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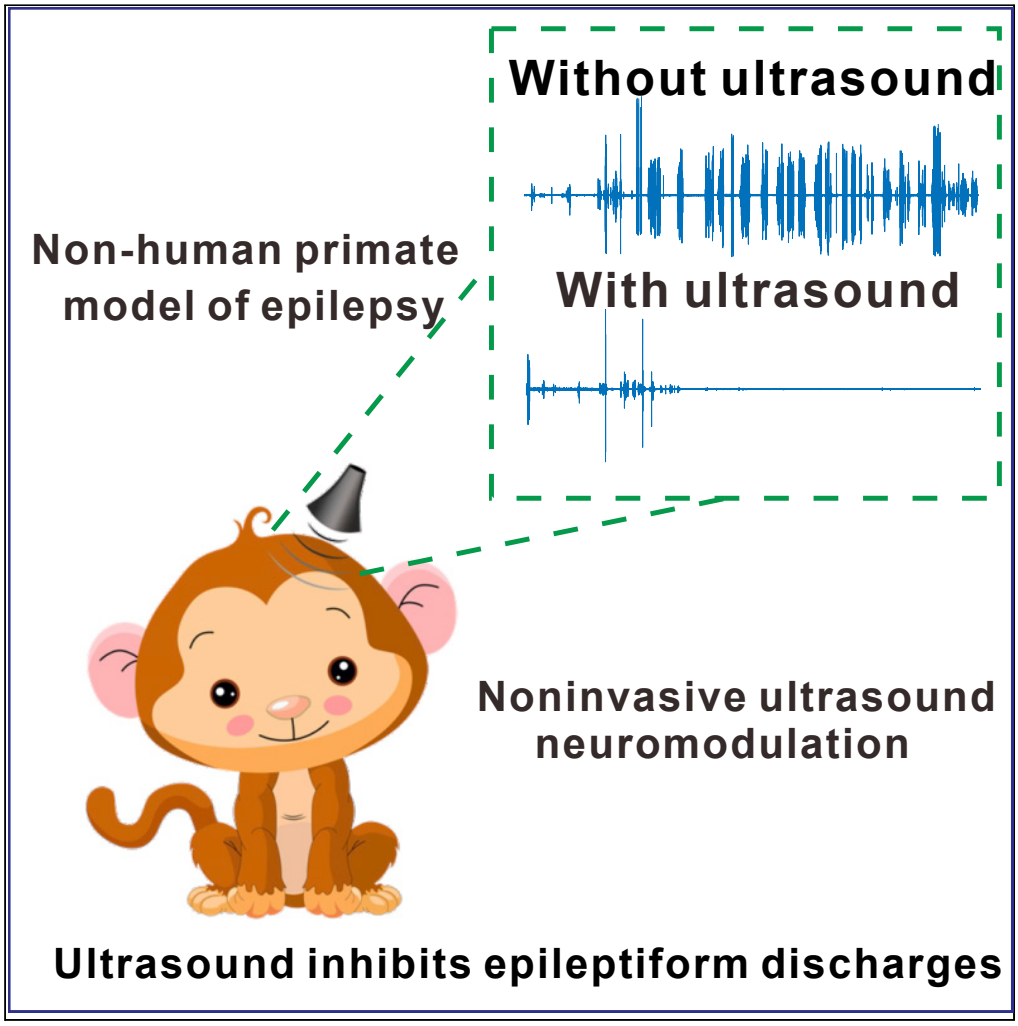

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HIGHLIGHTS

Ultrasound stimulation can inhibit seizures in non-human primates with epilepsy

Ultrasound stimulation reduces the number and duration of seizures

Ultrasound stimulation is a safe, noninvasive therapeutic method for epilepsy

Zou et al., iScience *23*, 101066
May 22, 2020
https://doi.org/10.1016/j.isci.2020.101066

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Ultrasound Neuromodulation Inhibits Seizures in Acute Epileptic Monkeys

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SUMMARY

Ultrasound stimulation has recently emerged as a non-invasive method for modulating brain activity in animal and human studies with healthy subjects. Whether brain diseases such as Alzheimer's disease, epilepsy, and depression can be treated using ultrasound stimulation still needs to be explored. Recent studies have reported that ultrasound stimulation suppressed epileptic seizures in a rodent model of epilepsy. These findings raise the crucial question of whether ultrasound stimulation can inhibit seizures in non-human primates with epilepsy. Here, we addressed this critical question. We confirmed that ultrasound stimulation significantly reduced the frequency of seizures in acute epileptic monkeys. Furthermore, the results showed that the number and duration of seizures were reduced, whereas the inter-seizure interval was increased after ultrasound stimulation. Besides, no significant brain tissue damage was observed by T2-weighted MR imaging. Our results are of great importance for future clinical applications of ultrasound neuromodulation in patients with epilepsy.

INTRODUCTION

Epilepsy is one of the most prevalent neurological disorders characterized by recurrent seizures resulting from excessive excitation or inadequate inhibition of neurons (Pavlov et al., 2013; Blumcke, 2017). Neuromodulation techniques have gained widespread attention owing to their therapeutic utility for epilepsy. They used physical means to modulate neuronal activity, thereby decreasing the frequency or duration of seizures (Theodore and Fisher, 2004).

Ultrasound neuromodulation has gained global attention in recent years owing to its bimodal modulatory effects with exquisite spatial specificity and depth penetration. The evidence from animal and human studies with healthy subjects illustrates that ultrasound can penetrate the skull to the specific brain regions causing behavioral change and improving sensory discrimination abilities (Tufail et al., 2010; Legon et al., 2014; Folloni et al., 2019; Fouragnan et al., 2019). Recent studies have demonstrated that ultrasound stimulation can inhibit the epileptic seizures in a rodent model of epilepsy (Hakimova et al., 2015; Li et al., 2019). Min et al. showed that low-intensity, pulsed ultrasound sonication suppressed the number of epileptic signal bursts using the acute epilepsy model in the rat (Min et al., 2011). Also, Hakimova et al. indicated that ultrasound stimulation effectively inhibited acute seizure activity, including status epilepticus, and subsequent recurrent seizures in the chronic period in a kainate-induced mouse model of mesial temporal lobe epilepsy (Hakimova et al., 2015). Recently, Li et al. reported that low-intensity ultrasound could effectively modulate nonlinear dynamics in acute epileptic mice (Li et al., 2019). These findings suggested a potential role for ultrasound in the treatment of epilepsy, but it has not yet been tested whether ultrasound stimulation can inhibit seizures in nonhuman primates with epilepsy.

We aimed to determine whether ultrasound stimulation was capable of functionally modulating brain activity in non-human primates with epilepsy. The effectiveness of ultrasound neuromodulation was identified by a penicillin-induced epilepsy model in non-human primates (Lin et al., 2020). The results indicated that the number of seizures was significantly reduced, whereas the inter-seizure interval was increased after ultrasound stimulation. The present study suggested that ultrasound may offer a non-invasive method for the treatment of epilepsy.

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https://doi.org/10.1016/j.isci.2020.101066

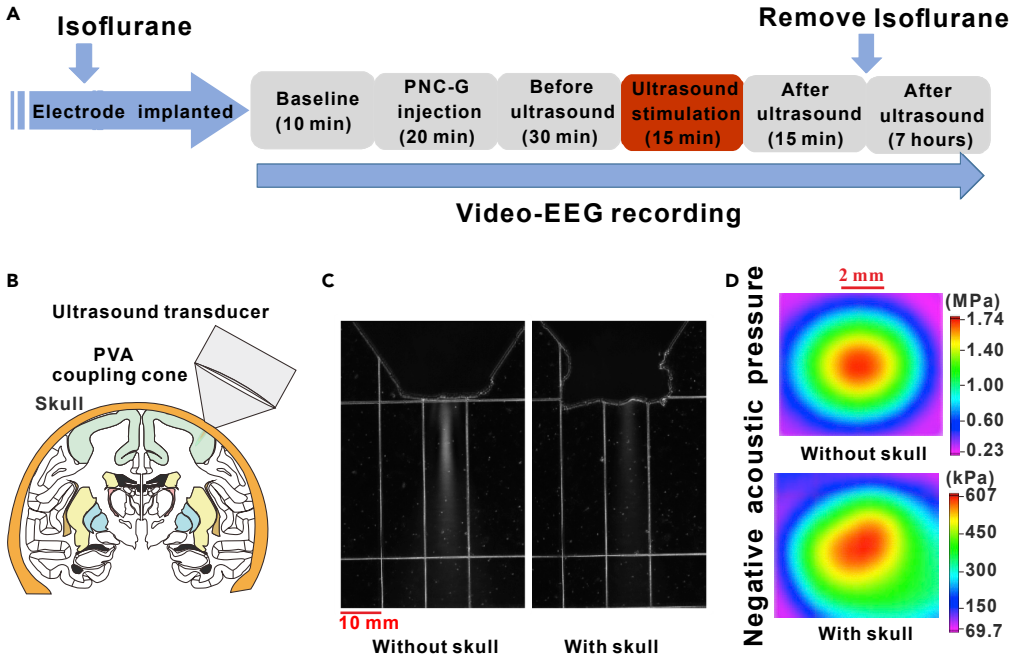

Figure 1. Schematic of the Ultrasound Neuromodulation System

(A) The experimental process. (B) Ultrasound transducer was placed on the scalp and fixed to the mechanical arm. The coupling cone was filled with PVA phantom. (C) Acoustic field distributions in longitudinal plane without and with mouse monkey skull measured by the OptiSon Ultrasound Beam Analyzer (Onda, USA). Scale bar, 10 mm. (D) Acoustic pressure distribution in axial plane without and with mouse monkey skull measured by a calibrated hydrophone. Scale bar, 2mm.

RESULTS

Examination of the Effect of Ultrasound Parameters on Epileptic Seizures

The sonication parameters were selected based on real-time monitoring of behavior and electroencephalograph (EEG). Ultrasound waves with different frequencies and durations were delivered to the prefrontal motor cortex (Figure 1). We found that ultrasound stimulation at a frequency of 800 kHz, a pulse repetition frequency (PRF) of 500 Hz, a duty cycle of 36%, and an acoustic pressure of 1.74 MPa reduced the number of seizures compared with ultrasound stimulation at a frequency of 750 kHz (Figure 2A). In addition, the number of seizures was reduced when ultrasound was delivered at a frequency of 800 kHz, a PRF of 500 Hz, and an acoustic pressure of 1.74 MPa for 15 min (Figure 2B). Therefore, a frequency of 800 kHz and a duration of 15 min were used as the ultrasound parameters in subsequent experiments.

Ultrasound Neuromodulation Inhibits Behavioral Seizures

After penicillin injection, all monkeys were monitored for behavioral seizures by continuous video-EEG recording. The total seizure counts for 7 h (sham: 129.1 $\pm$ 13.42, ultrasound: 75.75 $\pm$ 6.527, t test, $p = 0.003$) were significantly reduced after 15 min of ultrasound treatment (Figure 2C). Seizure monkeys were randomly selected for ultrasound stimulation; the result revealed that the monkeys in the ultrasound stimulation group had a shorter seizure duration (sham: 112.1 $\pm$ 15.33 min, ultrasound: 71.38 $\pm$ 11.9 min, t test, $p = 0.0544$), as shown in Figure 2D. Figure 2E indicated that the mean interval between seizures was 307.9 $\pm$ 15.33 min in the sham group and 348.6 $\pm$ 11.9 min in the stimulation group ($p = 0.0544$). In addition, we observed that the mean number of seizures per hour in the ultrasound stimulation group was lower than that in the sham stimulation group (Figures 2F, 2G, and S1). These results showed a trend in the suppression of acute seizures in non-human primates by ultrasound stimulation.

The Safety of Ultrasound Neuromodulation

To evaluate the safety of ultrasound neuromodulation, we visualized the temperature change on the surface of the skull during ultrasound stimulation using a thermal infrared imager (R300, NEC Avio, Tokyo, Japan). After 15 min of ultrasound stimulation, the temperature rise was approximately 0.3°C (Figure S2). In addition, T2-weighted MR

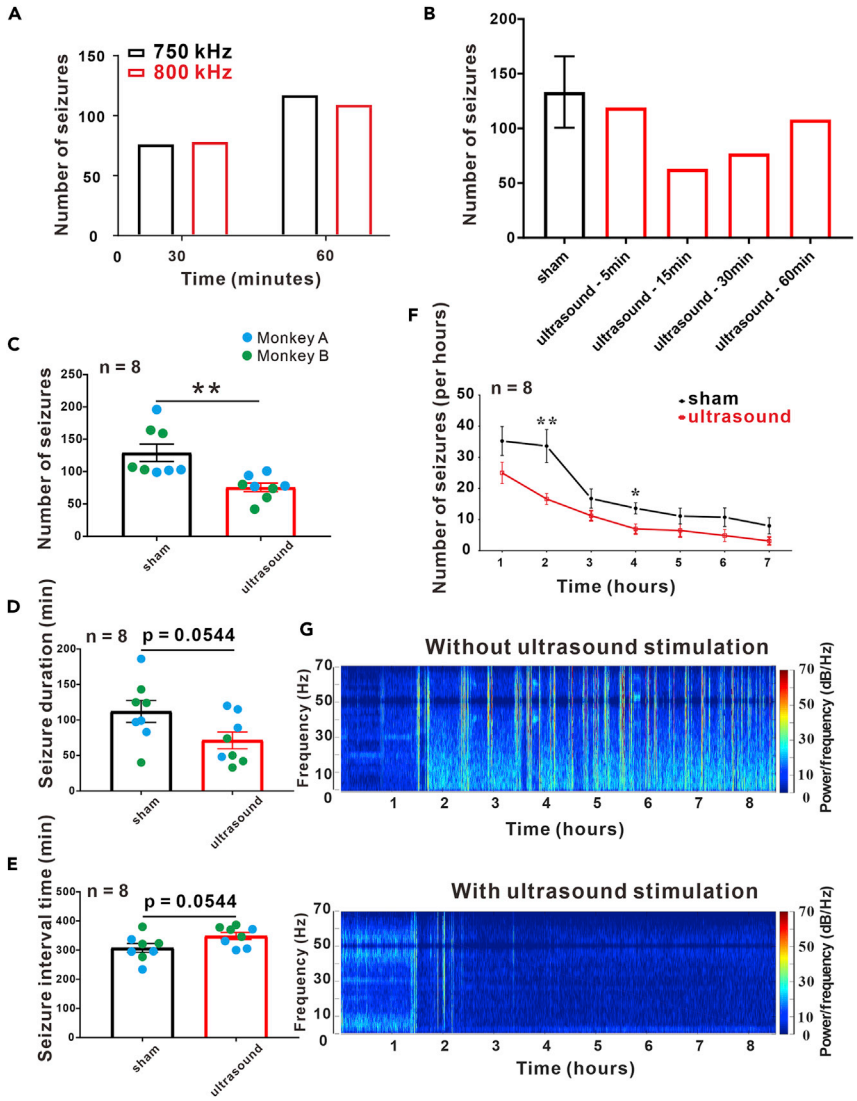

Figure 2. Ultrasound Parameters Selection and Video-EEG within 7 h after Ultrasound Stimulation

Ultrasound transducers with a frequency of 750 kHz and 800 kHz were used to stimulate for 30 min and 60 min. By observing the total number of epileptic seizures, we found that the two transducers had the same effect on epileptic EEG. B. An 800 kHz ultrasound transducer was used to stimulate epileptic monkeys with difference time, and under the action of different ultrasonic stimulation time, the number of seizures was as follow: sham (133.3 ± 16.36), 5 min (119), 15 min (63), 30 min (77) and 60 min (108). We found that 15 min ultrasound stimulation had an obvious inhibition effect. C. The total number of epileptic seizures was significantly reduced after ultrasound stimulation (sham: 129.1 ± 13.42, ultrasound: 75.75 ± 6.527, n = 8, independent-sample t-test, $p < 0.01$). D. The duration of epileptic seizures was decreased after ultrasound stimulation (sham: 112.1 ± 15.33, ultrasound: 71.38 ± 11.9, n = 8, independent-sample t-test, $p = 0.0544$). E. The inter-seizure interval was longer with ultrasound stimulation than sham stimulation (sham: 307.9 ± 15.33, ultrasound: 348.6 ± 11.9, n = 8, independent-sample t-test, $p = 0.0544$). F. The frequency of epileptic seizures per hour after 15 min of ultrasound stimulation. The number of seizures gradually decreased in both groups as time progressed. 1st hour (sham: 35.25 ± 4.636, ultrasound: 25 ± 3.423, n = 8, independent-sample t-test, $p = 0.1265$, 2nd hour (sham: 33.625 ± 5.305, ultrasound: 16.625 ± 1.802, n = 8, independent-sample t-test, $p = 0.0085$), 3rd hour (sham: 16.750 ± 3.098, ultrasound: 11.250 ± 1.623, n = 8, independent-sample t-test, $p = 0.0889$), 4th hour (sham: 13.625 ± 1.812, ultrasound: 7.000 ± 1.604, n = 8, independent-sample t-test, $p = 0.0318$), 5th hour (sham: 11.125 ± 2.539, ultrasound: 6.500 ± 2.104, n = 8, independent-sample t-test, $p = 0.2506$), 6th hour (sham: 10.750 ± 2.975, ultrasound: 4.875 ± 1.922, n = 8, independent-sample t-test, $p = 0.1633$), 7th hour (sham: 8.000 ± 2.619, ultrasound: 3.125 ± 1.274, n = 8, independent-sample t-test, $p = 0.1218$). G. The total number of seizures and the EEG power density with time in two groups. Data are represented as mean ± sem.

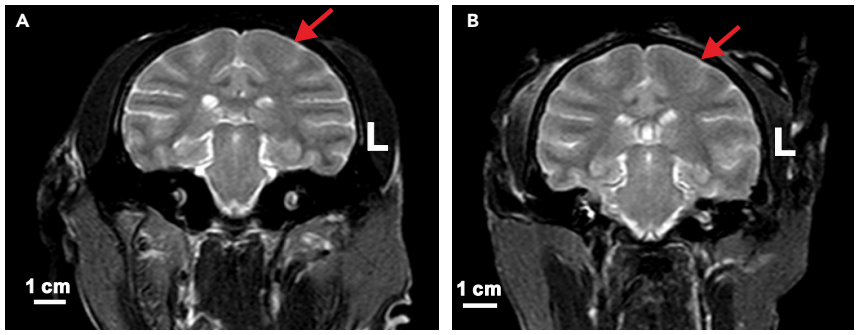

Figure 3. MRI images of monkeys stimulated by ultrasound.

MRI images of monkeys stimulated by ultrasound. T2-weighted MR imaging was performed after ultrasound stimulation. The red arrows indicated where the stimulation was applied to. No pathological damage was found in each monkey after ultrasound stimulation. Scale bar, 1 cm.

imaging showed that there was no tissue damage or bleeding after ultrasound stimulation (Figures 3A and 3B, Videos S1 and S2).

DISCUSSION

This study demonstrated that noninvasive ultrasound stimulation could inhibit acute seizures in monkeys. Video-EEG recordings from the epileptic foci tend to show that ultrasound neuromodulation reduced the frequency and duration of seizures and increased the inter-seizure interval in a penicillin-induced epilepsy nonhuman primate model.

Epilepsy is a prevalent neurological disorder resulting in disruptive seizures and is often associated with pharmaco-resistance. Neuromodulation techniques have recently been employed to modulate aberrant neuronal activity and decrease the frequency or duration of seizures. These techniques employ physical means to modulate neuronal activity, thereby decreasing the frequency or duration of seizures (Liebetanz et al., 2006; Krook-Magnuson et al., 2013; Salanova et al., 2015; Bauer et al., 2016, 2017). Compared with deep brain stimulation and optogenetics, ultrasound can noninvasively penetrate the skull to reversibly modulate neuronal activity and does not require the implantation of an electrode or optical source (Li and Cook, 2018; Deffieux et al., 2013; Wang et al., 2017). Ultrasound neuromodulation has a higher spatial resolution and offers deeper tissue penetration than non-invasive neuromodulation methods, such as transcranial magnetic stimulation and transcranial direct current stimulation (Bystritsky et al., 2011; Folloni et al., 2019; Fouragnan et al., 2019). In this study, we found that ultrasound could noninvasively stimulate the prefrontal motor cortex and inhibit behavioral seizures in monkeys. Overall, low-intensity ultrasound neuromodulation is a promising noninvasive brain stimulation tool that appears to have neuromodulatory effects associated with behavioral changes.

In this study, we found that the inhibitory effect could last for 7 h after 15 min of ultrasound stimulation (Figure 2). In addition, Davide Folloni et al. indicated that 40-s of ultrasound stimulation could cause brain activity of macaque monkeys for more than 1 h (Folloni et al., 2019). These suggest that ultrasound may offer possible non-invasive treatment of epilepsy.

The mechanism by which ultrasound inhibits seizures was not examined in this study. Most authors champion nonthermal mechanical mechanisms of ultrasound neuromodulation. Recently, we reported that ultrasound could open the *Escherichia coli* mechanosensitive channel of large conductance (MscL) to control neuronal activities (Ye et al., 2018). Moreover, Huang et al. indicated that the therapeutic mechanism of ultrasound neuromodulation could possibly be attributed to promoted brain-derived neurotrophic factor (BDNF) expression (Huang et al., 2017). In addition, we found that ultrasound conferred neuroprotection in Parkinson's disease mice (Zhou et al., 2019a, 2019b). An important study on the mechanism of ultrasound to suppress epileptic seizures is confirmed by our recent research (Lin et al., 2020). We used patch-clamp to record brain slices of patients with epilepsy and found that ultrasound stimulation can inhibit neuronal

excitability in brain slices from epileptic patients, and the inhibition efficiency is more than 65%. In addition, we observed increased expression of c-Fos protein in GABAergic neurons, suggesting that ultrasound stimulation may enhance GABAergic neuron activity and increase the inhibitory postsynaptic inputs. But, owing to the different ultrasound parameters used in two studies, the potential mechanism of ultrasound stimulation for treatment of epilepsy still needs to be studied.

Magnetic resonance imaging showed that ultrasound neuromodulation did not cause any tissue damage, which may suggest that ultrasound is a safe neuromodulation tool. Previous studies have shown that ultrasound was able to mitigate focal cerebral ischemia in rats (Guo et al., 2015; Li et al., 2017), reduce essential tremors in rats (Sharabi et al., 2019), and modulate brain function in humans (Monti et al., 2016). Our recent studies have indicated that ultrasound stimulation can improve motor function in Parkinson's disease model mice (Zhou et al., 2019a, 2019b). In a study of 54 cases of ultrasound regulating the central nervous system, only two had ultrasound-related injuries (Blackmore et al., 2019). Another study on magnetic resonance acoustic radiation force imaging also pointed out that localized brain regions did not cause tissue damage after ultrasound stimulation (Gaur et al., 2020). Therefore, ultrasound may be a safe, noninvasive therapeutic method for the modulation of neurological disorders, including epilepsy and Parkinson's disease.

Limitations of Study

Our research also has several limitations that should be addressed in the future. First, there were only two animals used in our study; to further verify the role and mechanism of ultrasound neuromodulation technology in non-human primate epilepsy models, we will need to increase the number of animals in the future. Second, from the current experimental results, the ultrasound duration time plays a significant role in the effect of ultrasound to inhibit seizures. We should focus on more point-in-time of ultrasound in the future. Different combinations of ultrasonic parameters were also crucial. Third, the acute epilepsy model was used in our experiments. This model is helpful for us to study the role of ultrasound neuromodulation in epilepsy. However, chronic human temporal epilepsy is more common in human diseases. The other models were studied in the next study. In addition, to reduce the attenuation of the skull, the array ultrasound transducer with low frequency will be developed to deliver ultrasound energy to the targeted region.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND CODE AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article and its supplemental materials.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101066.

ACKNOWLEDGMENTS

The work was supported by the National Natural Science Foundation of China, China (Grant No. 81527901, 11774371, 11574341, 11674347, 11904380, 81671193). Natural Science Foundation of Guangdong Province, China (Grant No. 2017A030313879); Guangdong-Hong Kong-Macao Greater Bay Area Center for Brain Science and Brain-Inspired Intelligence Fund, China (No. 2019024); Guangdong grant "Key technologies for treatment of brain disorders" (No. 2018B030332001, No. 2018B030331001) and The Guangdong Provincial Clinical Medical Centre for Neurosurgery, China (No. 2013B020400005). CAS Key Laboratory of Health Informatics Fund, China (2011DP173015).

AUTHOR CONTRIBUTIONS

J.Z., L.N., L.M., and H.Z. designed experiments. W.M., Y.W., X.H., Z.L., Y.Q., C.T., and J.Z. conducted the experiments. J.Z., Y.C., and Y.G. designed and performed epilepsy surgery. J.Z., L.N., L.M., T.Y., and H.Z. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: October 27, 2019
Revised: January 20, 2020
Accepted: April 13, 2020
Published: May 22, 2020

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iScience, Volume *23*

Supplemental Information

Ultrasound Neuromodulation Inhibits Seizures in Acute Epileptic Monkeys

Junjie Zou, Long Meng, Zhengrong Lin, Yangzi Qiao, Changjun Tie, Yibo Wang, Xiaowei Huang, Tifei Yuan, Yajie Chi, Wen Meng, Lili Niu, Yanwu Guo, and Hairong Zheng

Transparent Methods

Experimental Animals Details

Two adult male rhesus monkeys (named “A” and “B”) participated in this study and were obtained from Guangdong Landau Biological Technology Co., Ltd., Guangzhou, China. All animals were housed individually under a 12 h/12 h light/dark cycle at 24°C ± 1°C and 55 ± 5% humidity with sufficient water and food supply. All animal protocols described in this work (Certificate number: LDACU20170306-01) were approved by the Institutional Ethical Committee of Animals Experimentation of Guangdong Landau Biological Technology Co., Ltd. All efforts were made to minimize pain and/or discomfort of the animals.

Electrode implantation and transcranial injection of penicillin

Surgery was performed under general anesthesia using isoflurane (1.5-3%, 1.5 L/min, R51022, RWD), and salivation was reduced using atropine (0.05 mg/kg, IM). The heads of the monkeys were fixed, and surgery was performed using a stereotaxic apparatus (68901, RWD) for non-human primates. Under aseptic conditions, a midline linear incision of approximately 4 cm was made, the muscle and the periosteum were separated, and the skull was exposed. The stereotactic coordinates of the right hand movement area were calculated and determined according to “A Combined MRI and Histology: Atlas of the Rhesus Monkey Brain in Stereotaxic Coordinates”(Logothetis, 2012). The right hand movement area was located at the following stereotactic coordinates: 30 mm anterior and 15 mm lateral relative to the interaural line and 3 mm dorsoventral relative to the dura. In order to accurately **position the transducer over the stimulation site,** the MRI was used to **obtain the anatomical structure**. A dental bur was used to drill 2 holes in the skull, one at the location for penicillin injection and the other at the location for electrode implantation, 5 mm anterior to the right hand movement area (Figure 1B). **At the end of the experiment, we closed these two holes with bone wax and used bone wax as a marker for our localization. Besides, in order to ensure that ultrasound stimulation is performed at the same location each time, bone wax is attached to the surface of the skull as a marker to mark the position of the ultrasonic transducer.** After the electrode implantation and EEG was recorded for 10 minutes, penicillin (2500 IU, 250 IU/μl, H44022446, Baiyunshan, Guangzhou, Guangdong province) was injected into the right hand

movement area by a microsyringe (50 μl, 1705RN, Hamilton) at 1 μl/min.

Video-EEG recording

An EEG recording system (Solar 1848, Solar) was used in these trials. Video-EEG recordings were continuously collected throughout the experiment. The EEG electrodes were fixed on the prefrontal cortex, and then the EEG signals and video recording are transmitted to the computer simultaneously. The length of each recording was approximately 8.5 hours. A portion of the 8-channel electrode was placed into the brain to record the ECOG single. The sampling rate was 500 Hz. The bandpass filter for data acquisition was set between 0.01 and 100 Hz. The number of seizures, seizure duration, inter-seizure interval time and the number of seizures per hour were calculated for trials with ultrasound stimulation and compared with trials without stimulation for each session and experimental condition explored in the study for each monkey.

Ultrasound neuromodulation

Two ultrasound transducers with fundamental frequencies of 800 kHz (IMASONIC, Voray sur l'Ognon, France) and 750 kHz (Sonic Concepts, Woodinville, CA) were used to stimulate the epileptic monkeys. A coupling cone was filled with polyvinyl alcohol (PVA). The frequency was set to 500 Hz, and the pulse duration was set to 100 ms using a first function generator, with rise and fall times set to 1 ms using a second generator (AFG3101, Tektronix) connected to the amplitude modulation entry of the first generator. A 100 W amplifier (2100L, EI, NY, USA) was used to deliver the required power to the transducer. The pressure amplitude at the focus was 1.74 MPa. An acoustic coupling device was required between the ultrasonic transducer and the monkey skull. In this study, we used a solid gel-type coupling material made of PVA(Maurice et al., 2005, Joe et al., 2019). We first designed a coupling cone based on the focal length of the ultrasonic transducer, printed it in a 3D printer, and placed the 10% PVA(363065, Sigma) solution in the coupling cone; after vacuuming, it passed a freeze-thaw cycle (12 hours of freezing and 12 hours of thawing). Finally, a fixed PVA coupling cone was obtained and stored in ultrapure water. The transducer was placed on the scalp and fixed to the mechanical arm.

Skull transmission was estimated on several clean and degassed primate skulls. These trials allowed

us to estimate the acoustic pressure at 1.74 MPa in the brains of the monkeys. The corresponding intensity spatial peak pulse average (I_{SPPA}) was 119.78 W/cm^2. By taking into account a minimum 5 s pause between each ultrasonic pulse, we also estimated the corresponding spatial peak time average intensity (I_{SPTA}) at less than 1431.71 mW/cm^2. Through a fresh monkey skull, acoustic pressure decreased about 65%. Low-intensity pulsed ultrasound was delivered for 15 minutes after penicillin injection for 30 minutes and was focused on the right hand movement area between the electrode location and the penicillin injection location. The animals were fixed on the primate chair for 7 hours after ultrasound stimulation. After each experiment, the animals were allowed to recover completely, and ceftriaxone sodium (1 g daily, IM) was administered to prevent postoperative infection for 7 days. **There are 14-day intervals in each experiment for recovery. Therefore, we considered sessions as random effects.**

Experimental process

The monkeys were anesthetized by isoflurane (1.5%, 1.5L/min) and implanted with the EEG recording electrode (Figure 1A). EEG was collected as a baseline for 10 minutes. After penicillin was injected into the prefrontal cortex with a microsyringe, spikes were recorded for 30 min. Then, the monkey received 15 min ultrasound stimulation. After 15 minutes of stimulation, the anesthesia was removed. Video-EEG was recorded for 7 hours. There are 14-day intervals in each experiment for recovery

Parameter selection

Before starting the ultrasonic stimulation experiment, we made hydrophone measurements and laser sound field measurements using the ultrasonic probe. We calibrated the energy before ultrasound and after ultrasound. These measurements showed that the ultrasound was attenuated to 65% on the monkey skull, and there was no significant change in focal position of the shape of the focal point. Our previous research have proved that ultrasound stimulation for 30min can decrease the number of spike during acute seizure(Zhengrong Lin, 2020). The monkey A and B were respectively selected to perform the experience about ultrasound stimulation time and compared the difference with the two-ultrasound transducer. There are 4 time points (5min, 15min, 30min and 60min) were set up to choose the best one.

Evaluation of temperature and safety

To evaluate the thermal effect of ultrasound in our study, the temperature in the monkey skull was recorded using a thermal infrared imager (R300, NEC Avio, Tokyo, Japan). To observe whether ultrasound stimulation can cause edema and necrosis of monkey brain tissue, T2-weighted MR imaging was performed after 15 min ultrasound stimulation.

Statistical analysis

All data are expressed as the mean ± sem. All analyses were conducted using SPSS statistics, version 22. All data were analyzed by an unpaired Student's t test. The level of statistical significance was set to a p value ≤ 0.05.

Supplemental reference lists

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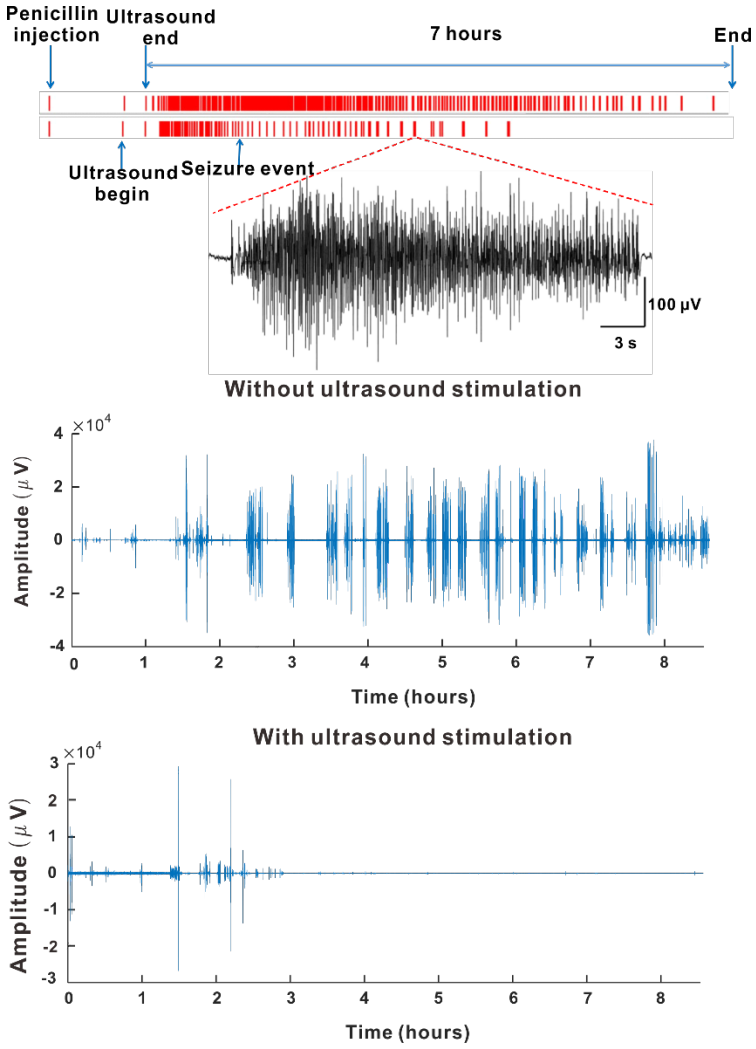
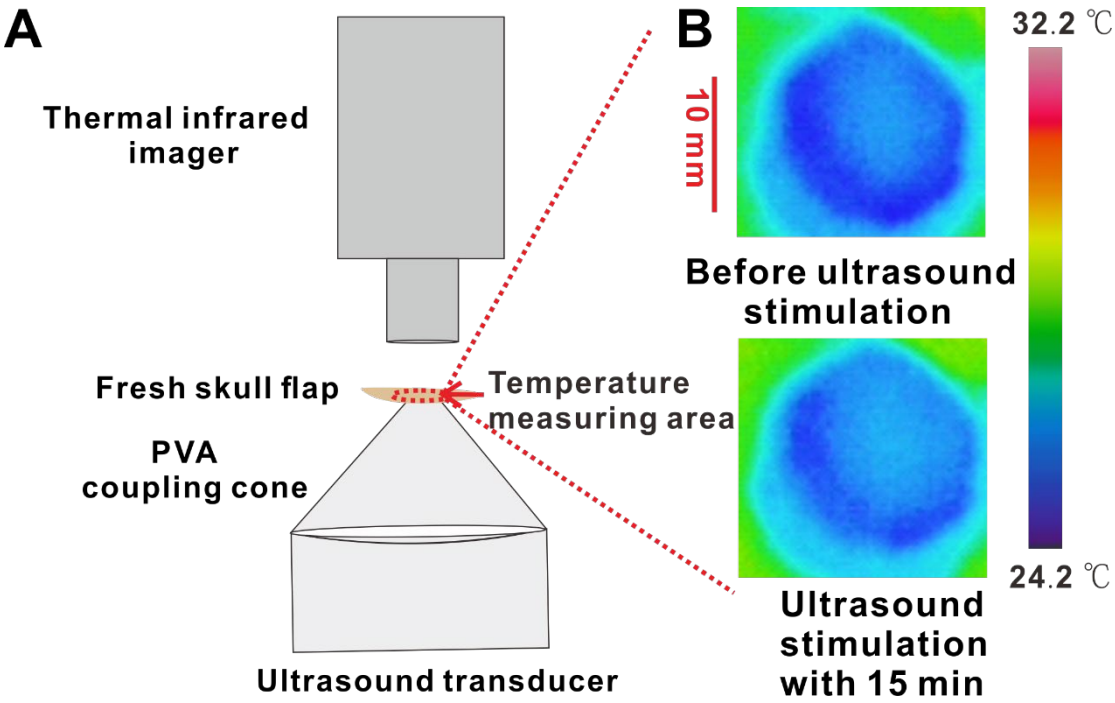
Supplementary Figure 1.



Supplementary Figure 1. The number of seizures was recorded for 7 hours after ultrasound stimulation or sham stimulation, related to Figure 2.

By quantifying the number of epileptic seizures in the EEG records, the number of episodes was reduced in monkeys after ultrasound stimulation compared with in monkeys that did not receive ultrasound stimulation.

Supplementary Figure 2.



Supplementary Figure 2. Sketch of the ultrasound stimulation site and the temperature change map after ultrasound stimulation, related to Figure 3.

A.The ultrasound transducer transmitted ultrasound stimulation, and the electroencephalogram recording electrode recorded signals from the left prefrontal cortex.

B. The temperature evaluation of the skull through which the ultrasound stimulation passed showed that, after 15 minutes of ultrasound stimulation (lower), the temperature of the skull surface did not rise more than 0.3°C above the temperature before stimulation (upper), which was within a safe temperature range.