre-kindling and post-kindling parameters, each analysis was based on the data averaged from a 45-sec continuous stable recording just

before and after the stimuli, respectively. The burst duration and inter-burst interval were measured and analyzed in pCLAMP 10. The sample size denotes the number of neurons.

Statistics

Mann-Whitney U test and Wilcoxon signed-rank test were used for statistical comparison (Prism 6, GraphPad, USA), which were two-sided and the p-values < 0.05 were accepted as significant difference. The software used to analysis are Prism 6.01 (GraphPad, USA) and PASW statistics for windows, version 18.0 (SPSS, USA) for in-vivo and in-vitro data, respectively. All data are shown as means ± SEM.

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