Int J Clin Exp Med 2014;7(11):4091-4098
www.ijcem.com /ISSN:1940-5901/IJCEM0002155

Original Article

Therapeutic effect of low frequency electric stimulation on the epileptogenic focus in amygdale-kindled rats

Yanfei Han[1,2], Yuping Wang[1]

[1]*Department of Neurology, Xuanwu Hospital of Capital Medical University, 100053, China;* [2]*Department of Neurology, Beijing Friendship Hospital, Capital Medical University, 100050, China*

Received August 28, 2014; Accepted October 23, 2014; Epub November 15, 2014; Published November 30, 2014

Abstract: This study was to examine the therapeutic effect of low-frequency electric stimulation (LFS) on the epileptogenic focus in amygdale-kindled rats, and to find out the optimal stimulus parameters. A microelectrode was implanted into the right amygdale of adult male rats. After fully kindling, LFS was delivered to the right amygdale (through the electrode) between seizures to induce stimulus trains (10 repetitive sequences). Next, we undertook controlled experiment in order to exclude the influence of seizure induction intervals on seizure. Fully kindled rats experienced trials for 4 days, the intervals of the repetitive seizure inducing stimulation was randomized for 5 min, 10 min, 15 min, and 20 min respectively. Finally, we applied an orthogonal design to test the 4 factors of parameters (frequency, pulse duration, current intensity and persistence time), in order to find out the best stimulus parameters. Results showed that compared to control group, the stage-4 seizure induction rate decreased dramatically in LFS group and animals in experiment group were more likely to be non-responsive to seizure-inducing stimuli. There were no statistical differences in the different seizure induction intervals. Significant differences were observed in different stimulus frequencies and stimulus train persistence times on the stage-4 seizure induction rate. These findings indicated that 1 Hz LFS is the best. Training for 5 min is more efficacious in controlling seizure. Thus, our results suggest that LFS applied directly to the site of seizure was effective and further studies are required to explore the most effective parameters for developing implanted stimulator.

Keywords: Kindling, low-frequency electric stimulation, best parameters, orthogonal design

Introduction

Stimulus frequency has been shown to be critical in the prevention of seizure [1]. Although many investigators have successfully used high-frequency patterns as a therapeutic strategy in studies as early as in 1969 [2-4], Goddard et al found that high-frequency stimulation had kindling effect in fact [5]. Several years later, Gaito et al found that low-frequency stimulation (LFS) could decrease or prevent seizure activity [6], and the term "quenching" was used to describe the interference in the kindling process after stimulation [7]. Goodman et al reported a dramatic decrease in the incidence of stage-5 seizures in fully kindled animals after LFS [8]. Recent years, research on LFS to suppress seizures continued to emerge [9-13], but stimulation site and stimulation parameters varied, difficult to directly employ.

The source of paroxysmal discharge of focal epilepsy was considered as some regions of brain tissue, named epileptogenic focus. Electrical stimulation of the epileptogenic focus can interfere with the nervous activity, and then interrupt the seizures. From the theoretical point, we can estimate that stimulation of the epileptogenic focus may produce a more direct and evident seizure-preventing effect than other targets. Although some data has certificated the positive effect of LFS of the epileptogenic focus [14], few reports comparing the anti-seizure difference of various parameters have been published.

In the present study, we established a amygdale-kindling model. After proving the anti-seizure effect of LFS to the epileptogenic focus, orthogonal design was adopted to find the best stimulus parameters, with the purpose of providing

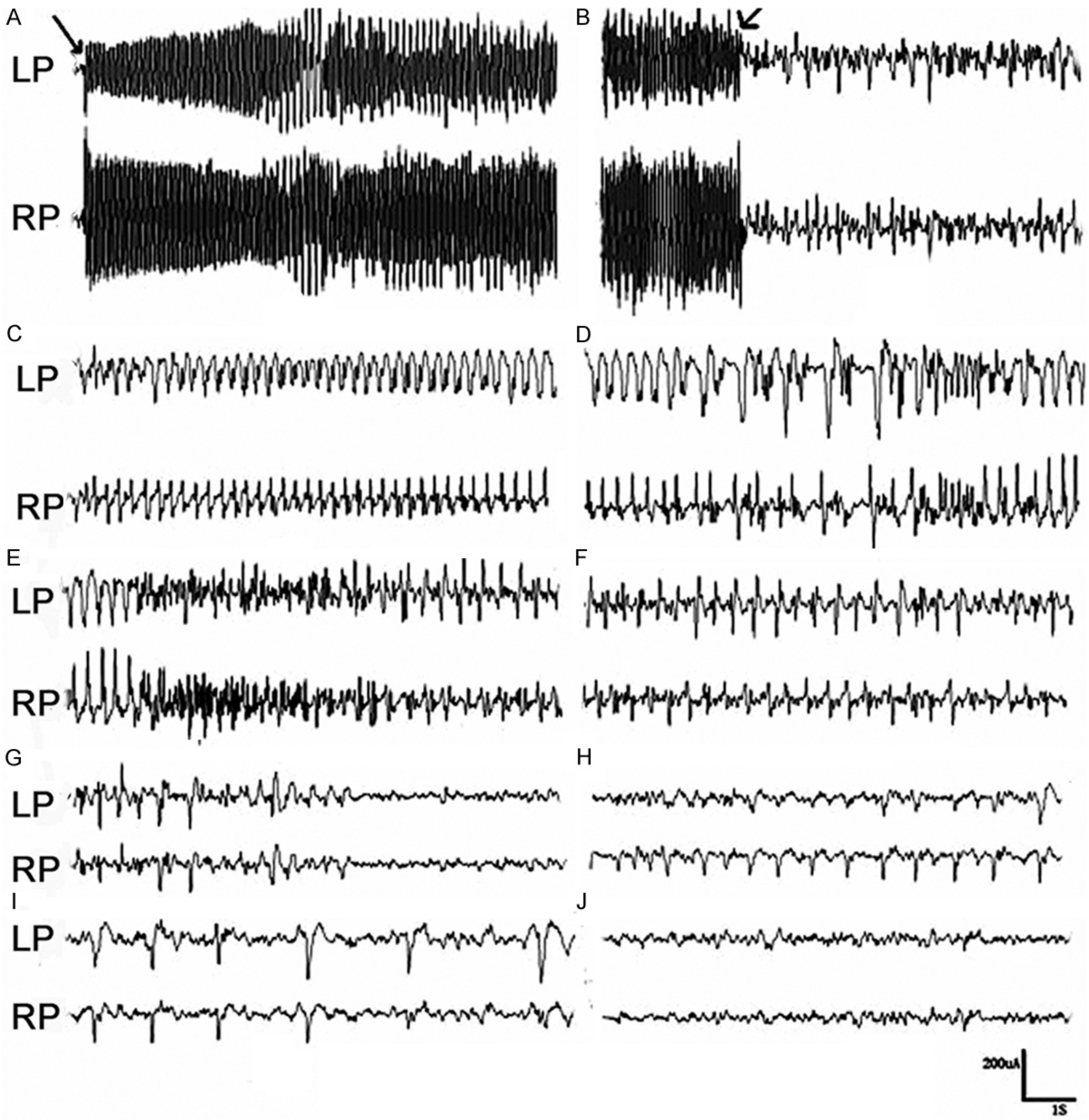

Figure 1. Rat EEG including a stage 4 seizure. The arrows indicated the start and end of the kindling stimulation. A to J are the consecutive recording of EEG. LP and RP are the electrodes located on the left and right cortex. High frequency discharges are recorded at the initial phase of the seizure after the kindling stimulation. The discharge frequency decreased gradually during the seizure.

rationale for the development of implanted stimulator.

Methods

Design

In this study, all animals were first implanted with electrodes, and then kindled one week later. Fully kindled rats were selected for further studies. We first investigated in 8 rats if low-frequency electric stimulation (LFS) delivered to the kindling site could ameliorate or relieve seizures induced by kindling stimulation. Then, the effects of different kindling intervals on seizure induction rates were observed in 4 rats. Finally, the optimal parameters of the low frequency stimulation were investigated in another 32 rats.

Animals

Male Sprague-Dawley rats (250-300 g) were separately housed under 12 h light/dark cycles with food and water ad libitum. All experiments were carried out in accordance with the Health Guide for the Care and Use of Laboratory Animals of Xuanwu Hospital. Furthermore, attempts were made to minimize the number of animals used in the study and their suffering.

Surgery

After absolute diet for 8 h, each animal was anesthetized with Chloral Hydrate (0.3 ml/100 g) and fixed in a stereotaxic apparatus. A midline incision was made in the scalp, calvarium was exposed. A bipolar, teflon-coated, stainless steel electrode (Kindling and stimulating electrode) was implanted into the right amygdale. The coordinated measures for it from bregma were as follows: AP 2.0 mm, ML 5.0 mm from the midline, DV 8.0 mm from the dura. Other three electrodes (EEG recording electrodes) were fixed in the skull on bilateral parietal areas (AP 5.0 mm, ML ± 2.0 mm, DV 1.0 mm), and the anterior fontanel (AP). Three screws were fixed into cranial bone. After this, the wound was covered with acrylic dental cement ensuring that all electrodes and screws were covered with the cement. Then, a plastic cap was fixed to protect all the electrodes.

After surgery, the rat was placed under a radiant heat source until it recovered from the anesthetic. All animals were allowed to recover at least one week before kindling stimulation.

Electroencephalography and seizure evaluation

Electroencephalographic (EEG) activity was monitored using an amplifier (0.1-100 Hz) connected with the electrodes embedded in the skull in each kindling day. We started the recording of EEG five minutes before each kindling or experiment. The acquisition of EEG lasted to the end of the whole procedure of the day. The amplified EEG signal was stored on hard disc in a computer for off-line analyzing. EEG after discharge duration (ADD) was used as a parameter for seizure evaluation.

A video camera was run simultaneously for capturing seizure behavior activity. Seizure severity was classified by rat's behavior according to Racine criteria [15]: stage 1: facial movement; stage 2: head nodding; stage 3: unilateral forelimb clonus; stage 4: bilateral forelimb clonus and rearing; stage 5: bilateral forelimb clonus, rearing and falling.

Kindling

For inducing seizures, kindling stimulation was given to each rat through the bipolar amygdale electrodes. Every ten minutes, we gave a string of electric pulses of square wave (160 pulses of 16-Hz) generated from a stimulator (Grass 48, America) to each rat. The pulse was 1-ms wide with an intensity of 400-uA. After several string of electric stimulation, the animal might show seizures of different degrees when receiving kindling stimulation. Seizure was confirmed by both behavior and EEG changes. Usually the seizure evolves from mild, minor, partial one into major generalized ones after receiving further several string of inducing stimulation. When the animal developed into a seizure of Racine's stage-4 for 3 consecutive times or into a seizure of stage-5, the animal was regarded as fully kindled. **Figure 1** shows the rat EEG, which includes an episode of stage-4 seizure. In each day, a maximum of 12 strings of kindling stimulation was given to each animal. Those animals that were not fully kindled were left to the next day for further kindling. We carried out LFS experiment 2 days after the rat was fully kindled.

Effect of LFS on epileptic seizure

In the experiment day, each kindled animal was moved into a cabinet. EEG electrodes were connected to the amplifier, and then EEG signal was stored on hard disk. The bipolar electrodes were connected to the stimulator. First, all animals were assigned for measuring the threshold of stage-4 seizure. The seizure threshold was measured by the minimal electric strength employed for inducing reliable seizures through the bipolar electrode stimulation. The test was started with a string of electric pulses (160 pulses of 16-Hz) of current intensity of 60 uA with a step increase of 20 uA until a stage-4 seizure was observed. This current intensity was named as seizure threshold.

Eighteen fully kindled rats were randomly divided into two groups: control group (n = 9) and experimental group (n = 9). In control animals, 10 seizures were consecutively induced with a 15-minutes interval. The seizure inducing stimuli were pulse train of 10 s with 16 Hz. The pulse was 1-ms wide with 50 uA higher than seizure threshold. Ten seizures were also consecutively induced in experimental animals

Table 1. $L_{16}4^5$ orthogonal table

Group	Frequency (Hz)	Current intensities (uA)	Pulse durations (ms)	Train persistence time (min)
1	0.5	50	0.2	5
2	0.5	Half seizure threshold	0.4	10
3	0.5	Seizure threshold	0.6	15
4	0.5	50 higher seizure threshold	1	20
5	1	50	0.4	15
6	1	Half seizure threshold	0.2	20
7	1	Seizure threshold	1	5
8	1	50 higher seizure threshold	0.6	10
9	2	50	0.6	20
10	2	Half seizure threshold	1	15
11	2	Seizure threshold	0.2	10
12	2	50 higher seizure threshold	0.4	5
13	4	50	0	10
14	4	Half seizure threshold	0.6	5
15	4	Seizure threshold	0.4	20
16	4	50 higher seizure threshold	0.2	20

with the same parameters. However, they also received LFS through the same kindling electrode at the seizure induction intervals. The LFS parameters were 1 Hz, 0.2-ms wide pulse train with half of the seizure threshold for 15 min. The ADD and the behavioral seizure score were measured for each animal after each seizure induction.

Influence of seizure induction intervals on seizure

The preparing procedure was the same as above. Four fully kindled rats experienced 10 seizures each day for 4 consecutive days with different seizure induction intervals. Seizure threshold was measured, and seizures were induced in each animal (pulse trains of 16 Hz for 10 s, 1-ms pulse width, 50 uA higher than seizure threshold). Four different train intervals (5 min, 10 min, 15 min, or 20 min) were applied in different animals in the four days. The 4 intervals were tested in each rat in a counter-balanced sequence. The behavioral seizure was scored for each animal after seizure induction.

Orthogonal design to find out the best stimulus parameters

To search the most effective stimulus parameters of LFS, including the different frequencies, pulse durations, current intensities and train persistence times, orthogonal design, a very efficient protocol, was used. Thirty-two fully kindled animals were randomly delivered into 16 groups. After testing the seizure threshold of stage-4, ten seizures were induced in each animal with the following parameters: pulse trains of 16 Hz for 10 s, 1-ms pulse width, 50 uA higher than the seizure threshold. Persistent LFS was given at the seizure induction intervals. The parameters of LFS for each group was displayed at the **Table 1**, each row corresponded with each group. The behavioral seizure was scored for each animal after each seizure induction.

Histological examination

Electrode location was determined at the end of the experiments. Each animal was anesthetized with Chloral Hydrate (0.3 ml/100 g) and sacrificed by perfusion with 4% paraformaldehyde through the aorta for 10 min. The brain was removed, mounted by mineral wax and sectioned on a vibratome. Each section was 50 um thick and stained by H&E. A successful electrode implantation for the stimulating electrodes was defined as an electrode tip within the basolateral amygdale or directly adjacent to the basolateral amygdale. With these criteria we found that in all the fully kindled animals the electrode tips were successfully implanted.

Statistics analysis

First, to verify the effect of LFS to the seizure onset site, we select Chi-square Test to detect the difference of seizure induction rate between the control and the experiment group. Then we choose Independent-Sample T Test to compare the difference of ADD between the two groups. Second, Chi-square Test was also used to compare the differences among the four conditions

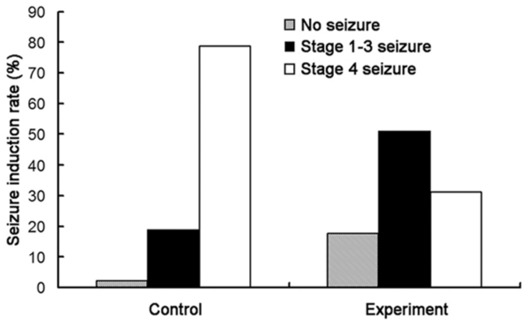

Figure 2. Y-axis represents the seizure reduction rate, and X-axis represents the two groups. LFS can decrease the rate of stage-4 seizure dramatically, and increase the rate of stage 1-3 seizure in the experiment group ($P < 0.01$).

in order to find whether there were any variances about the stage-4 seizure induction rate when the seizure induction intervals were not the same. Third, analysis of variance (ANOVA) was used to find out the best parameters of LFS. For all analysis, a $p < 0.05$ was considered significant.

Results

Effect of LFS to the seizure onset site

Nine animals were divided into each group, in which, each animal received ten sequences of seizure inducing stimuli in our experiment. Therefore, 90 times of inducing stimuli were carried out to each group indeed. Using Chi-square Test, compared to the control group, we found the stage-4 seizure induction rate decreased dramatically in LFS group. ($P < 0.01$, **Figure 2**).

The value of relative ADD (by subtracting ADD evoked by each of the seizure induction stimulus during test from the ADD obtained by seizure threshold stimulation before the test of each animal) was selected for the comparison between the two groups with the purpose of excluding the individual difference. However, no statistical difference was found using Independent-Sample T Test.

Effect of different seizure induction intervals

Each animal experienced 4 days' experiments of 4 different seizure induction intervals respectively. No statistical difference in stage-4 seizure induction rate was found among the four conditions using Chi-square Test.

Best parameters of LFS

According to the $L_{16}4^5$ orthogonal table, we completed the test of the 16 groups. We found statistical difference in factors of stimulus frequency ($P < 0.01$) and train persistence time ($p < 0.05$) (**Table 2**; **Figures 3**, **4**). Frequency of 1 Hz was found to be the best among the four levels in stimulus frequency. Train duration of 5-minute was more effective than other durations. No statistical difference was found among different pulse durations and current intensities.

Discussion

Before brain stimulation can be used as a new therapy for epilepsy, we have to answer fundamental questions of where to stimulate and what are the most effective parameters. This study demonstrates that LFS to the epileptogenic focus can block seizure evolving and we have found better parameters through an orthogonal design.

Effect of LFS of the seizure onset site

The initially tested target of electrical stimulation for seizure controlling was cerebellum [16], and cooper's attempt made us believe that electrical stimulation could inhibit seizures. Some investigators carried out animal experiments subsequently, in order to find out the eligible targets, including caudate nucleus, thalamus nucleus and epileptogenic focus. Because of the different test methods and assessment standards, the results are various.

Electrical stimulation of the amygdala in rats could disrupt seizures derived from repeated stimulation of the same site (kindling) [17]. The same author later reported that a DC current leakage from the stimulating apparatus was responsible for the protective effect they termed "quenching" [7]. This may be the first evidence published about electrical stimulation onto epileptogenic focus. Electrical stimulation of the epileptogenic focus can interfere with the nervous activity, and then interrupt the seizures. From the theoretical point, we can estimate that stimulation of the epileptogenic focus may produce a more direct and evident effect than other targets.

The decrease in kindled seizures observed in the present study after LFS can be explained by

Table 2. The result of ANOVA about stage-4 seizure induction rate.

	Level 1	Level 2	Level 3	Level 4
Frequency[#]	0.488 (0.5 Hz)	0.338 (1 Hz)[*]	0.588 (2 Hz)	0.600 (4 Hz)
Intensity	0.475 (50 uA)	0.425 (1/2 AT)	0.600 (AT)	0.513 (AT + 50 uA)
Pulse duration	0.513 (0.2 ms)	0.475 (0.4 ms)	0.475 (0.6 ms)	0.550 (1 ms)
Time[##]	0.363 (5 min)[*]	0.513 (10 min)	0.525 (15 min)	0.613 (20 min)

Footnotes: Factors of parameters (including frequency, current intensity, pulse duration and persistence time) with the four levels were investigated. We found statistical differences in frequency ($p < 0.01$) and persistence time ($p < 0.05$) on the rate of stage-4 seizure. Frequency of 1 Hz is the best and 5 min is the best in persistence time respectively. However, no statistical difference was found about pulse duration and current intensity. [*]The best level; [#]$P < 0.01$; [##]$P < 0.05$ among the four levels of this factor.

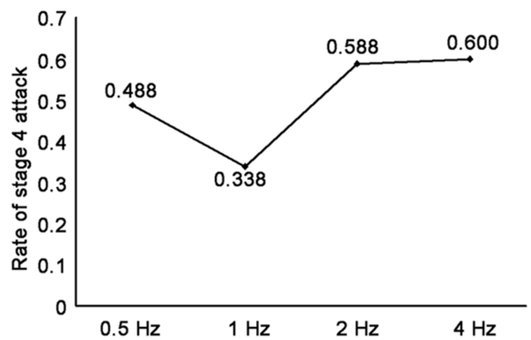

Figure 3. This plot illustrates the factor of frequency. The X-axis represents different frequencies (0.5 Hz, 1 Hz, 2 Hz, and 4 Hz) and the Y-axis means the rate of stage-4 seizure. The 1 Hz stimulation is the most useful frequency for diminishing the stage-4 seizure.

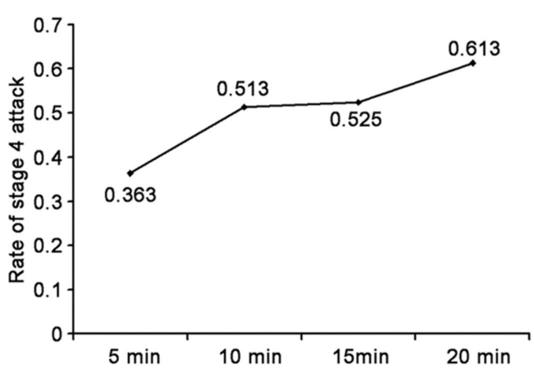

Figure 4. This plot illustrates the factor of persistent time. The X-axis represents different persistence times (5 min, 10 min, 15 min, and 20 min) and the Y-axis means the rate of stage-4 seizure. The 5 min stimulus train duration is the most useful in diminishing the stage-4 seizure.

the findings of Carrington and colleagues [14]. They observed a 200% increase in AD threshold that lasted for 2 to 3 days after the low frequency stimulation of the kindling focus. Obviously, an elevation in seizure threshold would make it less likely that a given kindling stimulus would elicit a seizure. Ghobdin [18] found that application of LFS at the kindling site almost completely prevented the devlopment of epilesy.

The mechanism for the AD threshold-elevating effect of LFS is largely unknown, but some investigators found LFS can cause neurotransmitter changing in the stimulated site. In 2004, Lopez-Meraz et al. revealed that LFS applied twice daily in normal rats increased the BDZ receptor binding levels in basolateral amygdala and thalamus ipsilateral to the place of stimulation and in contralateral temporal cortex [19]. In contrast, LFS produced a decrease of BDZ receptor binding levels in the contralateral frontal cortex. They also found repetitive LFS in normal rats produced a significant decrease of theμreceptor binding in the sensorimotor and temporal cortices ipsilateral to the place of the stimulation, the contralateral CA1 field of hippocampus, and bilateral dentate gyrus [19]. Considering that some of these structures are implicated in the generation and propagation of epileptic activity, the LFS-induced BDZ andμreceptor binding changes in these areas may restrain the spread of epileptic activity.

Effect of different seizure induction intervals

We concluded the seizure induction intervals from 5 min to 20 min can produce no difference in excitability of animals, which served as the basis for orthogonal design to find out the best stimulus parameters.

The most effective parameters of LFS

The most efficient frequency we found is 1 Hz, then is 0.5 Hz, and 4 Hz is the worst. This can be explained by the viewpoint of Goddard et al., that high-frequency stimulation had kindling effect in fact [5]. Conversely, too low-frequency was not power enough to change the neural activity in the kindling site.

The most effective persistence time of LFS is 5 min among the four levels. We supposed that too long persistence time stimulation might cause toleration of tissues, so the effect was decreased. In fact, short persistence time stimulation can inhibit seizure attack. Magdaleno-Madrigal et al. analyzed the effect of electrical stimulation of the nucleus of solitary tract, the persistence time is 1 min, they found it can prolong the days of kindling prominently [20]. Carrington et al found that a persistence time of 30 s can increase ADT dramatically [18]. Besides, the stimulation as short as 1 s can also block seizure attack. Shi et al. reported that substantia nigra pars reticulate stimulation for 1 s can block seizure attack by 43.5% [21].

Conclusion

Our study confirmed the therapeutic effect of LFS to the epileptogenic focus and made an initial attempt for searching the best parameters. In the present study, we found 1 Hz stimulation is more effective. The best parameters about stimulus train duration, intensity, and pulse width should be tested in more samples with more levels. The best parameters will guide the development of implanted stimulator, which will be useful for intractable epilepsy patients.

Acknowledgements

This work was supported by National Natural Science Funds (30770744).

Disclosure of conflict of interest

None.

Address correspondence to: Yuping Wang, Department of Neurology, Xuanwu Hospital of Capital Medical University, No. 45 Changchun Street, Beijing 100053, China. Tel: 86-10-83198273; Fax: 86-10-83157841; E-mail: wangyuping_xuanwu@163.com

References

[1] Lado FA, Velisek L and Moshe SL. The effect of electrical stimulation of the subthalamic nucleus on seizures is frequency dependent. Epilepsia 2003; 44: 157-164.

[2] Zhang Q, Wu ZC, Yu JT, Zhong XL, Xing YY, Tian Y, Miao D and Tan L. Anticonvulsant effect of unilateral anterior thalamic high frequency electrical stimulation on amygdala-kindled seizures in rat. Brain Res Bull 2012; 87: 221-226.

[3] Velasco F, Velasco M, Jimenez F, Velasco AL and Marquez I. Stimulation of the central median thalamic nucleus for epilepsy. Stereotact Funct Neurosurg 2001; 77: 228-232.

[4] Hodaie M, Wennberg RA, Dostrovsky JO and Lozano AM. Chronic anterior thalamus stimulation for intractable epilepsy. Epilepsia 2002; 43: 603-608.

[5] Goddard GV, McIntyre DC and Leech CK. A permanent change in brain function resulting from daily electrical stimulation. Exp Neurol 1969; 25: 295-330.

[6] Gaito J, Nobrega JN and Gaito ST. Interference effect of 3 Hz brain stimulation on kindling behavior induced by 60 Hz stimulation. Epilepsia 1980; 21: 73-84.

[7] Weiss SR, Eidsath A, Li XL, Heynen T and Post RM. Quenching revisited: low level direct current inhibits amygdala-kindled seizures. Exp Neurol 1998; 154: 185-192.

[8] Goodman JH, Berger RE and Tcheng TK. Preemptive low-frequency stimulation decreases the incidence of amygdala-kindled seizures. Epilepsia 2005; 46: 1-7.

[9] Rashid S, Pho G, Czigler M, Werz MA and Durand DM. Low frequency stimulation of ventral hippocampal commissures reduces seizures in a rat model of chronic temporal lobe epilepsy. Epilepsia 2012; 53: 147-156.

[10] Shahpari M, Mirnajafi-Zadeh J, Firoozabadi SM and Yadollahpour A. Effect of low-frequency electrical stimulation parameters on its anticonvulsant action during rapid perforant path kindling in rat. Epilepsy Res 2012; 99: 69-77.

[11] Shen FZ, Wang F, Yang GM, Miao L, Wang EJ, Xu J, Xu YL and Sun T. [The effects of low-frequency electric stimulus on hippocampal of Effects of low-frequency electric stimulus on hippocampal of alpha5 subunit of extra synapse GABAA receptor in kainic acid-induced epilepsy rats]. Zhonghua Yi Xue Za Zhi 2013; 93: 550-553.

[12] Kile KB, Tian N and Durand DM. Low frequency stimulation decreases seizure activity in a mutation model of epilepsy. Epilepsia 2010; 51: 1745-1753.

[13] Yamamoto J, Ikeda A, Satow T, Takeshita K, Takayama M, Matsuhashi M, Matsumoto R, Ohara S, Mikuni N, Takahashi J, Miyamoto S, Taki W, Hashimoto N, Rothwell JC and Shibasaki H. Low-frequency electric cortical stimulation has an inhibitory effect on epileptic focus in mesial temporal lobe epilepsy. Epilepsia 2002; 43: 491-495.

[14] Carrington CA, Gilby KL and McIntyre DC. Effect of focal low-frequency stimulation on amygdala-kindled afterdischarge thresholds and seizure profiles in fast- and slow-kindling rat strains. Epilepsia 2007; 48: 1604-1613.

[15] Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 1972; 32: 281-294.

[16] Cooper IS. Effect of chronic stimulation of anterior cerebellum on neurological disease. Lancet 1973; 1: 206.
[17] Weiss SR, Li XL, Rosen JB, Li H, Heynen T and Post RM. Quenching: inhibition of development and expression of amygdala kindled seizures with low frequency stimulation. Neuroreport 1995; 6: 2171-2176.
[18] Ghotbedin Z, Janahmadi M, Mirnajafi-Zadeh J, Behzadi G and Semnanian S. Electrical low frequency stimulation of the kindling site preserves the electrophysiological properties of the rat hippocampal CA1 pyramidal neurons from the destructive effects of amygdala kindling: the basis for a possible promising epilepsy therapy. Brain Stimul 2013; 6: 515-523.
[19] Lopez-Meraz ML, Neri-Bazan L and Rocha L. Low frequency stimulation modifies receptor binding in rat brain. Epilepsy Res 2004; 59: 95-105.
[20] Magdaleno-Madrigal VM, Valdes-Cruz A, Martinez-Vargas D, Martinez A, Almazan S, Fernandez-Mas R and Fernandez-Guardiola A. Effect of electrical stimulation of the nucleus of the solitary tract on the development of electrical amygdaloid kindling in the cat. Epilepsia 2002; 43: 964-969.
[21] Shi LH, Luo F, Woodward D and Chang JY. Deep brain stimulation of the substantia nigra pars reticulata exerts long lasting suppression of amygdala-kindled seizures. Brain Res 2006; 1090: 202-207.