Low-frequency repetitive transcranial magnetic simulation prevents chronic epileptic seizure

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Research Highlights
1. Electroencephalography is currently an important means to diagnose epilepsy and to evaluate treatments. To some extent, using a nonlinear analysis may overcome the interference of the unstable electroencephalography signals that are caused by mental activity.

2. In this study, we collected electroencephalography signals and analyzed the changes in nonlinear parameters after rats with chronic epilepsy received repetitive transcranial magnetic simulation. An innovative method was used to simultaneously view several parameters.

Abstract
Although low-frequency repetitive transcranial magnetic simulation can potentially treat epilepsy, its underlying mechanism remains unclear. This study investigated the influence of low-frequency repetitive transcranial magnetic simulation on changes in several nonlinear dynamic electroencephalographic parameters in rats with chronic epilepsy and explored the mechanism underlying repetitive transcranial magnetic simulation-induced antiepileptic effects. An epilepsy model was established using lithium-pilocarpine intraperitoneal injection into adult Sprague-Dawley rats, which were then treated with repetitive transcranial magnetic simulation for 7 consecutive days. Nonlinear electroencephalographic parameters were obtained from the rats at 7, 14, and 28 days post-stimulation. Results showed significantly lower mean correlation-dimension and Kolmogorov-entropy values for stimulated rats than for non-stimulated rats. At 28 days, the complexity and point-wise correlation dimensional values were lower in stimulated rats. Low-frequency repetitive transcranial magnetic simulation has suppressive effects on electrical activity in epileptic rats, thus explaining its effectiveness in treating epilepsy.

Key Words
neural regeneration; repetitive transcranial magnetic stimulation; electroencephalogram; nonlinear analysis; nonlinear parameters; epilepsy; epileptic seizure; epileptic discharge; grant-supported paper; neuroregeneration
INTRODUCTION

Epilepsy is a clinical syndrome caused by abnormal discharge of brain neurons\([1-3]\). At present, drug treatment is dominant, but 20–30% of patients may develop intractable epilepsy even if they have received antiepileptic drugs\([4]\). Furthermore, a combination of several drugs can produce adverse reactions that are physically unbearable for patients. Although surgical treatment may be effective for some patients, preoperative evaluation and surgery are invasive and costly, thus hindering wide application. Vagal stimulation and deep brain electrical stimulation are both invasive, expensive, and require high-level surgical technology. Electroencephalographic biofeedback and ketogenic diet therapy have the disadvantage of being largely ineffective long-lasting treatments with many undesirable side effects, thus restricting clinical application\([2, 4]\).

Repetitive transcranial magnetic stimulation is a painless, non-invasive, safe, easily operated, and relatively cheap means for regulating nerves using knowledge from basic and clinical neurologic research. Repetitive transcranial magnetic stimulation can be used to excite or inhibit the cerebral cortex, and the transcranial magnetic field does not decay. Although increasing evidence indicates that low-frequency repetitive transcranial magnetic stimulation has antiepileptic effects\([5-8]\), the mechanism remains unclear. Understanding the underlying mechanism may provide insight into the pathophysiological mechanism of epilepsy and the effects of repetitive transcranial magnetic stimulation on brain function, improve antiepileptic therapy, and help avoid potential risks\([9]\). Repetitive transcranial magnetic stimulation is typically divided into low-frequency (< 1 Hz) and high-frequency (≥ 1 Hz) categories. High-frequency repetitive transcranial magnetic stimulation can lead to cerebral cortex long-term potentiation, and is used to treat depression because it excites the cortex. In contrast, low-frequency repetitive transcranial magnetic stimulation leads to cerebral cortex long-term depression and is used to treat epilepsy\([10]\).

Although epilepsy has an unclear pathogenesis and numerous seizure subtypes, all the subtypes share the common electrophysiological mechanisms. Under normal conditions, electrical activity of single neurons across the brain is relatively independent, random, and nonlinear. Brain electrophysiological activity is a spatial accumulation of slow postsynaptic potentials on cortical pyramidal cells. An electroencephalography signal is therefore produced by a system consisting of a large number of interconnected nonlinear elements, and a distinctive "chaos" is characteristic of the signals\([11-12]\).

Electroencephalography is a noninvasive technology that can amplify and record the complex electrical signals generated by neurons through special electrodes placed on the surface of the skull. Seizure activity can be easily captured by electroencephalography, thus making electroencephalography the most important electrophysiological method for the diagnosis and therapeutic evaluation of epilepsy\([13-14]\). In order to analyze objectively the information provided by an electroencephalography recording many computational methods have been developed, the majority of which are based on the hypothesis that brain electrophysiological activity is generated by a highly complex linear system. In fact, an electroencephalography signal is time-varying and non-stationary, presents varying frequency components at different time points, and is easily affected by mental activity\([15]\). Thus, existing methods for analyzing electroencephalography data cannot accurately reflect the changes in the brain’s state\([15]\), and a nonlinear analysis may overcome these shortcomings\([8]\).

Jing and Takigawa\([16]\) compared electroencephalography correlation dimensions in the human brain following 20-Hz repetitive transcranial magnetic stimulation, and results showed that the correlation dimension began to increase 2 minutes after stimulation. Several-day transcranial magnetic stimulation

\[2567\]
also increased the correlation dimension in the frontal lobe, temporal lobe, and hippocampi in rats with temporal lobe epilepsy\textsuperscript{[17]}. After 15 normal rats were given 100 pulses of suprathreshold stimulation at 0.5 Hz, the correlation dimension significantly decreased, and no change was observed in the epilepsy group\textsuperscript{[18]}. These results are quite different, and therefore the influence of low-frequency repetitive transcranial magnetic stimulation on electroencephalography nonlinear indexes remains elusive, as does the electrophysiological mechanism of repetitive transcranial magnetic stimulation-induced antiepileptic effects\textsuperscript{[18]}.

In summary, we obtained electroencephalography signals from normal and chronically epileptic rats that were or were not subjected to repetitive transcranial magnetic stimulation, analyzed the change in several nonlinear parameters, and investigated the electrophysiological mechanism by which repetitive transcranial magnetic stimulation produces antiepileptic effects when used to treat chronic epilepsy.

**RESULTS**

**Quantitative analysis of experimental animals**

Forty-five healthy male Sprague-Dawley rats were randomly divided into a control group, an epilepsy group, and a transcranial magnetic stimulation group, with 15 rats in each group. Rats from both epilepsy and transcranial magnetic stimulation groups received lithium-pilocarpine injection to induce epilepsy, and the transcranial magnetic stimulation group solely received repetitive transcranial magnetic stimulation treatment after the injection. Initially, eleven rats failed to develop epilepsy and five died. However, alternative rats were used, and finally data from 45 rats were included in the analyses.

**Behavioral changes**

The control group did not show any spontaneous seizures.

In the epilepsy group, after rats were intraperitoneally injected with lithium chloride 127 mg/kg or scopolamine 1 mg/kg, no abnormal behaviors were observed. After pilocarpine 30 mg/kg was given intraperitoneally for 3 minutes, movements became stiff and less frequent, with increased gazing and staring upward. At 10 minutes, rats exhibited nodding, swallowing, teeth grinding, and occasionally displayed wet-dog tremor. At 15 minutes, rats raised their heads, occasionally fell and jumped, experienced tail stiffness and clonus of bilateral forelimbs or hind limbs. Peripheral cholinergic excitatory symptoms such as nosebleeds, bloody tears, fecal incontinence, and sweating were also observed. Subsequent to developing chronic epilepsy, rats entered a toxic stage for 3–5 days, showing few or unceasing movement in the cage, no appetite, and extreme sensitivity and irritability to surrounding stimuli.

All deaths occurred during this stage. After a silent period (10–20 days), some rats showed spontaneous seizures that were graded as II–IV according to the Racine classification system\textsuperscript{[19]}. The seizures lasted 10–20 seconds and were all self-terminated.

The transcranial magnetic stimulation group showed no clear spontaneous seizures after stimulation.

**Changes in electroencephalography**

In the control group, electroencephalography signals contained primarily alpha and beta waves, followed by theta waves. Wave amplitude was less than 75 μV, without any epileptiform discharge.

In the epilepsy group, after rats were injected with pilocarpine, several forms of epileptiform discharge (scattered, paroxysmal, persistent sharp wave, and spike wave) were observed in the electroencephalography. In chronic epileptic models, sharp waves and either sporadic or paroxysmal spike waves were traced.

In the transcranial magnetic stimulation group, the electroencephalography signals contained primarily alpha and beta waves, with occasional spikes and slow spike waves.

**Nonlinear electroencephalography analysis**

At 7, 14, and 28 days after low-frequency repetitive transcranial magnetic stimulation, electroencephalography nonlinear parameters (correlation dimension, point-wise correlation dimension, Lyapunov exponent, and Kolmogorov entropy) were significantly lower in the epilepsy and transcranial magnetic stimulation groups compared with the control group ($P < 0.05$). At days 7, 14, and 28, the correlation dimension and Kolmogorov entropy were significantly lower in the transcranial magnetic stimulation group compared with the epilepsy group ($P < 0.05$).

Additionally, at day 28, the point-wise correlation dimension and complexity were lower in the transcranial magnetic stimulation group compared with the epilepsy group ($P < 0.05$; Table 1).
DISCUSSION

Under normal conditions, neurons in the brain present spontaneous electrical activity, which has a “chaotic” characteristic. This indicates that the electrical activity of single neurons is relatively independent and random across the brain as a whole. The brain waves are thus complex and can be desynchronized, showing high-dimensional “chaotic” levels\(^4,20\). At this time, values of nonlinear kinetic parameters (correlation dimension, point-wise correlation dimension, Lyapunov exponent, and Kolmogorov entropy) that can assess neuronal network complexity are high. At the epileptic seizure stage, electroencephalography reveals that activity from a large number of neurons tends to enter into a spontaneous electrical rhythm, resulting in spontaneous discharge and the synchronization of neuronal excitability. Subsequently, neuronal excitation becomes more constantly synchronized, spontaneous electrical activity of single neurons enters the low-dimensional “chaotic” level, the complexity of electroencephalography waves continuously reduces, and electroencephalographic parameters (correlation dimension, point-wise correlation dimension, Lyapunov exponent, and Kolmogorov entropy) reach the minimum upon epileptic seizures\(^11,21\). At the epileptic interictal stage, the large amount of synchronous neuronal discharge terminates, spontaneous electrical activity of single neurons recovers, and electroencephalographic parameter values begin to increase. Our findings showed that the complexity of electroencephalography signals rose at the early stage, decreased during the middle stage, and rose again at the later stage during the seizure process, even at the interictal stage when electroencephalographic parameters were still lower than normal rats. This observation was consistent with previous studies\(^6,11,21-25\).

Electroencephalography signals obtained via scalp electrodes are mainly collected from cerebral cortex, and changes in cortical excitability will inevitably affect the generation of action potentials and transmission of excitability. The resting motor threshold is an indicator of motor cortex excitability at rest, and reflects the degree of membrane excitability in cortical motor cells\(^23\). Devaux et al\(^24\) found that the resting period of the motor cortex in the affected hemisphere of epileptic patients was shorter than that in the contralateral side, and that hyperexcitability in the affected side resulted from damaged inhibitory function of the motor cortex. In nine healthy volunteers who underwent 0.3 Hz repetitive transcranial magnetic stimulation, the cortical resting period was significantly prolonged compared with baseline\(^25\). Further, the resting motor threshold of epileptic patients was shown to be lower than that of volunteers in a normal control group, and that of untreated epileptic patients. Additionally, the resting motor threshold of the epileptogenic side was lower than that of the non-epileptogenic side\(^26\). These data suggest that the cerebral cortex of epileptic patients is overexcited, and that low-frequency repetitive transcranial magnetic stimulation can reduce the susceptibility to epileptic seizures by inhibiting the cerebral cortex. We speculate that the inhibitory effects of low-frequency repetitive transcranial magnetic stimulation on the cerebral cortex may have directly affected the electroencephalography signals collected via scalp electrodes from the transcranial magnetic stimulation group, resulting in the lower correlation-dimension and Kolmogorov-entropy values we observed in the transcranial magnetic stimulation group.

<table>
<thead>
<tr>
<th>Time after treatment (day)</th>
<th>Group</th>
<th>Correlation dimension</th>
<th>Point-wise correlation dimension</th>
<th>Lyapunov exponent</th>
<th>Kolmogorov entropy</th>
<th>Complexity</th>
<th>Approximate entropy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>7.03±0.37</td>
<td>6.30±0.12</td>
<td>23.76±1.49</td>
<td>171.10±21.6</td>
<td>0.99±0.06</td>
<td>1.31±0.04</td>
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<td></td>
<td>Epilepsy</td>
<td>5.32±0.25</td>
<td>5.13±0.49</td>
<td>16.82±3.08</td>
<td>132.12±47.87</td>
<td>1.01±0.08</td>
<td>1.29±0.02</td>
</tr>
<tr>
<td></td>
<td>TMS</td>
<td>3.48±0.41</td>
<td>4.09±0.46</td>
<td>16.04±1.90</td>
<td>89.94±30.89</td>
<td>0.80±0.10</td>
<td>1.22±0.06</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Control</td>
<td>6.88±0.59</td>
<td>6.84±0.49</td>
<td>20.14±9.12</td>
<td>1.06±0.07</td>
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<td></td>
<td>Epilepsy</td>
<td>4.79±0.62</td>
<td>4.55±0.76</td>
<td>16.07±2.68</td>
<td>122.41±50.89</td>
<td>0.89±0.17</td>
<td>1.27±0.05</td>
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<tr>
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<td>TMS</td>
<td>3.67±0.30</td>
<td>4.24±0.40</td>
<td>13.37±2.26</td>
<td>90.24±27.16</td>
<td>0.84±0.09</td>
<td>1.22±0.07</td>
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<td></td>
<td>28</td>
<td>Control</td>
<td>6.81±0.71</td>
<td>6.51±0.91</td>
<td>20.48±5.79</td>
<td>169.49±61.37</td>
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<tr>
<td></td>
<td>Epilepsy</td>
<td>4.94±0.92</td>
<td>5.07±0.81</td>
<td>14.48±3.92</td>
<td>119.30±21.71</td>
<td>1.11±0.05</td>
<td>1.31±0.04</td>
</tr>
<tr>
<td></td>
<td>TMS</td>
<td>3.90±0.46</td>
<td>4.21±0.46</td>
<td>14.01±4.24</td>
<td>88.28±24.28</td>
<td>0.74±0.12</td>
<td>1.21±0.09</td>
</tr>
</tbody>
</table>

\( *P < 0.05\), vs. control group; \( ^{ab}P < 0.05\), vs. epilepsy group. Chronic epilepsy was established in the epilepsy and TMS groups using a rat model for chronic epilepsy. The TMS group subsequently received TMS treatment. There were 15 rats in each group at each time point, and data are expressed as mean ± SD. Differences between measurement data were compared with one-way analysis of variance.
This is consistent with previous research\textsuperscript{[18]}, which showed that the correlation dimension decreased significantly after 15 normal rats received 100 pulses of suprathreshold stimulation at 0.5 Hz, but did not change in a sham stimulation group.

Theiler et al\textsuperscript{[15]} found that, electrical activity of the brain is nonlinear, rather than low-dimensional chaos. While the Lyapunov exponent and point-wise correlation dimension are suitable for analysis of low-dimensional chaotic systems, analysis of a high-dimensional chaotic system like the brain has limitations. This may explain the insensitivity of the Lyapunov exponent and the point-wise correlation dimension in this study. Complexity is defined as the maximal ratio of the shortest sequence length (bit) to the graphical structure or symbol sequence (when the latter approaches infinity), and is the first proposed method to evaluate the system complexity from the point of internal structure or change in the system. However, the complexity measurement is not only associated with uniform disorder, but also contains ordered structure, so it can be regarded as the superposition of randomness and regularity, which is the characteristic of a linear system\textsuperscript{[20]}. This concept explains the observed insensitive complexity in this experiment. Approximate entropy is a type of entropy as defined by Pincus\textsuperscript{[20]} that has good robustness, and is insensitive to few abnormal data in the time sequence, thus fitting for the analysis of physiological signals\textsuperscript{[20]}. In our experiments, all rats received chloral hydrate anesthesia prior to the acquisition of electroencephalography signals. Although chloral hydrate has little impact on electroencephalography signals\textsuperscript{[27]}, Koskinen et al\textsuperscript{[28]} found significant differences in electroencephalography approximate-entropy before and after propofol anesthesia, and the approximate entropy value was lower than in the awake state. This discrepancy can explain the insensitivity of approximate entropy in this experiment.

In summary, according to chaos theory, low-frequency repetitive transcranial magnetic stimulation may lead to rat brain synchronization, increase the complexity of neural networks, and raise nonlinear parameters that are indicators of brain complexity. Due to the inhibitory effect of low-frequency repetitive transcranial magnetic stimulation, the correlation dimension and Kolmogorov entropy of chronic epileptic rats in the transcranial magnetic stimulation group were lower than in the epilepsy group. Low-frequency repetitive transcranial magnetic stimulation has a certain inhibitory effect on the brain electrical activity of epileptic rats, and its antiepileptic effects may be related to this inhibition.

\begin{center}
\textbf{MATERIALS AND METHODS}
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\textbf{Design}

A randomized, controlled animal experiment.

\textbf{Time and setting}

Experiments were performed at the Animal Experimental Center of North Sichuan Medical College for model establishment and at the Institute of Neurological Disease of North Sichuan Medical College in China for obtaining electroencephalography signals and nonlinear analysis from May to December in 2011.

\textbf{Materials}

Healthy male Sprague-Dawley rats, aged 9 weeks, weighing 300–350 g, were provided by the Experimental Animal Center of North Sichuan Medical College, China (license No. SYXK (Chuan) 2008-076). All rats were housed for 1 week prior to experiments at a controlled temperature between 21 and 27°C, a humidity between 40 and 70%, a noise level < 60 dB, with a 24-hour light/dark cycle, and free access to food and water.

\textbf{Methods}

\textit{Establishment of the lithium-pilocarpine-induced epileptic model}

Chronic epilepsy models were established in rats as previously described\textsuperscript{[7]}, by the Institute of Neurological Disease, North Sichuan Medical College in China. In brief, rats were intraperitoneally injected with 127.17 mg/kg lithium chloride (1.5 mol/L). After 18 hours, they were injected with 1 mg/kg scopolamine (0.25 g/L; Sigma-Aldrich, St. Louis, MO, USA) and 30 minutes later, with 30 mg/kg pilocarpine (7.5 g/L; Sigma-Aldrich). Control group rats were injected with physiological saline. Rat behavior changes were observed for 90 minutes following the pilocarpine injection and evaluated according to the classification system described by Racine\textsuperscript{[19]}:

- grade 0: normal behavior;
- grade I: facial and ear convulsions;
- grade II: slightly trembling;
- grade III: apparent trembling, tail rigidity, difficulty in walking;
- grade IV: forelimb clonus, standing;
- grade V: complete tetanus, clonus, and tumbilng;
- grade VI: status epilepticus;
- grade VII: death. Status epilepticus was determined as the appearance of frequent or persistent limb clonus or complete tetanus for more than 30 minutes (i.e., grade IV and above).

If no seizure of grade IV or higher occurred within 30 minutes after pilocarpine injection, rats were injected with 10 mg/kg pilocarpine \textit{via} intraperitoneal injection,
and received an additional injection every 30 minutes until status epilepticus occurred. Rats in which epilepsy failed to be induced using 50 mg/kg injection were excluded and replacement rats were used in their stead. When status epilepticus was observed for 60 minutes, rats were given intraperitoneal injections of 10% chloral hydrate (3.5 mL/kg; Fuchen Chemical Reagent Factory, Tianjin, China) to terminate status epilepticus. Rats that died during the modeling process were also replaced. Chronic epileptic models were regarded successful upon the behavioral observation of spontaneous seizures and epileptiform discharges detected by electroencephalography using a ZN16E digital electroencephalography monitoring analyzer (Intelligent Electronic Industrial Company, Chengdu, China)\cite{29}.

**Repetitive transcranial magnetic stimulation**

Epilepsy-onset latency for the lithium-pilocarpine model was previously reported to be 11–69 days (mean 33 days)\cite{14}. The transcranial magnetic stimulation group rats therefore began to receive repetitive transcranial magnetic stimulation treatment 33 days after lithium-pilocarpine injection. The stimulation was given using a Magpro-R30 magnetic stimulator (Medtronic, Minneapolis, MN, USA) equipped with an MCF-75 toroidal coil (outer diameter 6.5 cm, inner diameter 1 cm, maximal output intensity 4.2 T; Medtronic). The stimulation parameters were as follows: 0.5 Hz, 40% motor threshold, 100 impulses/series 5 series per day, at an interval of 30 seconds, for 7 consecutive days. The coil center was aligned to the sagittal suture central point so that the coil plane was parallel to the parietal bone and close to the scalp.

**Behavioral observation and electroencephalography signal acquisition**

Raw electroencephalography signals were collected from the rats fed previously. Other electronic equipment (including a mobile phone and notebook computer) were turned off. In order to avoid interference from the sampling circuit and recording equipment, trapped waves were opened throughout the entire procedure. Rats were housed in separate labeled cages, and their attacks and times of spontaneous epileptic seizures were recorded using a video camera that recorded continuously. For electroencephalography recording, rats were intraperitoneally anesthetized with 3.5 mL/kg of 10% chloral hydrate, their scalp hair was removed and skin was defatted using alcohol cotton balls, and their heads were fixed with a brain stereotaxic apparatus (KD Scientific Corporation, Houlston, MA, USA). Two silver electrode needles were symmetrically inserted into the bilateral temporal region, and another needle was inserted into the nose as a reference electrode. The three electrodes were connected to the electroencephalogram monitoring device through a wire. Electroencephalography signals were collected 7, 14, and 28 days after the low-frequency repetitive transcranial magnetic stimulation.

**Electroencephalography nonlinear analysis**

The correlation dimension, point wise correlation dimension, Lyapunov exponent, Kolmogorov entropy, complexity, and approximate entropy were subject to nonlinear analysis using software in the ZN16E digital electroencephalography monitoring. Raw electroencephalography signals were analyzed using 80-Hz high-frequency filtering to attenuate EMG interference, while some electroencephalography signals that were contaminated with artifacts were analyzed using low-frequency filtering (less than 3.98 Hz) to attenuate the artifacts. One hundred-and-twenty-second electroencephalography signals with stable waveforms and minimal artifacts were used for analysis, and the average value of each parameter was calculated.

**Statistical analysis**

Data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Differences were compared with one-way analysis of variance with $P < 0.05$ considered statistically significant.

### REFERENCES


[7] Ke S, Zhao HN, Wang XM. Pretreatment with low-frequency repetitive transcranial magnetic stimulation may influence neuronal Bcl-2 and Fas protein expression


(Reviewed by Philips A, Raye W, Zhao CS, Zhang GM)
(Edited by Mu WJ, Yang Y, Li CH, Song LP, Liu WJ, Zhao M)