Effect of Electroacupuncture Stimulation of Hindlimb on Seizure Incidence and Supragranular Mossy Fiber Sprouting in a Rat Model of Epilepsy

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Abstract: Recently, electroacupuncture (EA) has been gaining more and more attention as a treatment for epilepsy. However, concrete evidence is needed to better understand its antiepileptic effect and the mechanism underlying this effect. The present study was designed to assess the effect of EA stimulation of hindlimb on the incidence of behavioral seizures (spontaneous recurrent seizures, [SRS]) and electroencephalogram (EEG) seizures, and the extent of supragranular mossy fiber sprouting (MFS) using the lithium-pilocarpine rat model of epilepsy. Sham EA at the same point without electrical stimulation was set as the control. EA and the sham EA were performed bilaterally (at the symmetrical Zusanli acupoints on both hind legs) 30 times every two days. The numbers of behavioral seizures and EEG seizures were then analyzed to evaluate the antiepileptic effect. After confirmation of the antiepileptic effect, MFS in the dentate gyrus (DG) supragranular layer was investigated by Timm's staining. The results showed that the EA stimulation of hindlimb significantly reduced the behavioral seizures, EEG seizures, and supragranular MFS; however, the sham EA without electrical stimulation showed no significant effect on seizures or supragranular MFS. The findings indicate that EA stimulation of hindlimb possesses an antiepileptic effect, which is probably related to its suppressive effect on aberrant MFS in DG.

Key words: electroacupuncture, epilepsy, spontaneous recurrent seizure, electroencephalogram (EEG), mossy fiber sprouting.

Acupuncture, one of the oriental medical therapeutic techniques that can be traced back at least 2,500 years, is gaining popularity in the West as an alternative and complementary therapeutic intervention. A long history of clinical practice has proved that acupuncture has many advantages, such as safety, efficiency, convenience, and few side effects. Classic acupuncture is administered by an insertion needles into acupoints and a swift manipulation of rotating (or lifting and thrusting) them. Nowadays, electroacupuncture (EA), a modern acupuncture approach using electrical stimulation instead of manual manipulation, has been widely applied.

Epilepsy, with a prevalence of about 1%, is a chronic neurological disorder characterized by the recurrent appearance of spontaneous seizures as a result of neuronal hyperactivity in the brain. The hippocampus is thought to be an epileptogenic focus in human temporal lobe epilepsy (TLE) [1], which is among the most common types of human epilepsy. The most consistently and severely affected region of the hippocampus is dentate gyrus (DG), and supragranular mossy fiber sprouting (MFS) is one of the basic neuropathological changes in dentate gyrus in response to epileptic seizures [1]. Reversely, aberrant supragranular MFS can affect functional characteristics of the DG network, and it has been widely proposed as an etiologic factor in humans TLE [1].

Various treatment options apply to epilepsy, including conventional pharmacological and nonpharmacological methods. Recently, concerns regarding the side effects of pharmacological approaches have aroused increasing interest in the use of nonpharmacological techniques, including electroacupuncture.

Several experimental studies showed antiepileptic effects of EA and found some underlying meaningful changes in response to it. An increase of melatonin [2] and taurine transporter [3, 4] and a decrease of nitric oxide synthases [5] after EA treatment were found to be involved in the antiepileptic effect of EA. A study by Dos Santos and colleagues [6] showed that EA stimulation of hindlimb prevented atrophy of some limbic structures and...
improved cognitive deficits in pilocarpine-epilepsy model rats, and these effects depended on the serotonergic system. More recently, our previous work showed that EA stimulation of hindlimb significantly reduced the frequency of spontaneous recurrent seizure until different time points (days 30, 45, and 60); moreover, EA stimulation of hindlimb also significantly elevated the expression of GAD67 mRNA in the dentate gyrus granule cell layer at those same points [7].

In this randomized controlled study, the effects of EA on the incidence of behavioral seizures and EEG seizures were investigated first to confirm the antiepileptic effect; the effect of EA on the mossy fiber sprouting was then investigated to explore the possible mechanism underlying the confirmed antiepileptic effect. The lithium-pilocarpine–induced rat model of epilepsy, which reproduces the main characteristics (including the behavioral, EEG, pathological features) of human temporal lobe epilepsy [8], was used in this study. The so-called Zusanli acupoint was chosen as the stimulated point because it is one of the most widely used and effective points in traditional Chinese medicine, and also because previous studies showed that the Zusanli acupoint was a practical choice when using EA to treat epilepsy [6, 7]. Nonelectrical-EA (sham EA at the same point without electrical stimulation) was administered simultaneously to examine the electrical stimulation specificity.

METHODS

Animals. Male Sprague-Dawley rats weighting 120 to 160 g were used in the present experiment. All procedures for the care and use of animals in the present experiment were performed under the guidelines of the Wuhan University Laboratory Animal Center. The Principles of Laboratory Animal Care (NIH publication No. 80-23, revised 1996) were followed. All the animals were kept at a controlled temperature (25°C ± 1°C) under a standard 12 h/12 h cycle (light/dark, lights on from 07:00 a.m. to 19:00 p.m.), with free access to food and water. All the animals were randomly divided into the 4 groups: normal; epilepsy EA; epilepsy nonelectrical-EA (without EA); epilepsy non-EA (without EA); epilepsy EA; epilepsy nonelectrical-EA (without EA); epilepsy non-EA (without EA).

Electrode implantation. All rats underwent monopolar recording electrode implantation. Briefly, they were lightly anesthetized with sodium pentobarbital (40 mg/kg, i.p.) via two clip electrodes; for sham EA, the two needles were only left in the muscles without connection to the electrotherapeutic apparatus. The needle point is the so-called Zusanli acupoint according to traditional Chinese medicine, which is located about 10 mm below the knee joint and about 5 mm lateral from the midline in the anterior surface of either hind leg. For EA, the two needles were attached bilaterally to an electrotherapeutic apparatus (Model G6805-2, SMIF, Shanghai, China) via two clip electrodes; for sham EA, the two needles were lowered via the hole into the left dentate gyrus at a depth of 2.9 mm from the neocortex. Also, a stainless steel microscrew was driven into the posterior cranium over the cerebellum to serve as a reference electrode. The electrodes were anchored to the skull with dental cement.

EA treatment. EA treatment was given to the animals 30 times, once every two days (18:30–19:00 p.m.) from day 2. Before EA treatments, all rats (including normal ones) were lightly anesthetized with sodium pentobarbital (40 mg/kg, i.p.) to minimize restraint stress. One stainless acupuncture needle 10 mm long with an outer diameter of 0.25 mm (Model A-7, Wujiang Jia Chen Acupuncture Devices Co., Jiangsu, China) was inserted obliquely 8 mm into the tibialis anterior muscle of each leg. For EA, the two needles were attached bilaterally to an electrotherapeutic apparatus (Model G6805-2, SMIF, Shanghai, China) via two clip electrodes; for sham EA, the two needles were only left in the muscles without connection to the electrotherapeutic apparatus. The needle point is the so-called Zusanli acupoint according to traditional Chinese medicine, which is located about 10 mm below the knee joint and about 5 mm lateral from the midline in the anterior surface of either hind leg. Before insertion of the needle, rat hair around the insertion point was cut to obtain a better location of the insertion point. Each EA or sham EA lasted 30 min. The stimulation parameters were set as a frequency of 4 and 20 Hz, alternatively, and an intensity of 1–20 mA, strong enough to elicit only slight twitches of the lower limbs.

J. GUO et al.
Monitoring of behavioral seizures. Since the frequency of spontaneous recurrent seizures (SRSks) in the pilocarpine model is much higher during the light (diurnal) period than during the dark (nocturnal) period [9], all SRS recordings were done during the light period. The animals were video monitored for eight hours a day (8:00 a.m.–12:00 a.m., 14:00 p.m.–18:00 p.m.) and recorded by disks. Light-sensitive black-white cameras (CCD-Kamera-Modul, Conrad Electronic, Germany) were used. SRS of 4 and 5 stages occurred in each animal were counted by a person using the video disks who was blind to the group assignment. The monitoring began on day 2 and was performed every day until day 60.

Monitoring of EEG seizures. On day 61, the EEG activity of dentate gyrus was recorded for 120 min (9:30 a.m.–10:30 a.m., 15:30 p.m.–16:30 p.m.) using a computerized EEG apparatus (RM6240; Chengdu Instrument Factory, Chengdu, China). The time constant was set at 0.2 s, the velocity at 200 ms/div, the sensitivity at 100 μV/cm, and the high-cutoff frequency at 30 Hz. The files were later reviewed by an electroencephalographer unaware of the experimental design who counted EEG seizures occurring in dentate gyrus during the 120 min. The seizures were defined according to previous studies [10, 11] as rapid rhythmic spiking at a frequency > 1/s and persisting for at least 10 s.

Tissue collection and processing. After being anesthetized with sodium pentobarbital (60 mg/kg, i.p.), the rats were perfused through the ascending aorta with 200 ml of sodium sulfide perfusion medium (2.925 g of Na₂S, 2.975 g of Na₂CO₃, 47 g/100 ml H₂O, 10 ml citric acid (47 g/100 ml H₂O), 3.47 g hydroquinone in 60 ml, and 30% sucrose solution at 4°C for 12 h). For each brain, frozen transverse sections of 20 μm through the entire extent of the hippocampus were cut by a freezing microtome (Leica, Nussloch, Germany) and thaw-mounted onto slides pretreated with poly-L-lysine. The sections were then stored at –70°C until assay. Every 10th section (with an interval of 200 μm) was stained for mossy fibers using Timm’s staining method.

Timm’s staining. Timm’s staining is a histochemical technique that labels the synaptic terminals of the mossy fibers because of their high content of Zn. After being washed, the slides were dehydrated in alcohol, cleared in xylene, and coverslipped with Permount.

For Timm’s staining, the sections were independently examined under a light microscope (Olympus, Japan) by three observers who were unaware of the experimental design. Timm scores for mossy fiber sprouting at supragranular layer was rated according to the widely used Cavazos scale: (0: no granules; 1: occasional granules in the supragranular region occurring in patchy distribution; 2: numerous granules occurring in patchy distribution; 3: occurring in near-continuous distribution; 4: highly concentrated band of granules appearing either in continuous or near-continuous distribution; 5: continuous dense laminar band of granules from the crest to the tip of the dentate). For each section, three Timm scores were determined independently by the three examiners; for each rat, one score was calculated by averaging all the independently derived values from one rat.

Quantitative analysis. The results (behavioral seizure numbers, EEG seizure numbers, and Timm scores) were analyzed with SPSS. Data were expressed as the mean ± standard error of the mean (SEM) and were analyzed using one-way ANOVA followed by Tukey’s HSD post hoc multiple comparison tests. Differences were considered to be statistically significant for \( P < 0.05 \), and all tests were used two-sided.

RESULTS

Behavioral changes induced by pilocarpine

After pilocarpine injection, the rats exhibited gradually aggravating syndromes. First, the animals were motionless; they stared, crouched on all limbs, and their ears were cocked back. Then came the second phase, which included chewing, teeth chattering, body shivering, and...
yawning, all associated with mild salivation. Finally, self-sustained limbic status epilepticus was reached, including early rearing, upper extremity clonus and falling, with intense salivation. Immediately after an injection of diazepam, the rats turned peaceful. Eight of the lithium-pilocarpine–treated rats did not develop status epilepticus, and 14 rats died during status epilepticus. Those that showed no status epilepticus or couldn’t survive in status epilepticus were eliminated from their groups. Ultimately, the numbers of remaining rats in each group were 7 in epilepsy non-EA, 8 in epilepsy EA, 11 in epilepsy nonelectrical-EA, and still 15 in the normal group.

**EA stimulation of hindlimb reduces behavioral seizure numbers**

All remaining epileptic rats showed SRS, but normal rats showed no SRS. The data analysis of the SRS is shown in Fig. 1. One-way ANOVA tests showed signifi-
Beneficial Effects of Electroacupuncture on Epilepsy

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Cant differences of the mean SRS numbers (over about 2 months) among all 3 epilepsy groups ($F = 7.489, P = 0.003$). Tukey’s HSD post hoc multiple comparison tests revealed that (i) versus the epilepsy non-EA group (29.29 ± 1.80, $n = 7$), the epilepsy EA group (21.63 ± 1.62, $n = 8$) showed a significant decrease of mean SRS numbers ($P = 0.011$), and the epilepsy nonelectrical-EA group (29.18 ± 1.57, $n = 11$) failed to show any change of mean SRS numbers ($P = 1$). (ii) Versus the epilepsy nonelectrical-EA group, the epilepsy EA group showed a significant difference in mean SRS number ($P = 0.005$).

EA stimulation of hindlimb reduces EEG seizure numbers that occurred in dentate gyrus

Although normal rats accidentally showed some spikes in their electroencephalograms, no rapid rhythmic spiking (EEG seizure) was found. The data analysis of EEG seizures is shown in Fig. 2. Figure 3 demonstrates typical EEG seizures that occurred in dentate gyrus.

The data analysis of EEG seizures for a better understanding of the effect. For each rat, the number of EEG seizures that happened during 120 min after the last EA treatment (on day 61) was obtained; whereas the number of behavioral seizures that happened during 59 consecutive days (from day 2 to day 60, 8 hours per day) was

DISCUSSION

In the present work, we first investigated whether EA can reduce the incidence of behavioral seizures or EEG seizures to evaluate its antiepileptic effect. We analyzed both data of behavioral seizures and EEG seizures for a better understanding of the effect. For each rat, the number of EEG seizures that happened during 120 min after the last EA treatment (on day 61) was obtained; whereas the number of behavioral seizures that happened during 59 consecutive days (from day 2 to day 60, 8 hours per day) was
obtained cumulatively, because the frequency of behavior
torial seizures is comparatively low and a comparatively
long time is needed to “capture” enough behavioral sei-
zure. Our results of seizure recordings clearly showed
that EA of the hindlimb significantly reduced the behav-
ioral seizures and EEG seizures in dentate gyrus, but the
sham EA needleling at the same point without electrical
stimulation failed to show any effect on seizures. These
findings manifest that EA of the hindlimb possesses some
anti-epileptic effect and that this effect had stimulation
specificity (not simply the result from the insertion of
acupuncture needles).

After the anti-epileptic effect test, we observed the
change of dentate gyrus supragranular MFS caused by
EA. It has been widely accepted that aberrant supragran-
ular MFS is an essential basis to explain the hyperexcitabil-
ity of epileptic tissue and is one of the major causes of in-
creased seizure susceptibility in both human temporal
lobe epilepsy and animal models of human temporal lobe
epilepsy [12]. Normally, the granule cells do not innervate
other granule cells. Once initial injuries cause aberrant su-
pragranular MFS in their “own field,” they establish new
synaptic connections (recurrent excitatory circuits) amon-
g themselves in the supragranular region, which is
very prone to recurrent seizure discharges [1, 13]. There-
fore treatment strategies that restrain the aberrant MFS af-
after hippocampal injury have considerable significance in
regard to the development of apt therapy for temporal lobe
epilepsy. For example, it has been found that in kainic-
acid–induced adult rats, a ketogenic diet could decrease
the frequency of spontaneous recurrent seizures and pre-
vent MFS in the dentate gyrus, suggesting that a ketogenic
diet might play an antiepileptic role by suppressing MFS
[14]. Based on the close and direct correlation between
seizure susceptibility and aberrant supragranular MFS, we
hypothesized that—accompanied by the suppressing ef-
effect on seizure occurrence—EA of hindlimb could also
have a suppressing effect on supragranular MFS.

Our data analysis of Timm scores for MFS clearly
showed that a lithium-pilocarpine injection causes serious
supragranular MFS, in consistency with previous studies,
and the EA of hindlimb significantly reduced this aberrant
supragranular MFS. However, the sham EA at the same
point without electrical stimulation failed to show any
effect on supragranular MFS. The results manifest that EA
of the hindlimb can suppress supragranular MFS, and this
effect also had stimulation specificity. This finding
verifies our hypothesis and indicates that the antiepileptic
effect of EA of hindlimb may be related to its suppressive
effect on MFS.

It was widely believed that acupuncture (including
electroacupuncture) acts as a neuromodulating input into
the central nervous system, yielding ultimate therapeutic
effects [15, 16]. Zusanli acupoint is located in the muscu-
lus tibialis anterior innervated by the deep peroneal nerve,
Some studies [17, 18] confirmed that the peroneal nerve
was part of the afferent route for impulses arising from
Zusanli acupoint. In regard to epilepsy, a laboratory ani-
mal experiment was reported that came to this conclusion:
all the observations lead to the presumption that when ap-
plied at Zusanli acupoint, electroacupuncture exerts sei-
zure-suppressing effects through a pathway of the hypo-
thalamic arcuate nucleus to the brain stem raphe nucleus,
and finally to hippocampus [19].

Various kinds of afferent fibers have been found to be
involved in the afferent route of signal transmission of Zu-
sanli stimulation. It seems there is no doubt about the pre-
dominant role of large afferent fibers, because the thresh-
old of electrical intensity needed to activate them (groups
I, II and III) is much lower. An early study by Lu found
that local afferent fiber composition at Zusanli acupoint
contained a more myelinated fibers, more large-sized fi-
bers, and more A-beta fibers (equate to group II fibers)
then nonacupoints; and when the Zusanli acupoint was
stimulated electrically, a larger-than-normal proportion of
A-beta fibers was activated [18]. So Lu suggested the pre-
dominance of large afferent fibers in the composition and
activity of Zusanli acupoint might be one of the funda-
mental characteristics of the point in regard to its structure
and function and that it might contribute to its powerful
acupuncture effect. Another related study by Xiao and Li
utilizing compound action potentials to identify fiber
types found that group I, II, and III fibers were activated
with weak currents, but stronger currents were needed to
activate group IV fibers in the deep peroneal nerve [20].
Recently, however, more and more studies have clearly
shown that group IV fibers also take a part in the signal
transmission process. The study by Liu demonstrated that
some C fibers (equating to group IV fibers) could be excit-
ed when the stimulation intensity reached the threshold
of those C fibers [21]. The study by Kagitani showed that all
four (I, II, III, and IV) groups of somatic afferents were
activated by acupuncture stimulation at Zusanli acupoint
[22]. The study by Mori showed that electroacupuncture
stimulation of Zusanli acupoint could excite group III and
group IV afferent fibers [23].

Different kinds of beneficial changes in the brain have
been found as responses to EA when treating epilepsy (as
we have discussed). Those beneficial changes could be in-
terrelated with one another, and the acupunctural antiepi-
leptic effect may be an overall result from those multiple
interrelated factors. It has been shown that EA of hindlimb
possesses a neuroprotective effect in animal models of ep-
ilepsy [6] and other animal models of brain disease [24,
25]. It is also known that EA of hindlimb markedly
improved blood supply and microcirculation in the cerebral
tissues, which indicated that EA stimulation of hindlimb
could alleviate hypoxia-ischemia–associated neuron loss
and thus possess a neuroprotective effect [26, 27]. Since
supragranular MFS is largely attributed to loss of the gran-
ule cells in the dentate gyrus [28], EA stimulation of hindlimb may suppress MFS through its neuroprotective effect, finally contributing to the antiepileptic effect.

This study has several limitations. First, it was unable to perform EA or sham EA in unanesthetized epileptic rats because they tended to be easily agitated and slow to habituate to environmental conditions while awake. Consequently an accurate localization of needling points was difficult. So they received EA or sham EA under lightly anesthetized conditions. But this may have an influence on brain neurotransmitters and synapse transmission, and also the EA effect. To minimize the influence, we administered light anaesthesia to the rats, in which the dose of sodium pentobarbital (40 mg/kg, i.p.) is lower than the normal dose (60 mg/kg, i.p.). Those who received no stimulation (rats in the normal and epilepsy non-EA groups) were also lightly anesthetized to eliminate the difference elicited by the anesthetic among groups. Second, the values of SRS numbers, EGG seizure numbers, and MFS scores between all 3 epilepsy groups are comparatively close, even though some meaningful significant differences are present. That provides the information that the EA treatment cannot completely cure epilepsy. However, our data still can provide evidence that the EA treatment possesses some beneficial effect symptomatically and pathologically. Actually, in practice EA stimulation is mostly considered as a supplementary treatment for epilepsy.

In conclusion, our findings indicate that electroacupuncture stimulation of hindlimb possesses an antiepileptic effect that is related to its suppressive effect on aberrant supragranular mossy fiber sprouting in the lithium-pilocarpine rat model of epilepsy. These findings could provide concrete evidence for a more detailed investigation about the acupunctural antiepileptic effect and would have clinical implications.

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\textbf{Beneficial Effects of Electroacupuncture on Epilepsy}

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