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Effects of transcranial focal electrical stimulation alone and associated with a sub-effective dose of diazepam on pilocarpine-induced status epilepticus and subsequent neuronal damage in rats

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ABSTRACT

Experiments were conducted to evaluate the effects of transcranial focal electrical stimulation (TFS) applied via tripolar concentric ring electrodes, alone and associated with a sub-effective dose of diazepam (DZP) on the expression of status epilepticus (SE) induced by lithium-pilocarpine (LP) and subsequent neuronal damage in the hippocampus. Immediately before pilocarpine injection, male Wistar rats received TFS (300 Hz, 200-μs biphasic square charge-balanced 50-mA constant current pulses for 2 min) alone or combined with a sub-effective dose of DZP (0.41 mg/kg, i.p.). In contrast with DZP or TFS alone, DZP plus TFS reduced the incidence of, and enhanced the latency to, mild and severe generalized seizures and SE induced by LP. These effects were associated with a significant reduction in the number of degenerated neurons in the hippocampus. The present study supports the notion that TFS combined with sub-effective doses of DZP may represent a therapeutic tool to induce anticonvulsant effects and reduce the SE-induced neuronal damage.

1. Introduction

Epilepsy is one of the most common brain disorders worldwide with no age, racial, social class, national nor geographic boundaries. It affects about 67 million people, 85% of whom live in developing countries [1]. Despite decades of research, new antiepileptic drugs (AEDs) and advances in surgical therapy, many patients suffer from refractory epilepsy or the side effects of AEDs and surgical treatment [2].

Pharmacoresistant epilepsy, as well as other conditions such as brain tumors, ischemic brain injury and alcohol withdrawal, is associated with an increased likelihood of status epilepticus (SE), a neurologic emergency that requires immediate vigorous treatment in order to prevent brain injury [3–6]. Yet, strategies to prevent SE and its consequences in patients at high risk for SE are limited. Notably, the efficacy of diazepam (DZP) and similar first-line abortive SE treatments is incomplete and SE often continues after administration of these drugs [7,8]. Indeed, many of the medications used to stop SE have several well-known and potentially serious adverse effects, such as respiratory depression, sedation, hypotension and cardiac dysrhythmias [9].

The use of brain stimulation in the treatment of pharmacoresistant epilepsy has a long history, but few studies have focused on its acute effects to prevent SE and its consequences. Indeed, it is suggested that the protocols effective in the termination of SE are different from those used in the prevention of seizures [10].

We previously demonstrated that noninvasive transcranial focal electrical stimulation (TFS) was able to reduce the expression of pilocarpine-induced SE in Sprague–Dawley rats when applied during seizure activity via tripolar concentric ring electrodes (TCREs) (Fig. 1) [11]. The effects of TFS may last for hours and are associated with desynchronization at the beta and gamma frequencies, but not with motor contractions or pain [12,13]. These results support the notion that TFS has the potential to be a viable noninvasive therapy for SE. However, at present, it is unclear if TFS is able to prevent the SE and the subsequent neuronal damage.

The identification of therapeutic strategies that prevent SE and its consequences constitutes a major clinical need. Therefore, for the present study, we investigated if TFS associated with DZP may represent a good approach to avoid the expression of this disorder and the subsequent neuronal damage. Experiments in rats were designed to investigate if TFS alone or associated with a sub-effective dose of DZP was able to prevent the lithium-pilocarpine-induced (LP) SE and consequent cell damage in the hippocampus when applied before the pilocarpine injection. We studied the hippocampus because it is an area of the brain prone to generating seizure activity [14] and presents

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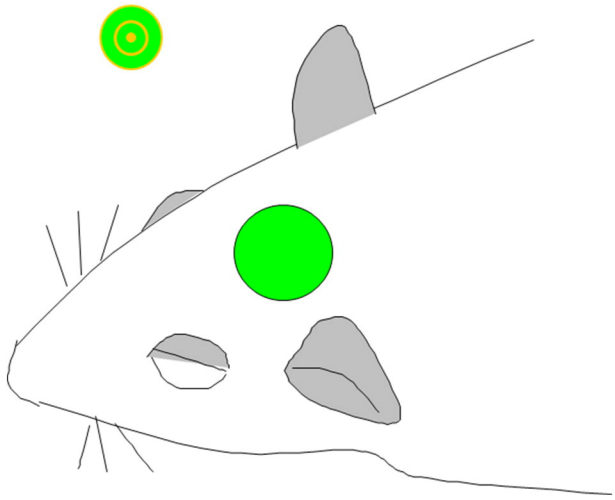

Fig. 1. Schematic representation of the electrode placement. The TFS was applied between the outer ring and the central disc of electrode.

early and late cellular events associated with neuronal damage and cognitive impairment following pilocarpine-induced SE [15,16].

2. Methods

2.1. Animals

Adult male Wistar rats initially weighing 250–300 g were used in the present study. They were individually housed at 22 °C and maintained on a 12-h light/dark cycle. Rats had free access to food and water. Procedures involving animal care were conducted in agreement with the Mexican Official Standard (NOM-062-ZOO-1999) and the Ethical Committee of the Center for Research and Advanced Studies (Protocol #222/04).

2.2. Transcranial focal electrical stimulation

The TFS consisted of 200-μs symmetrical biphasic square charge-balanced constant current pulses at a rate of 300 Hz and at an intensity of 50 mA. We employed a custom stimulator designed and built by our group, with programmable frequency, phase, and time duration of the TFS output signals. The TFS was controlled by a Parallax Basic Stamp 2P24® which had the specific TFS pattern pre-programmed to run automatically for 2 min when triggered [11,12].

The TCRE was placed on the shaved scalp centered on the top of the head, as close to 5 mm behind the bregma as possible. Approximately 2 mm of Ten-20 electrode paste was used for skin-to-electrode impedance matching. Then, TFS was applied through the outer ring (external diameter of 1 cm) and disc of a TCRE (with the middle ring floating).

2.3. Experimental groups

2.3.1. LP-TFS + DZP group ($n = 13$)

Rats received daily administration of saline solution (1 ml/kg, i.p.) for 5 days to habituate them to manipulations. Twenty-four hours after the last saline injection, the animals received lithium chloride (3 mEq/kg, i.p.). Twenty-four hours later, the scalp was shaved and TFS was applied as described above. Immediately after, the animals received the administration of pilocarpine (35 mg/kg, i.p.) and a sub-effective dose of DZP (0.41 mg/kg, i.p.). This sub-effective dose of DZP, defined as the dose reducing 30% or less the number of animals presenting LP-induced severe generalized seizures, was determined from dose–response studies carried out in our laboratory (data not shown). Then, the following parameters were assessed during 3 h of continuous behavioral monitoring by an author blinded to the treatment condition: latency to the first forelimb clonus and generalized seizure, as well as establishment of SE, and percentage of animals presenting mild (rearing and upper extremity clonus) and severe generalized seizures (rearing, upper extremity clonus, and falling), as well as SE. We utilized the definition of SE commonly used in the rat pilocarpine model, i.e., continuous motor seizures (stage 3 to 5 seizures according to Racine [17]) persisting for at least 30 min and associated with unresponsiveness to any environmental stimuli [18].

2.3.2. LP-DZP group ($n = 10$)

Rats were manipulated as indicated previously for LP-TFS + DZP group, except that they did not receive TFS.

2.3.3. LP-TFS group ($n = 14$)

Animals were manipulated as described above for LP-TFS + DZP group, except that they received vehicle administration instead of DZP.

2.3.4. LP group ($n = 24$)

Animals were manipulated as described earlier for LP-DZP group, except that they received vehicle administration instead of DZP.

2.3.5. TFS group ($n = 5$)

Rats received TFS as described above for LP-TFS group followed by saline injection, instead of LP.

2.3.6. Control group ($n = 5$)

Animals were manipulated as described above for LP group, except that they received vehicle administration instead of LP.

Rats from all pilocarpine-treated groups that went into SE received an injection of DZP (10 mg/kg i.p.) 2 h after its onset to stop the seizures, standardize the duration of continuous seizure activity and reduce the mortality rate.

2.4. Histology

Animals that survived 24 h after LP-induced SE or manipulation were injected with an overdose of pentobarbital and were transcardially perfused with 0.1-M phosphate buffered saline (PBS) and 4% paraformaldehyde solution in PBS. Then, the brains were removed and postfixed for one week at 4 °C and processed for embedding in paraffin. Coronal sections were then cut (12-μm thickness) with the aid of a microtome (Leica RM2125 RT, Germany) and mounted onto gelatin-coated slides. The sections were deparaffinized and hydrated in water for their subsequent processing for Nissl and Fluoro-Jade (FJ) staining. Fluoro-Jade is a fluorescent marker that binds to irreversibly damaged neurons and allows identification of degenerating neurons [19].

Fluoro-Jade staining was performed as follows. The slides were first immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 min. This was followed by 2 min of incubation in 70% alcohol and 2 min in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 20 min and then rinsed in distilled water for 2 min. Thereafter, the slides were incubated in FJ for 2 h. The 0.0001% working solution of FJ was prepared by adding 1 ml of stock FJ solution (0.01%) to 99 ml of 0.1% acetic acid in distilled water. Then, the slides were rinsed for 1 min in each of three distilled water washes and dried. The slides were immersed in xylene for 1 min and mounted in synthetic resin (Merck Lab.). Sections from the dorsal and ventral hippocampus corresponding to 3.30 mm and 5.60 mm from bregma, respectively, [20] were examined.

2.5. Cell counting

Fluoro-Jade positive (FJ+) neurons were counted in the dentate gyrus, CA1 and CA3. All images were digitized using an Evolution MP freeze camera (Media Cybernetics, USA) connected to an Axiolab microscope (Zeiss, Germany) and Image-Pro Plus 5.1 software to analyze the images and count the cells. The average cell density per unit volume was determined with the optical fractionator method [21,22]. This procedure allowed the determination of the fraction of tissue in which neurons were counted. The complete sectioning of the hippocampus resulted in approximately 50 to 60 sections, and every third section was sampled (for a total of 20 sections). Then, the first sampling fraction was 1/3; this is called the section sampling fraction or ssf. A volume fraction of each tissue was taken and the area sampling fraction (asf) = area (frame) / area (x y) was the area of counting frame (220 × 180 μm), relative to the area associated with each field in the computer monitor. The third sampling fraction reflected that cells were not counted in the entire thickness of the tissue at each sampling location. Instead, a three-dimensional probe of a known height was placed in the tissue. The thickness of the tissue (12 μm) divided by the height of the dissector was the third sampling fraction. This is called the tissue sampling fraction or tsf. The estimate of the total cell number was therefore the sum of cells counted ($\sum$ Q–), multiplied by the reciprocal of the three fractions of the brain region sampled as represented by the equation:

$$N = \sum Q - (1/ssf)(1/asf)(tsf)$$

where N is the estimate of the total cell number and $\sum$ Q– is the number of counted cells on all sections. In order to standardize the counting, the same volume fraction was used for each experimental group. The reader is directed to West and colleagues [21,22] for a detailed description of the optical fractionator. The qualitative assessment of FJ+ neurons was performed by an author blinded to the treatment condition.

2.6. Statistical analysis

Results of latency to the behavioral changes produced by LP in all experimental groups were compared using a one-way ANOVA and post-hoc Tukey's multiple comparison test. The percentage of animals showing mild and severe generalized seizures, as well as SE as a consequence of LP administration, was statistically analyzed using Fisher's exact test. To evaluate the cell loss in specific hippocampal regions after the different treatments, we performed ANOVA followed by Bonferroni post-hoc test. In all statistical comparisons, a $p < 0.05$ or lower was considered significant.

3. Results

3.1. Control and TFS groups

Animals from control and TFS groups did not demonstrate behavioral changes after manipulation. Evaluation of FJ staining demonstrated low numbers of degenerating neurons in the hippocampus in either group (Fig. 2, Table 1).

3.2. LP-induced SE

Nearly all the animals from the LP group (95.8%) showed mild and severe generalized seizures, which evolved into SE. Latencies to behavioral changes evaluated after pilocarpine administration were first forelimb clonus at 23.9 ± 1.6 min, first generalized seizure at 25.6 ±1.6 min, and establishment of SE at 30.3 ± 1.6 min (Figs. 3 and 4). In all rats that went into SE, we observed an extensive FJ+ staining in CA1, CA3 and hilus of dentate gyrus (Fig. 2, Table 1).

3.3. Effects of TFS on LP-induced SE

A lower percentage of animals from the LP-TFS group showed mild (78.5%) and severe generalized seizures (78.5%) as well as SE (71.4%). However, these values as well as latencies to the first forelimb clonus, first generalized seizure and establishment of SE were not significantly different when compared with the LP group (Figs. 3 and 4). The LP-TFS group demonstrated a lower number of FJ+ neurons, an effect that was significant in CA3 (20%, $p < 0.01$) and dentate gyrus (16%, $p < 0.05$) of rats without SE, when compared with the LP group (Fig. 2, Table 1).

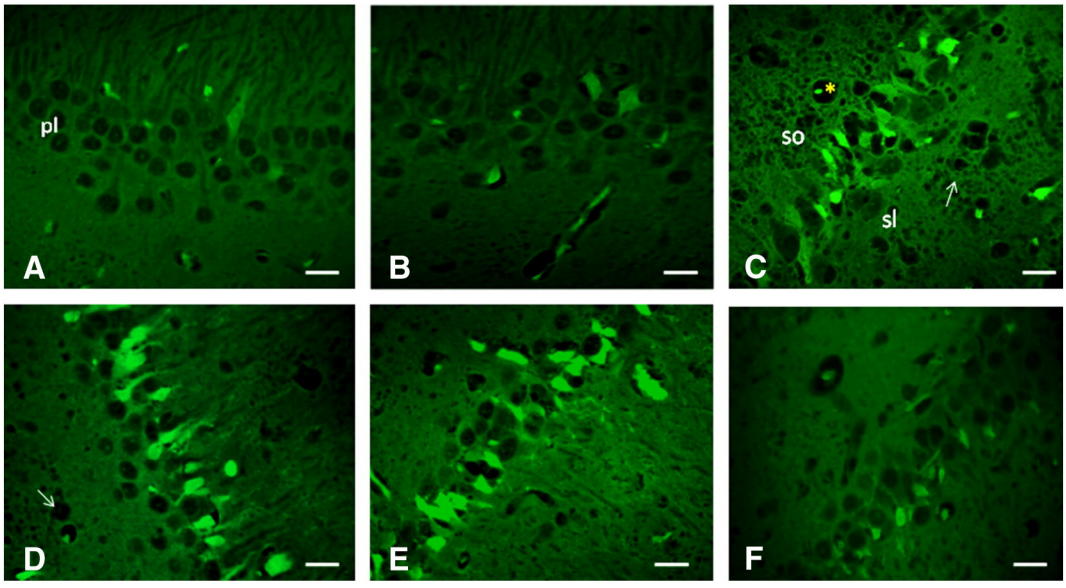

Fig. 2. Example of FJ staining in CA1 under control conditions (A) and after TFS alone (B), as well as following LP-induced SE alone (C), and associated with TFS (D), DZP (E) and TFS plus DZP (F). Animals were sacrificed 24 h after SE or manipulation. Note the low number of FJ+ cells in A, B and F and elevated FJ+ cells in C, D and E. Asterisk and arrows indicate the presence of vacuolations evident in the stratum oriens (so) and stratum lucidum (sl) in C and D. Scale bars, 20 μm.

Table 1
Number of cells in degeneration in selective hippocampal regions 24 h after manipulation or SE induced by lithium-pilocarpine model.

Group	CA1	CA3	Dentate gyrus
Control	165 ± 23	165 ± 27	140 ± 16
TFS	195 ± 23	152 ± 8	135 ± 4
LP	1008 ± 42	913 ± 21	1070 ± 28
LP-TFS (SE)	943 ± 27	802 ± 31	952 ± 28
LP-TFS (no SE)	958 ± 38	739 ± 43**	896 ± 40*
LP-DZP	694 ± 36***	588 ± 22***	899 ± 30*
LP-DZP + TFS (SE)	429 ± 66***,@@	512 ± 16***	578 ± 49***,@
LP-DZP + TFS (no SE)	402 ± 15***,@@	539 ± 41***	594 ± 56***,@@

DZP, diazepam; LP, lithium-pilocarpine; no SE, animals without status epilepticus; SE, status epilepticus; TFS, transcranial focal electrical stimulation.
Values express number of cells per mm^3.
Analysis of data by one-way ANOVA revealed a significant group difference in CA1 ($F(7,40) = 82.8$, $p < 0.0001$), CA3 ($F(7,40) = 53.23$, $p < 0.0001$) and dentate gyrus ($F(7,40) = 78.40$, $p < 0.0001$).
* $p < 0.05$ when compared with LP group.
** $p < 0.01$ when compared with LP group.
*** $p < 0.001$ when compared with LP group.
@ $p < 0.01$ when compared with LP + DZP group.
@@ $p < 0.001$ when compared with LP + DZP group.

3.4. Effects of DZP on LP-induced SE

All rats pretreated with a sub-effective dose of DZP (LP-DZP group) had LP-induced seizures and SE, and non-significant changes were found in latencies to the different behavioral alterations when compared to the LP group (Figs. 3 and 4). In contrast with the LP group, rats from the LP-DZP group demonstrated a significant reduction in the number of FJ+ neurons in all the hippocampal areas evaluated (CA1, 31%, $p < 0.001$; CA3, 35%, $P < 0.001$; dentate gyrus, 16%, $p < 0.05$) (Fig. 2, Table 1).

3.5. Effects of TFS combined with DZP on LP-induced SE

The pretreatment with a sub-effective dose of DZP combined with TFS produced total protection against LP-induced seizures and SE in 61.6% of animals of the LP-TFS + DZP group, an effect that was significant when compared with the LP and LP-DZP groups ($p < 0.0001$, $p = 0.003$, respectively) and nearly significant ($p = 0.054$) for protecting against mild and generalized seizures in contrast with the LP-TFS group (Fig. 3). Animals from the LP-TFS + DZP group that went into SE also demonstrated significantly increased latencies to the first forelimb clonus ($p < 0.001$), generalized seizure ($p < 0.001$) and establishment of SE ($p < 0.001$), when compared with the LP, LP-DZP and LP-TFS groups (Fig. 4). Histological evaluation revealed a significant diminution in the number of FJ+ neurons in all hippocampal areas examined of animals from the LP-TFS + DZP group, a situation that was more evident when values were compared with the LP and LP-DZP groups. Analysis revealed that rats from the LP-TFS + DZP group with total protection against LP-induced seizures exhibited similar number of degenerating cells (CA1, 57%; CA3, 44%; dentate gyrus, 46%) when compared with those animals from the LP-TFS + DZP group showing SE during 2 h (CA1, 60%; CA3, 41%; dentate gyrus, 44%) (Fig. 2, Table 1).

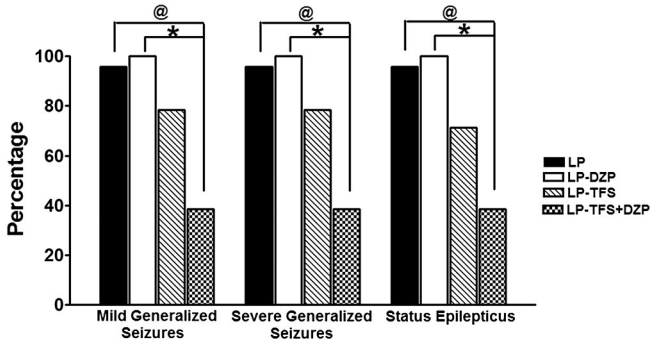

Fig. 3. Percentage of rats presenting mild and severe generalized seizures as well as status epilepticus following lithium-pilocarpine administration alone (LP) and combined with TFS (LP-TFS), a sub-effective dose of DZP (LP-DZP) and TFS plus a sub-effective dose of DZP (LP-TFS + DZP). @$p < 0.003$; *$p < 0.0001$ according to Fisher's exact test.

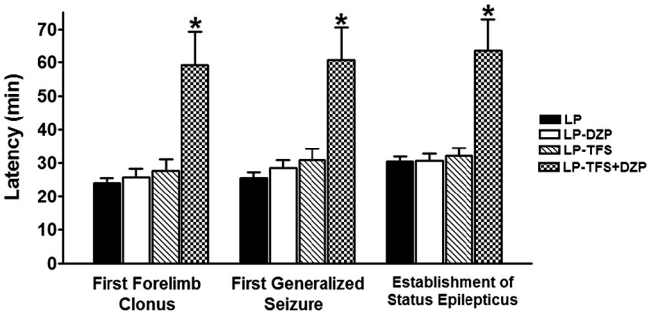

Fig. 4. Latencies in minutes (mean ± S.E.M.) to the behavioral changes induced after lithium-pilocarpine administration alone (LP) and combined with TFS (LP-TFS), a sub-effective dose of DZP (LP-DZP) and TFS plus a sub-effective dose of DZP (LP-TFS + DZP). Analysis of data by one-way ANOVA revealed a significant group difference in first forelimb clonus ($F(3,45) = 15.00$, $p < 0.001$), first generalized seizure ($F(3,45) = 15.81$, $p < 0.001$) and establishment of status epilepticus ($F(3,44) = 17.97$, $p < 0.001$). The asterisk refers to a statistically significant difference of $p < 0.001$ according to post-hoc Tukey's multiple comparison test.

4. Discussion

Previously, we demonstrated that TFS applied during pilocarpine-induced SE was able to reduce the seizure activity [11]. The results of the present study reveal that TFS applied before pilocarpine administration, by itself, induces a trend toward its effectiveness for preventing SE and neuronal damage. The effects are statistically significant when TFS neuromodulation is combined with sub-effective doses of DZP. Our data support the notion that TFS combined with DZP can represent a good noninvasive prophylactic strategy to avoid or reduce the expression of seizure activity and neuronal damage induced by SE.

Benzodiazepines are the first-line treatment for termination of SE in humans [23]. However, SE and seizure activity result in a reduction in the GABA receptor (GABAR)-mediated inhibition of hippocampal principal neurons [24–26] and a reduced response of these neurons to neuroprotective effects of benzodiazepines [27]. These changes during SE can be explained by the impaired function and internalization of $GABA_A$ receptors [28–30]. The selective reduction in the $GABA_A$ receptor γ2 subunit gene expression, which is required for DZP sensitivity, has been proposed as a mechanism inducing a downregulation of benzodiazepine-binding sites, reduction in GABAR-mediated inhibition, and an extensive loss of hippocampal neurons [31,32]. In contrast, the enhancement of GABA neurotransmission through a continuous increase in hippocampal GABA extracellular levels, or through an enhancement in sensitivity to GABA by administration of DZP, may reduce the ischemic CA1 damage [33,34]. Our results indicate that TFS can potentiate the anticonvulsant and neuroprotective effects mediated by the pretreatment with sub-effective doses of DZP. This situation may be explained by an augmented GABA-gated chloride influx through $GABA_A$ receptor-regulated ion channels that restricts interictal spike propagation under epileptic conditions and maintains an inhibitory input sufficient for neuronal survival. Future experiments should be designed to determine if TFS modifies GABA neurotransmission, a situation that could, in part, prevent the reduction of GABAR-mediated inhibition induced by SE.

An important finding from the present study was that TFS alone was able to reduce the LP-induced neuronal damage in CA3 and

dentate gyrus, but not in CA1, of those animals without SE. In contrast, all rats receiving TFS plus DZP showed a significant reduction in the neuronal damage of CA1, CA3 and dentate gyrus subsequent to pilocarpine administration, even though some of the rats went into SE. It is known that muscarinic cholinergic activation is involved in the initiation of seizure activity, whereas the neuronal loss induced by SE is associated with glutamatergic excitotoxicity mediated by NMDA receptors [35–37]. According to this information, it cannot be ruled out that changes in glutamatergic neurotransmission contribute to the protective effect of TFS on LP-induced neuronal damage.

The present study supports the idea that sub-effective doses of DZP plus TFS can represent a good strategy to prevent SE and neuronal damage subsequent to brain insults. There are three areas that need to be explored for future work on this topic. Other AEDs and also smaller doses of DZP need to be tested in the paradigm used in the present study to realize the best AED and its minimal sub-effective dosage to be applied in order to avoid SE and neuronal damage. We also need to determine the duration for the effectiveness of the TFS at potentiating the AEDs' effects or vise versa. It is important to notice that although the LP model is not a chronic animal model of epilepsy, it can represent the first approach to determine if TFS combined with AEDs is a potential therapeutic intervention to prevent SE and the subsequent permanent brain damage. It is also relevant to evaluate the effects of TFS in experimental models of epileptogenesis to determine if it represents a good strategy for seizure prophylaxis.

Under our experimental conditions and using the parameters previously found to modify seizure activity [11–13], it is possible to support the conclusion that TFS is a promising noninvasive stimulation method to prevent SE. However, an important limitation of the present study is the lack of electrographic brain recordings during and after the SE, a situation that could help identify the brain areas involved in TFS-induced effects. Future experiments should be designed to enable the application of TFS and online monitoring of its neurophysiologic effects [38]. This situation would allow one to associate behavioral with neurophysiologic effects of the stimulation and make it safer and more effective.

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