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Comparing the anticonvulsant effects of low- and high-frequency electrical stimulation of olfactory bulb in rats

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Abstract

Deep brain stimulation (DBS) is an alternative treatment for epilepsy. In the present study, we investigated the anticonvulsant effects of low- and high-frequency stimulation (LFS and HFS) of olfactory bulbs on kindled seizures. Full hippocampal kindled male rats received either LFS (1 Hz) or HFS (130 Hz) in the olfactory bulb at 5 min, 6, 24, and 30 h after the last kindling stimulation. Applying HFS and LFS alleviated the seizure severity and restored the memory impairment in kindled animals. Applying both LFS and HFS in full-kindled animals decreased the excitatory postsynaptic currents (EPSCs), while increased the inhibitory postsynaptic currents (IPSCs). The excitation-to-inhibition (EPSC/IPSC) ratio decreased following LFS and HFS in kindled animals through pre-and post-synaptic mechanisms. The expression of NR2A (NMDA receptor subunit) and GluR2 (AMPA receptor subunit) genes was increased while the expression of γ2 ($GABA_A$ receptor subunit) gene was decreased in kindled animals. Applying both LFS and HFS in kindled rats restored these changes. Obtained results suggested the olfactory bulb as a suitable DBS target for treating epilepsy. In addition, applying DBS at both low- and high-frequencies exerts similar anticonvulsant effects, showing that LFS may also be as effective as HFS in epilepsy treatment.

Keywords: Epilepsy; Deep brain stimulation; Olfactory bulb; Hippocampus; Seizure

Significance Statement

Deep brain stimulation (DBS) is a new treatment for drug-resistive epilepsies. However, finding the optimum DBS' parameters, including its frequency and the stimulation target, are under research. Although high-frequency stimulation (HFS) of different brain areas is commonly used in epilepsy patients, there are a lot of documents showing that low-frequency stimulation (LFS) is also effective in suppressing the seizures. In addition, some preliminary experiments showed that olfactory system may be a suitable target for DBS. Therefore, we applied HFS and LFS in olfactory bulb of kindled animals and showed that LFS was as effective as HFS in reducing seizures. Both LFS and HFS reduced seizure severity and restored the seizure-induced changes in: memory, synaptic transmission, glutamate and GABA-A receptors gene expression, brain waves power and histological parameters. These results suggest that LFS may be a good pattern of DBS in epilepsy treatment.

1. Introduction

Temporal lobe epilepsy is a common form of medically refractory epilepsy that affects the temporal lobe. Over 20-30% of patients still have uncontrolled seizures (Löscher et al., 2013). Although surgery is often recommended in this subset of refractory patients, most of them are not good candidates since they do not have a single seizure focus or it is difficult to determine the exact seizure focus (Engel Jr, 2018). Hence, alternative therapeutic approaches to controlling intractable temporal lobe epilepsy are essential.

DBS has been considered an alternative therapy for treating epilepsy, with many advantages, including reversibility, adjustability, and low complication risk (Fisher & Velasco, 2014; Li & Cook, 2018). Depending on the target and stimulation parameters, DBS has different therapeutic actions (Fisher, 2011; Laxpati et al., 2014). The stimulation frequency is an essential factor in determining the anticonvulsant action of DBS. In the clinic, DBS is applied at high-frequency stimulation (HFS, usually >100 Hz) to exert anticonvulsant effects (Chabardès et al., 2002; Gross, 2004). However, there are a lot of clinical (van Gompel et al., 2015; Velasco et al., 2005) and preclinical studies showing the effectiveness of low-frequency DBS (LFS) (Khodadadi et al., 2022; Manahan-Vaughan, 2000).

DBS target is also very important in determining its anticonvulsant action. Brain areas involved in seizure generation or propagation may be suitable targets for DBS. The anterior nucleus of the thalamus and the hippocampus are among the main DBS targets in epilepsy patients (Laxpati et al., 2014; Salanova et al., 2015). In addition, according to preclinical studies, many other brain areas, such as subthalamic nucleus (Wu & Sharan, 2013), hippocampus (Klinger & Mittal, 2016)

and olfactory bulb (Khodadadi et al., 2022) may also be considered as targets for anticonvulsant action of DBS.

The olfactory bulb exhibits important anatomical connections with brain structures linked to epilepsy. Through the entorhinal cortex, it establishes a connection with the hippocampal formation (Vanderwolf, 1992) and reciprocally receives direct synapses from the ventral region of the hippocampus (Aqrabawi & Kim, 2018). Notably, olfactory auras are commonly associated with temporal lobe epilepsy (Foldvary-Schaefer & Unnwongse, 2011), indicating the significant involvement of the olfactory bulb in this condition. It has been found that the severity of seizures increases after olfactory bulbectomy in mice, highlighting the pivotal role of the olfactory bulb in epilepsy (Jiang et al., 2015). Furthermore, temporal lobe epilepsy patients often present with olfactory deficits, accompanied by a reduction in olfactory bulb volume (Hummel et al., 2013).

Following determining a suitable target, if the therapeutic effects of HFS and LFS in treating a neural disease are the same, LFS will be the preferred pattern. This is because the energy transferred to the brain, tissue damage, and battery assumption will be lower following LFS application compared to HFS (Kile et al., 2010; Koss et al., 2005).

According to the above explanations, we considered the olfactory bulb as a promising target for the anticonvulsant action of DBS. Our preliminary experiments showed that applying LFS in olfactory bulb had anticonvulsant effects against hippocampal kindled seizures (Khodadadi et al., 2022). However, more experiments need to shed light on the effectiveness of olfactory bulb-DBS as an effective manner for treating drug-resistant epilepsy. Therefore, in the present study, we investigated

the anticonvulsant action of olfactory bulb-DBS at both low (LFS) and high (HFS) frequencies.

2. Materials and methods

2.1. Animals

Male Wistar rats (200-280 g, 2-4 months old) were purchased from Pasteur Institute (Tehran, Iran). The animals were held under a 12-h light/12-h dark cycle (lights on from 8:00 a.m.–8 p.m.) and free access to water and food at 22–25°C. They were kept separately after surgery. All experiments and animal care procedures were approved by the "Tarbiat Modares University Ethical Committee for Animal Research" by IR.MODARES.REC.1399.088 ethical code. The authors confirm that all experiments were performed in accordance with relevant guidelines and regulations.

2.2. Surgery

The subjects were anesthetized by injection of ketamine/xylazine mixture anesthesia (100/10 mg/kg, i.p.). Once fully anaesthetized, a monopolar recording electrode was twisted with a bipolar stimulating electrode and was stereotaxically implanted in the right ventral hippocampus (5.3 mm posterior to bregma, 5.2 mm lateral to right, 7.5 mm below dura). These electrodes were used for kindling stimulations and afterdischarge (AD) recording. In addition, two bipolar stimulating electrodes were inserted in the right and left olfactory bulb (7.5 mm anterior to bregma, 1.0 mm lateral to right or left, 1.6 mm below dura) for applying DBS. A monopolar recording electrode was also positioned in the right olfactory bulb (twisted to those mentioned above bipolar stimulating electrode) and the piriform cortex (0.8 mm posterior to bregma, 4.9 mm lateral to the right, and 8.8 mm below the skull).

The electrodes were teflon-coated stainless steel wires (0.125 mm bare diameter, 0.5 mm tip distance, Cat No. 791500, A-M Systems). The reference and ground electrodes were connected to the skull by stainless-steel screws. Each electrode was soldered to a metal pin, and all pins were put in a plastic multichannel socket. Then, the socket was installed on the skull by dental acrylic. Histological verification was carried out at the end of the experiments to confirm the electrode placement in animals.

2.3. Kindling procedure

Kindling stimulations were applied after 7-10 days post-surgical recovery period. A train of stimuli (1 ms monophasic square pulses, 50 Hz for 2 s) was applied to determine the AD threshold. The stimulus intensity was 30 μA. If ADs were not recorded, the intensity was increased in steps of 10 μA at 20 min intervals until ADs were recorded for 15 s. The discharge fluctuations with the frequency > 1 Hz and amplitude > 2×baseline were considered as ADs. The subjects received 6 stimulations daily at the AD threshold at 20-min intervals. Following kindling stimulations, the epileptiform ADs were continuously captured using a PC-based data acquisition system (BIODAC ES1721, TRITA Health Technology CO., Tehran, Iran). Behavioral seizure stages 1–5 were categorized according to the Racine scales. Stages 1–3 were considered focal, and stages 4–5 as generalized seizures. To be considered fully kindled, animals had to undergo three consecutive stage 5 seizure. In addition to seizure stages, AD duration (ADD), and stage 5 duration were recorded.

2.4. Applying DBS in the olfactory bulb

To examine the effect of olfactory bulb DBS on hippocampal kindled seizures, fully kindled rats received four packages of bilateral DBS in the olfactory bulb

through stimulating electrodes. The 1st DBS package was applied immediately after the last kindling stimulation when the ADs were finished. The 2nd DBS was applied 6 h later. The 3rd DBS package was applied at the next day (18-20 h after the 1st DBS), and the 4th DBS was 6 h later. Each DBS package consisted of 4 trains of monophasic square waves with 0.1 ms pulse width. The duration of each train was 200 s, and the inter-train interval was 100 s. These trains were applied as low-frequency stimulation (LFS; 1 Hz, each train contained 200 pulses) or high-frequency stimulation (HFS; 130 Hz, each train contained 26000 pulses). LFS and HFS parameters were determined according to our previous experiments (Ghafouri et al., 2016; Sadeghian et al., 2020). Animals received 6 kindling stimuli/day at 24 h and one week after termination of LFS or HFS.

2.5. Whole-cell patch clamp recording

For whole cell recordings, ventral and dorsal hippocampal slices were prepared 48 hours after the last kindling stimulation (Ghafouri et al., 2019). Rats were deeply anesthetized by CO_2 (100%) inhalation, decapitated by a guillotine, and their brain was quickly extracted. The right hemisphere was separated in an ice-cold cutting solution composed of (in mM): 238 sucrose, 2.5 KCl, 0.5 $CaCl_2$, 2 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$ and 11 D-glucose, pH of 7.3–7.4, and Osmolality equilibrated to 290–300 mOsm. The cutting solution was bubbled with 95% oxygen and 5% carbon dioxide. Acute 300 µm horizontal hippocampal slices were obtained from the right hemisphere using a VT1200S vibrotome (Leica, Nussloch, USA). Then, slices were continuously superfused with ACSF solution containing (in mM): 125 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, and 10 D-glucose. Slices were incubated in ACSF at 32–35 °C for 1 h, pH of 7.3–7.4 with osmolality of 290–300 mOsm, and bubbled with 95% oxygen and 5% carbon dioxide. Following

incubation, slices were kept at room temperature (23–25 °C) for a minimum of 30 min, then transferred to a submerged recording chamber on the fixed stage of an upright microscope (Axioskop 2 FS MOT; Carl Zeiss, Germany). Slices were continuously superfused with the standard ACSF at the rate of 2 ml/min.

Whole-cell, voltage-clamp recordings were made from ventral and dorsal hippocampal CA1 pyramidal neurons that were visually identified by an infrared CCD camera (IR-1000, MTI, USA) with a 40X water immersion objective lens. Recording microelectrodes (4–6 MΩ 1.5 mm outer diameter, filamented borosilicate glass, Sutter, USA) were pulled with a horizontal puller (P-97, Sutter Instrument, USA). EPSCs and IPSCs were recorded with an internal solution containing (in mM): 102 $CsMeSO_4$, 3.7 NaCl, 2 Mg ATP, 0.3 NaGTP, 0.2 EGTA and 20 HEPES, 5 TEA-Cl, 11 BABTA, 5 QX-314. The pH of internal solution was 7.3–7.4 (equilibrated by CsOH) and its osmolality was 290–300 mOsm.

Synaptic currents were evoked by a bipolar stimulating electrode (A-M Systems, Cat No. 791500) placed 100-150 μm from the soma of recorded neurons. The stimulating current was applied at five min after creating the whole-cell patch clamp configuration. Its intensity was adjusted by an Isolated Pulse Stimulator (Model 2100, A-M Systems, Inc.) synchronized with the Clampfit 10 data acquisition software (Molecular Devices). The monophasic square wave stimulating currents were applied at a frequency of 0.05 Hz, with a pulse duration of 0.1 ms, at an intensity equal to 1.5-fold of the threshold to record evoked EPSCs and IPSCs.

To calculate the excitation to inhibition ratio, both EPSC and IPSC have to be recorded from the same hippocampal pyramidal neuron. For this purpose, the reverse potentials of the excitatory and inhibitory currents were calculated. To obtain the I-V curve of EPSCs, the evoked EPSCs were recorded in the presence of

bicuculline (20 μM) as the $GABA_A$ receptors antagonist. The I-V curve of IPSCs was obtained in the presence of CNQX (20 μM) and APV (50 μM) as AMPA and NMDA receptors antagonist, respectively. Using the I-V curves, the reverse potential (the potential at which the recorded current was equal to zero) of glutamate receptors was +10 mV, and of the $GABA_A$ receptors was -52 mV. Then, the glutamatergic currents were recorded at the holding potential of -52 mV and GABAergic currents were recorded when the potential was clamped M at +10 mV. The excitatory and inhibitory nature of the currents was confirmed by removing the relevant currents in the presence of their blockers.

Paired-pulse ratio for EPSCs and IPSCs were monitored using interstimulus intervals of 100 ms. For obtaining coefficient of variation (CV), failure rate, synaptic efficacy, and synaptic potency, the slices were stimulated at an intensity equal to the threshold (minimal stimulation) for at least 10 times. CV was measured by dividing the standard deviation to mean of EPSC or IPSC amplitude. Failure rate was considered as the percentage of unsuccessful responses, synaptic efficacy was determined as the averaged amplitude of success and unsuccess responses, and synaptic potency was calculated as the mean amplitude of success responses. These parameters were calculated as an index of cell excitability and an index of pre- or post-synaptic mechanisms (Álvarez-Ferradas et al., 2015). Spontaneous IPSCs were also recorded for 10 min. The recorded currents were handpicked and analyzed by Mini Analysis Program using a threshold of 12 pA.

Synaptic currents were monitored by Multiclamp 700B amplifier and digitized by Digidata 1440 A/D converter (Molecular Devices, CA, USA). Data were collected at 10 kHz and low pass filtered at 2 kHz. The electrode capacitance and series

resistance were compensated, and recordings were discarded when the series resistance was higher than 20 GΩ or was changed by more than 25%.

2.6. Y-maze test

Y-maze test was done for checking the changes in working memory. The maze was made from black plexiglass and contained three arms connected to each other by 120° degrees. The length and width of each arm were 50 cm and 16 cm respectively. The high of all arms as 32 cm. The test was started by putting the animal at the end of one arm and letting it to explore the whole maze for 8 min. The number of arm entries (i.e., when all four limbs of the animal were inside the arm) were counted. When the animal entered three arms consecutively, it was considered as a correct alternation. The percentage of spontaneous alternations was calculated as an index of working memory according to below equation:

$$Spontaneous\ altarnation\% = \frac{Number\ of\ correct\ alternations}{Total\ entries-2} \times 100$$

The animal movements were video monitored and analyzed by Ethovision software 11 (Noldus Information Technology, Wageningen, The Netherlands).

2.7. Barnes maze test

To evaluate spatial learning and memory, animals were trained by Barnes maze test. The maze consisted of a 90 cm height round dark plexiglass platform with the diameter of 122 cm. The platform contained 18 holes at its surrounds and the diameter of each hole was 9 cm. All holes were covered by a dark plexiglass plate in beneath except one of them that contained the escape (goal) box (30 cm in length, 12 cm in width and 15 cm in height). The light of the environment was very high so that the animal tended to go to the goal box. During four training days, the animal learned the spatial position of the goal box according to the environmental cues.

For starting the test, the rat was put in a cubed start box (20.5 cm× 20.5 cm× 24 cm) in the center of platform. The light was turned on and the start box was removed at 10 s later. Animal was free to explore the maze for up to 5 min to find the goal box. After entering the rat into the goal box, the training was finished and light was turn off. The rat was let to stay in goal box for 1 min. The learning procedure included four trials a day, each trial was up to 5 min and the inter-trials interval was 15 min. The platform was completely cleaned by ethylic alcohol (70%) after each trial. After training, to check the memory retention, the animals underwent a probe test in which the goal box was replaced by a dark plexiglass plate. The animal behavior was monitored by a video camera mounted to the ceiling.

The following parameters were measured in Barnes maze test: a) Primary latency was the time from removing the start box till finding the goal box; b) Total latency was measured as the time from removing the start box till entering to the goal; c) Primary errors was defined as the number of holes that were explored incorrectly before finding the goal box; d) Total errors was the number of explored holes before entering the goal box; e) Velocity and total distance during a trial (were calculated by using EthoVision software); f) The strategy that animal used to find the goal box and was categorized as direct, serial, and random; g) The goal sector (GS) frequency that was the number of exploration the target and its neighbor holes divided by 3; h) The non-goal sector (NGS) frequency that was the number of exploration of 15 non-goal holes divided by 15 and, i) Target-seeking activity that was the number of total hole explorations divided by 18.

2.8. Real-time PCR

The gene expression was measured by quantitative real-time PCR (qRT-PCR). Subjects were deeply anesthetized by CO2 (100%) inhalation, decapitated by

a guillotine, and their brain was removed at 48 h after the last kindling stimulation. The hippocampus was isolated and preserved at -80°C. A phenol-chloroform extraction method (Ausubel et al. 1992) was used to isolate total mRNA for gene expression studies using RIboEX (GenAll, Korea). The final pellet of total RNA was air-dried and suspended it in 30 L of DEPC (diethylpyrocarbonate)-treated water. For the spectrophotometric determination of RNA concentration at 260 and 280 nm, 1 µL of total RNA was used. The integrity and quality of RNA samples were determined by using 2 µL of total RNA electrophoresed (Akhtarian, Iran) on a 1% agarose gel (Fermentaz, Germany). cDNA synthesis was performed on the extracted RNA. For each sample, cDNA synthesis was performed using 2 µg of total RNA, 10 µL reaction buffer (Parstous, Iran), and 2 µL reverse transcriptase (Parstous, Iran) according to the manufacturer's instructions.

Quantitative RT-PCR was performed using the qRT-PCR Master Mix (Ampliqon, Herlev, Denmark) on a Rotor-Gene Q device (Qiagen, Hilden, Germany). Initial heating was at 95˚C for 15 min; 40 cycles of amplification, each composed of 15 s at 94˚C, 20 s at the annealing temperature, and 30 s at 72˚C. The annealing temperature for NR2A, GluR2, γ2, and GAPDH was 60˚C. Expression levels were normalized to GAPDH as endogenous internal control. The cycle threshold was measured as an index of gene expression. In our experiments, there was no significant difference in cycle threshold for GAPDH in different experimental groups; therefore, we considered it a suitable housekeeping gene. Primer sequences for NR2A, GluR2, γ2, and GAPDH were purchased from CinnaGen, Iran (Table 1).

2.9. Nissl staining

Staining was performed using Cresyl violet acetate (0.1%; IHC world, USA) on 8 µm thick sections of hippocampal tissue. The brain samples were passed

through three ethanol gradients (96%, 80%, and 70%) each for one min. Following washing with tap water for 2–3 min, samples were stained for 1.5 min with Cresyl fast violet. After washing in water, samples were taken to ethanol 70%, 80%, and 90% for 15 s, then to ethanol 96% for 4 min, and then to ethanol 100% and 90% each for 2 min. Then, samples were placed in Xylol for five min. Each group consisted of three rats and three sections per rat, which were stained and photographed using an Olympus BX-51 microscope and a DP72 camera.

We measured the number of dying neurons in the CA1 region. The intact neurons were defined as the ones with the pale nuclei and discrete nuclei and were not basophilic. A dying neuron is characterized by abnormal morphology, including shrunken and hyperbasophilic features (Lin et al., 2021; Ooigawa et al., 2006).

Table 1. The sequences of primers that are used in real-time polymerase chain reactions.

Gene	Primer Sequence (5'->3')	product length	Accession number
GAPDH	F:AGGGGCCATCCACAGTCTTC R:AGAAGGCTGGGGCTCATTTG	258	NM-001394060.2
GluR2 AMPA	F:GTGTTTGCCTACATTGGAGTG R:TTGGCTGTGTATGAGGAGATG	269	NM-017261.2
NR2A NMDA	F:TGGAGAAGCACAACTACGAGA R:CATTCCTGATACCGAACCCAC	27	NM-012573
gama2 GABAA	F:ACCATAGCCCGGAAGTCTCT R:CCTTGCTTGGTTTCCGGTTG	145	NM-183327.1

2.10. Local field potential analyses

Local field potentials (LFPs) were recorded from the ventral hippocampal CA1 area, olfactory bulb and piriform cortex by placing the animals in a plexiglas recording box inside a Faraday cage. Video recordings were also made of the subjects' behavior and reviewed to observe the state of resting. A custom-written MATLAB (MathWorks) routine was used for all analyses. LFP analyses were performed at 48 to 52 hours after the last kindling stimulation.

LFP signals were amplified (1000x gain), low-pass filtered at 250 Hz, notch filtered at 50 Hz, and digitized at 1 kHz through by the amplifier (BIODAC-ESR18622, TRITA Health Technology Co). Power spectral density (PSD) was calculated for 10 s (when the animal was in resting state) using Welch's periodogram function in MATLAB (Hentschke et al., 2007). Power spectrum calculations were performed on 10 s segments of data using 1 ms Hamming windows with 90% overlap.

2.11. Experimental design

In the first experiment, the effects of applying LFS and HFS in olfactory bulbs were evaluated on seizure parameters in fully kindled animals. Animals were assigned into 3 groups: In the kindled+LFS group, full kindled animals received four packages of LFS and then received 6 daily kindling stimulations at 48 h and one week after the last kindling stimulation and their seizure parameters were assessed. In the kindled+HFS group, full kindled animals were treated similarly but received HFS instead of LFS. In both kindled+LFS and kindled+HFS groups, DBS was applied at two different intensities (100 and 200 μA). In kindled group, full kindled animals were treated similarly, but they did not receive LFS or HFS.

In the second experiment, the effects of applying HFS and LFS on learning and memory were evaluated in another groups of animals. Subjects were divided into control and kindled groups. Kindled animals were divided into three subgroups

including kindled+LFS (received LFS), kindled+HFS (received HFS), and kindled groups (did not receive DBS). Similarly, control animals were divided into three subgroups. Animals in the control group underwent the surgical procedure, but neither DBS nor kindling stimulations were received. In two other subgroups of control animals, named control+LFS and control+HFS groups, the experimental procedures were similar to kindled+LFS and kindled+HFS groups, but no kindling stimulation was applied. Y-maze test and LFP recordings in resting state were done in kindled animals at three times: before starting kindling stimulation, after achieving full kindled state and after receiving HFS or LFS. Barnes maze test was only done after receiving HFS or LFS in kindled animals. These tests were run in control animals at the equivalent times. At the end of Barnes maze test (i.e., one week after the last kindling stimulation) the rats' brain were removed and for histological evaluations. Histological experiments were done in control, kindled, kindled+HFS and kindled+LFS groups.

In the third experiment, we evaluated the impact of LFS and HFS on synaptic currents in fully kindled animals. The experimental groups were similar to second experiment. Hippocampal slices were prepared 48 h after the last kindling stimulation for patch clamp recording in kindled, kindled+LFS, and kindled+HFS groups or at the equivalent time duration in the control, control+LFS, or control+HFS groups.

In the fourth experiment, the gene expressions were measured in separate experimental groups including control, kindled, kindled+HFS and kindled+LFS. The hippocampal formation was sampled 48 to 52 h after the last kindling stimulation (or equal time in control group) for qRT-PCR experiments.

2.12. Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 8 for Windows (GraphPad Software, USA). Using two-way ANOVA with post-hoc Tukey's tests, we evaluated seizure severity and local field potential (LFP) recording analysis in different groups. To compare different experimental groups in Y-maze and Barnes tests, a three-way ANOVA followed by the Tukey's test was used. A one-way ANOVA was done to compare the measured parameters in the whole-cell patch clamp recording, gene expression, and histological staining between different groups of animals. For all analyses, $p<0.05$ was considered the significance level.

3. Results

There was no significant difference in the number of stimulations to achieve full kindled seizures in kindled (9.0±0.93 days), kindled+LFS (11.13±1.57 days), and kindled+HFS (10.86±0.63 days) groups. The AD threshold was 78.33±14.70 μA in kindled, 105.0±16.37 μA in kindled+LFS, and 68.57±8.57 μA in kindled+HFS groups. In addition, the AD duration following the first stimulation of animals at the AD threshold was 185.09±16.70 s in kindled, 200.07±16.23 s in kindled+LFS, and 182.22±14.71 s in kindled+HFS groups. These results showed no significant difference in seizure susceptibility among different experimental groups.

3.1. Applying both HFS and LFS in olfactory bulb had anticonvulsant effects

To evaluate the anticonvulsant action of olfactory bulb DBS, full kindled rats were stimulated by HFS (kindled+HFS) and LFS (kindled+LFS). Then, the animals received 6 daily kindling stimulations at 24 h and one week after the last DBS. Calculating the percent changes in maximum ADD and maximum stage 5 seizure duration showed that both LFS and HFS had anticonvulsant effects when applied at

high (200 μA) but not at low (100 μA) intensity (Fig. 1A, B). Therefore, in the next experiments, LFS and HFS were applied only at the intensity of 200 μA.

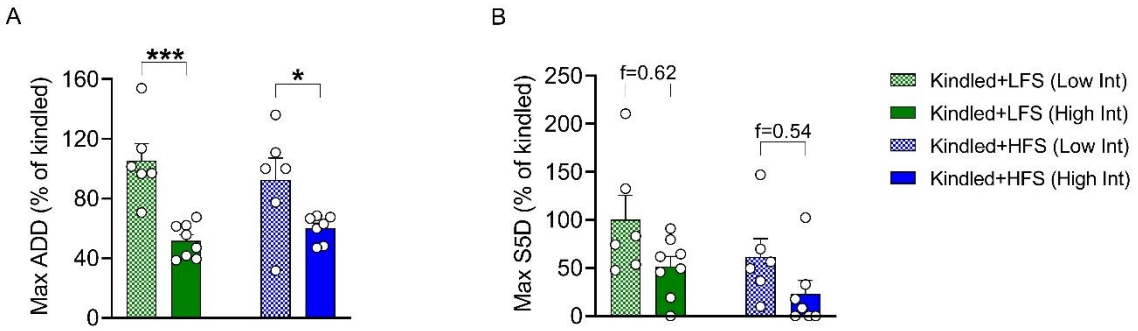


Fig. 1. Comparing the anticonvulsant action of applying LFS and HFS at low- and high-intensities in olfactory bulb. A: Comparing the effects of LFS and HFS on maximum ADD (afterdischarge duration) at low (100 μA) and high (200 μA) intensities. B: Comparing the effects of LFS and HFS on maximum S5D (stage 5 seizure duration) at low (100 μA) and high (200 μA) intensities. Both LFS and HFS were more effective at high-intensity. Int: Intensity. Data are presented as mean ± SEM. * P<0.05, *** P<0.001.

Then, the anticonvulsant effects of high-intensity LFS and HFS were compared at 24 h and one week after the last kindling stimulation (Fig. 2). A two-way ANOVA showed that there was a significant difference between experimental groups following LFS and HFS application ($F_{(4,36)}$=7.97, P<0.001). A Tukey post-hoc test showed that both LFS and HFS had similar anticonvulsant action and reduced maximum ADD at 24 h after DBS compared to respected groups before DBS (P<0.001 in kindled+LFS and P<0.05 in kindled+HFS group) and compared to kindled group (P<0.001) (Fig. 2B). This reducing effect was also observed at one week after DBS in both kindled+LFS and kindled+HFS compared to the kindled group (P<0.001). A two-way ANOVA also showed significant changes in the maximum S5D ($F_{(4,36)}$=2.80, P=0.04) among experimental groups. The post-hoc

Tukey test showed a significant difference between both kindled+LFS and kindled+HFS groups compared to kindled group at 24 h after DBS (Fig. 2C). However, when maximum S5D ($P<0.05$) were compared to before DBS data, there was a significant decrement only in kindled+HFS group (Fig. 2C).

Interestingly, there was no significant difference between kindled+LFS and kindled+HFS groups in different seizure parameters, and calculating the Cohen's f value showed a minor effect size ($f<0.54$) between the two groups (Fig. 2B, C).

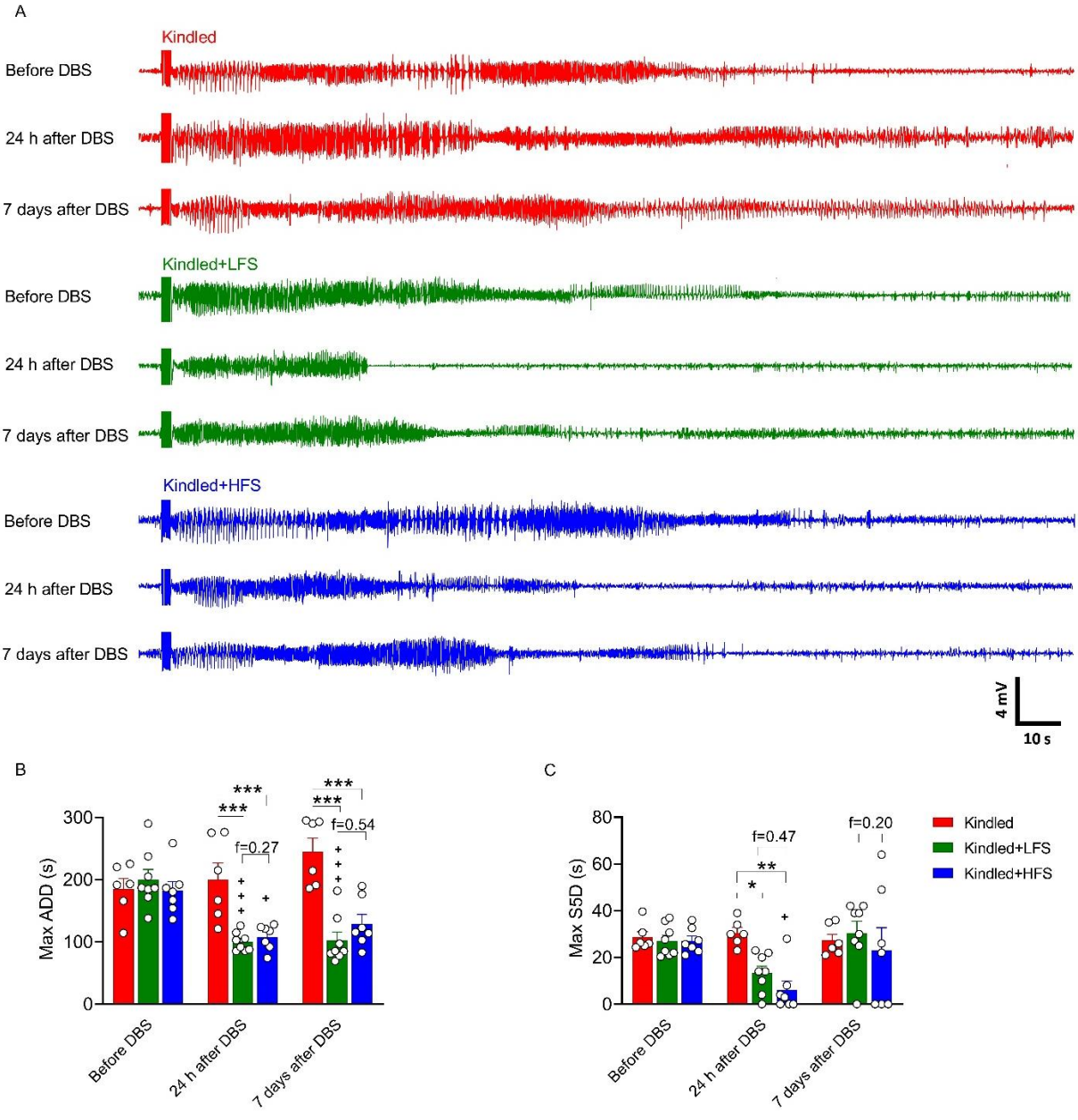


Fig. 2. Applying LFS and HFS in olfactory bulb had anticonvulsant effects. A: Sample records of ADs in different experimental groups at three times: before applying DBS (LFS or HFS) (top), 24 h after applying DBS (middle) and 7 days after applying DBS (bottom). B and C: Applying both LFS and HFS reduced the Max ADD (maximum afterdischarge duration), and maximum stage 5 seizure duration (Max S5D). There was no significant difference between the effects of LFS and HFS. The data are presented as mean ± SEM. * P<0.05, ** P<0.01 and *** P<0.001.

3.2. Applying DBS in olfactory bulb restored the seizure-induced impairment in learning and memory

The changes in spontaneous alternation percentage were compared in different control and kindled groups at three time-courses: a) before kindling, b) after kindling and before applying DBS and c) after DBS. A three-way ANOVA showed a significant difference among experimental groups ($F_{(10,72)}$=5.56, P<0.001). Post-hoc Tukey test revealed that there was no significant difference in spontaneous alternation percentage in Y-maze test among different experimental groups before starting the kindling procedure in kindled groups or at equivalent time in control groups. Kindling procedure decreased the working memory in kindled, kindled+LFS and kindled+HFS animals significantly (P<0.001). Applying both LFS and HFS restored the impairment in working memory and returned the spontaneous alternation percentage to control values in kindled+LFS and kindled+HFS groups (Fig. 3A). To compare the activity of animals, the number of arm entries in Y-maze test and open field test were compared among all experimental groups. No significant difference was observed in the number of arm entries (Fig. 3B).

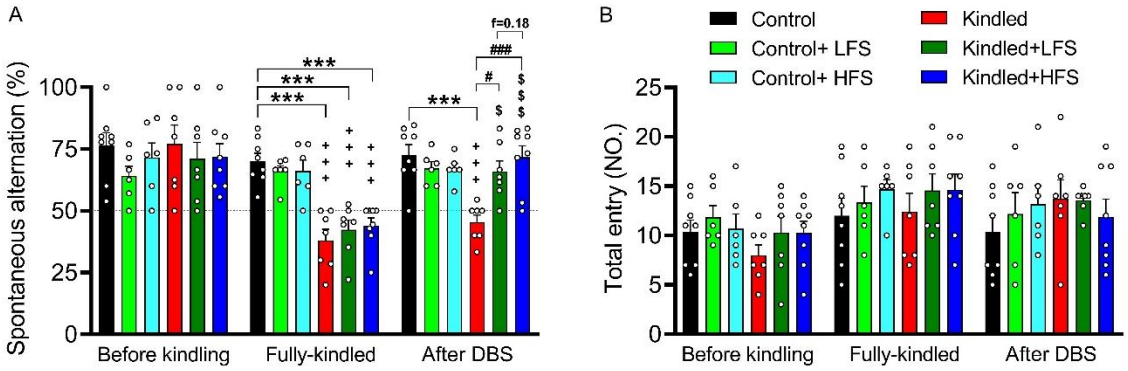


Fig. 3. Applying LFS and HFS in olfactory bulb restored the seizure-induced impairment in working memory in Y-maze test. A: The percentage of spontaneous alternation in Y-maze test in different experimental groups. At “Before kindling”, there was no significant difference between groups. The Spontaneous alternation reduced significantly in kindled animals at “Full kindled” time. Applying LFS and HFS restored the working memory at “After DBS” time. B: Total entries into the Y-maze arms was similar in all experimental groups. The data are presented as mean ± SEM. ***P<0.001, #P<0.05, ###P<0.001, +++ P<0.001 compared to respected group at “Before kindling”; $ P<0.05 and $$$ P<0.001 compared to respected group at “Full kindled”. There was no significant difference between the effects of LFS and HFS.

To evaluate the effect of olfactory bulb DBS on spatial learning and memory in kindled animals, Barnes maze test was used. Similar to previous reports, there was a significant impairment in learning and memory in kindled compared to control group. A three-way ANOVA followed by Tukey test showed a significant increase in the primary error (P<0.05) and total errors (P<0.01) in kindled compared to control group on training day 1 (Fig. 4A, B), ($F_{(15,108)}$=1.22, P=0.27). The direct strategy %, GS exploration (P<0.01) and GS/NGS ratio (P<0.05) were also reduced in kindled animals compared to control group (Fig. 4E-J), ($F_{(5,36)}$=4.51, P<0.002). Applying LFS and HFS in kindled animals (kindled+LFS and kindled+HFS groups) restored the impairment in above mentioned parameters (Fig. 4A-K).

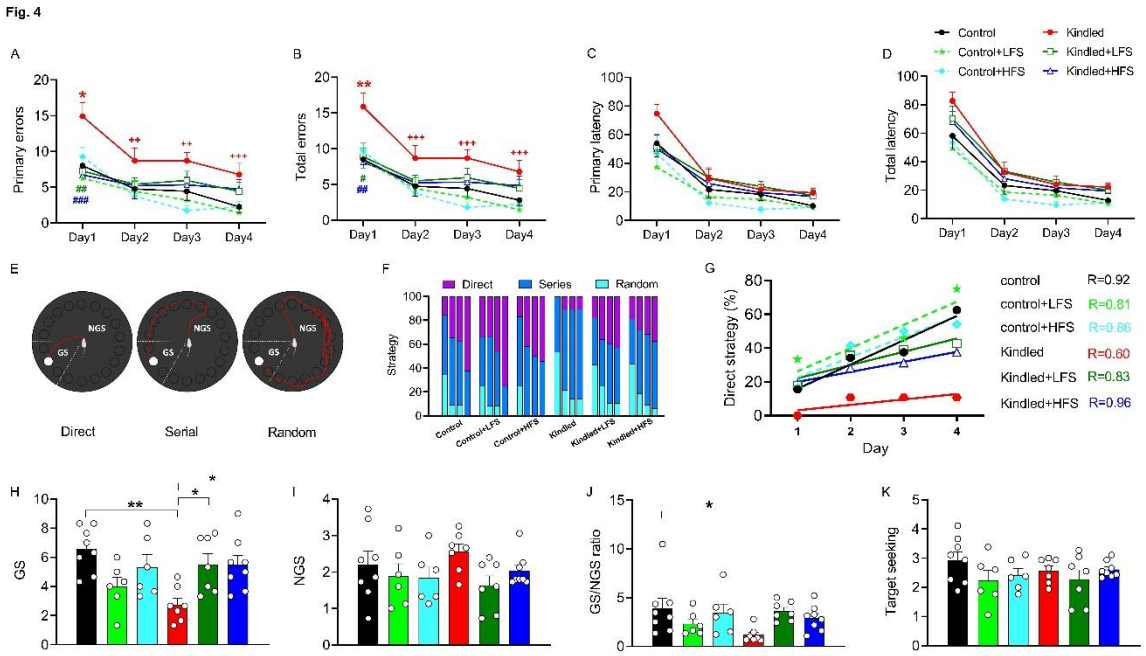


Fig. 4. Applying LFS and HFS in olfactory bulb restored the seizure-induced impairment in special learning and memory in Barnes maze test. A-D: Applying both LFS and HFS restored the kindling-induced impairment in spatial learning and memory and reduced the number of primary errors and total errors (but not latencies) in kindled+LFS and kindled+HFS groups compared to kindled animals.E: Three types of searching strategies during trail days by experimental groups. F: Changes in percentage of three types of searching strategies during trail days. G: Correlation graph representing the relation between percentage of direct strategy and trail days. There was small correlation in direct strategy in kindled animals compared to control. Application of LFS or HFS improved this parameter in kindled animals. H-K: Application of LFS and HFS improved the goal sector (GS) frequency in the kindled+LFS and kindled+HFS groups compared to kindled group. I: No changes were observed in non-goal sector (NGS) frequency among experimental groups. In Addition, a significant decrease of GS/NGS ratio was seen in kindled animals and was removed in kindled+LFS and kindled+HFS groups. Data are presented as mean ± SEM. In A and B, * P<0.05, ** P<0.01, compared to control group; # P<0.05, ## P<0.01 and ### P<0.001, compared to kindled group; ++ P<0.01 and +++ P<0.001, compared to first day of respected group. There was no significant difference between the effects of LFS and HFS.

3.3 Applying DBS in olfactory bulb decreased the excitation/inhibition ratio in kindled animals

To determine the effect of LFS and HFS on excitatory and inhibitory post-synaptic currents, whole-cell patch clamp recording was performed in ventral hippocampal slices at 24 h after DBS. Data were obtained from 9-12 slices from

three subjects in all experimental groups. EPSCs were recorded when the membrane voltage was clamped in IPSC's reverse potential. A one-way ANOVA showed a significant change in eEPSC amplitude ($F_{(3,42)}$=7.25, P=0.0005), area under the curve ($F_{(3,42)}$=4.77, P=0.0059), and rise slope ($F_{(3,42)}$=3.75, P=0.0178). Post-hoc Tukey test showed a significant increase in eEPSC amplitude (P<0.001), area under the curve (P<0.01), and rising slope (P<0.05) in pyramidal cells of kindled animals compared to the control group. Applying both LFS and HFS in the kindled+LFS and kindled+HFS groups restored these changes. There was no significant difference in eEPSC parameters between kindled+LFS or kindled+HFS compared to the control group (Fig. 5B, C, D).

After EPSC recording, the IPSC was also recorded from the same CA1 neuron when the membrane voltage was clamped in EPSC's reverse potential. One-way ANOVA test showed a significant change in eIPSC amplitude ($F_{(3,42)}$=4.72, P=0.0063), area under the curve ($F_{(3,42)}$=4.21, P=0.0108), and rising slope ($F_{(3,42)}$=3.16, P=0.0338) among all experimental groups. Although there was no significant difference in eIPSCs' parameters among kindled and control groups, applying HFS increased the IPSC's parameters in kindled+HFS groups. Tukey's post hoc test showed a significant increase in eIPSC amplitude (P<0.01), area under the curve (P<0.01), and rising slope (P<0.05) only in kindled+HFS compared to the kindled group. Calculating the Cohen's f value showed a large effect size between both kindled+LFS or kindled+HFS and kindled group (Fig. 5E, F, G).

Then, the excitation/inhibition (E/I) ratio was calculated as EPSC/IPCS parameters. One-way ANOVA and Tukey post-hoc test showed that kindling significantly increased the E/I ratio (P<0.01 for amplitude and area under the curve) compared to the control group. Applying both LFS and HFS restored the E/I ratio,

and a significant decrease was observed in the amplitude-E/I ratio, area under curve-E/I ratio, and rise slope-E/I ratio in both kindled+LFS and kindled+HFS groups. Tukey post-hoc test did not show any significant difference between kindled+LFS and/or kindled+HFS and the control groups. On the other hand, a significant difference was observed among kindled group and both kindled+LFS and kindled+HFS groups (Fig. 5H, I, J). Applying either LFS or HFS in control animals had no significant effect on measured parameters.

Although the olfactory bulb is mainly connected to the ventral hippocampus, considering the important role of the dorsal hippocampus in seizure propagation and cognitive functions, we measured the same parameters by whole cell recording from CA1 neurons in dorsal hippocampal slices. The obtained results were very similar to those get from ventral CA1 neurons (Fig. 5L-T). One-way ANOVA showed a significant change in eEPSC amplitude ($F_{(3,38)}$=3.77, P=0.0182) and rising slope ($F_{(3,38)}$=4.24, P=0.0113). Post-hoc Tukey test showed a significant increase in eEPSC amplitude (P<0.05) and rising slope (P<0.05) in pyramidal cells of kindled animals compared to the control group. Application of both LFS and HFS in the kindled+LFS and kindled+HFS groups restored these changes, and there was no significant difference in eEPSC parameters between kindled+LFS or kindled+HFS compared to the control group (Fig. 5L, M, N).

Analyzing the IPSCs parameters by one-way ANOVA showed a significant change in the eIPSC area under the curve ($F_{(3,38)}$=3.08, P=0.039). Tukey post-hoc test revealed a significant decrease in IPSC area under curve in the kindled compared to the control group (P<0.05). However, application of both LFS and HFS restored this parameter and there was a significant increase in eIPSC area under the curve in kindled+LFS (P<0.01) and kindled+HFS (P<0.05) compared to the kindled

group (Fig. 5P). Although no significant difference was observed in IPSC amplitude and rise slope between different groups, calculating the Cohen's f values showed a medium effect size between kindled and both kindled+LFS and kindled+HFS groups (Fig. 5O, Q).

Similar to the ventral hippocampal area, one-way ANOVA and Tukey's test showed a significant increase in E/I ratio in kindled compared to control group. Applying both LFS and HFS restored the changes in E/I ratio (Fig. 5R, S, T). Interestingly, in the dorsal CA1 neurons, there was no significant difference between the restoring effects of LFS and HFS on different parameters in kindled+LFS and kindled+HFS groups.

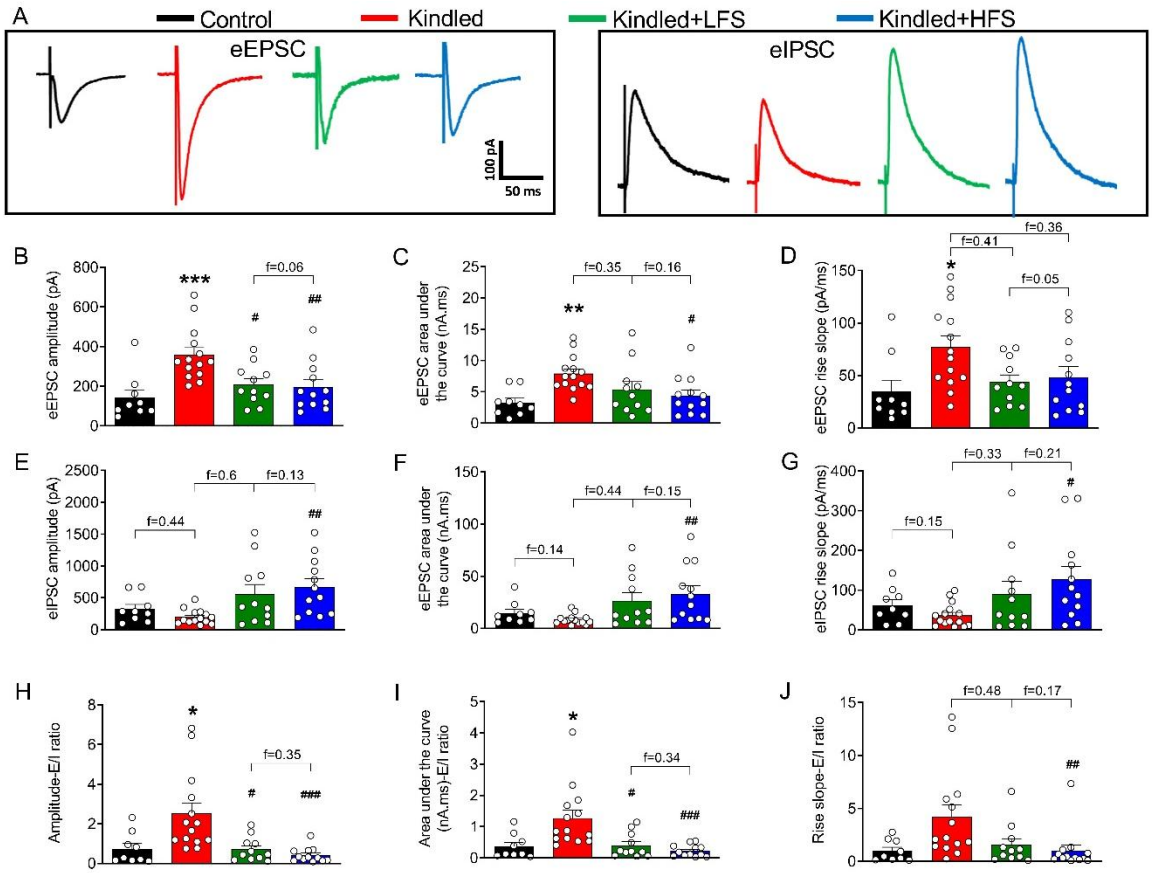


Fig. 5. Applying LFS and HFS in olfactory bulb decreased the excitation/inhibition ratio in kindled animals. A: Sample records of eEPSC (holding potential=-52 mV) and eIPSC (holding potential=+10 mV) in ventral pyramidal CA1 neurons when the stimulating electrodes were placed in the Shaffer collaterals in different experimental groups. B-D:

Applying LFS and HFS decreased the eEPSC amplitude, area under the curve and rise slope in kindled animals. E-G: LFS and HFS administration increased the eIPSC amplitude, area under the curve and rise slope in kindled animals. H-J: Applying both LFS and HFS decreased the excitation/inhibition (E/I) ratio in kindled animals. K-T: The same parameters in dorsal pyramidal CA1 neurons when the stimulating electrodes were placed in the Shaffer collaterals in different experimental groups. Results were very similar to ventral hippocampus. The data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, #P<0.05, ##P<0.01, ###P<0.001. There was no significant difference between the effects of LFS and HFS.

3.4. Applying DBS in olfactory bulb changed the synaptic transmission through pre- and post-synaptic mechanisms

To find the involvement of presynaptic- or post-synaptic mechanisms in LFS and HFS actions, paired-pulse ratio (PPR) was calculated for eEPSC and eIPSC amplitude. In the ventral and dorsal hippocampal slices, a one-way ANOVA test did not show a significant difference in eEPSC PPR ($F_{(3,42)}$=2.31, P=0.089) and eIPSC PPR ($F_{(3,42)}$=0.24, P=0.866) among experimental groups. Calculating Cohen's f also did not show a large effect size among different experimental groups (Fig. 6A2-D2).

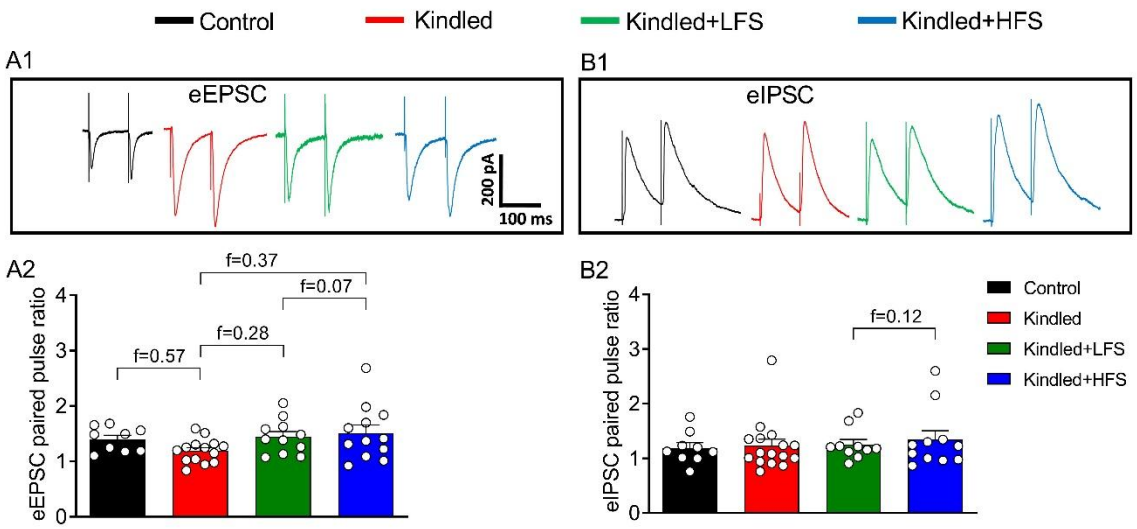


Fig. 6. Paired-pulse ratio did not change in ventral and dorsal hippocampus of kindled animals or following LFS and HFS application in olfactory bulb of kindled animals. A1 and B1: Sample traces of paired eEPSCs (holding potential=-52 mV) and paired eIPSCs (holding potential=+10 mV) recorded from ventral pyramidal CA1 neurons in different experimental

groups in response to paired pulsed at 100 ms inter-pulse interval. A2: Applying LFS and HFS did not change the eEPSC paired-pulse ratio. B2: Applying LFS and HFS did not change the eIPSC paired-pulse ratio. C-D: Similar results obtained from dorsal hippocampus. Data are presented as mean ± SEM.

Then, the coefficient of variance (CV), failure rate, efficacy and potency were measured for eEPSCs and eIPSCs. One-way ANOVA test showed no significant difference in CV among experimental groups in ventral and dorsal hippocampal slices (Fig. 7B, G, L, Q). Calculating the Cohen's f values also did not show a large effect size. The lack of change in PPR and CV indicates the involvement of a postsynaptic mechanism in LFS and HFS action.

A significant difference was observed in failure rate ($F_{(2,19)}$=7.35, P=0.0043), efficacy ($F_{(3,24)}$=12.32, P<0.0001), and potency ($F_{(3,24)}$=5.76, P=0.0041) of eEPSCs among experimental groups in ventral hippocampus (Fig. 7C, D, E). The failure rate was decreased (P<0.01), and the efficacy (P<0.01) and potency (P<0.05) were increased in a kindled group compared to the control group significantly. Applying both LFS and HFS removed these differences so that there was no significant difference in failure rate, efficacy, and potency between kindled+LFS and kindled+HFS and control groups. No significant difference was observed in these parameters when eIPSCs were recorded in ventral hippocampus (Fig. 7H-J). These results may show the role of presynaptic mechanisms in DBS action (see Discussion).

In the dorsal hippocampal slices, one-way ANOVA showed a significant difference in eEPSC failure rate percentage among experimental groups ($F_{(2,22)}$=4.91; P=0.0172). Similar to ventral hippocampal data, applying both LFS and HFS restored the kindling-induced decrease of eEPSP failure rate (Fig. 7M). There was no significant difference in eIPSC failure rate, efficacy and potency between

kindled and control groups, however, applying both LFS and HFS increased the eIPSC efficacy and potency in kindled+LFS ($P<0.05$) and kindled+HFS groups ($P<0.01$ for efficacy and $P<0.05$ for potency) compared to kindled group. (Fig. 7R, S, T).

No significant difference was observed between the kindled+LFS and kindled+HFS groups. In addition, calculating Cohen's f also demonstrated a small effect size (Fig. 7). Applying either LFS or HFS in control animals had no significant effect on measured parameters.

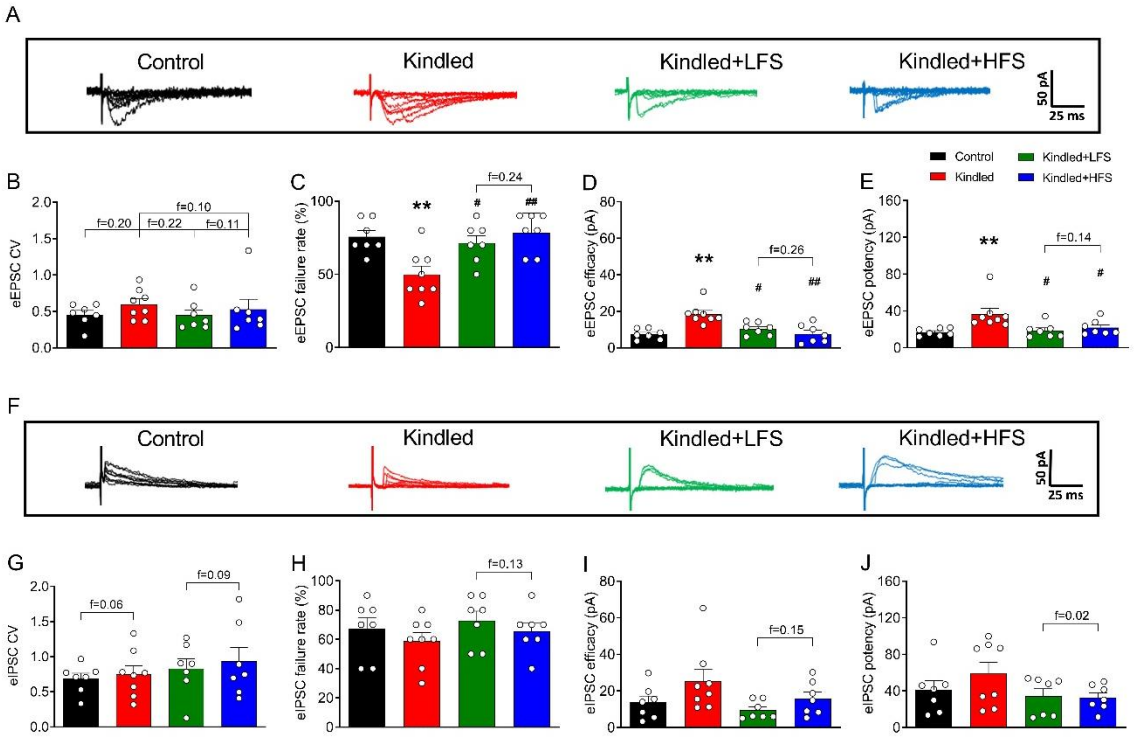


Fig. 7. Applying LFS and HFS in olfactory bulb did not change coefficient of variation (CV), but restored the changes in failure rate, efficacy and potency of eEPSCs and eIPSCs in ventral and dorsal hippocampus in kindled animals. A: Sample traces of 10 superimposed eEPSCs (holding potential=-52 mV) recorded from ventral pyramidal CA1 neurons in response to threshold intensity in different experimental groups. B: Calculating the CV showed no significant changes among experimental groups. C-E: Both LFS and HFS application restored the kindling -induced changes in failure rate, efficacy and potency of eEPSCs. F: Sample traces of 10 superimposed eIPSCs (holding potential=+10 mV) recorded from ventral pyramidal CA1 neurons in in response to threshold intensity in different experimental groups. G-J: There was not any significant difference in CV, failure

rate, efficacy and potency of eIPSCs among experimental groups. K-O: The same parameters of eEPSCs recorded from dorsal pyramidal CA1 neurons in response to threshold intensity in different experimental groups. Applying LFS and HFS restored only the kindling-induced changes in failure rate. No change was observed in other parameters. P-T: The same parameters of eIPSCs recorded from dorsal pyramidal CA1 neurons in response to threshold intensity in different experimental groups. There was not any significant difference between kindled and control groups, however, application of both LFS and HFS in kindled animals significantly increased the efficacy and potency of eIPSCs. Data are presented as mean ± SEM. *P<0.05 and **P<0.01 compared to control group. #P<0.05 and ##P<0.01 compared to kindled group. There was no significant difference between the effects of LFS and HFS.

Spontaneous EPSCs (sEPSCs) and IPSCs (sIPSCs) were also recorded from ventral and dorsal hippocampus. It was not possible to analyze sEPSCs because the number of these events was very low. Therefore, only the effect of DBS application on sIPSCs was analyzed in CA1 pyramidal cells of the ventral and dorsal hippocampus in kindled animals. One-way ANOVA and post-hoc Tukey test showed a significant increase in the inter-event interval of sIPSCs in pyramidal cells in kindled rats compared to the control group (P<0.05) in ventral (but not in dorsal) CA1 neurons (Fig. 8). Application of LFS and HFS in kindled animals prevented the increase in this parameter. There was a significant difference between kindled and both kindled+LFS (P<0.01) and kindled+HFS groups (P<0.001), ($F_{(3,45)}$=6.88; P=0.0006). No significant difference was observed in sIPSCs amplitude in ventral or dorsal hippocampal CA1 neurons (Fig 8.). Changes in inter-event intervals, but not in amplitude of sIPSCs indicates the involvement of presynaptic mechanisms.

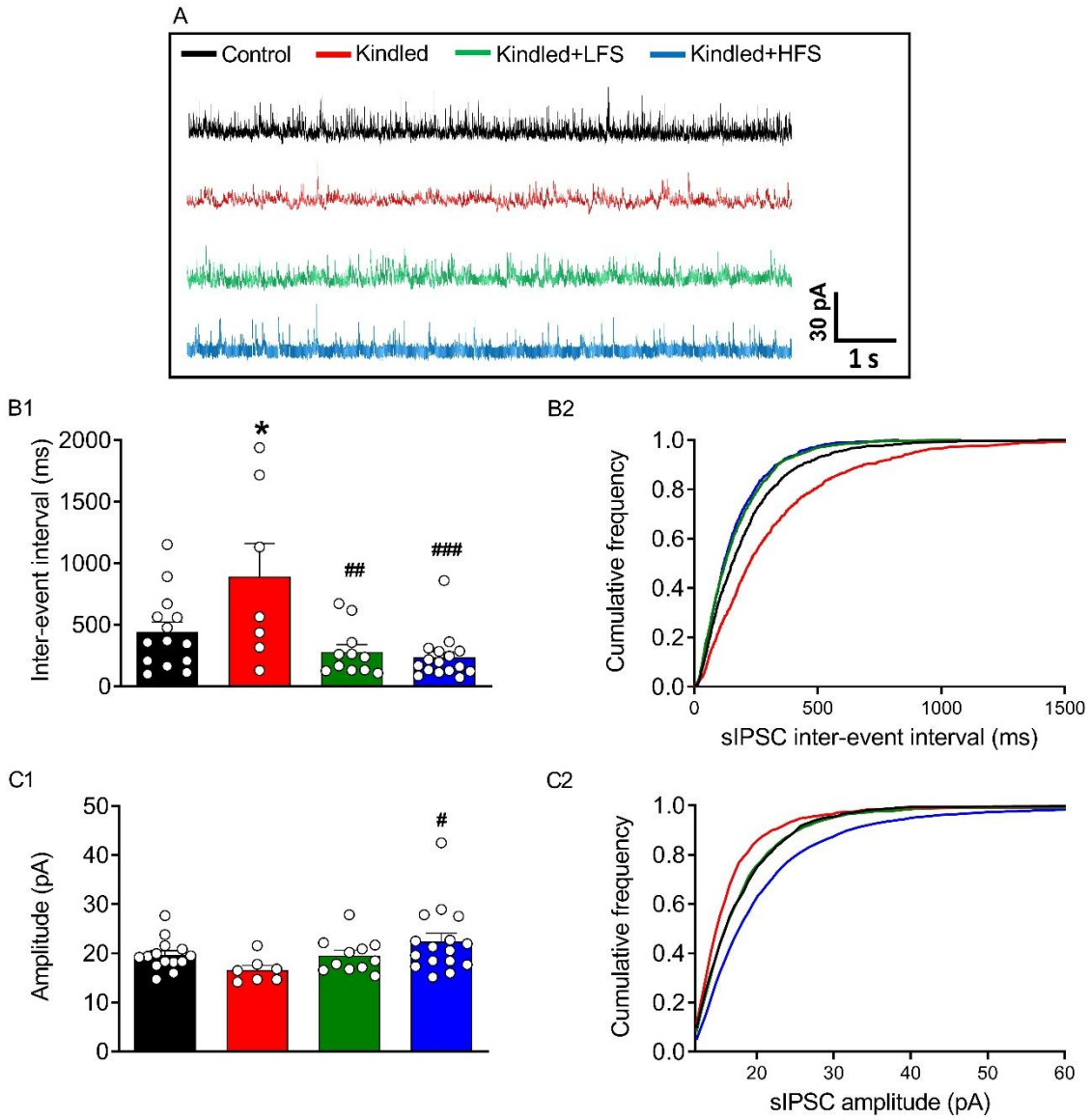


Fig. 8. Applying LFS and HFS in olfactory bulb restored the changes in spontaneous IPSCs (sIPSCs) amplitude in ventral hippocampus of kindled animals. A: Sample traces of sIPSCs recorded from ventral (left) and dorsal (right) pyramidal CA1 neurons. B1 and B2: sIPSCs inter-event intervals increased in kindled animals significantly, but returned to control level following application of both LFS and HFS.
C1 and C2: Kindling did not change the sIPSCs amplitude. Applying HFS, but not LFS significantly increased this parameter. D and E: Neither kindling, nor applying LFS and HFS changed the sIPSC interevent-intervals and amplitude in different experimental groups. Data are presented as mean ± SEM * P<0.05 compared to control group; # P<0.05, ## P<0.01 compared to kindled group.

3.5. Applying DBS in olfactory bulb changed the NR2A, GluR2, and γ2 gene expression

In the next step, gene expression of the NR2A subunit of the NMDA receptor, the GluR2 subunit of the AMPA receptor, and the γ2 subunit of the $GABA_A$ receptor were measured. A one-way ANOVA and Tukey post hoc test revealed that NR2A and GluR2 genes expression were significantly greater in the kindled compared to control group (P<0.01). Applying LFS and HFS in kindled animals decreased the expression of these genes in kindled+LFS and kindled+HFS groups compared to kindled group (for NR2A: $F_{(3,12)}$=8.16; P=0.003; for GluR2: $F_{(3,12)}$=15.14; P=0.0002) (Fig. 9A, B). The expression of γ2 gene decreased in the kindled compared to control group (P<0.05). Applying both LFS and HFS somehow restored the change in γ2 expression and there was no significant difference between the kindled+LFS and kindled+HFS groups and the control groups (Fig. 9C) ($F_{(3,12)}$=4.83; P=0.0198). As Fig. 9 shows, calculating Cohen's f also showed a small size effect when we compared kindled+LFS and kindled+HFS groups. These data showed a similar effect of HFS and LFS application on gene expression (Cohen's f<0.2).

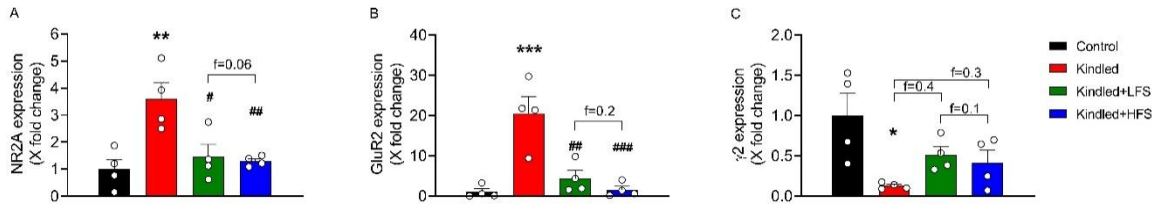


Fig. 9. Applying LFS and HFS in olfactory bulb restored the seizure-induced changes in the gene expression of NR2A subunit of NMDA receptor (A), GluR2 subunit of AMPA receptor (B), and γ2 subunit of $GABAA_A$ receptor (C). Data are presented as mean ± SEM. * P<0.05 and ** P<0.01 compared to control group; # P<0.05 and ## P<0.01 compared to kindled group. There was no significant difference between the effects of LFS and HFS.

3.6. Applying DBS in OB prevents hippocampal neuronal death

We next investigated whether LFS and HFS confer their anticonvulsant effects by preventing hippocampal cell death. To do so, we performed Nissl staining to highlight any neuropathological changes in the CA1 and CA3 regions of the dorsal and ventral hippocampus in rats at one week following the last kindling stimulation (Fig. 10). In the ventral hippocampus CA3 area, a one-way ANOVA showed significant difference among experimental groups ($F_{(3,8)}$=22.91; P=0.0003). In the kindled group, the cell death percentage in the CA3 regions was significantly higher than control group (P<0.001). Applying LFS significantly decreased the amount of cell death in CA3 region compared to kindled group (P<0.001). However, in the kindled+HFS group, the reduction of cell death was not significant compared to the kindled group (Fig. 10A and B).

One-way ANOVA test also showed a significant difference in the percentage of cell death in dorsal hippocampal CA3 area among experimental groups ($F_{(3,8)}$=6.62; P=0.0146). Tukey test showed that the amount of cell death was significantly increased in the CA3 regions in the kindled compared to the control group (P<0.05). Applying LFS, but not HFS, significantly decreased the number of cell death in the dorsal CA3 region when applied in full kindled animals compared to kindled group (P<0.05) (Fig. 10E).

No significant difference was observed in the number of cell death in CA1 area in dorsal or ventral hippocampus among experimental groups.

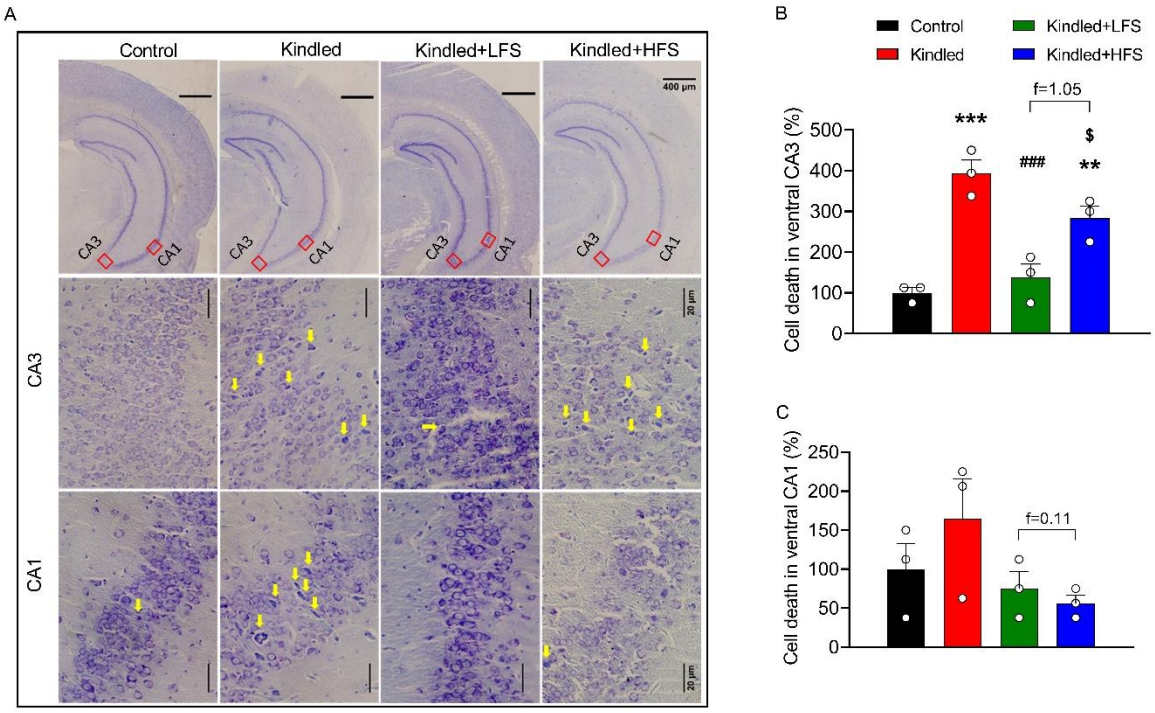


Fig. 10. Applying LFS and HFS in olfactory bulb had protective effect against cell death in CA1 and CA3 regions of ventral and dorsal hippocampus in kindled animals. A: Representative Nissl staining images of the CA3, and CA1 areas of the ventral hippocampus in different groups. B: The cell death percentage was increased in CA3 area of kindled animals. Applying LFS, but not HFS, significantly reduced the cell death. There was a significant difference between kindled+LFS and kindled+HFS groups. C: No significant changes was observed in cell death percentage in CA1 area among experimental groups. D-F: Similar results obtained in dorsal hippocampus. Of course, there was no significant difference among kindled=LFS and kindled+HFS groups in dorsal CA3 area. Data are resented as mean ± SEM. * P<0.05, ** P< 0.001 and *** P< 0.0001 compared to control group; # P<0.05 and ### P<0.001 compared to kindled group; $ P<0.05 compared to kindled+LFS group.

3.6. Applying DBS in olfactory bulb restored the kindling-induced changes in brain oscillations

The power of delta (<4 Hz), theta (4–12 Hz), beta (12–30 Hz), low gamma (30-45 Hz) and high gamma (80–120 Hz) waves (Hentschke et al., 2007) were evaluated in the hippocampus at three time points: before kindling (as baseline), before DBS in full kindled animals, and 24 h after DBS. Data obtained before DBS and 24 h after DBS were normalized to the baseline (before kindling). As Fig. 11

shows, almost the power of all mentioned waves was significantly higher in the hippocampus of animals in kindled (P<0.01 for delta and theta, P<0.001 for beta, P<0.01 for low gamma and P<0.05 for high gamma waves), kindled+LFS (P<0.01 for delta, P<0.05 for theta and high gamma waves), and kindled+HFS groups (P<0.05 for theta, P<0.01 for beta and P<0.05 for high gamma waves) compared to the control group. After DBS, the power of these oscillations in kindled+LFS and kindled+HFS groups was significantly decreased compared to kindled group (for delta: $F_{(3,22)}$=3.27; P=0.04; for theta: $F_{(3,22)}$=2.92; P=0.056; for beta: $F_{(3,22)}$=1.81; P=0.174; for low gamma: $F_{(3,22)}$=1.32; P=0.292; for high gamma: $F_{(3,22)}$=2.4; P=0.095), (Fig. 11). There was no difference between kindled+LFS and kindled+HFS groups (Cohen's f<0.17). Applying LFS and HFS in control animals did not affect the power of brain oscillations.

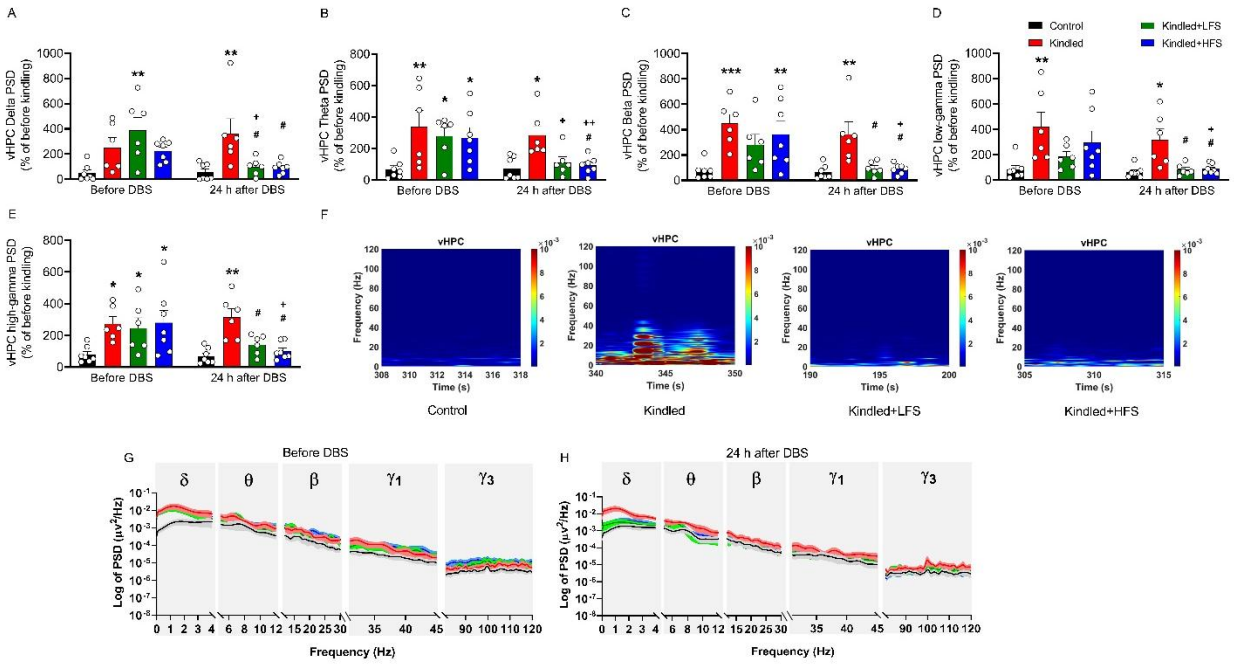


Fig. 11. Applying LFS and HFS in olfactory bulb restored the seizure-induced changes in the power spectral density (PSD) of different brain rhythms in ventral hippocampus. A-E: PSDs were calculated before and after LFS and HFS application as % of before kindling situation. Kindling significantly increased PSD of delta, theta, beta and gamma waves compared to

control. Applying both LFS and HFS restored the changes in brain waves in kindled animals at 24 h after application. F: Color maps showing an obvious increase (red and yellow colors) in the PSD brain rhythms during 10 s of LFP recording when the animal was at rest and awake in different experimental groups. The increased PSD did not observe in kindled+LFS and kindled+HFS groups. G and H: PSD (normalized as the percentage of the baseline at before kindling) of all brain rhythms was higher in kindled animals compared to control. LFS and HFS restored the PSD of brain rhythms at 24 h after their application. Data were presented as mean ± SEM. * P<0.05, ** P<0.01 and *** P<0.001 compared to control group; # P<0.05, ## P<0.01, ### P<0.001 compared to kindled group. There was no significant difference between the effects of LFS and HFS.

4. Discussion

Results of the present study showed that electrical stimulation of the olfactory bulb at the frequencies of 1 and 130 Hz reduced seizure activity in the kindled rats. Therefore, the olfactory bulb may be considered a potential target for seizure control in temporal lobe epilepsy.

DBS frequency has a significant impact on its anticonvulsant effects (Mina et al., 2013). An important result of the present study was that the anticonvulsant effects of LFS were similar to those of HFS. The mechanism of this frequency-dependent effect in brain stimulation is still not fully understood. Although the anticonvulsant effect of HFS has been widely studied in clinical and preclinical research (Al-Otaibi et al., 2011; Fisher & Velasco, 2014; Han et al., 2014), few studies have compared the anticonvulsant effects of LFS and HFS in similar situation. Some researchers showed that HFS is more effective than LFS. Wyckhuys et al. showed that 130 Hz stimulation is more effective than 5 Hz stimulation in reducing excitability in epileptic mice (Wyckhuys et al., 2010). The beneficial effect of HFS (130 Hz), but not LFS (5 Hz), was described by Boëx et al. in non-lesional temporal lobe epilepsy (Boëx et al., 2007). In contrast to above reports, Albensi et al. demonstrated that DBS at both 1 Hz and 100 Hz reduced seizure-like activity in

living hippocampal slices (Albensi et al., 2004). In another study, Lim et al. showed that applying both LFS (5 Hz) and HFS (145 Hz) resulted in 45% reduction in seizure frequency across all patients with temporal lobe epilepsy (Lim et al., 2016). However, there are limited clinical studies that supports the efficacy of LFS in epilepsy patients (Chkhenkeli et al., 2004; Koubeissi et al., 2013; Schrader et al., 2006; Yamamoto et al., 2002). Therefore, it seems that LFS maybe potentially considered as an effective anticonvulsant pattern of DBS.

We observed that DBS restored the seizure-induced changes in brain waves' power. The increase in delta power is a good indicator for identifying seizure foci (Gambardella et al., 1995; Geyer et al., 1999). In line with our results, Chen et al. showed that applying LFS in the globus pallidus reduced delta power and had anticonvulsant effects via activating GABAergic neurons (Cheng et al., 2015). Our results showed that applying both LFS and HFS increased GABAergic currents in pyramidal neurons and enhanced the expression of the γ2 subunit of $GABA_A$ receptors in kindled animals. Therefore, it may be suggested that applying LFS and HFS may decrease delta power through increasing the GABAergic activation.

Our study showed that, in addition to the power of low-frequency waves, gamma wave power also increased in the hippocampus of kindled, but restored to normal level following application of both LFS and HFS. Previous findings also indicated that olfactory pathway stimulation through nasal air puffs had significant effect on the power of gamma frequency bands (Riazi et al., 2024; Salimi et al., 2022). It has been shown that the increase in gamma power may be due to the synchronization of neuronal activity and is related to the simultaneous activation of inhibitory interneurons and excitatory glutamatergic neurons in the pyramidal cell layer (Bartos et al., 2007). Our whole cell recording's data showed the recovering

effect of LFS and HFS on both excitatory and inhibitory synaptic currents, which may lead to restoring of gamma power. Considering the role of brain waves in learning and memory (Bartos et al., 2007; Harmony, 2013), it may be suggested that the observed improving effects of both LFS and HFS in working and special memory are somehow due to restoring effects of DBS on brain waves' power.

The preventing effect of DBS on cell death in the hippocampal CA3 (and to some extent in the CA1) region of both the dorsal and ventral hippocampus may also be involved in the restoring effects of LFS on brain waves and learning and memory (Takei et al., 2016).

All therapeutic actions of HFS and LFS were accompanied with their reducing effect on E/I ratio, that was primarily through the suppression of EPSCs in kindled animals. Therefore, our data confirmed the "synaptic modulation" hypothesis of DBS mechanism. Our previous research has also shown that neuromodulators such as dopamine (Rezaei et al., 2023; Sadeghian et al., 2020) and adenosine (Shojaee et al., 2020) are involved in the DBS action. In line with previous reports (Ahmadi et al., 2024; Kohr et al., 1993), kindling lead to an increase in glutamatergic currents (which were mainly AMPA currents because EPSCs were recorded in negative voltages) and in gene expression of the NR2A subunit of NMDA receptors and the GluR2 subunit of AMPA receptors. These changes restored after applying DBS in kindled rats.

Of course, some changes were also observed in IPSCs including the reduction of IPSC's area under the curve in dorsal hippocampus and the decrease in the frequency of sIPSCs in the kindled group compared to the control group. Meanwhile, in accord with the previous studies (Bonansco & Fuenzalida, 2016; Titulaer et al., 1995), a significant reduction in the expression of γ2 subunit of the

$GABA_A$ receptor was also observed in kindled animals. Both LFS and HFS recovered the changes in IPSCs in kindled animals.

Both presynaptic and postsynaptic mechanisms were involved in the synaptic modulation effects of LFS and HFS in kindled rats. The lack of changes in PPR, changes in the expression of glutamate and GABA receptor subunits, the lack of changes in the coefficient of variation (CV) for excitatory and inhibitory currents (Clements, 1990; van Huijstee & Kessels, 2020), support the role of postsynaptic mechanisms in observed synaptic changes. In addition, changes in the failure rate of EPSCs (that were restored by LFS and HFS) may indicate modifications in silent synapses and represent a postsynaptic phenomenon (Clements 1990).

However, some evidences of the present study show the involvement of presynaptic mechanism in DBS action. First, the decrease in the EPSC's potency and the increase in IPSC's potency. This is likely due to pre-synaptic changes in neurotransmitter release or changes in the number of excitatory and inhibitory inputs (Oertner et al., 2002). This finding is consistent with a previous study that showed that with an increase in the number of mitral cell inputs to granule cells in the hippocampus, both the probability of neurotransmitter release and potency also increased (Buckmaster et al., 1996; Hashimotodani et al., 2017). Second, applying HFS and LFS increased the cumulative frequency of sIPSC's inter-event intervals (but not amplitude). These results indicated the involvement of presynaptic mechanisms in the effects of LFS and HFS.

Conclusion

Our findings demonstrated that the olfactory bulb is a potential target for DBS in the epilepsy treatment. Interestingly, applying both LFS and HFS in the olfactory bulb had anticonvulsant effect and restored the increased E/I ratio in CA1 pyramidal

neurons. Therefore, it can be concluded that application of LFS may be a good substitute for HFS in the treatment of epilepsy. In addition, the anticonvulsant effects following application of LFS and HFS in olfactory bulb, leads to the suggestion that stimulation of the olfactory epithelium (as a non-invasive method for treating seizures in drug-resistant epileptic patients) may also yield appropriate therapeutic effect in epilepsy patients. Given that both LFS and HFS in the olfactory bulb induced significant anticonvulsant effects, and considering that HFS may be uncomfortable to be applied to the olfactory epithelium, LFS potentially be introduced as a promising candidate for this therapeutic approach in the future.

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- **Author contributions**

  Parisa Zarei carried out the experiment and analyzed the data and wrote the first draft of the manuscript; *Sareh Rostami* and *Fatemeh Bakhtiarzadeh* and *Mahmoud Rezaei* participated in the methodology, *Farideh Faramarzi* and *Mehrdad Behmanesh* were involved in PCR experiments and data analysis, *Motahareh Heibatollahi* and *Razieh Rohani* were involved in histological analysis, *Victoria Barkley* participated in reviewing and editing the manuscript, *Mohammad Reza Raoufy* and *Mohammad Javan* and *Amir Shojaei* and *Yaghoub Fathollahi* participated in the design of the study and in the results interpretation, *Javad Mirnajafi-Zadeh* contributed in the design of the experiments, data analysis, writing the manuscript and preparing the funds. All authors reviewed the manuscript.

- **Data availability**

  The datasets used and analysed during the current study available from the corresponding author on reasonable request.

- **Additional Information**

  The authors declare no competing financial interests.

Figure legends

Fig. 1. Comparing the anticonvulsant action of applying LFS and HFS at low- and high-intensities in olfactory bulb. A: Comparing the effects of LFS and HFS on maximum ADD (afterdischarge duration) at low (100 μA) and high (200 μA) intensities. B: Comparing the effects of LFS and HFS on maximum S5D (stage 5 seizure duration) at low (100 μA) and high (200 μA) intensities. Both LFS and HFS were more effective at high-intensity. Int: Intensity. Data are presented as mean ± SEM. * $P<0.05$, *** $P<0.001$.

Fig. 2. Applying LFS and HFS in olfactory bulb had anticonvulsant effects. A: Sample records of ADs in different experimental groups at three times: before applying DBS (LFS or HFS) (top), 24 h after applying DBS (middle) and 7 days after applying DBS (bottom). B and C: Applying both LFS and HFS reduced the Max ADD (maximum afterdischarge duration), and maximum stage 5 seizure duration (Max S5D). There was no significant difference between the effects of LFS and HFS. The data are presented as mean ± SEM. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

Fig. 3. Applying LFS and HFS in olfactory bulb restored the seizure-induced impairment in working memory in Y-maze test. A: The percentage of spontaneous alternation in Y-maze test in different experimental groups. At “Before kindling”, there was no significant difference between groups. The Spontaneous alternation reduced significantly in kindled animals at “Full kindled” time. Applying LFS and HFS restored the working memory at “After DBS” time. B: Total entries into the Y-maze arms was similar in all experimental groups. The data are presented as mean ± SEM.

***P<0.001, #P<0.05, ###P<0.001, +++ P<0.001 compared to respected group at “Before kindling”; $ P<0.05
and $$$ P<0.001 compared to respected group at “Full kindled”. There was no significant difference between the effects of LFS and HFS.

Fig. 4. Applying LFS and HFS in olfactory bulb restored the seizure-induced impairment in special learning and memory in Barnes maze test. A-D: Applying both LFS and HFS restored the kindling-induced impairment in spatial learning and memory and reduced the number of primary errors and total errors (but not latencies) in kindled+LFS and kindled+HFS groups compared to kindled animals. E: E: Three types of searching strategies during trail days by experimental groups. F: Changes in percentage of three types of searching strategies during trail days. G: Correlation graph representing the relation between percentage of direct strategy and trail days. There was small correlation in direct strategy in kindled animals compared to control. Application of LFS or HFS improved this parameter in kindled animals. H-K: Application of LFS and HFS improved the goal sector (GS) frequency in the kindled+LFS and kindled+HFS groups compared to kindled group. I: No changes were observed in non-goal sector (NGS) frequency among experimental groups. In Addition, a significant decrease of GS/NGS ratio was seen in kindled animals and was removed in kindled+LFS and kindled+HFS groups. Data are presented as mean ± SEM. In A and B, * P<0.05, ** P<0.01, compared to control group; # P<0.05, ## P<0.01 and ### P<0.001, compared to kindled group; ++ P<0.01 and +++ P<0.001, compared to first day of respected group. There was no significant difference between the effects of LFS and HFS.

Fig. 5. Applying LFS and HFS in olfactory bulb decreased the excitation/inhibition ratio in kindled animals. A: Sample records of eEPSC (holding potential=-52 mV) and eIPSC (holding potential=+10 mV) in ventral pyramidal CA1 neurons when the stimulating electrodes were placed in the Shaffer collaterals in different experimental groups. B-D: Applying LFS and HFS decreased the eEPSC amplitude, area under the curve and rise slope in kindled animals. E-G: LFS and HFS administration increased the eIPSC amplitude, area under the curve and rise slope in kindled animals. H-J: Applying both LFS and HFS decreased the excitation/inhibition (E/I) ratio in kindled animals. K-T: The same parameters in dorsal pyramidal CA1 neurons when the stimulating electrodes were placed in the Shaffer collaterals in different experimental groups. Results were very similar to ventral hippocampus. The data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, #P<0.05, ##P<0.01, ###P<0.001. There was no significant difference between the effects of LFS and HFS.

Fig. 6. Paired-pulse ratio did not change in ventral and dorsal hippocampus of kindled animals or following LFS and HFS application in olfactory bulb of kindled animals. A1 and B1: Sample traces of paired eEPSCs (holding potential=-52 mV) and paired eIPSCs (holding potential=+10 mV) recorded from ventral pyramidal CA1 neurons in different experimental groups in response to paired pulsed at 100 ms inter-pulse interval. A2: Applying LFS and HFS did not change the eEPSC paired-pulse ratio. B2: Applying LFS and HFS did not change the eIPSC paired-pulse ratio. C-D: Similar results obtained from dorsal hippocampus. Data are presented as mean ± SEM.

Fig. 7. Applying LFS and HFS in olfactory bulb did not change coefficient of variation (CV), but restored the changes in failure rate, efficacy and potency of eEPSCs and eIPSCs in ventral and dorsal hippocampus in kindled animals. A: Sample traces of 10 superimposed eEPSCs (holding potential=-52 mV) recorded from ventral pyramidal CA1 neurons in response to threshold intensity in different experimental groups. B: Calculating the CV showed no significant changes among experimental groups. C-E: Both LFS and HFS application restored the kindling -induced changes in failure rate, efficacy and potency of eEPSCs. F: Sample traces of 10 superimposed eIPSCs (holding potential=+10 mV) recorded from ventral pyramidal CA1 neurons in in response to threshold intensity in different experimental groups. G-J: There was not any significant difference in CV, failure rate, efficacy and potency of eIPSCs among experimental groups. K-O: The same parameters of eEPSCs recorded from dorsal pyramidal CA1 neurons in response to threshold intensity in different experimental groups. Applying LFS and HFS restored only the kindling-induced changes in failure rate. No change was observed in other parameters. P-T: The same parameters of eIPSCs recorded from dorsal pyramidal CA1 neurons in response to threshold intensity in different experimental groups. There was not any significant difference between kindled and control groups, however, application of both LFS and HFS in kindled animals significantly increased the efficacy and potency of eIPSCs. Data are presented as mean ± SEM. *P<0.05 and **P<0.01 compared to control group. #P<0.05 and ##P<0.01 compared to kindled group. There was no significant difference between the effects of LFS and HFS.

Fig. 8. Applying LFS and HFS in olfactory bulb restored the changes in spontaneous IPSCs (sIPSCs) amplitude in ventral hippocampus of kindled animals. A: Sample

traces of sIPSCs recorded from ventral (left) and dorsal (right) pyramidal CA1 neurons. B1 and B2: sIPSCs inter-event intervals increased in kindled animals significantly, but returned to control level following application of both LFS and HFS. C1 and C2: Kindling did not change the sIPSCs amplitude. Applying HFS, but not LFS significantly increased this parameter. D and E: Neither kindling, nor applying LFS and HFS changed the sIPSC interevent-intervals and amplitude in different experimental groups. Data are presented as mean ± SEM * P<0.05 compared to control group; # P<0.05, ## P<0.01 compared to kindled group.

Fig. 9. Applying LFS and HFS in olfactory bulb restored the seizure-induced changes in the gene expression of NR2A subunit of NMDA receptor (A), GluR2 subunit of AMPA receptor (B), and γ2 subunit of $GABAA_A$ receptor (C). Data are presented as mean ± SEM. * P<0.05 and ** P<0.01 compared to control group; # P<0.05 and ## P<0.01 compared to kindled group. There was no significant difference between the effects of LFS and HFS.

Fig. 10. Applying LFS and HFS in olfactory bulb had protective effect against cell death in CA1 and CA3 regions of ventral and dorsal hippocampus in kindled animals. A: Representative Nissl staining images of the CA3, and CA1 areas of the ventral hippocampus in different groups. B: The cell death percentage was increased in CA3 area of kindled animals. Applying LFS, but not HFS, significantly reduced the cell death. There was a significant difference between kindled+LFS and kindled+HFS groups. C: No significant changes was observed in cell death percentage in CA1 area among experimental groups. D-F: Similar results obtained in dorsal hippocampus. Of course, there was no significant difference among kindled=LFS

and kindled+HFS groups in dorsal CA3 area. Data are resented as mean ± SEM. * P<0.05, ** P< 0.001 and *** P< 0.0001 compared to control group; # P<0.05 and ### P<0.001 compared to kindled group; $ P<0.05 compared to kindled+LFS group.

Fig. 11. Applying LFS and HFS in olfactory bulb restored the seizure-induced changes in the power spectral density (PSD) of different brain rhythms in ventral hippocampus. A-E: PSDs were calculated before and after LFS and HFS application as % of before kindling situation. Kindling significantly increased PSD of delta, theta, beta and gamma waves compared to control. Applying both LFS and HFS restored the changes in brain waves in kindled animals at 24 h after application. F: Color maps showing an obvious increase (red and yellow colors) in the PSD brain rhythms during 10 s of LFP recording when the animal was at rest and awake in different experimental groups. The increased PSD did not observe in kindled+LFS and kindled+HFS groups. G and H: PSD (normalized as the percentage of the baseline at before kindling) of all brain rhythms was higher in kindled animals compared to control. LFS and HFS restored the PSD of brain rhythms at 24 h after their application. Data were presented as mean ± SEM. * P<0.05, ** P<0.01 and *** P<0.001 compared to control group; # P<0.05, ## P<0.01, ### P<0.001 compared to kindled group. There was no significant difference between the effects of LFS and HFS.

Table 1. The sequences of primers that are used in real-time polymerase chain reactions.

Gene	Primer Sequence (5'->3')	product length	Accession number
GAPDH	F:AGGGGCCATCCACAGTCTTC R:AGAAGGCTGGGGCTCATTTG	258	NM-001394060.2
GluR2 AMPA	F:GTGTTTGCCTACATTGGAGTG R:TTGGCTGTGTATGAGGAGATG	269	NM-017261.2
NR2A NMDA	F:TGGAGAAGCACAACTACGAGA R:CATTCCTGATACCGAACCCAC	27	NM-012573
gama2 GABAA	F:ACCATAGCCCGGAAGTCTCT R:CCTTGCTTGGTTTCCGGTTG	145	NM-183327.1

Supplementary Files

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