

Deep brain stimulation effects on learning, memory and glutamate and GABA_A receptor subunit gene expression in kindled rats

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Epileptic seizures are accompanied by learning and memory impairments. In this study, the effect of low frequency stimulation (LFS) on spatial learning and memory was assessed in kindled animals and followed for one month. Fully kindled rats received LFS at 4 times (immediately, 6 h, 24 h and 30 h following the final kindling stimulation). Applying LFS improved kindled animals' performance in the Barnes maze test. This LFS action was accompanied by a decrease in NR2B gene expression, an increase in the gene expression of the α subunit of calcineurin A and an increased NR2A/NR2B ratio in kindled animals. In addition, the gene expression of the GABA_A receptor γ_2 subunit increased at 2–3 h after applying LFS. The increase in NR2A/NR2B ratio was also observed 1 week after LFS. No significant changes were observed one month after LFS administration. Field potential recordings in the hippocampal CA1 area showed that kindling-induced potentiation of the field EPSP slope returned to near baseline when measured 2–3 h after applying LFS. Therefore, it may be postulated that applying LFS in kindled animals reduced the seizure-induced learning and memory impairments, albeit time-dependently. In tandem, LFS prevented kindling-induced alterations in gene expression of the described proteins, which are potentially important for synaptic transmission and/or potentiation. Moreover, a depotentiation-like phenomenon may be a possible mechanism underlying the LFS action.

Key words: seizure, deep brain stimulation, learning and memory, GABA, NMDA, evoked potentials

INTRODUCTION

Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy. TLE is characterized by recurrent, spontaneous seizures that originate from the temporal lobe (Falconer et al., 1964). The main therapeutic intervention for epileptic patients is antiepileptic drugs, but 20–30% of epileptic patients are drug resistant (Schmidt and Schachter, 2014). Therefore, more research has been dedicated to finding new therapies for epilepsy.

Over the last decade, deep brain stimulation has been suggested as a treatment for patients with drug-resistant epilepsy and patients who are not surgical resection candidates (Jobst et al., 2010). Low frequency stimulation (LFS), in the range of 0.5 to 5 Hz, has been shown to suppress seizures in both epileptic patients (Yamamoto et al., 2002; 2006) and animal models of epilepsy (Gaito et al., 1980; Goodman et al., 2005). It has been suggested that LFS shares similar mechanisms with long-term depression (LTD) to accomplish its antiepileptic action (Toprani and Durand, 2013) or depotentiation (Rogawski, 2002). NMDA receptors

(NMDAR), GABA receptors (GABAR) and calcineurin may be involved in these types of synaptic plasticity.

Many patients with TLE suffer from memory impairments (Giovagnoli and Avanzini, 1999) that are commonly attributed to neural hyperexcitability in the hippocampus following seizure activity (Mazara-ti, 2008). Many studies have shown that kindling leads to time-dependent memory deficits (Leung and Shen, 1991; Leung et al., 1994; Gilbert et al., 2000).

Learning and memory are highly complex processes. Each is accompanied by changes in brain activity, including synaptic plasticity in the form of long-term potentiation (LTP) and LTD (Kemp and Manahan-Vaughan, 2007). Previous studies showed that NMDARs play an important role in synaptic plasticity (Hunt and Castillo, 2012), learning and memory (Li and Tsien, 2009), and epileptogenesis (Frasca et al., 2011). Changes in the NR2A to NR2B subunits ratio affect the functional diversity of NMDARs (Cui et al., 2013). This ratio plays an important role in synaptic plasticity and memory processes, and an increase in this ratio induces LTD in synapses (Cui et al., 2013). It has been reported that blockade of NMDARs inhibits seizure activity (Albertson et al., 1980), prevents epileptogenesis in kindled rats (Trommer and Pasternak, 1990), and impairs learning and memory (Tsien et al., 1996).

GABA, as the main inhibitory neurotransmitter in the brain, has a critical role in the suppression of seizure activity. Significant changes in the expression of GABA_A receptor (GABA_AR) subunits have been reported in the hippocampus of epileptic patients (Baulac et al., 2001) and kindled animals (Sperk et al., 2004). Additionally, the GABAergic system involves in memory processing (Givens and Olton, 1990; Chrobak and Napier, 1992) and blocking GABA_ARs leads to spatial and working memory impairments (Rao et al., 2000).

In addition to glutamate and GABA receptors, protein phosphatases also have an important role in learning and memory. Calcineurin is a calcium/calmodulin-dependent phosphatase that is composed of catalytic and regulatory subunits and is highly enriched in neural tissue (Klee et al., 1979; Pallen and Wang, 1985). Calcineurin is associated with many brain disorders, including epilepsy (Kipanyula et al., 2016), and may regulate the activity of other receptors, such as GABA_ARs (Huang et al., 2001) and NMDARs (Shi et al., 2000).

Our previous studies showed that the application of LFS following kindling stimulations prevented kindling-induced potentiation (Mohammad-Zadeh et al., 2007; Jahanshahi et al., 2008). In addition, applying LFS to the hippocampal CA1 region prevented seizure-induced increases in spontaneous glutamatergic transmission and a decrease in spontaneous GABAergic

transmission. These changes may be involved in the anticonvulsant mechanism of LFS (Ghafouri et al., 2016). Applying LFS in kindled animals ameliorated the apparent cognitive impairments in short-term memory in the Y-maze test (Ghafouri et al., 2016) and long-term memory in the Morris water maze (Esmaeil-pour et al., 2017a).

Accordingly, the present study was an attempt to investigate the time-dependent effect of LFS on seizure-induced impairments in spatial learning and memory and changes in gene expression of NR2A (*Grin2a*) and NR2B (*Grin2b*) NMDAR subunits, the GABA_AR γ_2 subunit (*Gabrg2* gene) and the α subunit of calcineurin A (*PPP3CA* gene), up to 1 month after LFS administration. Moreover, to further address whether LFS' restorative effects on learning and memory are exerted through changes in seizure-induced synaptic potentiation, we investigated the effect of LFS application on evoked field potential responses in the CA1 region of the hippocampus.

METHODS

Animals

Five- to six-week-old male Wistar rats were housed with water and food ad libitum, under controlled temperature conditions (22–25°C) and light/dark cycle (12–12 h, lights on 7 am). All experimental and animal care procedures were performed according to the ethical guidelines set by the Ethical Committee of Faculty of Medical Sciences, Tarbiat Modares University (reference #: 52D/6589), which were completely in accordance with the “NIH Guide for the Care and Use of Laboratory Animals”.

Kindling procedure and LFS application

The rats underwent stereotaxic surgery under (100 mg/kg) ketamine and (10 mg/kg) xylazine (i.p.) anesthesia and received a tripolar stimulating/recording electrode in CA1 region of the right hippocampus at the following coordinates: 3.2 mm posterior and 2 mm lateral from bregma, and 2.3 mm below dura (Paxinos and Watson, 2013). The tripolar electrode was made by twisting three strands of Teflon-coated stainless-steel wires with a diameter of 203 μ m (A-M Systems, USA). A monopolar reference/ground electrode was connected to the skull by a miniature screw. All electrodes were connected to the pins of a small multi-channel plastic socket as a head stage and affixed to the skull with dental acrylic.

We allowed a one-week recovery period following the surgical operation before starting any experiments. As ketamine and xylazine have a short half-life (2 h and 1 h respectively) (Veilleux-Lemieux et al., 2013), the effects of both drugs were minimized (or removed) by the long recovery period. Afterdischarge (AD) threshold was measured to determine the stimulus intensity. For this purpose, rats were stimulated at the intensity of 30 μ A (1 ms monophasic square pulses, 50 Hz for 3 s). If no AD was triggered, the stimulus intensity was increased at 10 μ A steps at 20 min intervals until AD duration (ADD) evoked for at least 20 s. Twenty minutes after setting the stimulus intensity, the kindling procedure was started by delivering 6 daily stimulations at 20 min intervals. The local field potentials were recorded for at least 200 s after stimulation, and the behavioral seizure stages were rated according to Racine's scales (Racine, 1972). Animals received kindling stimulations until they achieved a fully-kindled state, defined as the appearance of stage 5 seizures on three consecutive days.

LFS was applied in four sessions (immediately, 6 h, 20–24 h and 26–30 h after the final kindling stimulation). It included 5 trains of 200 monophasic square wave pulses at 1 Hz with 1 ms pulse duration. LFS intensity was equal to the AD threshold for each kindled rat. The LFS pattern was achieved according to our previous study (Ghafouri et al., 2016).

Barnes maze

Spatial learning and memory was evaluated using the Barnes maze test (Barnes, 1979). The maze consisted of a black Plexiglas circular platform, 122 cm in diameter on a 90 cm stand with 18 evenly spaced 9-cm-diameter holes arranged in a clock face manner around the circumference, with an escape box (30 × 11.5 × 15 cm) placed under one of the holes (target hole). Other holes were blocked by a black Plexiglas plate 12 cm in diameter. The surface of the maze was illuminated by a lamp. Four visual cues were placed around the table, 20 cm away from the circular platform to facilitate spatial orientation for the rats. Animals performed 3 trials per day with a 20-min inter trial interval on 3 consecutive days for training. At day 4, the escape box was replaced with a black Plexiglas plate and animals performed a probe test.

Twenty to thirty minutes before the test, the animals' home cages were put in the lab for habituation. Then, each animal was placed in the center of the maze in the start box (20.5 × 19.5 × 24.5 cm) for 10 s. The start box was removed, the animal was released to search for the target hole for 3 min, and then it was placed inside

the escape box for 30 s by the experimenter. The platform and escape box were cleaned with 70% ethanol after each trial. The escape box and visual cues were rotated 90 degrees from their original place each day. The probe trial was performed on the fourth day, and performance was recorded for 180 s.

The behavioral parameters were measured by offline analyses of the video recordings using EthoVision XT 11 (Noldus Information Technology, Wageningen, The Netherlands). A reviewer who was blind to group identity evaluated the video recordings.

The measured parameters included (a) Primary latency: the time that an animal took to find the target hole for the first time (during 3 days of training); (b) Number of errors: number of incorrect holes explored by the animal, exploration was indicated by the rat lowering its nose and sniffing the table surface; (c) Hole exploration frequency in the goal sector (GS): the sum of the explorations of the target hole and its adjacent right and left holes divided by 3, during the probe test; (d) Hole exploration frequency in the non-goal sector (NGS): the sum of explorations of the 15 non-goal holes divided by 15, in the probe test; (e) Goal sector preference: the ratio of GS to NGS explorations — this parameter provides an index of remembered spatial orientation of the GS in animals; (f) Target-seeking activity: the sum of explorations for all holes in the probe test, divided by 18 (i.e., mean explorations per hole); (g) Strategies: tracking patterns that an animal chooses to find the target hole. Strategies were classified as (1) random: animals moved back and forth across the platform until the escape box was found, (2) serial: animals traveled around periphery of the maze until they found the correct hole and (3) direct: animals moved to escape box directly or with less than two errors.

RNA extraction, cDNA synthesis and quantitative real-time PCR experiments

For quantitative real-time PCR, animals were sacrificed and their right dorsal hippocampus was removed immediately. Considering the fact that we applied the kindling stimulation and LFS to the right dorsal hippocampal CA1 area, and because different regions of dorsal hippocampus have important roles in spatial learning and memory, brain tissue sampling was made from right dorsal hippocampus for qPCR. Then, samples were frozen on dry ice and stored at -80°C until further processing.

(a) *RNA preparation and reverse transcription*: According to the manufacturer's instructions, the phenol-chloroform extraction method (Chomczynski and

Sacchi, 1987) and a total RNA extraction kit (Parstous, Iran) was used for total mRNA isolation. The total RNA was suspended in 30 μ l of DEPC (diethyl-pyrocabonate) treated water. A spectrophotometer (NanophotometerNP80, Germany) was used for determination of the RNA purity and concentration. 3 μ l of total RNA, 2 μ l Oligo-dT primer (Parstous, Iran) and 10 μ l reverse transcriptase (Parstous, Iran) were used for cDNA synthesis with the RT-PCR technique.

(b) *Real-time PCR*: A quantitative PCR instrument (Rotor-Gene Q, Qiagen, Hilden, Germany) and SYBR Green real-time Master Mix Kit (Ampliqon, Herlev, Denmark) was used for this assessment. Each 20 μ l reaction mixture contained SYBR Green PCR Master Mix, the primers, and a 3 μ l cDNA sample. Real-time PCR was performed in 36 well plates with initial heating for 15 min at 95°C and then 35 cycles of 95°C (60s); 60°C (60 s); and 72°C (60 s). GAPDH was used as an endogenous control to normalize expression level of target genes by the $2^{-\Delta\Delta CT}$ method. Melting curve analysis was used for all PCR reactions to confirm the amplification specificity. Specific primers for rat *Grin2a* and *Grin2b*, *Gabrg2*, *Ppp3ca* and *Gapdh* were designed and purchased from CinnaGen, Iran (Table I). We calculated the *Grin2a* to *Grin2b* ratio after estimating the changes in mRNA expression.

Evoked field potential recording

For field potential recordings, animals underwent stereotaxic implantation of a monopolar recording electrode in the CA1 stratum radiatum (2.8 mm posterior to bregma, 1.8 mm lateral to the midline, 2.3–2.5 mm below dura) and a bipolar stimulating electrode in the Schaffer collaterals of the right dorsal hippocampus (3.1 mm posterior to bregma, 3.5 mm lateral to the midline, 3.1–3.5 mm below dura) (Paxinos and Watson, 2013). The electrode depths were adjusted using electrophysiological responses. Once the location of the

electrodes was verified, the electrodes and entire assembly (including two stainless steel screws as reference and ground electrodes and multichannel socket) were sealed and fixed to the skull with dental acrylic.

After a post-surgery recovery of 7–10 days, field potentials were recorded from freely moving animals. Signals were bandpass filtered with cutoff frequencies of 1–3000 Hz and digitized at 10 kHz. Responses were evoked using monophasic square wave pulses (120 pulses, 0.1 ms pulse duration, 0.1 Hz) at test pulse intensity. Test pulse intensity was determined the day before starting the kindling procedure as 50% of the maximum response according to input-output relationship. This intensity was used for field EPSP (fEPSP) recording in all sessions. Recordings were carried out at four time points: a) before starting the kindling stimulations—baseline recording; b) on the last day of kindling procedure—after kindling recording; c) after applying 4 packages of LFS in kindled + LFS groups or at the equivalent time in sham, kindled and LFS groups – after LFS recording – and d) 30–60 min after completing the Barnes maze test – after Barnes recording. The fEPSP slope was calculated as the slope of fEPSP rising phase in linear portion (10–90% of amplitude).

Experimental design

Animals were assigned to 5 groups: control, sham, kindled, kindled + LFS and LFS. In the kindled group, animals received daily kindling stimulations until they exhibited stage 5 seizures on three consecutive days. In the kindled + LFS group, animals were treated in the same manner, but LFS was applied in 4 sessions, as described above. In the LFS group, animals were manipulated similarly to the kindled + LFS group, but received only LFS (without kindling stimulations). Another group of animals underwent surgery and stereotaxic electrode implantation but did not receive any kindling or LFS stimulation – the sham group. In this group,

Table I. Nucleotide sequences of the primers used for real-time PCR.

Gene name	Primer	Product length	Accession number
GAPDH	F: TCCCCATTCTTCCACCTTTGATGCT R: ACCCTGTGCTGTAGCCATATTCAT	104	NM-017008
Calcineurin _(A-α)	F: TGACCACTTCTGTTCACTTTTTTT R: GCAAGAACATCCAAGTCTGAG	80	NM-017041.1
NR2A	F: CAATCTGACTGGATCACAGAGC R: CTGTCTTCCCTTGAAAGGATC	207	NM-012573
NR2B	F: CGGATGACACCCGACCAAGAA R: GGACTCATCTTATCCGCCA	122	NM_012574.1
GABA _{A(γ2)}	F: ACCATAGCCCGGAAGTCTCT R: CCTTGCTTGTTTCCGGTTG	145	NM-183327.1

the elapsed time between surgery and other tests was similar to animals in the kindled and/or kindled + LFS group. Additionally, a control group was included for behavioral and gene expression experiments, in which animals did not undergo surgery and did not receive any stimulation. As no significant differences were found between sham and control groups, their data were merged and considered together as control group. The number of animals used for learning and memory experiments was $n=6$ for control, kindled and kindled + LFS groups and $n=5$ for the LFS group. For gene expression studies, $n=5$ for control and $n=4$ for kindled, kindled + LFS and LFS groups. In the field potential recording experiments, 5 animals were assigned to each group. The number of animals were determined so that the power was equal or greater than 0.8.

To assess the time-dependent effects of LFS on kindling-induced learning and memory impairment and gene expression, all experimental groups were divided into three subgroups in which the behavioral test or hippocampal sampling were done at 2–3 h, 1 week, and 1 month after applying LFS in kindled + LFS and LFS groups or equivalent time in kindled and control groups. When the Barnes maze test was started at 2–3 h after LFS, the LFS' significant restorative effect on learning and memory was observed and continued for 4 days. Consequently, field potentials recordings were continued until the end of this procedure. The selected times were selected according to our previous study (Esmailpour et al., 2017b), in which we observed short- (at 24 h) and long-term (at 1 week) effects of LFS on kindling-induced cognitive impairments.

Statistical analyses

Data were analyzed by GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA, USA) and presented as mean \pm SEM. A two-way repeated measures ANOVA followed by a Tukey's *post-hoc* test was used to compare Barnes test data among different groups of animals during experiments. In addition, a one-way ANOVA and Tukey's *post-hoc* test were employed to compare parameters of the Barnes probe test, as well as real-time PCR data. For the field potential recordings, the mean response value obtained on the day preceding the first kindling procedure was considered baseline, and subsequent data were expressed as the percent changes from the baseline. A two-way ANOVA followed by Tukey's *post-hoc* test was used to assess for changes in fEPSP slope. $P<0.05$ was considered the threshold for statistical significance. In addition, in order to evaluate the effect of learning on the fEPSP slope in kindled and kindled + LFS group more precisely, the effect size index

was determined by computing the Cohen's *d* before and after the Barnes maze test. According to Cohen's *d*, effect size was classified as small ($d=0.2$), medium ($d=0.5$) and large ($d\geq 0.8$).

RESULTS

Inhibitory effect of LFS on kindled seizures

After the first kindling stimulation, there were no significant differences between kindled and kindled + LFS groups for AD threshold (63.31 ± 27.47 μ A in kindled and 51.17 ± 16.17 μ A in kindled + LFS group) or ADD (195.5 ± 17.91 s in kindled and 188.7 ± 25.19 s in kindled + LFS group). In addition, the mean number of stimulations to achieve a fully kindled state was similar in the kindled (48.41 ± 4.63) and the kindled + LFS (48.37 ± 3.45) groups. Therefore, there was no significant difference in seizure susceptibility between the animals used in these two experimental groups.

Our previous study showed that the LFS pattern used in the present study (4 packages on two consecutive days) had anticonvulsant effects in fully kindled animals. Applying LFS decreased ADD and increased latency to the onset of a stage 4 seizure, compared to the same animals before LFS application (Esmailpour et al., 2017a).

Effect of LFS on kindling-induced impairment of spatial learning and memory

The effect of LFS on kindling-induced impairments in spatial learning and memory was assessed by the Barnes maze test 2–3 h following LFS and followed up by assessment 1 week and 1 month later in separate groups of animals.

Spatial learning took place in all experimental groups at 2–3 h, 1 week and 1 month following LFS, as a decrease in primary latency was observed over the 3 days of the training phase (Fig. 1A). A two-way repeated measures ANOVA analysis followed by a Tukey's *post-hoc* test revealed no significant difference between the kindled and the kindled + LFS groups. To evaluate changes in primary latency among experimental groups more precisely, we calculated the primary latency at day 3 of the training phase as a percentage of day 1. One-way ANOVA indicated a significant increase in this parameter in the kindled ($39.10\pm 4.97\%$; $n=6$) compared to the control group ($17.63\pm 2.74\%$; $n=6$) but only soon after kindling (equivalent to 2–3 h after applying LFS) (Fig. 1B). A significant decrement was also observed in the kindled + LFS group compared to the

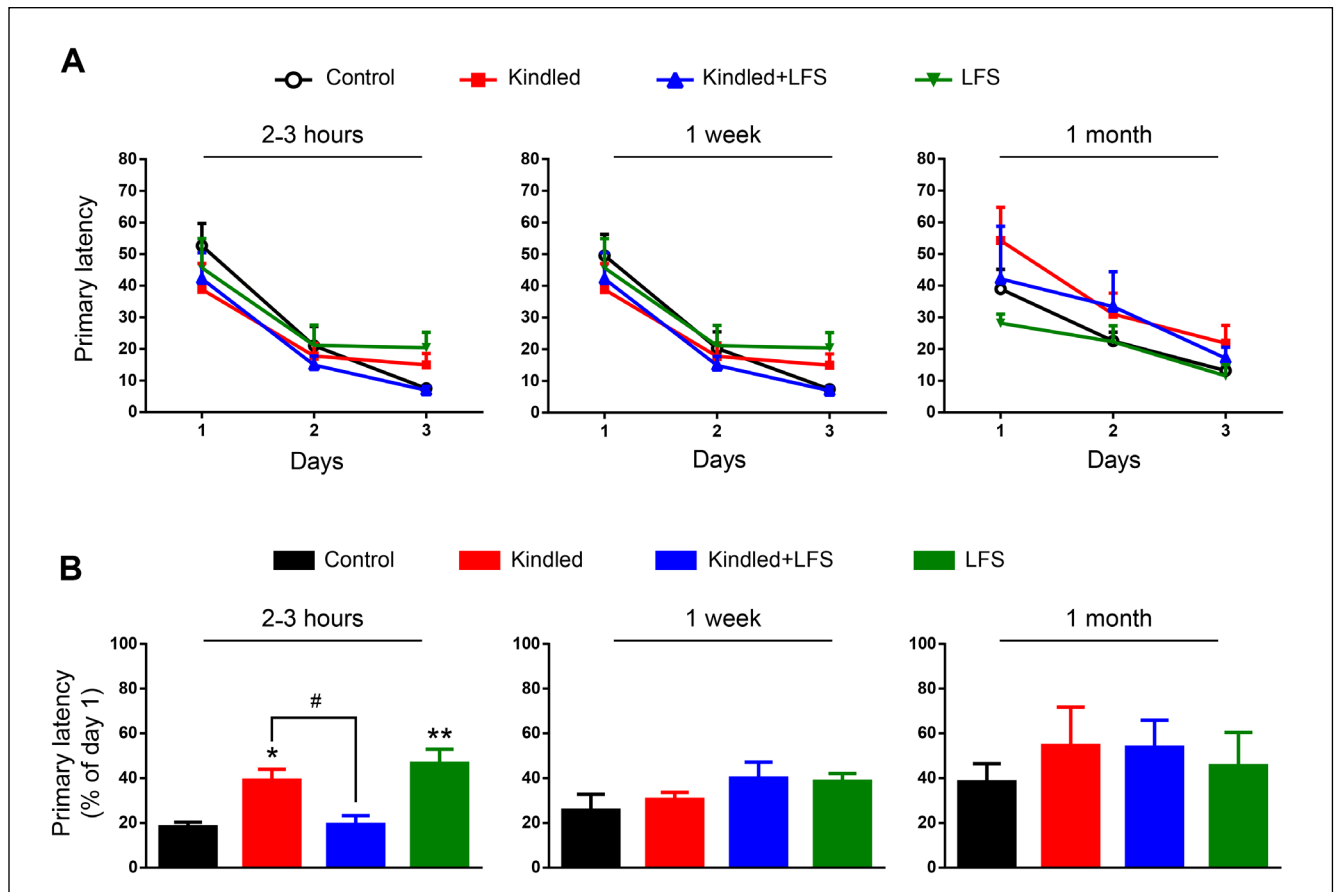


Fig. 1. Effect of LFS on spatial learning and memory in the Barnes maze test. (A) Primary latency at 2–3 h, 1 week and 1 month after LFS. (B) Primary latency as a percentage of day 1 during the same times as in A. All data shows mean \pm SEM. * $P < 0.05$ compared to control group. # $P < 0.05$ compared to kindled group (n=6 in control, kindled and kindled + LFS and n=5 in LFS groups).

kindled animals at this time (19.26 ± 4.07 , $n=6$; $P < 0.05$). Similar to the kindled animals, LFS alone increased the primary latency at day 3 as a percentage of day 1 when the Barnes maze test was carried out 2–3 h after applying LFS (46.60 ± 6.29 , $n=5$; $P < 0.01$).

A two-way repeated measures ANOVA using data obtained 2–3 h after administration of LFS revealed a significant effect of treatment ($F_{3,19}=5.76$; $P < 0.01$) and time ($F_{2,38}=12.01$; $P < 0.0001$) on the number of errors (Fig. 2A). The results showed a significant increase in the number of errors at this time in the kindled, compared to control, animals on day 1 ($P < 0.05$) and day 3 ($P < 0.01$) of training. Application of LFS significantly decreased errors in kindled + LFS animals on day 3 compared to kindled animals at the same time ($P < 0.05$).

Similarly, measuring the number of errors 1 week after the final kindling stimulation revealed a significant impairment in the performance of kindled animals on the third training day compared to control ($P < 0.05$). Administration of LFS significantly decreased the number of errors in kindled + LFS animals compared to kindled animals on the third training day ($P < 0.05$)

(Fig. 2B). There were no significant differences between experimental groups 1 month after applying LFS.

Exploration frequency in the GS was also evaluated during the probe test. GS exploration was significantly decreased in kindled animals compared to controls at the time points equivalent to 2–3 h ($P < 0.001$) and 1 week ($P < 0.01$) after LFS application. Applying LFS improved animals' performance so that a significant increase in GS exploration frequency was observed in kindled + LFS animals ($P < 0.05$) compared to the kindled group at 2–3 h and 1 week after applying LFS ($P < 0.05$) (Fig. 3A). No significant changes were observed after 1 month. In addition, for kindled animals, NGS exploration frequency was significantly higher than in the control group 2–3 h after LFS ($P < 0.05$). LFS treatment in the kindled + LFS group significantly decreased NGS exploration compared to the kindled group as measured 2–3 h post LFS ($P < 0.05$). No significant changes were found at 1 week and 1 month after LFS administration (Fig. 3B).

GS preference (GS/NGS ratio) was also evaluated, as a spatial memory index. GS preference was significant-

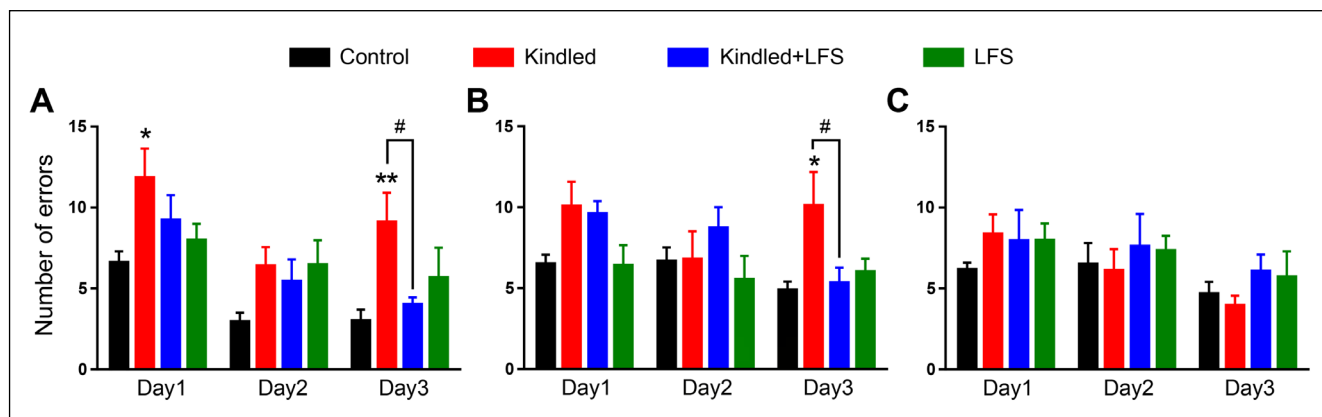


Fig. 2. Effect of LFS on the number of errors during training days in the Barnes maze test at 2–3 h (A), 1 week (B) and 1 month (C) after applying LFS. All data are mean \pm SEM. * $P < 0.05$; ** $P < 0.01$ compared to control group. # $P < 0.05$ compared to kindled group ($n = 6$ in control, kindled and kindled + LFS and $n = 5$ in LFS groups).

ly reduced in kindled animals compared to controls at the time points equivalent to 2–3 h and 1 week following LFS ($P < 0.001$). Applying LFS in kindled + LFS animals significantly enhanced GS preference compared to the kindled group at 2–3 h and 1 week after LFS ($P < 0.001$) (Fig. 3C).

Next, we analyzed target-seeking behavior across the experimental groups. The data revealed a significant reduction in target-seeking activity in kindled animals compared to controls at the time points equivalent to 2–3 h and 1 week after LFS ($P < 0.01$). Applying LFS following kindling stimulations improved target-seeking activity in the kindled + LFS group at 2–3 h ($P < 0.05$) and 1 week after the final LFS session (although not significantly) compared to the kindled group (Fig. 3D).

Chi-square analysis indicated a significant difference in search strategy distribution in all experimental groups at all 3 time points ($P < 0.05$ for 2–3 h and 1 week; $P < 0.01$ for 1-month group). As previously described, the tracking strategy that an animal used to find the target hole was either direct, serial or random (Fig. 4A). The search pattern of control animals at all time points changed through training days, and it was accompanied by a decrease in random and serial strategies and increase in the use of a direct strategy over 3 days of training (Fig. 4).

In kindled groups, animals primarily used a serial strategy to find the escape box; by day 3 of training, employing a direct search pattern was abandoned at all evaluated times. In kindled + LFS animals, the LFS application led to animals utilizing a direct search strategy (Fig. 4).

There was no significant difference in mean velocity during the training phase among experimental groups at different time points. This implies that differences in performance were not dependent on an animal's locomotor activity (data not shown).

Effect of LFS on mRNA expression of *Grin2a*, *Grin2b*, *GABRG2* and *PPP3CA*

The Barnes maze test revealed that LFS application improved spatial learning and reduced the spatial memory impairments in kindled animals. Then, we evaluated whether these LFS-moderated improvements were accompanied by changes in gene expression. Thus, in the next experiment, we investigated the mRNA expression levels of *Grin2a*, *Grin2b*, *Gabrg2* and *Ppp3ca* in the different experimental groups at 2–3 h, 1 week and 1 month after applying LFS.

In kindled animals, there was a significant increase in *Grin2b* expression (kindled: $1,607 \pm 460.60$, $n = 4$; control: 114.50 ± 22.76 , $n = 5$; $P < 0.01$) and significant decrease in the NR2A to NR2B ratio (kindled: 0.02 ± 0.00 , $n = 4$; control: 1.03 ± 0.14 , $n = 5$; $P < 0.01$) soon after kindling (equivalent to 2–3 h after applying LFS). Kindling did not significantly affect *Gabrg2* mRNA expression compared to controls at this time (Fig. 5). At the same time point (2–3 h after applying LFS), *Grin2b* expression significantly decreased (35.38 ± 6.32 , $n = 4$; $P < 0.01$), and the *Grin2a* to *Grin2b* ratio (0.78 ± 0.20 , $n = 4$; $P < 0.01$) and the expression of *Gabrg2* (191.9 ± 37.46 , $n = 4$; $P < 0.05$) increased, in the kindled + LFS group compared to the kindled group. There was no significant difference in *Ppp3ca* expression among kindled, kindled + LFS and control groups. Applying LFS alone significantly increased *Gabrg2* (307.9 ± 19.51 , $n = 4$; $P < 0.0001$) and decreased *Ppp3ca* expression (20.41 ± 3.17 , $n = 4$; $P < 0.05$) compared to controls at 2–3 h after administration of LFS (Fig. 5E).

The NR2A to NR2B expression ratio was still significantly lower in the kindled compared to the control group at 1 week after the final LFS (kindled: 0.10 ± 0.03 , $n = 4$; control: 0.81 ± 0.17 , $n = 5$; $P < 0.05$). *PPP3CA* expression also significantly decreased in kindled compared to control group (kindled: 14.69 ± 3.51 , $n = 4$; control: 102.20 ± 30.49 ,

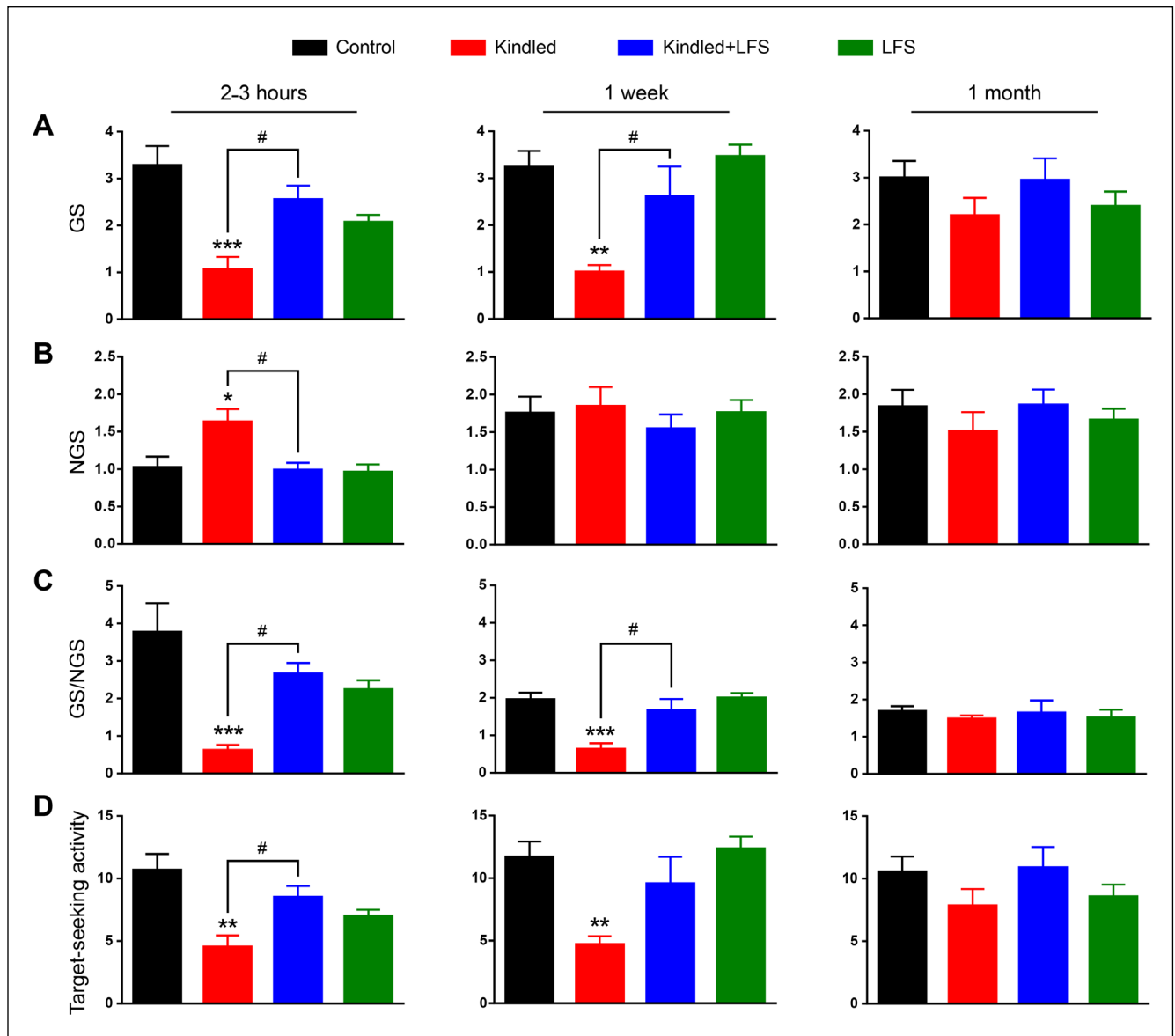


Fig. 3. Effect of LFS on hole exploration frequency in the goal sector – GS (A), hole exploration frequency in the non-goal sector – NGS (B), goal sector preference – GS/NGS (C) and target-seeking activity (D) in the Barnes maze test at 2–3 h (left), 1 week (middle) and 1 month (right). All data represents mean \pm SEM. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ compared to control group. # $P < 0.05$ compared to kindled group ($n = 6$ in control, kindled and kindled + LFS and $n = 5$ in LFS groups).

$n = 5$; $P < 0.05$) at this time point. A non-significant decrease in NR2A expression was also seen in the kindled group 1 week following the final stimulation. One week after its application in fully-kindled animals, LFS significantly increased the NR2A to NR2B ratio (0.77 ± 0.17 , $n = 4$; $P < 0.05$) and NR2A expression (194.60 ± 48.31 , $n = 4$; $P < 0.01$) compared to the kindled group. LFS application in fully-kindled animals also prevented a significant decrease in *Ppp3ca* expression (Fig. 5).

There was no significant difference in *Grin2a*, *Grin2b*, *Gabrg2* or *Ppp3ca* between experimental groups at 1 month after applying LFS (data not shown).

Effect of LFS on kindling-induced potentiation

As explained before, field potential recordings were performed at 4 time points during the experiments. These time points were before starting the kindling stimulations (baseline recording); on the final day of kindling procedures (after kindling); following application of LFS (after LFS, i.e., before starting the first session of Barnes maze test) and immediately after the final learning trial on the third day of the Barnes maze test in the kindled + LFS group or at equivalent times in other groups.

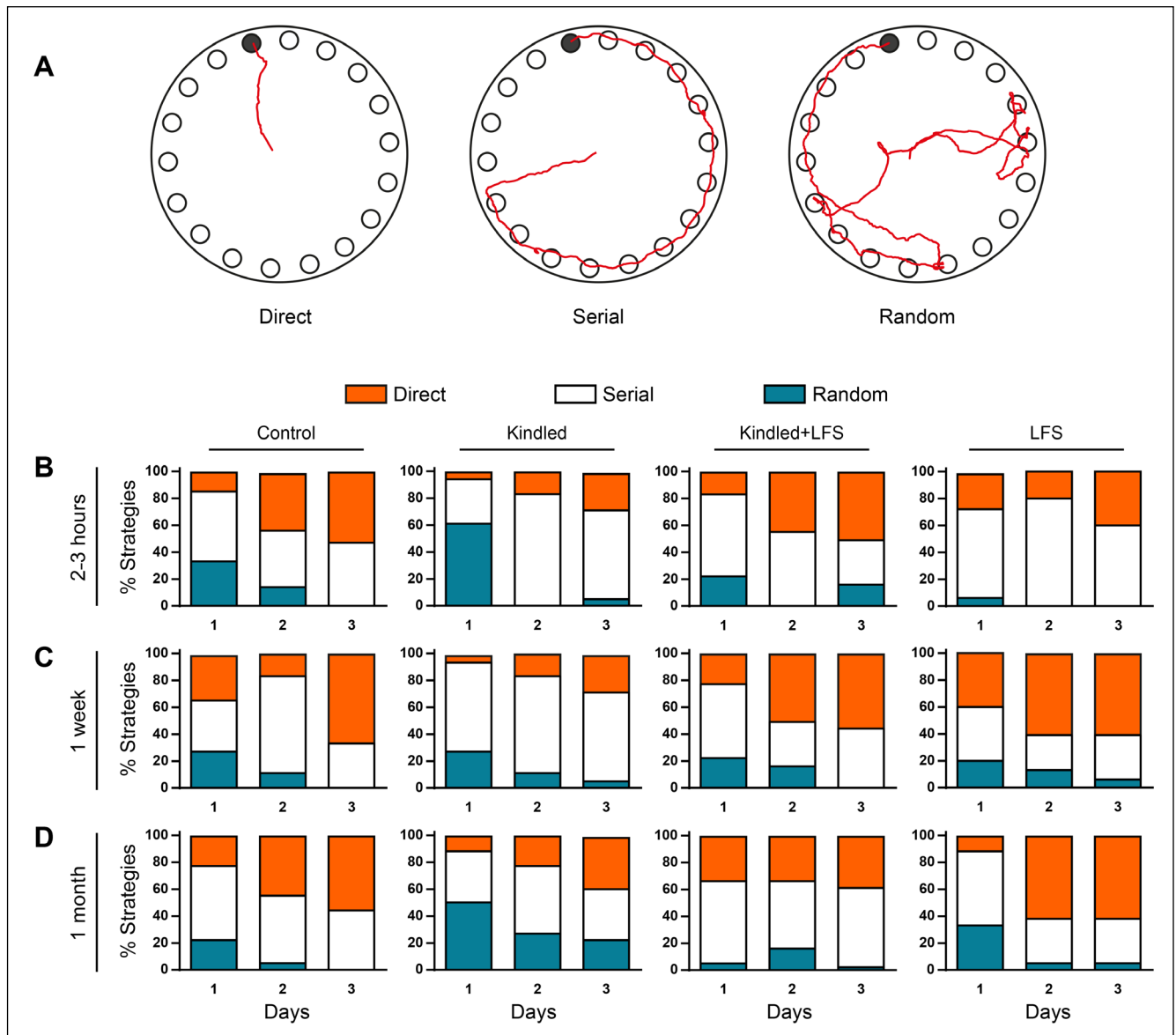


Fig. 4. Changes in Barnes maze strategies in different experimental groups. Strategies were divided into direct (A, left), serial (A, middle) and random (A, right). Quantitative comparison of data obtained at 2–3 h (B), 1 week (C) or 1 month after final LFS application are shown. $P < 0.05$ for 2–3 h and 1 week and $P < 0.01$ for the 1-month group ($n = 6$ in control, kindled and kindled + LFS and $n = 5$ in LFS groups).

There was no significant difference in test pulse intensity between the experimental groups (control: $300.00 \pm 45.52 \mu\text{A}$, kindled: $306.00 \pm 43.26 \mu\text{A}$, kindled + LFS: $285.27 \pm 41.92 \mu\text{A}$ and LFS: $274.80 \pm 31.65 \mu\text{A}$; $n = 5$). A two-way repeated measures ANOVA showed a significant effect of treatment ($F_{3,16} = 10.48$; $P < 0.001$) and time factor ($F_{2,32} = 3.40$; $P < 0.05$) and their interaction ($F_{6,32} = 7.77$; $P < 0.0001$) on fEPSP slope. As Fig. 6 shows, synaptic transmission was potentiated due to kindling acquisition and there was a significant increase in fEPSP slope in kindled and kindled + LFS groups compared to sham group after kindling procedure (kindled: $178.18 \pm 10.71\%$, kindled + LFS: $173.44 \pm 22.42\%$, sham:

$103.57 \pm 3.73\%$; $P < 0.001$). This potentiation was nearly reduced to the mean value of the sham group by applying LFS in the kindled + LFS group and resulted in a significant difference between kindled + LFS and kindled group (kindled + LFS: $122.64 \pm 14.44\%$, kindled: $182.62 \pm 13.32\%$, sham: $108.71 \pm 3.37\%$; $P < 0.001$).

LTP induction has been documented as necessary for normal learning and memory (Quinlan et al., 2004; Rioult-Pedotti et al., 2000; Whitlock et al., 2006). Therefore, to determine whether the ameliorating effect of LFS on learning and memory is accompanied by learning-induced synaptic potentiation, field potentials were recorded 30–60 min after the final learning trial

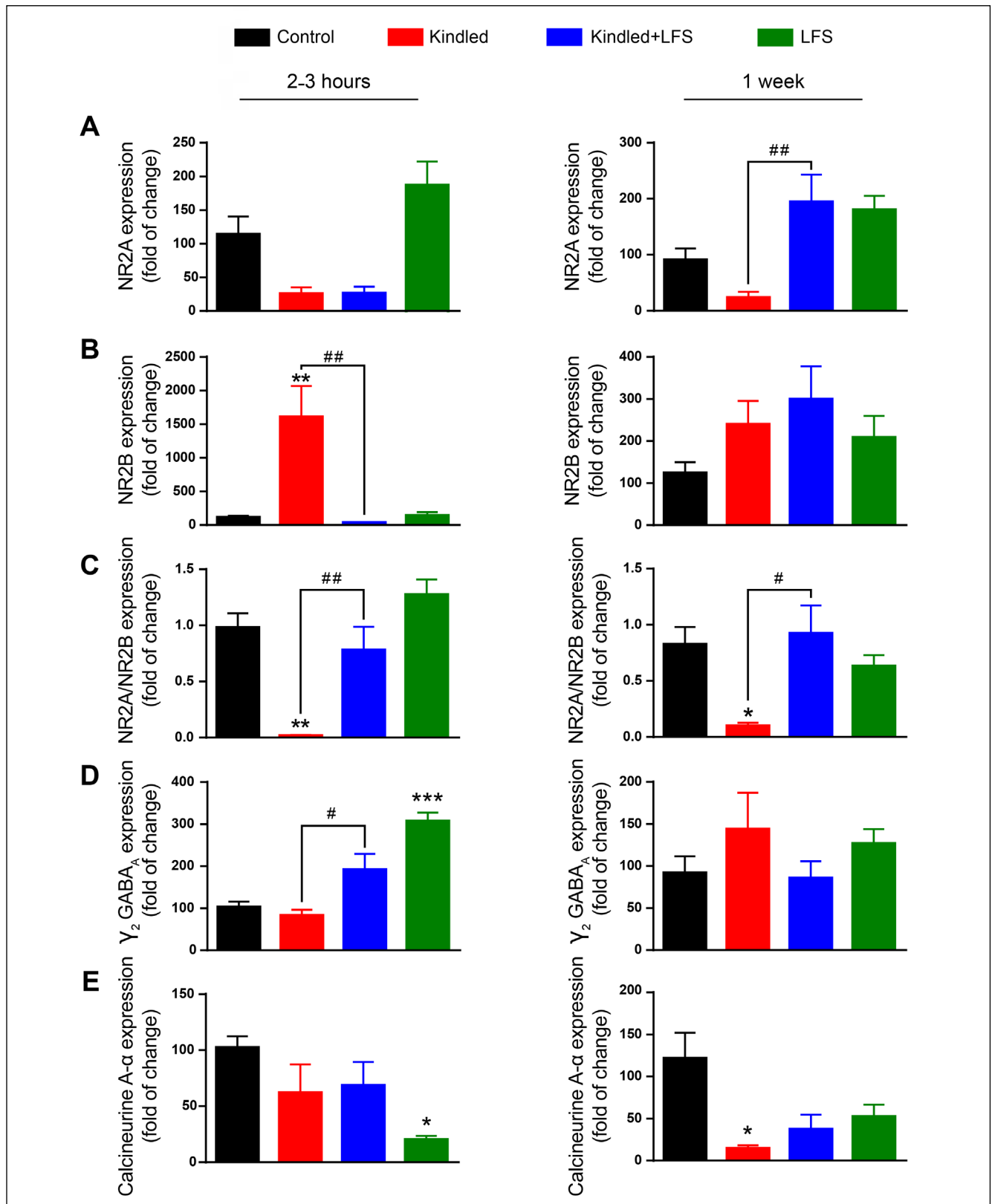


Fig. 5. Effect of LFS on gene expression at 2-3 h and 1 week following LFS administration. A) *Grin2a*, B) *Grin2b*, C) *Grin2a/Grin2b* ratio, D) *Gabrg2*, E) *Ppp3ca*. All data are represented as mean \pm SEM. * P<0.05; ** P<0.01; *** P<0.001 compared to control group. # P<0.05; ## P<0.01 compared to kindled group (n=5 in control and n=4 in kindled, kindled + LFS and LFS groups).

of the Barnes maze test. The fEPSP slope was reduced in the kindled group and enhanced in the kindled + LFS group following the Barnes maze test compared to before the Barnes maze test (i.e., after LFS). Although these alterations were not statistically signifi-

cant, examining the effect size by computing Cohen’s *d* showed a large effect of learning in the sham group ($d=1.01$), a medium effect of learning in the kindled + LFS group ($d=0.47$) and a small effect in the kindled group ($d=0.26$).

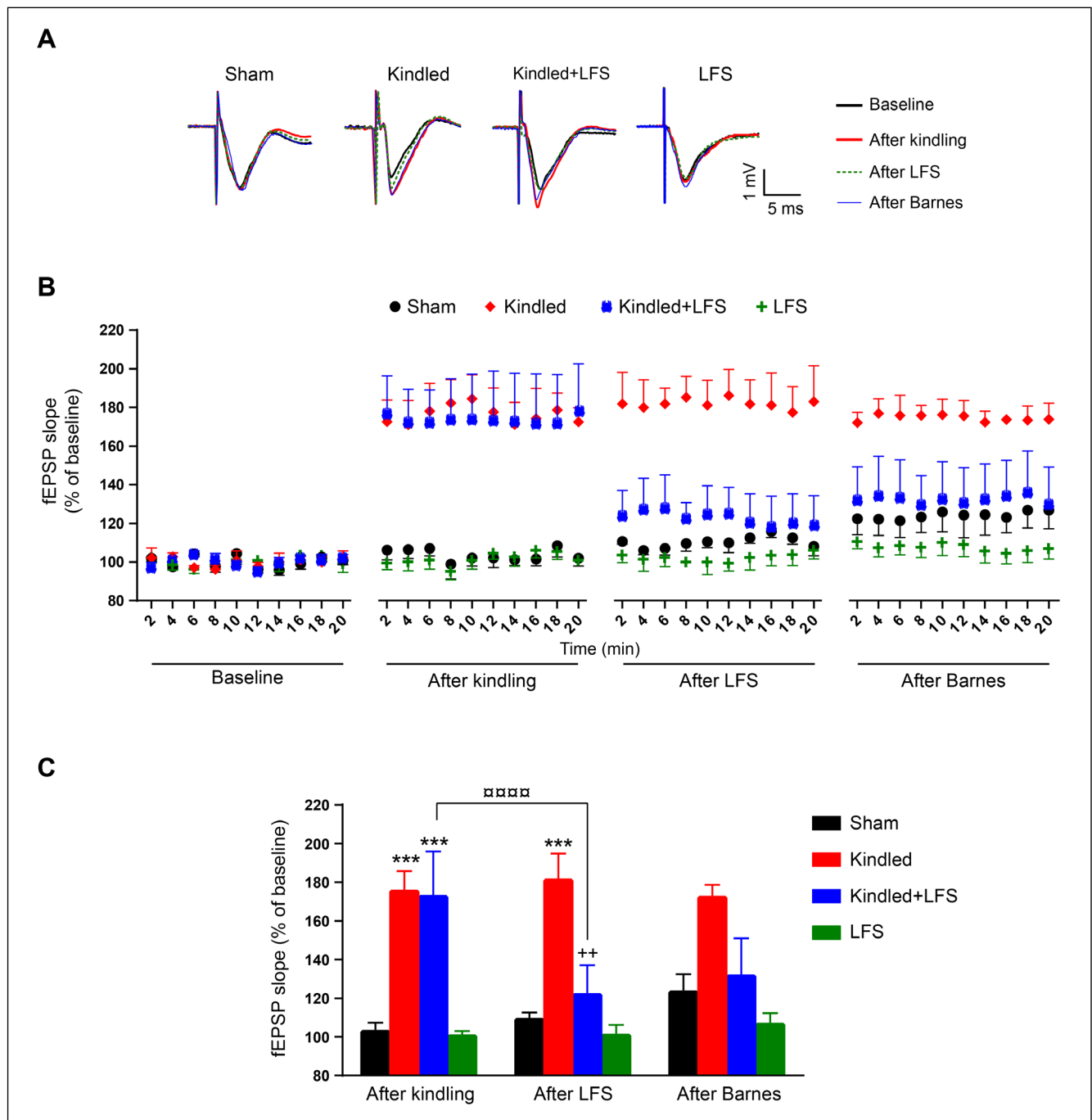


Fig. 6. LFS application removes kindling-induced potentiation and restores learning-induced potentiation in the CA1 area of hippocampus when examined 2–3 h after the LFS. (A) Sample traces of fEPSP responses (average of 12 waveforms) taken before starting the kindling procedure (baseline), after kindling achievement, following the LFS application and subsequent to the ending of the Barnes maze test learning period in different experimental groups. (B) Time-course diagrams showing the changes in fEPSP slope in different experimental groups ($n=5$) during 20 min of recording at various times of the experimental procedure. (C) Quantitative comparison of fEPSP slope among different experimental groups. Data are represented as mean \pm SEM. *** $P<0.001$ compared to control group, and # $P<0.01$ and **** $P<0.001$ ($n=5$).

Applying LFS alone did not affect synaptic transmission, as no significant changes in fEPSP slope were observed between LFS and sham groups in all recording sessions.

DISCUSSION

Our findings revealed that applying LFS to the hippocampal CA1 region of kindled animals in the same pattern that had previously achieved an anticonvulsant effect (Esmailpour et al., 2017a) improved seizure-induced spatial learning and memory impairments. These effects were accompanied by increases in the *Grin2a* to *Grin2b* expression ratio and *Gabrg2* and *Ppp3ca* expression. LFS application was also able to eliminate the kindling-induced enhancement of synaptic transmission and restore learning-induced potentiation at Schaffer collateral-CA1 synapses.

We observed a significant impairment in Barnes maze test performance in kindled animals at 2–3 h and 1 week after the final LFS. Therefore, our findings support the previous reports that CA1 kindling disrupts memory performance in animals (Leung and Shen, 1991; Gilbert et al., 1996; 2000). These results are also consistent with clinical reports that epileptic patients suffer from spatial learning and memory impairments (Giovagnoli and Avanzini, 1999; Amlerova et al., 2013).

In the present study, applying LFS to kindled animals restored the animals' performance in a Barnes maze test, compared to kindled animals at 2–3 h and 1 week after applying LFS. These findings support our previous results that applying LFS restored the kindling-induced impairment in Y-maze (Ghafouri et al., 2016) and Morris water maze tests (Esmailpour et al., 2017a). Of course, it must be noted that the Morris water maze test is accompanied by swimming stress for animals, which can significantly affect their spatial memory and performance (Hölscher, 1999; Sunyer et al., 2007). In addition, it has been shown that stress alone causes brain network excitability and increases seizure-induced impairments in learning and memory (Song et al., 2006; Dubé et al., 2015). Therefore, in this study we used the Barnes maze test for spatial learning and memory evaluation.

Spatial learning and memory impairments following seizure development may result from long-lasting potentiation of neurotransmission in hippocampal synapses (i.e., kindling-induced potentiation) (Cain, 1989; Barnes et al., 1994). This potentiation is mediated by an imbalance in excitatory to inhibitory transmission as a result of the changes in synaptic receptor expression (Bonansco and Fuenzalida, 2016). Apply-

ing LFS may ameliorate seizure-induced memory impairments by reducing synaptic potentiation through LTD or depotentiation-like mechanisms (Bashir and Collingridge, 1994; Burette et al., 1997). This effect leads to a restored capacity for synaptic plasticity and allows learning-induced synaptic potentiation. Consistent with this argument, in the present study, LFS treatment was able to largely eliminate kindling-induced potentiation of fEPSCs in Schaffer collaterals to CA1 pyramidal neurons' synapses. In addition, these synapses were potentiated by the learning period in the Barnes maze in the kindled + LFS group but not the kindled group.

The NR2A to NR2B ratio is an important regulator of synaptic plasticity properties. An increase in the NR2A to NR2B ratio enhances the likelihood of LTD induction (Cui et al., 2013) and a decrease in this ratio heightens susceptibility to LTP induction (Cho et al., 2009). A significant increase in NR2B and a non-significant decrease in the NR2A subunit expression after kindling in this study supports *in situ* hybridization studies that showed a minor decrease in NR2A in the dentate gyrus (Pratt et al., 1993) and significant increase in the NR2B subunit in the fascia dentata (Kamphuis et al., 1995a) of fully-kindled animals. A slight increase in the NR2B subunit has been also reported at 5 days following kindling (Pratt et al., 1993).

In the present study, the observed increase in the NR2A to NR2B ratio following LFS application may provide a scenario for induction of LTD or depotentiation. In addition, the reduction in NR2B gene expression soon after kindling stimulations is consistent with our previous report in which the NMDA current decreased after LFS administration in kindled animals (Ghafouri et al., 2019). Therefore, LFS (by increasing the NR2A to NR2B ratio) may act through depotentiation and/or LTD-like mechanism(s) to reduce synaptic strength toward its normal level and in this way restore the ability for new synaptic potentiation, learning and memory.

Unlike our results, some *in situ* hybridization studies indicated a significant increase in *Grin2a* in the fascia dentata (Kamphuis et al., 1995a) and a non-significant decrease in *Grin2b* in other areas (Pratt et al., 1993; Kamphuis et al., 1995a) 24 h after the last kindling stimulation. In addition, no significant changes were reported in the NR2A subunit at 5 days following kindling (Pratt et al., 1993). These inconsistent findings may be related to the difference in sensitivity of real-time PCR and *in situ* hybridization techniques, in which the real-time PCR is more sensitive (Biedermann et al., 2004). However, it should be considered that even if the gene expression of receptors does not change, receptor function may be altered by chang-

es in receptor sensitivity or in post-receptor signal transductions.

We did not observe any changes in *Ppp3ca* gene expression in kindled animals. These findings support previous studies that showed no significant changes in *Gabrg2* expression in the dorsal hippocampus of fully-kindled animals at 24 h and 1 week post kindling (Kamphuis et al., 1995b; Nishimura et al., 2005). The mRNA expression of *Gabrg2* significantly increased 2–3 h following LFS. These data are consistent with our recent study that LFS application could increase GABA currents (Ghafouri et al., 2019). On the other hand, changes in the GABA_AR $\gamma 2$ subunit appear to be a mechanism of the anticonvulsant action of LFS.

As widely accepted, disrupting inhibitory to excitatory transmission can lead to seizure activity in the brain. Additionally, other studies have revealed that GABA_ARs are necessary for LTD induction in hippocampal synapses (Wigström and Gustafsson, 1983; Wagner and Alger, 1995). Therefore, our results suggest that the applying LFS may relieve the kindled animals' impairments through increasing the inhibitory to excitatory ratio and preventing neuronal hyper-excitability and/or LTD mechanism(s) that reverse seizure-induced potentiation at 2–3 h following LFS.

Applying LFS in kindled animals prevented a decrease in calcineurin expression at 1 week post LFS. Calcineurin may be involved in LTD (Lin et al., 2003), so it is possible that LFS may influence the improvements in kindled animals' Barnes maze performance through LTD-like mechanism(s) at 1 week following the LFS. At the same time, the NR2A/NR2B subunits ratio was also increased significantly in kindled + LFS compared to kindled animals. Previous studies have shown that an increase in NR2A/NR2B ratio facilitates the occurrence of LTD and depotentiation by limiting Ca²⁺ entry through NMDARs (Yashiro and Philpot, 2008). Therefore, the changes in calcineurin and glutamate receptors align with preparation for LTD and/or depotentiation induction. In fact, changes in these two parameters are consistent with our main hypothesis that LFS application reduced the abnormal potentiation of kindled animals in the short- (2–3 h) and long-term (one week). However, some studies on the pilocarpine model of epilepsy have reported that an increase in calcineurin activity is not associated with a change in its gene expression following a seizure (Kurz et al., 2001; 2003).

In this study, kindled animals were followed up for 1 month, and no significant change in behavioral and molecular tests were seen at this time. These findings were in line with previous studies that memory impairments could be observed less than 3–4 weeks after the final kindling stimulation in animals with generalized

seizures (Lopes da Silva et al., 1986; Feasey-Truger et al., 1993; Gilbert et al., 2000). Similar to our results, no changes in the gene expression level of GABA_A (Kamphuis et al., 1995b; Nishimura et al., 2005) and NMDA (Kamphuis et al., 1995a; Corcoran et al., 2011) receptor subunits have been reported at 2–4 weeks following the last kindling stimulation.

It was also interesting that applying LFS in non-kindled rats significantly impaired their spatial learning and memory compared to controls. These data supported our previous studies that showed LFS alone impaired Morris water maze performance (Esmaeilpour et al., 2017a). This controversy regarding LFS' effects (an “improving” effect in kindled animals and a “destructive” effect in LFS alone) shows that LFS' impact on learning and memory performance depends on levels of neuronal excitability. It is important to consider that since we used the qPCR method to investigate the effect of LFS on gene expression, this study faced two main limitations. First, with this technique, we could not study the expression of genes separately for each hippocampal subregion, while the gene expression change in each subregion may be differentially affected by LFS and has a different role in improving learning and memory by LFS. Second, changes in gene expression were evaluated at the level of mRNA expression. Although the change in protein expression is usually parallel to the change in mRNA expression, for a more realistic interpretation of the effects of LFS on receptor subunit expression, it is best to look directly at the expression of proteins.

CONCLUSION

According to the results of the present study, LFS reduced spatial learning and memory impairments up to 1 week following its administration. This effect of LFS was accompanied by changes in subunit expression of NMDA and GABA_A receptors and calcineurin in the hippocampus. LFS treatment was also able to remove abnormal, kindling-induced potentiation of fEPSP in the CA1 area of hippocampus through a depotentiation-like mechanism and restore learning-induced synaptic potentiation. However, more studies are necessary to identify the precise mechanism of LFS action.

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