

Research Paper: Low-frequency Stimulation Decreases Hyperexcitability Through Adenosine A1 Receptors in the Hippocampus of Kindled Rats



Amir Shojaee¹, Parvin Zareian^{1*}, Javad Mirnajafi-Zadeh²

1. Department of Physiology, School of Medicine, AJA University of Medical Sciences, Tehran, Iran.

2. Department of Physiology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran



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ABSTRACT

Introduction: In this study, the role of A1 adenosine receptors in improving the effect of Low-Frequency Electrical Stimulation (LFS) on seizure-induced hyperexcitability of hippocampal CA1 pyramidal neurons was investigated.

Methods: A semi-rapid hippocampal kindling model was used to induce seizures in male Wistar rats. Examination of the electrophysiological properties of CA1 pyramidal neurons of the hippocampus using whole-cell patch-clamp recording 48 h after the last kindling stimulation revealed that the application of LFS as two packages of stimulations at a time interval of 6 h for two consecutive days could significantly restore the excitability CA1 pyramidal neurons evidenced by a decreased in the of the number of evoked action potentials and enhancement of amplitude, maximum rise slope and decay slope of the first evoked action potential, rheobase, utilization time, adaptation index, first-spike latency, and post-AHP amplitude. Selective locked of A1 receptors by the administration of 8-Cyclopentyl-1,3-dimethylxanthine (1 μ M, 1 μ l, i.c.v.) before applying each LFS package, significantly reduced LFS effectiveness in recovering these parameters.

Results: On the other hand, selective activation of A1 receptors by an injection of N6-cyclohexyladenosine (10 μ M, 1 μ l, i.c.v.), instead of LFS application, could imitate LFS function in improving these parameters.

Conclusion: It is suggested that LFS exerts its efficacy on reducing the neuronal excitability, partially by activating the adenosine system and activating its A1 receptors.

* Corresponding Author:

Parvin Zareian, PhD.

Address: Department of Physiology, School of Medicine, AJA University of Medical Sciences, Tehran, Iran.

Tel: +98 (21) 438220

E-mail: zareianj2011@yahoo.com

Highlights

- Epilepsy is one of the most prevalent chronic brain disorders worldwide;
- Selective locked of A1 receptors by administration of 8-cyclopenthyly dimethylexanthine significantly reduced LFS effectiveness in recovering these parameters.
- Patients diagnosed with refractory epilepsy may qualify for surgical resection of epileptic brain tissue.
- Recently, deep brain stimulation (DBS) is used in the treatment of refractory epilepsy.
- Low-frequency electrical stimulation (LFS) is an effective pattern of DBS in both clinical and experimental studies.

Plain Language Summary

Epilepsy is one of the most prevalent chronic brain disorders worldwide characterized by the presence of recurrent, spontaneous seizures; however, seizures do not respond well or are resistant to them all.

1. Introduction

Epilepsy is one of the most prevalent chronic brain disorders worldwide, characterized by the presence of recurrent, spontaneous seizures, resulting from the uncontrolled, synchronous excitation of a neuronal population within the brain. Despite numerous available antiepileptic drugs, seizures do not respond well or are resistant to them in more than 20%-40% of the patients (French, 2007). Patients diagnosed with refractory epilepsy may qualify for surgical resection of epileptic brain tissue. However, there are serious postoperative complications following neurosurgical resection, such as surgical mortality, permanent or temporary language and motor or psychologic deficits, and possibly, lack of amelioration or worsening in seizure activity (especially in patients with tumor-associated epilepsy) (Kwan & Brodie, 2000; Lin et al., 2011).

Furthermore, over 40% of the patients with refractory epilepsy do not meet the appropriateness criteria for the surgical treatment (Kwan & Brodie, 2000). Among the epilepsy syndromes, mesial temporal lobe epilepsy is the most frequent type of focal epilepsy with intractable seizures in adulthood. In this morbidity, seizures arise from different areas of the temporal lobe, such as the hippocampus, parahippocampal gyrus, and amygdala (Engel, 2001), and pathologically are characterized by specific morphological alterations in the hippocampus (Hinterkeuser et al., 2000; Temkin, 2009).

In recent years, there has been a growing interest in application of Deep Brain Stimulation (DBS) in the treatment

of refractory epilepsy. This therapeutic method involves the implantation of one or more electrodes into specific brain regions to deliver controlled electrical pulses to a target in brain to modulate neuronal activity. Although DBS is a recognized therapeutic tool that has received the U.S. Food and Drug Administration (FDA) approval for the treatment of refractory epilepsy; however, the antiepileptic mechanisms involved in its effectiveness are still largely unknown.

Low-Frequency electrical Stimulation (LFS) is an effective pattern of DBS, which has shown with anticonvulsive activity in both clinical (Kinoshita et al., 2004; Yamamoto et al., 2002; Yamamoto et al., 2006) and experimental (Gaito & Gaito, 1981; Gharib, Sayyahi, Komaki, Barkley, Sarihi, & Mirnajafi-Zadeh, 2018; Goodman, Berger, & Tchong, 2005; Mohammad-Zadeh et al., 2007; Shahpari, Mirnajafi-Zadeh, Firoozabadi, & Yadollahpour, 2012) studies. We have previously demonstrated that the application of LFS at a frequency of 1 Hz retards the onset or development of seizures in both in vivo and in vitro models of seizures (Ahmadirad et al., 2019; Ghasemi et al., 2019; Ghorbani, Mohammad-Zadeh, Mirnajafi-Zadeh, & Fathollahi, 2007; Jahanshahi Mirnajafi-Zadeh, Javan, Mohammad-Zadeh, & Rohani, 2009; Shahpari et al., 2012). This LFS paradigm was also able to improve learning and memory deficits following kindling acquisition (Esmailpour, Sheibani, Shabani, & Mirnajafi-Zadeh, 2017). Conventionally, LFS is a well-known research tool for the elimination of long-term potentiation, and induction of long-term depression of synaptic transmission. It has been proposed that LFS employs similar mechanisms to exert its inhibitory effect on seizure activity, such as endogenous adenosine system.

Adenosine, as an endogenous purine ribonucleoside, is a neuromodulator and a homeostatic regulator in the nervous system that mostly suppresses excitatory synaptic transmission and reduces neuronal excitability (de Mendonça & Ribeiro, 1997; Ribeiro, 1995). Adenosine is released during seizures and it is believed to have an important role in terminating the seizures (Dunwiddie, Hoffer, & Fredholm, 1981; During & Spencer, 1992; Ribeiro, Sebastiao, & de Mendonca, 2003; Zhou et al., 2018). Anticonvulsant activity of the adenosine system has been demonstrated in various animal models of epilepsy, and its dysfunction can cause a seizure (Alasvand Zarasvand, Mirnajafi-Zadeh, Fathollahi, & Palizvan, 2001; Boison, 2005, 2013; Chwalczuk, Rubaj, Swiader, & Czuczwar, 2008; Ekonomou, Angelatou, Vergnes, & Kostopoulos, 1998; Gasior, Borowicz, Kleinrok, & Czuczwar, 1996; Malhotra & Gupta, 1997; Siebel et al., 2015; Young & Dragunow, 1994). Antiepileptic effect of adenosine is mainly accomplished through the activation of its A1 receptors, which is the most abundant adenosine receptor in brain areas critical for epileptogenesis, such as the hippocampus (Fedele, Li, Lan, Fredholm, & Boison, 2006; Fredholm et al., 2001; Fredholm, Chen, Masino, & Vaugois, 2005; Hosseinmardi, Mirnajafi-Zadeh, Fathollahi, & Shahabi, 2007; Zeraati, Mirnajafi-Zadeh, Fathollahi, Namvar, & Rezvani, 2006; Zhou et al., 2018).

Our previous studies have shown that the anticonvulsant effect of LFS is partly mediated through adenosine A1 receptors (Jahanshahi et al., 2009; Mohammad-Zadeh et al., 2009). In addition, LFS employs adenosine A1 receptors to inhibit seizure-induced potentiation of Field Excitatory Postsynaptic Potentials (fEPSP) in dentate gyrus of the hippocampal formation (Mohammad-Zadeh et al., 2009).

In another study, we reported that the application of LFS can prevent seizure-induced hyperexcitability of CA1 pyramidal neurons in hippocampus (Ghotbedin, Janahmadi, Mirnajafi-Zadeh, Behzadi, & Semnani, 2013). Considering the important role of adenosine A1 receptors in the anti-epileptic effect of LFS, in this study, we investigated the role of these receptors in the effectiveness of LFS applied following kindling acquisition on seizure-induced hyperexcitability of hippocampal CA1 pyramidal neurons in rats. Accordingly, we employed a semi-rapid hippocampal kindling model to induce and develop the seizures and used the whole-cell patch-clamp recording to examine electrophysiological properties of the hippocampal CA1 pyramidal neurons.

2. Methods

2.1. Animals

Male Wistar rats (45 days old) were kept in normal conditions at $23\pm 2^{\circ}\text{C}$ temperature and a 12:12 light:dark schedule (lights on at 7:00 A.M) with ad libitum access to food and water. Adequate measures were taken to minimize pain or discomfort. All experiments were carried out based on the ethical guidelines set by the Ethical Committee of Faculty of Medical Sciences, Tarbiat Modares University and were entirely complied with the "NIH Guide for the Care and Use of Laboratory Animals".

2.2. Animal surgery

Animals were anesthetized with a ketamine/xylazine mixture (100/10 mg/kg, i.p.) and underwent stereotaxic implantation of a bipolar stimulating electrode in right Schaffer Collateral pathway (coordinates: A, 3.1 mm; L, 3.1 mm from Bregma and 2.8 mm below the dura), a monopolar recording electrode in CA1 area (coordinates: A, 2.8 mm; L, 1.8 mm from Bregma and 2.4 mm below the dura) of the hippocampus and a 23-gauge guide cannula was also located in the left lateral ventricle (coordinates: A, -0.9 mm; L, -1.5 mm from Bregma and 3.5 mm below the dura) (Paxinos & Watson, 2009). The exact position of the recording and stimulating electrodes was set to record a maximum slope of field EPSP in the stratum radiatum layer of the hippocampal CA1 area following the Schaffer Collateral pathway stimulation and also to confirm the correct electrodes placement. The electrodes composed of twisted stainless steel, Teflon coated wires with a diameter of 127 μm insulated, except for their tips (A-M Systems, Inc., WA, U.S.A.). Each reference and ground electrode consisted of a stainless-steel screw that was implanted over the frontal and parietal lobes. All electrodes were connected to an electrical socket and fixed to the skull with dental cement.

2.3. Semi-rapid kindling procedure

The rats were individually transferred from their home cage to a recording box (30 cm \times 30cm \times 30 cm) following a postoperative day 10. A flexible, shielded cable was plugged to the animal's socket while the animal was moving freely in the recording box. Then, kindling was induced in a semi-rapid manner. Electrical stimulation comprised a 1 ms monophasic square wave of 50 Hz with a 2 s train duration at the afterdischarge threshold. This stimulation paradigm was delivered 6 times daily at inter-train intervals of 20 min. For afterdischarge threshold determination, stimulations were started at an intensity of

10 μ A. The intensities were then increased in increments of 10 μ A (with 10 min intervals) until the stimulation could induce the ADs for at least 20 s. This intensity was considered as the afterdischarge threshold and employed for kindling stimulations. The afterdischarge was defined as spikes observed in local field potentials with a frequency of at least 1 Hz and an amplitude of at least twice the baseline activity recorded immediately following stimulation pulses. In this study, the afterdischarge threshold intensity of animals ranged from 20 to 100 μ A. Extracellular local field potential signals were boosted and sampled (at 10 kHz) using a PC-based data acquisition system (D3111 Data Acquisition, ScienceBeam Co., Iran) and a homemade software. The progression of kindling was examined by monitoring the behavioral seizure stage and estimating the afterdischarges duration following each stimulation. Behavioral seizures were rated according to the Racine's standard classification (Racine, 1972): stage 1. Facial clonus; stage 2. Head nodding; stage 3. Forelimb clonus; stage 4. Rearing and bilateral forelimb clonus; and stage 5. Rearing and falling. When the animals expressed stage 5 seizure over 3 consecutive days, they were regarded as fully kindled.

2.4. LFS application

In LFS-treated groups, LFS was administered at 5 min, 6, 18, and 24 h after the last kindling stimulation. LFS was applied as 4 packages of electrical pulses at 5 min intervals; every package comprised 200 monophasic square wave pulses with 0.1 ms in duration and 1 Hz in frequency. This LFS pattern was similar to our previous study (Ghafouri, Fathollahi, Javan, Shojaei, Asgari, A., & Mirnajafi-Zadeh, 2016). The intensity of pulses for LFS was equal to the afterdischarge threshold for each kindled rat.

2.5. Drug administration

1,3-dimethyl-8-cyclopentylxanthine (CPT; Sigma, UK), as a selective A1 receptor antagonist, and N6-cyclohexyladenosine (CHA; Sigma, UK), as a selective adenosine A1 receptor agonist, were dissolved in DMSO, and then diluted by adding artificial cerebrospinal fluid (aCSF; composition of aCSF has been mentioned in whole-cell patch-clamp procedure) to the desired concentration. The ultimate concentration of DMSO in solutions was 0.1% and their pH was adjusted to 7.35 - 7.40. The solutions were then sterilized through microfilters (0.2 μ m, Minisart, NML, Sartorius, Germany). Drugs were delivered to the left lateral ventricle via a 30-gauge cannula, which was 1 mm below the tip of the guide cannula. CPT at a dose of 2 mM and CHA at a dose of 10 mM were microinjected (1 μ l over 1 min) into the left

lateral ventricle 3 min before each LFS session in CPT and CHA receiving groups, respectively.

2.6. Whole-cell patch-clamp recording

Rats were killed by decapitation 48 h after the last kindling stimulation under ether anesthesia. Slice preparation and whole-cell patch-clamp recording were performed in the same way as described previously (Shojaei et al., 2014). Briefly, brains were rapidly isolated and the right hemisphere was horizontally sliced with 400 μ m in thickness using a vibroslicer (Leica VT 1200s, Leica Microsystems AG, Wetzlar, Germany) in ice-cold cutting artificial cerebrospinal fluid composed of (in mM) 238 sucrose, 2.5 KCl, 1 NaH₂PO₄, 0.5 CaCl₂, 2 MgSO₄, 26.2 NaHCO₃ and 11 D-glucose. Also, pH ranged from 7.35-7.45 after equilibration with 95% O₂ and 5% CO₂. Osmolarity was adjusted to 290-300 mOsm. Transverse hippocampal slices (400 μ m) were prepared using ...

Slices were immediately transferred to a Gibbs chamber containing ACSF and incubated at 32-35°C for 60 min. ACSF was composed of (in mM) 125 NaCl, 3 KCl, 2 CaCl₂, 1.3 MgCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, and 10 D-glucose continuously bubbled with 95% O₂ and 5% CO₂. The osmolality was 295 \pm 5 mOsm and pH was adjusted to 7.3-7.4. Before transferring the slices to the submerged recording chamber, they were kept at room temperature (23-25°C) for a minimum of 20 min. Hippocampal CA1 pyramidal neurons were observed using an upright microscope (Axioskop 2 FS MOT; Carl Zeiss, Germany) equipped with infrared CCD camera (IR-1000, MTI, USA). Recording chamber was continuously superfused with the ACSF at a rate of 3 ml/min.

Whole-cell patch-clamp recording under the current-clamp mode was made from CA1 pyramidal neurons. Recording microelectrodes with a tip resistance of 4-7 M Ω (1.5 mm O.D. and 0.86 mm I.D., Sutter, USA) were pulled from borosilicate glass with a horizontal puller (P-97, Sutter Instrument, USA) in 4 steps and filled with intracellular solution containing (in mM) 115 K-glucuronate, 20 KCl, 10 disodium-phosphocreatine, 2 EGTA, 10 HEPES, 2 MgATP and 0.3 Na₂GTP. We adjusted pH to 7.25-7.30 and osmolality was set to 290-295 mOsm. Pipette capacitance compensation and bridge balance were carried out and series resistance was compensated by 80%. Signals were recorded by a Multiclamp 700B amplifier and digitized with a Digidata 1440 A/D converter (Molecular Devices, CA, USA). Signals were filtered and digitized at 10 kHz. Electrophysiological analysis was performed using pClamp 10 software.

Measurement of the electrophysiological parameters was started 10 min after the establishment of whole-cell configuration. To estimate the firing properties of neurons, depolarizing current pulses (100 to 200 pA, 50 pA increment, 650 ms) were injected and the number of evoked action potentials (in each depolarizing current step), as well as the onset latency, amplitude of the first spike, adaptation index, instantaneous frequency and post-AHP amplitude (in the 150 pA depolarizing current step) were measured. Also, a depolarizing ramp current (0 - 200 pA during 1000 ms) was injected to calculate the rheobase and utilization time. Action potential amplitude was considered as the voltage difference between the baseline and peak of the first evoked action potential. First spike latency was defined as the time elapsed from the onset of depolarizing current injection to the threshold of the evoked action potential. Instantaneous frequency was defined as the frequency of first two spikes generated in response to applying depolarizing current. The adaptation index of spike frequency was calculated by dividing the average of the last three inter-spike intervals to the average of the first three inter-spike intervals of evoked action potentials.

The post-AHP was measured as the voltage difference between the baseline (before current injection) and the peak of hyperpolarization produced after cessation of depolarizing current injection. The rheobase was considered as the current injected for evoking the first action potential. The delay in firing the first action potential in this situation was measured as utilization time.

2.7. Experimental groups

After the recovery period, animals were divided into seven groups. In the kindled+LFS (KLFS) group (n=3), LFS was administered at 5 min, 6, 18, and 24 h after the last kindling stimulation, and 1 μ M ASCF containing 0.1 % DMSO was injected into the lateral ventricle as a vehicle (for 1 min) before each LFS session. In the KLFS+CPT groups (n=3), animals experienced the same procedure; however, they received CPT instead of the vehicle. Animals of the kindled+CHA (n=3) and kindled+CPT (n=3) groups received CHA or CPT, respectively, at the same time after the kindling procedure, LFS was not administered. In the kindled group (n=3), animals were subjected to only kindling stimulations and in the kindled+Vehicle group (n=3), they received vehicle following kindling acquisition. Animals of the LFS group (n=3) were treated with LFS alone. There were also two control groups, including the sham-operated (n=3) and naïve (non-operated) (n=3) rats. There was no statistically significant difference between

the kindled and kindled+vehicle, and also between the sham-operated and naïve groups; therefore, their data were combined into a kindled (n=6) and control (n=6) groups, respectively. Besides, as no statistical difference in various parameters was found between the kindled and kindled+CPT groups, the data of the kindled+CPT group is not shown.

2.8. Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (version 6.01, GraphPad Software, Ca, USA). Normal distribution of data was checked using Shapiro-Wilk and Levene's test, respectively. The number of evoked action potentials was compared by one-way ANOVA and other parameters were analyzed using a two-way ANOVA (both followed by a Tukey's post-hoc test). Values are presented as Mean \pm Standard Error of the Mean (SEM) and a P-value of less than 0.05 was considered statistically significant.

3. Results

The electrophysiological properties of CA1 pyramidal neurons were evaluated using whole-cell patch-clamp recording. To characterize the role of adenosine A1 receptors in the effectiveness of LFS on cellular hyperexcitability, the evoked firing activity of pyramidal cells was examined in response to the injection of depolarizing current pulses. A significant difference was observed in the number of action potentials evoked in response to injecting different depolarizing current steps (Figure 1A). A two-way ANOVA of the number of evoked action potentials revealed a significant difference in terms of the group ($F_{5,150}=35.28$, $P<0.001$) and current intensities ($F_{2,50}=115.9$, $P<0.001$). As Figure 1A shows and we reported in our previous studies (Ghotbedin et al., 2013; Moradi Chameh et al., 2015; Shojaei et al., 2014), the excitability of CA1 pyramidal neurons enhanced by kindling stimulations as evidenced by a higher number of action potentials triggered by an injection of 100 to 200 pA depolarizing currents in the kindled group compared with the control group (11.75 \pm 1.01 in 100 pA, 18.33 \pm 0.90 in 150 pA, and 21.92 \pm 0.96 in 200 pA in the kindled group vs. 5.33 \pm 0.99 in 100 pA, 11.50 \pm 1.08 in 150 pA, and 14.67 \pm 1.33 in 200 pA in the control group; $P<0.001$; n=12 cells in 6 slices from 6 rats; Figure 1A). This parameter indicated a significant reduction due to LFS application in the KLFS group (2.00 \pm 1.09 in 100 pA, 6.25 \pm 0.77 in 150 pA, and 10.34 \pm 0.73 in 200 pA; $P<0.001$ compared with the kindled group; n=8 cells in 6 slices from 3 rats). Antagonism of adenosine A1 receptors in KLFS+CPT group significantly alleviated the

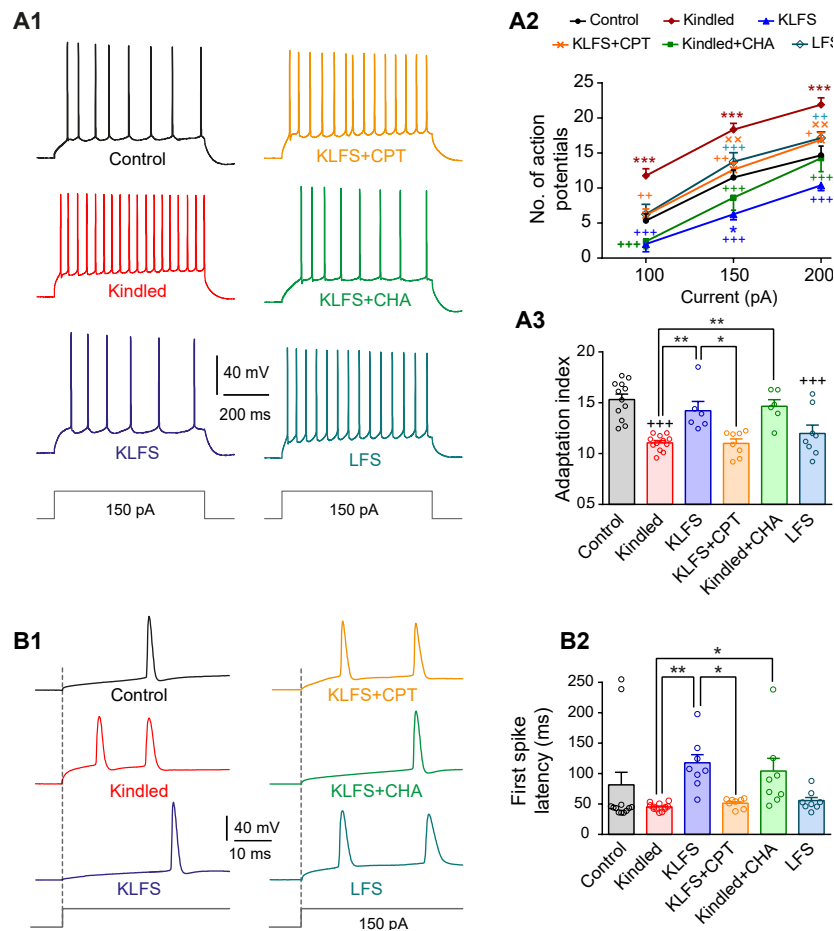


Figure 1. Role of A1 adenosine receptors in the effectiveness of LFS applied following kindling acquisition on the number of evoked action potentials, adaptation index, first spike latency and post-AHP amplitude in of hippocampal CA1 pyramidal neurons

A1: Sample traces of action potentials fired by hippocampal CA1 neurons in response to injecting 150 pA depolarizing current in different experimental groups.

A2: Quantitative comparison of the number of spikes evoked in response to the application of depolarizing current pulses of 100, 150, and 200 pA (650 ms) in CA1 pyramidal neurons among various experimental groups.

* P<0.05; and *** P<0.001 compared with the control group; + P<0.05, ++ P<0.01, and *** P<0.001 compared with the kindled group, ×× P<0.01 compared with the KLFS group, Mean±SEM.

A3: Comparing adaptation index of action potentials evoked in response to 150 pA depolarizing current pulse in different experimental groups. B1: Sample voltage traces from pyramidal neurons in response to 150 pA depolarizing current pulse to compare the first spike latency in various experimental groups. The dashed line shows the onset of depolarizing current.

B2: Quantitative comparison of latency to the first spike triggered in response to 150 pA depolarizing current pulse in CA1 pyramidal neurons in different experimental groups.

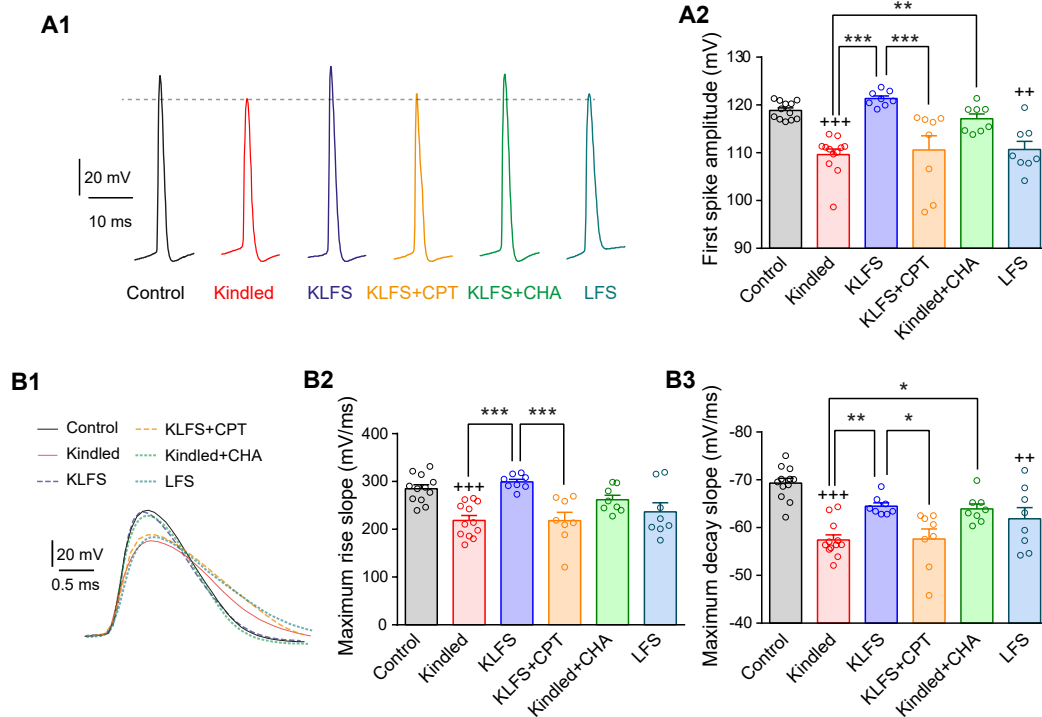
C1: The sample voltage traces shown in A1 on a new scale to further highlight differences in post-AHP amplitude in different experimental groups.

C2: Statistical comparing the amplitude of Post-AHP elicited in response to 150 pA depolarizing current pulse. + P<0.05 and *** P<0.001 compared with the control group;

* P<0.05, ** P<0.01, and *** P<0.001. KLFS: kindled+ low frequency stimulation; LFS: Low-frequency stimulation;

CPT: 1,3-dimethyl-8-cyclopentylxanthine; CHA: N6-cyclohexyladenosine

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Figure 2. Role of A1 adenosine receptors in the effectiveness of LFS applied following kindling acquisition on properties of first action potential evoked in response to 150 pA depolarizing current pulse

A1: Representative samples of the first spike evoked by injecting the 150 pA depolarizing current in pyramidal neurons in various experimental groups. A dashed is aligned with the peak of the kindled group spike to further highlight the difference in amplitude of the first spike between different groups.

A2: Quantitative comparison of first spike amplitude generated in response to depolarizing current injection of 150 pA between different experimental groups.

B1: Superimposed sample traces of the first spike elicited by injecting 150 pA depolarizing current in CA1 pyramidal neurons obtained from different experimental groups.

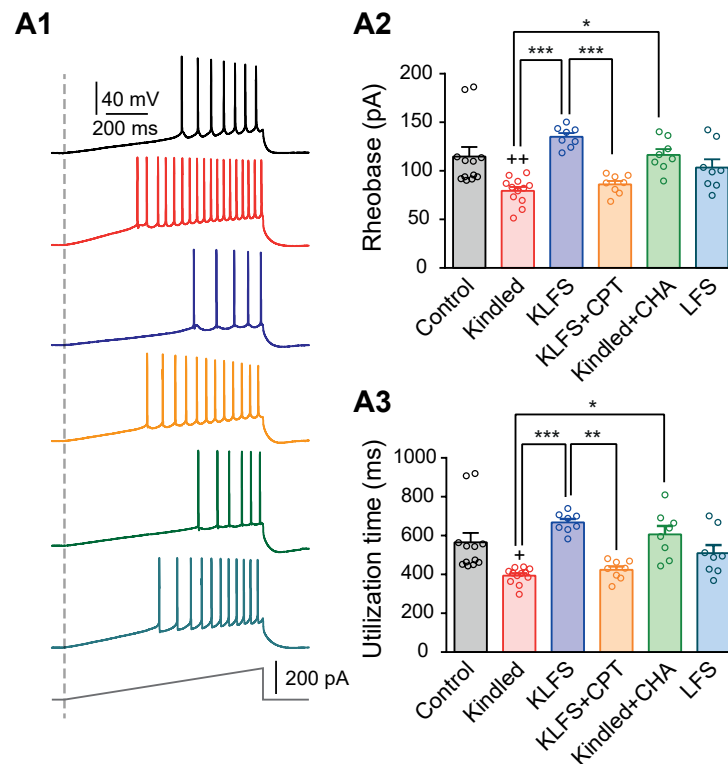
B2 and B3 show a comparison of the maximum rise slope and maximum decay slope of the first spike, respectively, in CA1 pyramidal neurons in response to 150 pA depolarizing current in different experimental groups.

++ P<0.01 and +++ P<0.001 compared with the control group; * P<0.05, ** P<0.01 and *** P<0.001.

KLFS: Kindled+ low-frequency stimulation; LFS: Low-frequency stimulation; CPT: 1,3-dimethyl-8-cyclopentylxanthine; CHA: N6-cyclohexyladenosine

suppressive effect of LFS on the number of evoked action potentials (6.12 ± 0.89 in 100 pA, 12.62 ± 0.10 in 150 pA and 16.87 ± 1.01 in 200 pA; $P < 0.01$ in 100 pA and 150 pA, $P < 0.05$ in 200 pA; $n = 8$ cells in 6 slices from 3 rats). Activation of these receptors following seizure development could mimic the ameliorative effect of LFS and result in lowering the number of fired action potentials in all depolarizing steps (2.37 ± 1.03 in 100 pA; 8.62 ± 1.77 in 150 pA and 14.25 ± 1.90 in 200 pA; $P < 0.001$ compared with the kindled group; $n = 8$ cells in 6 slices from 3 rats). This parameter didn't show significant changes in the LFS group compared to control (Figure 1A).

A one-way ANOVA showed a significant difference in latency to the first spike among different experimental groups ($F_{5,50} = 4.20$; $P < 0.01$, Figure 1B). In detail, kindling stimulations diminished this latency (from 81.53 ± 20.64 ms in control to 45.22 ± 1.43 ms in kindled group), but we couldn't find significant difference compare to control ($P = 0.32$; $n = 12$ cells in 6 slices from 6 rats). However, LFS administration to animals of KLFS group increased first spike latency (117.80 ± 13.36 ms; $P < 0.01$ compared to kindled group; $n = 8$ cells in 6 slices from 3 rats). Inhibition of adenosine A1 receptors prevented the effect of LFS on first spike latency (51.34 ± 2.30 ms; $P < 0.05$



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Figure 3. Role of A1 adenosine receptors in effectiveness of LFS on rheobase and utilization time.

A1: Sample voltage responses to injection of depolarizing ramp current recorded from CA1 pyramidal neurons in various experimental groups. A2 and A3: show the changes in rheobase and utilization time in different experimental groups, respectively.

+ $P < 0.05$; and ++ $P < 0.01$ compared with the control group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

KLFS: Kindled+ low-frequency stimulation; LFS: Low-frequency stimulation; CPT: 1,3-dimethyl-8-cyclopentylxanthine; CHA: N6-cyclohexyladenosine

compared to KLFS group; $n=8$ cells in 6 slices from 3 rats). Injection of CHA was also capable to recover this parameter in kindled+CHA group (104.30 ± 20.75 ms; $P < 0.05$ compared to kindled group; $n=8$ cells in 6 slices from 3 rats). Employment of LFS alone in LFS group had no significant effect on this parameter (55.76 ± 5.04 ms; $P=0.78$ compared to control) (Figure 1B).

Evaluation of spike accommodation demonstrated a statistically significant difference in adaptation index among experimental groups ($F_{5,46}=12.42$; $P < 0.001$; Figure 1A). This index showed a considerable reduction in kindled group (1.11 ± 0.02 in kindled vs 1.53 ± 0.05 in control group; $P < 0.001$; $n=12$ cells in 6 slices from 6 rats). LFS treatment of fully kindled animals in KLFS group could significantly restore this parameter near to control values (1.42 ± 0.09 ; $P < 0.01$ compared to kindled group; $n=6$ cells in 6 slices from 3 rats). Blockade of adenosine A1 receptors by CPT in the KLFS+CPT group completely

eliminated the effectiveness of LFS on the adaptation index (1.10 ± 0.04 ; $P < 0.05$ compared to KLFS group; $n=8$ cells in 6 slices from 3 rats). This parameter significantly enhanced by activation of adenosine A1 receptors in kindled+CHA group (1.46 ± 0.06 ; $P < 0.01$ compared to kindled group; $n=6$ cells in 6 slices from 3 rats). It showed a significant reduction by treatment of animals with LFS alone in LFS group (1.20 ± 0.08 ; $P < 0.001$ compared to control; $n=8$ cells in 6 slices from 3 rats) (Figure 1A).

We next measured AHP magnitude after a 150 pA current injection (post-AHP). Statistical analysis revealed that AHP had a significantly different amplitude than other experimental groups ($F_{5,50}=9.180$; $P < 0.001$; Figure 1C). This parameter significantly decreased due to kindling development (from -2.66 ± 0.25 mV in the control group to -1.63 ± 0.19 mV in the kindled group; $P < 0.05$; $n=12$ cells in 6 slices from 6 rats). However, it completely improved by the LFS application in the KLFS group

(-2.78 ± 0.23 mV; $P < 0.05$ compared with the kindled group; $n = 8$ cells in 6 slices from 3 rats). The amplitude of post-AHP in the KLFS+CPT group did not show an alteration following LFS treatment and a significant difference was observed compared with the KLFS group (-1.67 ± 0.15 mV; $P < 0.05$ than the KLFS group; $n = 8$ cells in 6 slices from 3 rats). Examination of this parameter in the kindled+CHA group showed that it recovered by adenosine A1 receptor activation (2.81 ± 0.14 mV; $P < 0.05$ in comparison with the kindled group; $n = 8$ cells in 6 slices from 3 rats). Administration of LFS also significantly decreased post-AHP amplitude in the LFS group (-1.25 ± 0.29 mV; $P < 0.001$ compared with the control; $n = 8$ cells in 6 slices from 3 rats) (Figure 1C).

Estimating the amplitude of the first spike evoked by 150 pA depolarizing current revealed that it declined by kindling stimulations in the kindled group (from 118.90 ± 0.53 mV in control to 109.60 ± 1.17 mV in the kindled group; $P < 0.001$ in comparison with control; $n = 12$ cells in 6 slices from 6 rats, Figure 2A) but LFS restored this parameter in the KLFS group (121.30 ± 0.55 mV; $P < 0.001$ compared with the kindled group; $n = 8$ cells in 6 slices from 3 rats). LFS application following CPT injection in the KLFS+CPT group failed to recover kindling-induced decrement of first spike amplitude (110.60 ± 2.97 mV; $P < 0.001$ than the KLFS group; $n = 8$ cells in 6 slices from 3 rats). Activation of adenosine A1 receptors by CHA in the kindled+CHA group could significantly increase this parameter in comparison with the kindled group (117.10 ± 1.00 mV; $P < 0.01$ vs. the kindled group; $n = 8$ cells in 6 slices from 3 rats). Similar to the effect of kindling stimulations, the application of LFS alone also significantly decreased amplitude of first spike (110.70 ± 1.70 mV; $P < 0.01$ compared with the control group; $n = 8$ cells in 6 slices from 3 rats) (Figure 2A).

Maximum rise slope and maximum decay slope of the first action potential were also decreased by kindling stimulations (from 285 ± 8.40 mV/ms in the control group to 219 ± 10 mV/ms in the kindled group for maximum rise slope and from -69 ± 1.00 mV/ms in the control group to -57 ± 1.10 mV/ms in the kindled group for maximum decay slope; $P < 0.001$; $n = 12$ cells in 6 slices from 6 rats; Figure 2B). LFS application in the KLFS group significantly recovered both parameters (299 ± 5.40 mV/ms for maximum rise slope and -64 ± 0.69 mV/ms for maximum decay slope; $P < 0.001$ for maximum rise slope and $P < 0.01$ for maximum decay slope compared with the kindled group; $n = 8$ cells in 6 slices from 3 rats). Administration of CPT and inhibition of adenosine A1 receptors before LFS application in the KLFS+CPT group significantly eliminated the efficacy of LFS in raising

these parameters (218 ± 17.00 mV/ms for maximum rise slope ($P < 0.001$) and -58 ± 2.10 mV/ms for maximum decay slope ($P < 0.05$) compared with the KLFS group; $n = 8$ cells in 6 slices from 3 rats). Post-kindling treatment of the animals with CHA and activation of adenosine A1 receptors in the kindled+CHA group significantly increased the maximum decay slope of the first evoked spike than the kindled animals (-64 ± 1.00 mV/ms; $P < 0.05$). Although the enhancement of maximum rise slope in this group was not significant compared with the kindled group, however, no significant difference was observed than the control group (262 ± 17.00 mV/ms; $P = 0.12$ vs. the kindled group and $P = 0.73$ vs. the control group; $n = 8$ cells in 6 slices from 3 rats). LFS application in the LFS group insignificantly declined maximum rise slope and significantly reduced maximum decay slope of the first evoked spike (237 ± 19.00 mV/ms for maximum rise slope ($P = 0.06$) and -62 ± 2.30 mV/ms for maximum decay slope ($P < 0.01$) compared with the KLFS group; $n = 8$ cells in 6 slices from 3 rats) (Figure 2B).

We then measured rheobase and utilization time parameters by applying ramp depolarizing currents. As we reported previously (Ghotbedin et al., 2013; Moradi Chameh, Janahmadi, Semnanian, Shojaei, & Mirnajafi-Zadeh, 2015), both parameters showed a significant reduction following kindling acquisition (from 114.70 ± 9.85 pA in the control group to 79.48 ± 3.99 pA in the kindled group for rheobase ($P < 0.01$), and from 565.40 ± 48.76 ms in the control group to 393.70 ± 77.92 ms in the kindled group for utilization time ($P < 0.05$); $n = 12$ cells in 6 slices from 6 rats; Figure 3).

LFS treatment of animals in the KLFS group significantly increased rheobase and utilization time (135.10 ± 3.77 pA for rheobase and 667.60 ± 17.80 ms for utilization time; $P < 0.001$; $n = 8$ cells in 6 slices from 3 rats). CPT injection in the animals of the kindled group significantly eliminated the efficacy of LFS treatment on recovering these parameters (86.13 ± 3.53 pA for rheobase ($P < 0.001$) and 423.80 ± 17.41 ms for utilization time ($P < 0.01$) in the KLFS+CPT group compared with the KLFS group; $n = 8$ cells in 6 slices from 3 rats). CHA administration and activation of adenosine A1 receptors could again mimic LFS effect on these parameters (116 ± 50 pA for rheobase ($P < 0.01$) and 606.40 ± 42.82 ms for utilization time ($P < 0.001$) in kindled+CHA group compared with the kindled group; $n = 8$ cells in 6 slices from 3 rats). The results obtained from the LFS group showed that LFS alone did not affect rheobase (103.50 ± 8.44 pA; $P = 0.085$) and utilization time (509.70 ± 41.70 ms; $P = 0.86$) in comparison with the control group; $n = 8$ cells in 6 slices from 3 rats) (Figure 3).

4. Discussion

In this study, we assessed the role of adenosine A1 receptors in recovering the seizure-induced hyperexcitability of CA1 pyramidal neurons by LFS treatment. The obtained results confirmed that the application of LFS can improve the deleterious effect of kindling on cellular excitability. Also, inhibition of adenosine A1 receptors decreased the effectiveness of LFS in the reduction of cellular excitability in kindled animals. Furthermore, restoring effect of LFS on cellular excitability could be mimicked to a great extent by activation of A1 adenosine receptor. We formerly showed that antiepileptogenic effects of LFS are mediated somehow through the activation of adenosine A1 receptors (Mohammad-Zadeh et al., 2009). In addition, LFS exerted its anticonvulsant effect, in part, by preventing the kindling-induced decrement of A1 receptor gene expression during epileptogenesis (Jahanshahi et al., 2009).

Data obtained from the present study confirmed that LFS could decrease neuronal excitability, indicated by reducing the number of evoked action potentials, extending first spike latency, increasing the adaptation index, post-AHP amplitude, maximum decay slope of the first evoked action potential, rheobase current, and utilization time parameters. Investigating the role of adenosine A1 receptors in these observations revealed that these receptors are involved in restoring the effect of LFS on the number of evoked action potentials.

This parameter is regulated by different voltage- or calcium-dependent outward potassium currents comprising A-type (Lien & Jonas, 2003C; Rudy & McBain, 2001; Storm, 1987; Wang et al., 1998) slowly inactivating D-type (Segal & Barker, 1984; Storm, 1990), slowly activating, non-inactivating M type (Gu, Vervaeke, Hu, & Storm, 2005; Marrion, 1997), large-conductance, calcium-activated (BK) potassium channels (Gu, Vervaeke, & Storm, 2007; Shao, Halvorsrud, Borg-Graham, & Storm, 1999; Storm, 1987) and small-conductance calcium-activated (SK) potassium channels (Engel, Schultens, & Schild, 1999; Pedarzani et al., 2005). These currents underlie neuronal excitability and the abnormality in their functions leads to increased neuronal excitability and seizure (Brenner et al., 2005; Du et al., 2005; Oliveira et al., 2010; Segal, Barker, 1984; Singh et al., 1998; Wu & Barish, 1999). Therefore, LFS may involve adenosine A1 receptors to affect the activity of one or more types of potassium currents.

For a better clarification, we also assessed the effect of LFS application in the presence of A1 adenosine receptor antagonist (CPT) on maximum decay slope of

the first spike, first spike latency, spike adaptation, and post-AHP amplitude in response to a depolarizing current injection. Maximum decay slope and onset latency of the first action potential parameters were hampered to be changed by LFS when adenosine A1 receptors were blocked. Maximum decay slope of the first action potential is dependent on the activity of A-type potassium channels (Bean, 2007; Kim, Wei, & Hoffman, 2005) and first spike latency is mainly determined by the function of both A- and D-type potassium currents (Storm, 1988; Yuan & Chen, 2006). These potassium channels play a pivotal role in the regulation of neuronal excitability (Nakajima, Nakajima, Leonard, & Yamaguchi, 1986; Rogawski, 1985; Segal & Barker, 1984). Based on the findings of this study, it can be suggested that the employed adenosine A1 receptors by LFS can affect A- and D- type potassium currents.

LFS also lost its efficacy on increasing the spike adaptation and post-AHP amplitude when adenosine A1 receptors were blocked. These parameters are mostly dependent on big- and small-conductance (BK and SK) potassium currents (Figenschou, Hu, & Storm, 1996; Gu, Vervaeke, & Storm, 2007). Therefore, the recovery of these currents by LFS may be mediated through adenosine A1 receptors.

The restoring effect of LFS on maximum rise slope of the first evoked action potential, rheobase current, and utilization time was stopped when adenosine A1 receptors were blocked. These parameters are mostly controlled by voltage-gated sodium channels. Considering the important role of these channels in the generation of action potentials (Offord & Catterall, 1989; Waxman, ib-Hajj, Cummins, & Black, 2000), changes in the activity of them by adenosine A1 receptors may also be considered as a possible LFS mechanism of action. In this regard, it has also been shown that the expression of four voltage-gated sodium channel subtypes (NaV1.1, NaV1.2, NaV1.3, and NaV1.6) is elevated in the hippocampus and temporal lobe cortex following epileptic seizures (Berkovic, Mulley, Scheffer, & Petrou, 2006; Blumenfeld et al., 2009; Mulley, Scheffer, Petrou, Dibbens, Berkovic, & Harkin, 2005; Waxman et al., 2000; Xu et al., 2013).

To confirm the possible involvement of adenosine A1 receptors in the ameliorating effect of LFS, we observed that the application of an A1 adenosine receptor agonist (CHA) could mimic the effect of LFS on cellular excitability in kindled animals. Previous studies have demonstrated that the activation of adenosine A1 receptors hyperpolarizes the cells by direct triggering inwardly-rectifying potassium channels (GIRKs) in postsynaptic

neurons and decreases cellular excitability (Nicoll, 1988; North, 1989; Rotermund et al., 2018; Thompson, Capogna, & Scanziani, 1993). GIRK1–3 are expressed relatively at high levels by hippocampal CA1 and CA3 pyramidal cells (Drake, Bausch, Milner, & Chavkin, 1997; Karschin, Dissmann, Stühmer, & Karschin, 1996; Liao, Jan, & Jan, 1996; Ponce et al., 1996). Activation of these currents through adenosine A1 receptors inhibits action potential generation in response to depolarizing current injection to CA1 neurons (Hargus, Bertram, & Patel, 2009).

Besides, activation of adenosine A1 receptors can activate calcium-dependent potassium-current flows through the SK channels (Gerber, Greene, Haas, & Stevens, 1989; Haas & Greene, 1984). These channels are controlled by cAMP and underlie the accommodation of firing seen in many hippocampal pyramidal neurons (Marrion & Tavalin, 1998). Furthermore, adenosine reduces the High Voltage-activated (HVA) calcium currents through adenosine A1 receptors in CA1 (Scholz & Miller, 1991) and CA3 (Mogul, Adams, & Fox, 1993) hippocampal pyramidal cells.

It should be noted that the activation of these receptors can also affect synaptic transmission. Adenosine A1 receptors exist at excitatory synapses in the hippocampus (Rebola, Pinheiro, Oliveira, Malva, & Cunha, 2003) and their activation has been demonstrated to hamper excitatory synaptic transmission in CA1 (Brundege & Dunwiddie, 1996) and CA3 pyramidal neurons. Altogether, such mechanisms may be employed by adenosine A1 receptors to affect sodium and potassium currents and reduce the neuronal excitability in kindled animals. However, further studies are required to elucidate this issue in more details.

In conclusion, according to the present data, it may be suggested that LFS involves adenosine A1 receptors to exert its efficacy on neuronal excitability following seizure development in the CA1 area of the hippocampus. The results of this study also confirm the fact that adenosine A1 receptors can be considered as a potential target for drug development against epilepsy.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article.

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Authors' contributions

All authors contributed equally in preparing all parts of the research.

Conflict of interest

The authors declared no conflict of interest regarding the publication of this article.

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