

Distinct patterns of electrical stimulation of the basolateral amygdala influence pentylenetetrazole seizure outcome

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ABSTRACT

Our working hypothesis is that constant interpulse interval (IPI) electrical stimulation would resonate with endogenous epileptogenic reverberating circuits, inducing seizures, whereas a random interinterval electrical stimulation protocol would promote desynchronization of such neural networks, producing an anticonvulsant effect. Male Wistar rats were stereotaxically implanted with a bipolar electrical stimulation electrode in the amygdala. Pentylenetetrazole (10 mg/ml/min) was continuously infused through an intravenous catheter to induce seizures while four different patterns of temporally coded electrical stimulation were applied: periodic stimulation (PS), pseudo-randomized IPI stimulation (LH), restrictively randomized IPI stimulation (IH), and bursts of 20-ms IPIs (burst). PS decreased the pentylenetetrazole threshold to forelimb clonus, whereas IH increased the threshold to forelimb clonus and to generalized tonic-clonic seizures. We hypothesize that PS facilitates forelimb clonus by reverberating with epileptogenic circuits in the limbic system, whereas IH delays forelimb clonus and generalized tonic-clonic seizures by desynchronizing the epileptic neural networks in the forebrain–midbrain–hindbrain circuits.

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1. Introduction

In about one-fourth of persons with epilepsy, seizures are not satisfactorily controlled with pharmacological treatment [1–3]. In addition, many of these patients with refractory epilepsy are not eligible for ablative surgery, which, in most cases, requires a readily identifiable epileptogenic focus [4–6]. A more recent alternative available for these patients is electrical stimulation (ES) of the nervous system [7]. ES may be applied peripherally in structures such as the vagus nerve (vagus nerve stimulation) [8–10] and the trigeminal nerve (trigeminal nerve stimulation) [11,12] or targeted to a variety of structures in the central nervous system (deep brain stimulation) [13–15], most predominantly the anterior nucleus of the thalamus [16,17], the subthalamic nuclei [18,19], and the epileptogenic focus itself [20]. Classically, continuous or intermittent high-frequency ES (high-frequency stimulation) is the overall adopted pattern for an anticonvulsant effect, whereas low-frequency ES (low-frequency stimulation) is generally believed to be proconvulsant [7].

Although targeting of different neural substrates with ES has proved to be an effective approach to controlling seizures [7], the

underlying mechanisms of seizure suppression are still poorly understood [21,22]. Classically, there are two main philosophies regarding neural substrate excitability to explain the clinical benefits of ES in epilepsy: (1) ES works by suppressing or inhibiting epileptogenic structures, which is analogous to the functional ablation performed by neurosurgery; or (2) ES works by activating or stimulating neural networks that would modulate seizure-like activity. In fact, it has been reported that changing either ES parameters (e.g., amplitude, frequency, and wave morphology) or the structure targeted may influence the effect of ES on seizure control, in some cases even resulting in seizure potentiation [23]. This work addresses a different paradigm, in which ES may also play a critical role in neural synchronization depending on the temporal pattern used (i.e., pulses not distributed evenly in time), even though overall frequency of stimulation, amplitude, and electrode placement are maintained constant. In particular, the use of temporally coded/low-frequency ES may bring significant improvement in the undesired side effects of ES of neural structures.

The existence of epileptogenic neural networks that gradually synchronize by means of oscillatory reverberating circuits has been suggested in animal models of epilepsy, for example, the Genetic Epilepsy Prone Rat (GEPR). Moraes et al. [24] proposed that the EEG spike morphology signature found in the GEPR-9 seizure is the result of sequential and recurrent involvement of

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forebrain–midbrain–hindbrain neural substrates. Moreover, in the GEPR tonic–clonic seizure, the interspike interval increases linearly after each spike [25], which would corroborate the theory of a reverberating circuit that would gradually compromise neural communication between elements in the loop due to either metabolic or neurochemical stress. Accordingly, the reverberating neural networks that oscillate and generate epileptiform activity would be modulated either by setting any element of the reverberating loop in a refractory state or by desynchronizing the sequential involvement of such elements. Our working hypothesis is that an ES protocol consisting of fixed-frequency ES that would resonate with endogenous epileptogenic reverberating circuits would induce seizures, whereas a nonperiodic (e.g., random) ES protocol would promote desynchronization of such neural networks, thus behaving as an anticonvulsant.

In the present report, the basolateral amygdala was chosen as the ES target using the pentylenetetrazole (PTZ) animal model of epilepsy. The amygdaloid complex was chosen because of its role in the modulation and transfer of epileptiform activity in several animal models of temporal lobe epilepsy [26–29]. Supramaximal ES of the amygdala has been shown to produce hippocampal after-discharge, whereas repetitive subthreshold ES induces plastic changes in the temporal lobe that culminate in epileptiform activity [30]. The amygdaloid complex has monosynaptic afferents to and efferents from the parahippocampal areas (e.g., entorhinal cortex and subiculum) [31], providing the anatomical substrates for transfer and modulation of epileptic activity. In this work, we tested the hypothesis that low-frequency ES (four stimuli per second) of the basolateral amygdala would differentially modulate seizure outcome in PTZ-treated animals if applied at either (1) constant intervals or (2) randomly spaced intervals.

2. Methods

All experiments were done in accordance with the Ethical Committee for Animal Experimentation (Comitê de Ética em Experimentação Animal—CETEA) of the Federal University of Minas Gerais (Universidade Federal de Minas Gerais—UFMG), and Procedures for animal care were previously approved by this organization under Protocol 150/06.

We designed and built an electrical stimulator composed of a constant-current isolation unit driven by a PC-programmable clocking system. C++ software was developed to program the stimulator with four patterns of temporally coded stimuli, all delivering a total of four pulses per second (to guarantee the same energy flow): (1) constant interspike intervals (IPs) of 250 ms (periodic); (2) bursts with 20-ms IPs (burst); (3) pseudo-randomized IPs (linear decay histogram [LH stimulation]); and (4) restrictively randomized IPs (inverse decay histogram [IH stimulation]). These temporal patterns are depicted in Fig. 1 along with their corresponding average histograms. The LH pattern was obtained by sorting four time stamps in a 1-s interval out of a uniform distribution using an internal library function. The IH pattern was obtained using the same built-in function with the following algorithm: (A) sort an interval T_1 in the range 20–940 ms, wait T_1 , and fire pulse; (B) sort an interval T_2 in the range $T_1 + 20$ ms to 960 ms, wait T_2 , and repeat pulse; (C) repeat B twice until four pulses are fired and wait to complete the second. A minimum biologically plausible separation of 20 ms between pulses was observed in all patterns. Pulses were square, positive monophasic waves of 100- μ s duration with amplitudes varying from 100 to 350 μ A.

A total of 74 male Wistar rats from Centro de Bioterismo (CEBIO) of UFMG were randomly assigned to each stimulus group on the day of the experiment (periodic $n = 19$, burst $n = 6$, LH $n = 9$, IH $n = 14$) and also to an extra nonstimulated

control group (control $n = 26$); adjustments were made to maintain a minimum number of animals in each group. Mean weight did not differ significantly between groups (control: 306 ± 11 g, periodic: 292 ± 12 g, burst: 245 ± 12 g, LH: 253 ± 13 g, and IH: 313 ± 13 g, one-way ANOVA). All animals underwent a surgical procedure for implantation of a bipolar stimulation electrode. Electrodes were made of a twisted pair of stainless-steel (0.005 in.), Teflon-coated wires (Model 791400, A-M Systems Inc., Carlsborg, WA, USA). Animals were anesthetized via systemic sodium thiopental injection (40 mg/kg) and locally with lidocaine chlorhydrate plus epinephrine (2%) and then positioned in a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA). Coordinates for the right basolateral amygdala (AP = 2.8 mm, ML = 5.0 mm referenced from the bregma suture and 7.2 mm from dura mater) were derived from the Paxinos and Watson's atlas for rats [32]. Animals were pretreated with pentobarbitals (19 mg/kg) and flunixin (2.5 mg/kg). After correct positioning, the electrode was fixed to the skull with zinc cement and soldered to a telephone jack (Model RJ-45 6x6), which was fixed onto the skull with dental acrylic. A 5- to 7-day post-operative recovery period was observed before stimulation began. On the day before the experiment, animals received very low frequency (0.25 Hz) ES of increasing current amplitude to determine the stimulus threshold, defined as the lowest amplitude of current capable of evoking observable and distinguishable twitching behavior. On the day of the experiment, animals were caudally cannulated for intravenous infusion of PTZ (Sigma) diluted in saline at a concentration of 10 mg/ml. The cannula was connected to an infusion pump set at the rate of 1 ml/min. The ES was initiated 10 s before beginning the infusion. The latencies to onset of forelimb clonus and tonic–clonic seizures were determined and were correlated with the injected PTZ dose. All experiments were recorded on VHS tape for behavioral analysis. After stimulation, brains were electrically lesioned (0.5 mA for 2 s) and immediately removed, sliced, and stained with neutral red (2%) for histological confirmation of electrode position. Animals that survived 60 min after the end of the experiment ($n = 4$) were anesthetized with urethane (140 mg/kg) and transcardially perfused with formaldehyde (4%) before brain removal and histology procedures. Animals with incorrect positioning of electrodes were not included in our analysis. PTZ dose data were normalized by body weight (PTZ threshold), and the results were analyzed with one-way ANOVA and assessed post hoc with the Tukey multiple comparison test. Survival ratio and occurrence of uncommon PTZ-induced behaviors, such as facial clonus, rearing and falling, and whole-body tonus, were assessed with contingency tables and Fisher's exact test. Data were considered to be statistically significant at $P < 0.05$.

3. Results

All animals, except for the saline-injected control group, displayed the convulsive behavior sequence typical of the PTZ model. This consisted of a first myoclonic jerk, followed by forelimb clonus (FC), generalized clonus, and generalized tonic–clonic seizures (GTCs) [33]. Some seizure-related behavior was confused with the twitching reaction to ES; thus, only the occurrence of forelimb clonus and tonic-clonic seizures could be adequately quantified.

Distinct patterns of ES differentially modulated the latency to FC ($P < 0.0001$, one-way ANOVA) and GTCs ($P < 0.0001$, one-way ANOVA), as shown in Figs. 2A and B, respectively. The PTZ threshold for FC was significantly reduced by periodic stimulation when compared with control ($P < 0.05$, post hoc Tukey test) and IH stimulation ($P < 0.001$, post hoc Tukey test). Neither burst nor LH

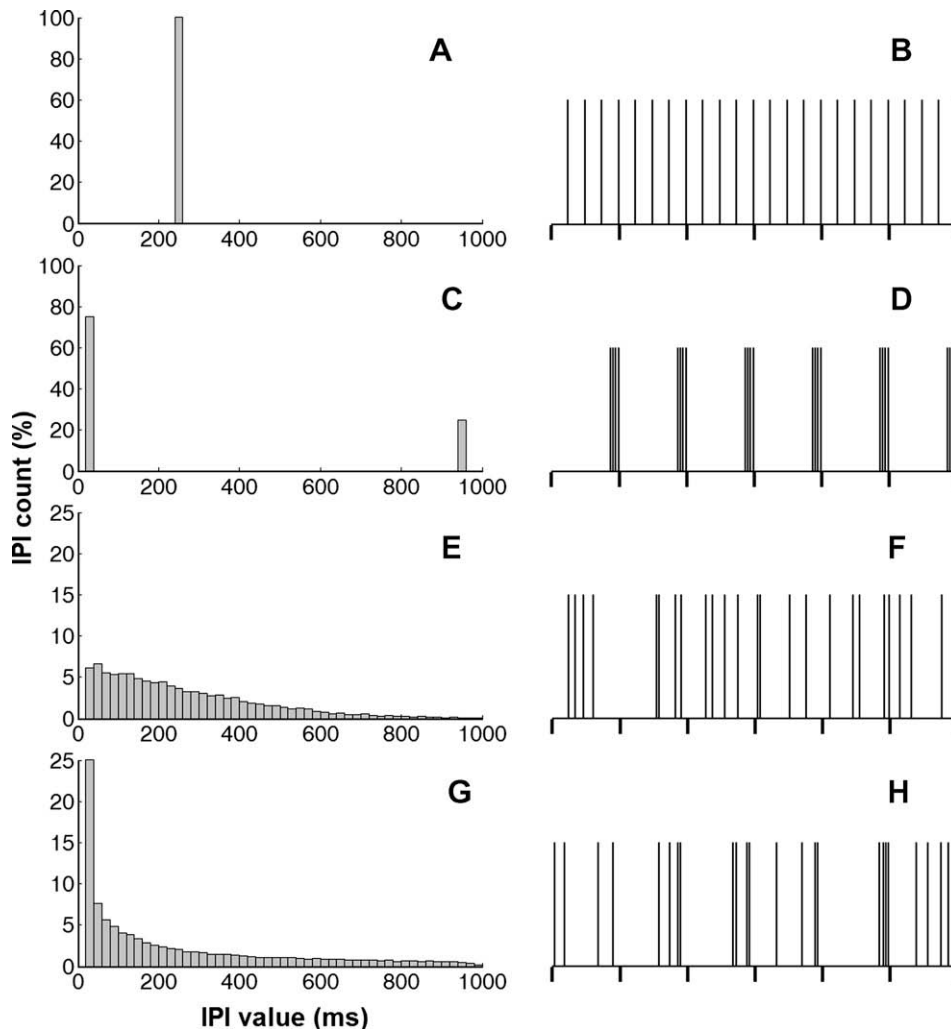


Fig. 1. Rats were stimulated with four different temporal patterns depicted here in descending order of rows: periodic (A, B), burst (C, D), LH (E, F), and IH (G, H). Left: IPI histograms. Right: typical realization of each pattern. The thick vertical lines below the x axis in the right panels mark the consecutive 1-s time windows considered for randomizing IPIs. Note there are always four pulses between each pair of these marks, guaranteeing the same mean energy flow for all patterns.

stimulation changed the FC PTZ threshold. Nevertheless, IH stimulation significantly increased threshold to FC when compared with all groups ($P < 0.05$ against control and burst, $P < 0.01$ against LH, and $P < 0.001$ against periodic, post hoc Tukey tests). Moreover, IH stimulation robustly increased (by almost twofold) the PTZ threshold to GTCSs when compared with all other groups ($P < 0.001$ against all groups, post hoc Tukey test). Periodic stimulation and other patterns had no overt effects on GTCSs when compared in a pairwise manner.

Rearing behavior, very uncommon in PTZ models [33], was observed in the periodic and IH groups (Table 1) when compared with the control group, with statistical significance ($P < 0.01$ and $P < 0.05$, respectively, Fisher's exact test). Finally, three animals in the IH group, one in the periodic group, and none in the other groups survived. Survival rate of the IH group was statistically greater than that for other groups pooled together, as assessed in Table 2 ($P < 0.05$, Fisher's exact test).

There were no statistically relevant correlations among weight, current, behavior threshold, and behavior occurrence in any groups (data not shown).

4. Discussion

The results clearly indicate that distinct temporal patterns of ES, when applied to the amygdala, differentially modulate PTZ-induced

seizure behavior in a rodent model. The effects observed in this study could be predicted by a comprehensive theory of ictogenesis which takes in consideration not only hyperexcitability, but also, and more importantly, hypersynchronism and reverberating neural networks. In this sense, all three mechanisms must be kept in mind to adequately explain the results described.

As PTZ is a GABAergic antagonist [34], it creates a nonspecific condition of hyperexcitability necessary to induce seizures evoked by multiple reverberating neural circuits that are gradually recruited into the epileptogenic process as the drug is absorbed. In fact, low doses of PTZ (<40/mg) typically evoke minimal seizures displaying myoclonic jerks, forelimb and head clonus, and chewing [33]. These behaviors are classically correlated with exacerbated activity of structures in the limbic system, including the amygdala and hippocampus [35], which represent a more restricted forebrain region. In contrast, higher doses of the drug evoke minor seizures followed by major seizures with or without a tonic phase and generalized tonic-clonic behavior [33], both correlated with activation of a broader brain territory that includes structures in the hind-brain, midbrain, and forebrain [24,35]. These local and broader neural circuits are connected and synchronized during ictogenesis, as suggested by electrophysiological data [25], by means of physiological neuroanatomical connections and a pathological hyperexcitable condition. This gives rise to a myriad of stereotypical convulsive behaviors and cognitive deficits [36]. Finally, some

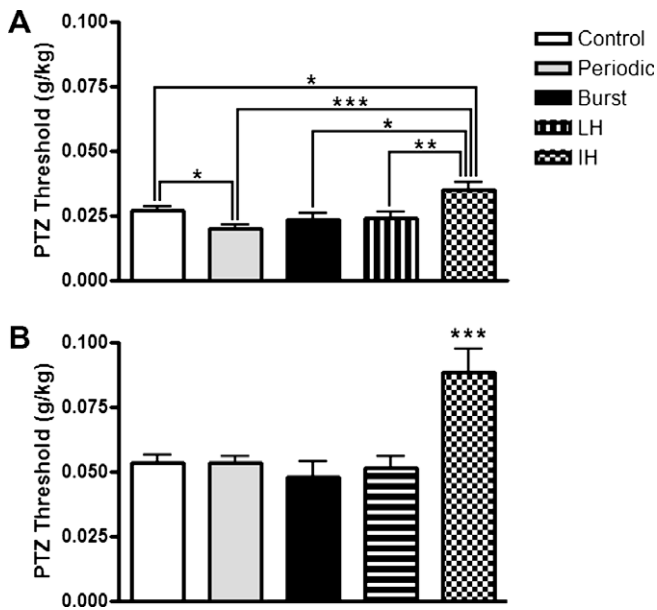


Fig. 2. PTZ threshold for two convulsive behaviors—forelimb clonus (A) and generalized tonic-clonic seizures (B)—according to stimulus pattern. IH stimulation increased drug threshold for both forelimb clonus and generalized tonic-clonic seizures when compared with all groups (both $P < 0.0001$, one way ANOVA; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, all post hoc Tukey). Moreover, periodic stimulation decreased drug threshold for forelimb clonus ($P < 0.05$, post hoc Tukey).

Table 1
Contingency table for occurrence of rearing behavior

	With rearing	Without rearing
Control	0	26
Periodic ^a	6	13
Burst	0	6
LH	0	9
IH ^b	4	10

^{a,b} Periodic and IH stimulated groups manifested more rearing behavior compared with the control group (^a $P < 0.01$ and ^b $P < 0.05$, respectively, Fischer's exact test).

Table 2
Contingency table for survival ratio^a

	Survived	Died
IH stimulus	3	11
Others	1	59

^a All other groups (periodic, burst, LH, and control) have been pooled together under "Others."

structures, such as the amygdala play a key role in this synchronization process, acting as a modulator and a center for the transfer of epileptiform activity [29].

Our understanding is that ES of the amygdala modulates convulsive behavior through direct modulation of neural epileptiform activity in local as well as broader neural circuits, in a manner similar to that by which the structure acts during normal activity. The periodic stimulation used in this study is in the frequency range of epileptiform activity, as revealed by electrophysiological studies [25] and, thus, may facilitate forelimb clonus by adding energy to the limbic system in a resonating fashion. In contrast, we hypothesize that IH stimulation impairs reverberation of the limbic system (local) and forebrain–midbrain–hindbrain (broader) circuits through an antiresonating effect in each of them and also through blockage of synchronizing mechanisms among them. The effect

results in the increase in PTZ threshold to forelimb clonus and generalized tonic-clonic seizures observed here.

Interestingly, rearing is a very uncommon behavior in the PTZ model and is said to be masked inside the GTCs that would predominate in the motor expression [35]. Nevertheless, periodic stimulation increases its occurrence probably due to a facilitation process capable of unmasking it from GTCs. By the same token, the increased rearing caused by IH stimulation is probably due to an unmasking process of delaying GTCs. One could hypothesize that if rearing was normally observed in the PTZ model, periodic stimulation would decrease its threshold, and IH would have no effects. A direct consequence of this reasoning is to design analogous experiments using different animal models of epilepsy that display limbic seizures (e.g., audiogenic kindling of WARs, amygdala electrical kindling, acute and spontaneous pilocarpine-induced seizures).

The difference in seizure threshold between LH and IH is not easily understood, and further manipulations of the temporal pattern of stimulation may help clarify the basis for this phenomenon. Although they have a distribution over a wide range of IPI values, the histograms of LH and IH have very distinct shapes. The LH histogram can be fit ($R^2 = 0.98$) by a linear equation of inclination close to $-1/100$, whereas IH is fit ($R^2 = 0.98$) by a power equation of exponent close to -1 (Figs. 3A and B, respectively). A first consequence of these different shapes is that each of them accumulates IPI occurrences in distinct ranges. Two differences are worth noting. First, LH has a higher count of IPI in the range 220–280 ms (Fig. 3C) than does IH. As mentioned before, this is in the range of epileptiform activity frequency of discharge, and thus, IPIs in this range are probably resonating and convulsant, as suggested by the periodic group. Additionally, IH has a considerably higher count of IPIs within the range 20–100 ms than does LH (Fig. 3D). One could assume that this means IH has a higher content of high-frequency stimulation, thus corroborating previous work, mainly on deep-brain stimulation, which would have anticonvulsant properties. However, this is not true, once high-frequency stimulation is delivered at frequencies over 100 Hz [7,21,35] to produce its inhibitory effects. Also, burst stimulation in this study, which is composed mainly of brief periods of high-frequency stimulation, was not effective in suppressing seizures. The authors propose an alternative hypothesis that relies on the recognition of different temporal codes of pulses or cortical motifs, also called *cortical songs* [37], by specialized neuronal circuits arranged, for example, as the synfire chains [38]. The existence of such circuits is strongly suggested by experimental studies [39–41]; a series of biologically plausible proposals have been described [42–45] and their features have been modeled and studied in silico [38,46–48]. According to these studies, pattern recognition circuits would reverberate when neuronal inputs have a specific well-defined temporal structure [38], and two or more of these circuits may synchronously couple when sharing the same temporal pattern of activity [49]. Finally, cortical motifs have strict timing constraints, and their constitutive pulses must be grouped within a certain time limit. Although there is no consensus on a value, some authors suggest that the whisking frequency (10 Hz or 100-ms period) is a restrictive time window for somatosensory processing [39]. In this sense, our understanding is that IH randomly activates multiple distinct circuits by "sending" randomized cortical motifs through efferents of the amygdala at a much faster rate than does LH, because it has a higher count of short IPIs in the range 20–100 ms. This would severely impair neural synchronization of local circuits, once they are activated by distinct patterns, preventing them from being coupled. It would also impair the transfer of epileptiform activity to broader circuits once the amygdala would fire in a pattern that may be not synchronized.

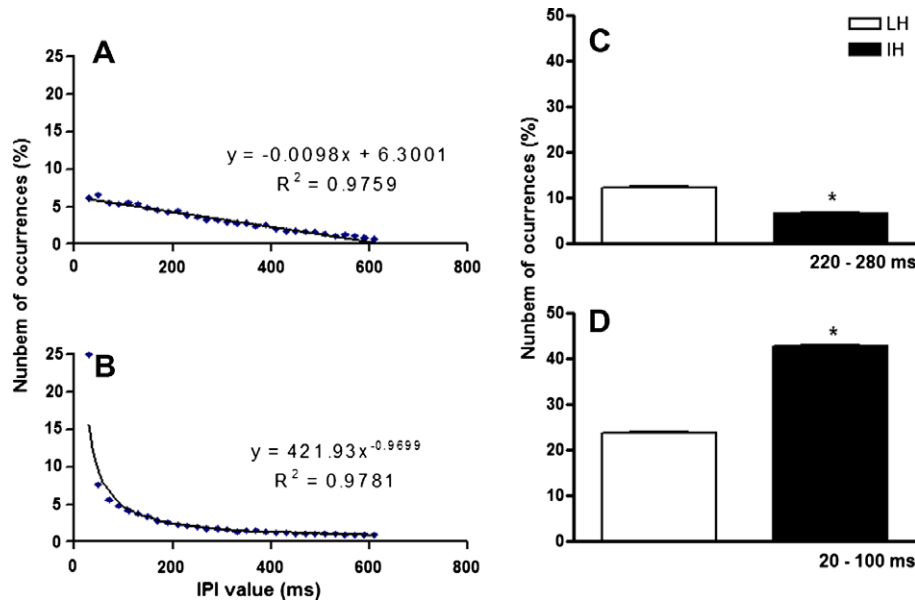


Fig. 3. Curve fitting for the mean histogram ($n = 8$ simulated histograms) of the two randomized stimulus patterns. The LH mean histogram is fit by a linear equation of inclination close to $-1/100$ (A), and the IH mean histogram is fit by a power curve of exponential close to -1 (B), both with high correlation factors ($R^2 = 0.9759$ and $R^2 = 0.9781$, respectively). This causes IH to have a lower IPI count in the epileptogenic range 220–280 ms (C) ($P < 0.0001$, Student's t test) and a higher IPI count in the range 20–100 ms (D) ($P < 0.0001$, Student's t test) compared with LH.

In short, a reasonable hypothesis for the difference between the two stimulation patterns is based on the proportions of resonating and antiresonating power present in each of them. The proportions of antiresonating and resonating power of LH stimulation patterns would not be high enough, probably close to one in a proper scale, canceling each other out. In contrast, the IH pattern would have a proportion such that the antiresonating power would overcome resonating power by far. This would make IH a seizure-suppressing stimulus, whereas LH would have no effect. A possible way to correlate antiresonating power with a randomized pattern construction algorithm would be to apply different limits around fixed 4-Hz time stamps (0, 250, 500, and 750 ms) for randomization of pulses and analyzing their effects in seizure suppression.

Our results suggest that desynchronizing neural activity by neurostimulation is an alternative to be considered in the treatment of epileptic disorders in clinical practice. A next logical step would be to run clinical trials of human deep-brain stimulation activated in LH and IH patterns. Such a strategy may overcome some unwanted collateral effects of classic high-frequency stimulation for the treatment of human epilepsy, such as a higher energy transfer to brain tissue. However, much work must be done in animal models to better assess the synchronizing/desynchronizing effects of ES. Moreover, behavior modulation through random patterns and other temporal codes of stimulation may provide fruitful insights into the mechanisms of seizure genesis, propagation, and termination.

Conflict of interest statement

The authors state that no other people or organization have inappropriately influenced this work. Therefore, there is no pertinent claim of a conflict of interest.

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