Epilepsy & Behavior xxx (xxxx) xxx

Contents lists available at ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

Noninvasive transcranial focal stimulation affects the convulsive seizure-induced P-glycoprotein expression and function in rats

Daniel Pérez-Pérez [a], José Luis Castañeda-Cabral [b], Sandra Orozco-Suárez [c], Julio Sotelo [d], Walter Besio [e], Luisa Rocha [b,*]

[a] Plan of Combined Studies in Medicine (PECEM), Faculty of Medicine, UNAM, México City, Mexico
[b] Pharmacobiology Department, Center for Research and Advanced Studies, México City, Mexico
[c] Unit of Medical Research in Neurological Diseases, Instituto Mexicano del Seguro Social, Centro Médico Nacional Siglo XXI, México City, Mexico
[d] National Institute of Neurology and Neurosurgery Manuel Velasco Suárez, México City, Mexico
[e] Department of Electrical, Computer, and Biomedical Engineering, University of Rhode Island, Kingston, RI, USA

ARTICLE INFO

Article history:
Received 12 October 2020
Revised 22 November 2020
Accepted 22 November 2020
Available online xxxx

Keywords:
P-glycoprotein
Neuromodulation
Drug-resistant seizures
Noninvasive electric stimulation
Transcranial focal stimulation

ABSTRACT

Transcranial focal stimulation (TFS) is a noninvasive neuromodulation strategy that reduces seizure activity in different experimental models. Nevertheless, there is no information about the effects of TFS in the drug-resistant phenotype associated with P-glycoprotein (Pgp) overexpression. The present study focused on determining the effects of TFS on Pgp expression after an acute seizure induced by 3-mercaptopropionic acid (MPA). P-glycoprotein expression was analyzed by western blot in the cerebral cortex and hippocampus of rats receiving 5 min of TFS (300 Hz, 50 mA, 200 μs, biphasic charge-balanced squared pulses) using a tripolar concentric ring electrode (TCRE) prior to administration of a single dose of MPA. An acute administration of MPA induced Pgp overexpression in cortex (68 ± 13.4%, $p < 0.05$ vs the control group) and hippocampus (48.5 ± 14%, $p < 0.05$, vs the control group). This effect was avoided when TFS was applied prior to MPA. We also investigated if TFS augments the effects of phenytoin in an experimental model of drug-resistant seizures induced by repetitive MPA administration. Animals with MPA-induced drug-resistant seizures received TFS alone or associated with phenytoin (75 mg/kg, i.p.). TFS alone did not modify the expression of the drug-resistant seizures. However, TFS combined with phenytoin reduced seizure intensity, an effect associated with a lower prevalence of major seizures (50%, $p = 0.03$ vs phenytoin alone). Our experiments demonstrated that TFS avoids the Pgp overexpression induced after an acute convulsive seizure. In addition, TFS augments the phenytoin effects in an experimental model of drug-resistant seizures. According with these results, it is indicated that TFS may represent a new neuromodulatory strategy to revert the drug-resistant phenotype.

1. Introduction

Transcranial focal stimulation (TFS) via tripolar concentric ring electrodes (TCRE) is a noninvasive neuromodulation strategy that consists of the application of alternating current at 300 Hz on the scalp [1]. Transcranial focal stimulation reduces the convulsive activity induced by pilocarpine, penicillin, and pentylenetetrazole [1–4]. Recently, it was described that TFS applied after each kindling electrical stimulation delays epileptogenesis in cats, an effect still evident a few weeks after TFS cessation [5]. Transcranial focal stimulation combined with a suboptimal dose of diazepam reduces the behavioral changes and neuronal damage induced by pilocarpine-induced *status epilepticus* [6]. The anticonvulsive and neuroprotective effects mediated by TFS [7] are partially explained by a decrease in the normally high release of glutamate during seizure activity [8].

The enhanced release of glutamate is a condition associated with drug-resistant epilepsy [9,10] due to an induced P-glycoprotein (Pgp) overexpression [11–13]. The overexpression of Pgp in the blood–brain barrier limits the penetration of anti-seizure drugs into the brain [14–16]. P-glycoprotein overexpression and resistance to antiepileptic drugs are conditions progressively induced during the induction of repetitive convulsive seizures [17–20]. However, at present, it is unknown if the enhanced glutamate release induced by a single convulsive seizure [21] facilitates the Pgp overexpression.

* Corresponding author at: Depto. Farmacobiología, Cinvestav-Sede Sur, Calzada Tenorios 235, Granjas Coapa, Mexico City, 14330, Mexico.
E-mail address: lrocha@cinvestav.mx (L. Rocha).

https://doi.org/10.1016/j.yebeh.2020.107659
1525-5050/

The blockage of Pgp expression and/or function is considered a therapeutic strategy to control drug-resistant seizures [19,20,22,23]. On the other hand, studies support that brain electrical stimulation, with neuromodulatory effects, can revert the drug-resistant phenotype, avoiding the Pgp function [10]. Supporting this notion, it was described that electrical stimulation at 50 Hz reduces the Pgp expression and its drug extrusion potency in tumor cells more effectively than tariquidar [24]. This group of evidence leads to suggest that under certain conditions, electrical stimulation may reduce the Pgp overexpression and/or function.

Considering that TFS lessens the high glutamate release during seizure activity [8], we hypothesized that this neuromodulatory strategy might reduce the Pgp overexpression and/or function after an acute seizure and in an experimental model of drug-resistant seizures. We used the administration of 3-mercaptopropionic acid (MPA) that results in clonic-tonic seizures as consequence of enhanced degradation and low synthesis of γ-aminobutyric acid (GABA) [25]. The repetitive administration of MPA results in resistance to phenytoin (PHT) through Pgp overexpression in brain areas such as cortex and hippocampus [18,26,27].

2. Materials and methods

2.1. Animals and manipulation

Male Wistar rats (250–300 g body weight), individually housed and maintained under environmentally controlled conditions (12-h light/dark cycles, 22 °C) with food and water *ad libitum*, were used in the present study. All experiments were approved by the institutional ethics committee (CICUAL 125-15) and were carried out according to the Mexican law for the care and use of laboratory animals (NOM-062-ZOO-1999) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were habituated to the manipulation with the administration of saline solution (SS, NaCl 0.9%, 1 ml/kg, i.p.) and 5 min of handling with a TCRE of 10 mm diameter, on the scalp. This procedure was applied every 24 h for 7 days.

2.2. Experiment 1. Pgp expression after an acute seizure and the effects of TFS

This experiment was designed to investigate if Pgp is overexpressed in the cortex and the hippocampus as a consequence of an acute MPA-induced convulsive seizure and if this effect is avoided by TFS (Fig. 1).

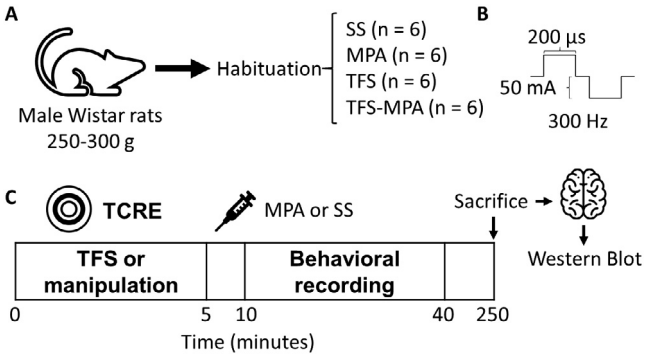

Fig. 1. Diagram of the experimental protocol used to evaluate P-glycoprotein (Pgp) expression after an acute seizure induced by 3-mercaptopropionic acid (MPA) and the effects of transcranial focal stimulation (TFS). A, Rats were submitted to 7 days of habituation. Then, they were randomly distributed among the different experimental groups. B, Diagram showing the parameters for TFS. C, Timeline of the experimental procedure. The hippocampus and cerebral cortex of the rats were obtained at the end of the experiment and used to determine the protein expression of Pgp by Western Blot. g, grams; SS, Saline Solution; mA, milliamperes; µs, microseconds; Hz, Hertz; TCRE, tripolar concentric ring electrode.

The prevalence and latency of the MPA-induced seizures were evaluated for 30 min after the drug administration. The intensity of the seizures was rated according to the following stages: 0, no behavioral changes; 1, isolated myoclonic jerks; 2, atypical (unilateral or incomplete) clonic seizures; 3, fully developed clonic seizures; 4, generalized clonic-tonic seizures with suppressed tonic phase; and 5, fully developed clonic-tonic seizures [28]. Stages 1 to 3 were considered minor seizures, whereas stages 4 and 5 were major seizures.

2.3. Experimental groups

TFS-MPA (*n* = 6). Twenty-four h after the end of habituation, rats received TFS (300 Hz, 50 mA, 200 µs, biphasic, and charge-balanced squared pulses) for 5 min using TCREs. MPA (37.5 mg/kg, i.p.) was applied 5 min after completion of TFS. Rats were sacrificed 4 h after the MPA administration. The cerebral cortex and the hippocampus were immediately obtained and stored at −70 °C until used to determine Pgp expression for western blot analysis (see Section 2.4).

MPA (*n* = 6). In order to determine the effects of MPA alone, the animals of this group were manipulated as the TFS-MPA group, except that they did not receive TFS.

TFS (*n* = 6). This group was designed to investigate the effects of TFS alone. Animals were manipulated as the TFS-MPA group, except that rats received SS instead of MPA.

SS (*n* = 6). These animals were manipulated as the TFS group, except that they did not receive TFS. The results obtained from this group were considered as the control condition for the experiment.

2.4. Western blotting

Cerebral tissue samples were homogenized in Radio Immuno Precipitation Assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM Ethylene-Diamine-Tetra acetic Acid, and 0.1% Triton X-100, pH 7.5) with protease inhibitor cocktail (Roche Diagnostics GmbH, Germany) in a cold bath at 4 °C. Then homogenates were centrifuged at 14,000g for 30 min at 4 °C and the supernatant (total protein extract) was immediately collected, aliquoted, and maintained at −70 °C. Protein concentration was determined in the extracts according to the Bradford method (Bio-Rad Laboratories, USA) using bovine serum albumin (Bio-Rad Laboratories, USA) as standard.

Samples (50 µg) of total protein extract were denatured by boiling for 5 min at 95 °C in Laemmli buffer (500 mM Tris-HCl pH 6.8, 2% Sodium Dodecyl Sulfate (SDS), 10% glycerol, 10% β-mercaptoethanol and 0.1% bromophenol blue). Electrophoresis was carried out in Tris/glycine/SDS running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3; Bio-Rad Laboratories, USA) at 85 V for 30 min and 100 V for 2 h using SDS-polyacrylamide gel electrophoresis (7.5%). Separated proteins were electroblotted onto polyvinylidene difluoride membrane (Immun-Blot, Bio-Rad Laboratories, USA) in a wet system at 0.6 A (constant current) for 30 min using transfer buffer (25 mM Trizma base, 250 mM glycine and 20% methanol, pH 8.3). Membranes with blotted proteins were incubated in 5% blocking solution (Blot-QuickBlocker, EMD Millipore, USA) diluted in Tris-buffered saline-Tween (TBS-T) buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at 4 °C. Then, the membranes were washed 3 times in TBS-T for 5 min each. Next, the membranes were incubated overnight with gentle shaking at 4 °C, with the following antibodies: rabbit monoclonal anti-Pgp (1:1000; Cat. ab170904, Abcam, USA) and rabbit monoclonal anti-actin (1:1000; Cat. ab179467; Abcam, USA). All primary antibodies were diluted in TBS buffer (20 mM Tris,

500 mM NaCl, pH 7.5). Then, membranes were washed 3 times in TBS-T for 5 min each, followed by incubation with corresponding secondary antibody HRP-goat anti-rabbit IgG (1:5000 and 1:10,000 for Pgp and actin, respectively) for 2 h at 4 °C diluted in TBS. Finally, the membranes were incubated in peroxide/luminol solution (Clarity Western ECL substrate, Bio-Rad Laboratories, USA) at room temperature for 5 min. The chemiluminescent data were normalized using actin as a constitutive protein, resulting in a relative expression ratio. Each sample was evaluated by duplicate.

2.5. Experiment 2. Effects of TFS on drug-resistant seizures induced by repetitive MPA

Phenytoin has been shown to protect animals from acute MPA-induced seizures [25]. However, the repetitive induction of generalized seizures with MPA results in Pgp overexpression and reduces brain bioavailability and anticonvulsant effects of phenytoin [18,27]. This PHT-resistant phenotype is reversed by the pharmacological inhibition of Pgp [19,20,22,23,26].

This experiment was designed to test the hypothesis that TFS can inhibit the drug resistance condition induced by the repetitive MPA administration, thus enhancing the anticonvulsant effects of PHT. For this purpose, rats ($n = 13$) received MPA administration every 12 h for 10 administrations. Initially, MPA was administered at 30 mg/kg, i.p. If the dose of MPA did not induce seizures greater than or equal to stage 3, the subsequent dose of MPA was increased by 2.5 mg/kg. This procedure was repeated up to a maximal dose of 37.5 mg/kg. Eight animals survived this experimental procedure and were randomly assigned to a crossover protocol [29] starting 24 h after the 10th dose of MPA. During the crossover protocol, each rat was exposed to one of four different treatments every 48 h. Twenty-four h after each treatment, the animals received a dose of MPA to maintain the drug-resistant phenotype (Fig. 2). At the end of the experiment, each animal crossed through all four different treatments. This strategy was used to evaluate the effects of different treatments in the same animal and reduce the number of experimental subjects. The different treatments during the crossover protocol were as follows:

PHT-TFS-MPA: TFS was applied in rats (see Section 2.2) pretreated with phenytoin (75 mg/kg, i.p., 50 min before TFS). Then, MPA (37.5 mg/kg, i.p.) was administered 5 min after the end of the TFS. Prevalence and latency of the MPA-induced behavioral changes were evaluated according to the same criteria as in experiment 1.

PHT-MPA: This treatment was designed to confirm that PHT did not induce anticonvulsant effects in animals with drug-resistant seizures. The manipulation was similar to PHT-TFS-MPA treatment, except that TFS was not applied.

TFS-MPA: This treatment was similar to PHT-TFS-MPA treatment, except that SS (pH 11.4) was applied instead of PHT. The results obtained were used to determine if TFS was able to modify the drug-resistant phenotype.

MPA: This treatment was similar to TFS-MPA treatment, except that TFS was not applied. This treatment was considered the control condition for experiment 2.

2.6. Statistical analysis

The sample size for continuous and categorical data was chosen according to Allgoewer & Mayer [30]. This strategy reduced the number of animals used in the experiments. Categorical variables are indicated as a percentage and interval variables are indicated as mean ± standard error. We used one-tailed Fisher's exact test, one-way ANOVA followed by pairwise t test with false discovery rate correction or Kruskal–Wallis, as necessary. We considered a statistically significant difference if the p-value was equal to or less

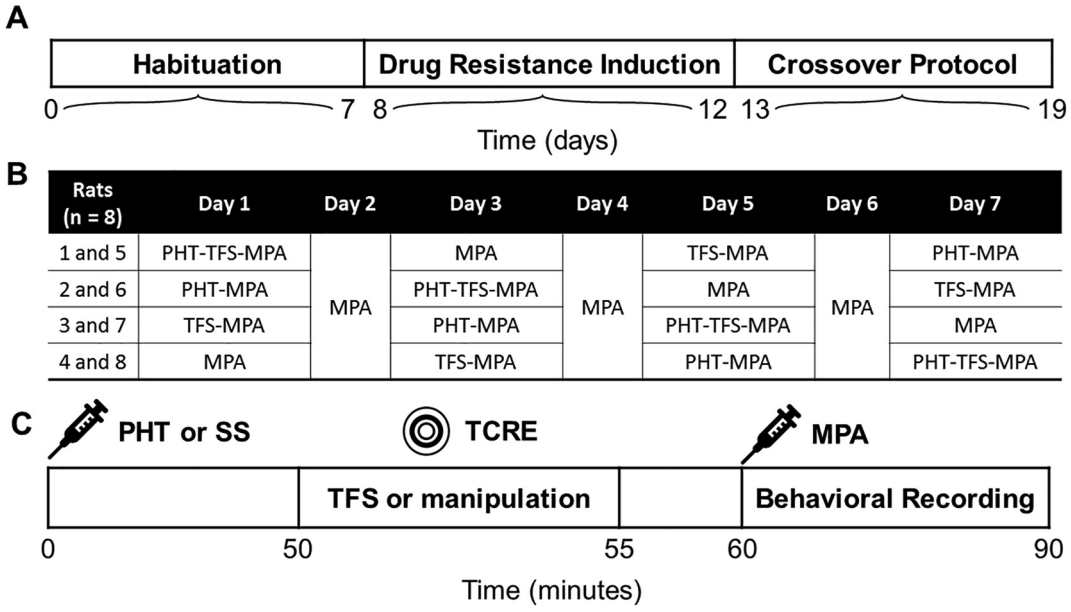

Fig. 2. Diagram of the experimental protocol used to evaluate different treatments in rats with drug-resistant seizures. A, Timeline of the complete protocol. Initially, animals were submitted to 7 days of habituation. Then, they received 3-mercaptopropionic acid (MPA) every 12 h for 5 days (days 8–12) to induce drug-resistant seizures. On day 13, animals were submitted to the crossover protocol. B, Schematic representation of the crossover treatments used for 8 rats with drug-resistant seizures. Twenty-four hours after completion of repetitive MPA administration, the animals were randomly assigned to a crossover protocol during which they received 4 different treatments. Twenty-four h after each treatment, the animals received a dose of MPA to maintain the drug-resistant phenotype. C, Diagram showing the timeline of procedures for the different treatments applied during the crossover protocol. Initially, phenytoin (PHT, 75 mg/kg, i.p.) or saline solution (SS, 1 ml/kg, i.p.) was administered. Then, a tripolar concentric ring electrode (TCRE) was used to apply transcranial focal stimulation (TFS) or manipulation, for 5 min. MPA (37.5 mg/kg, i.p.) was administered 5 min after TFS. The seizure activity was evaluated for 30 min after MPA injection.

than 0.05. All analyseswere performed using GraphPad Software version 6 (La Jolla, California, USA).

3. Results

3.1. TFS avoids the Pgp overexpression induced by a single MPA-induced convulsive seizure

In experiment 1, rats from the SS group did not show behavioral changes after manipulation. P-glycoprotein expression in their cerebral cortex and hippocampus was used as a control condition (100%) for further comparisons. The TFS-SS group presented no behavioral alterations during and after TFS. This group showed an increase of the Pgp expression in the cerebral cortex (67 ± 31.1%, $p = 0.03$) and no changes in the hippocampus (8.9 ± 15.9%, $p > 0.05$) (Fig. 3).

All animals (100%) from the MPA group presented minor and major seizures after MPA administration, with a latency of 306.5 ± 35 and 417.3 ± 75 s, respectively. In contrast to the SS group, the MPA group showed Pgp overexpression in both, cerebral cortex (68 ± 13.4%, $p < 0.05$) and hippocampus (48.5 ± 14%, $p < 0.05$) (Fig. 3). In the TFS-MPA group, MPA induced seizures as follows: minor seizures in 100% of rats with latency of 372.5 ± 37 s; major seizures in 83% of animals, with latencies 455.6 ± 71 s. These values were not significantly different when compared with those of the MPA group ($p > 0.05$). However, western blot experiments revealed that the Pgp expression of TFS-MPA group was similar to SS group in both, cerebral cortex ($p > 0.05$) and hippocampus ($p > 0.05$), and significantly lower when compared with MPA group (cerebral cortex, 68%, $p = 0.002$; hippocampus, 59%, $p = 0.003$) (Fig. 3).

3.2. TFS augments the effects of phenytoin in animals with drug-resistant seizures

In Experiment 2 and during the crossover protocol, the MPA treatment induced minor and major seizures in all animals (100%), with latencies of 474.5 ± 84 s and 481.6 ± 83.2 s, respectively. Rats with this treatment achieved a maximum seizure stage of 4.3 ± 0.2. The TFS-MPA treatment produced similar changes when compared with the MPA treatment: 100% ($p > 0.05$) showed minor seizures at 474 ± 29.3 s ($p > 0.05$), whereas 87.5% ($p > 0.05$) presented major seizures at 451.1 ± 19.9 s ($p > 0.05$). The highest seizure stage achieved under the TFS-MPA treatment was 4 ± 0.2 ($p > 0.05$ vs MPA) (Figs. 4 and 5).

The PHT-MPA treatment induced minor seizures in all the animals (100%) with a latency of 456.6 ± 55.7 s ($p > 0.05$ vs MPA). Although this treatment induced major seizures in all animals (100%), they had a longer latency (756 ± 64 s, $p = 0.04$ vs MPA). The maximal seizure stage achieved under PHT-MPA treatment was 4.4 ± 0.2 ($p > 0.05$ vs MPA) (Figs. 4 and 5). Finally, the PHT-TFS-MPA treatment induced the following changes: minor seizures in all the animals (100%) at 541.7 ± 57.7 s ($p > 0.05$ vs PHT-MPA); major seizures in 50% of the rats ($p = 0.03$ vs PHT-MPA) at 840 ± 97 s ($p > 0.05$ vs PHT-MPA) (Fig. 4). Under this treatment, rats achieved an average seizure stage of 3.2 ± 0.4, equivalent to minor seizures. This result was significantly lower when compared with MPA ($p = 0.04$) and PHT-MPA ($p = 0.02$) groups (Fig. 5).

4. Discussion

Our experiments revealed that Pgp is overexpressed in the cerebral cortex and hippocampus of rats after an acute convulsive seizure, an effect avoided by TFS. In addition, the data obtained support that TFS facilitates the effects of PHT in an experimental model of drug-resistant seizures.

The overexpression of ATP-binding cassette transporters such as Pgp is considered a mechanism of drug-resistance in epilepsy since it limits access of anti-seizure drugs to the brain [14–16]. P-glycoprotein overexpression is mediated by several circumstances such as excessive glutamate release [11,12,31], hypoxic-mimicking conditions [32], oxidative stress [12,13], and neuroinflammation [33]. P-glycoprotein overexpression is a condition produced by MPA-induced repetitive convulsive seizures [18,27]. Our results indicate for the first time that Pgp is overexpressed as a result of a single MPA-induced convulsive seizure. This effect can result from the changes induced by acute convulsive seizures such as excessive glutamate release [21], hypoxia [34], the activation of COX-2/prostaglandin E2 pathway [35], and high oxidative stress [36,37]. It is known that the repeated induction of convulsive sei-

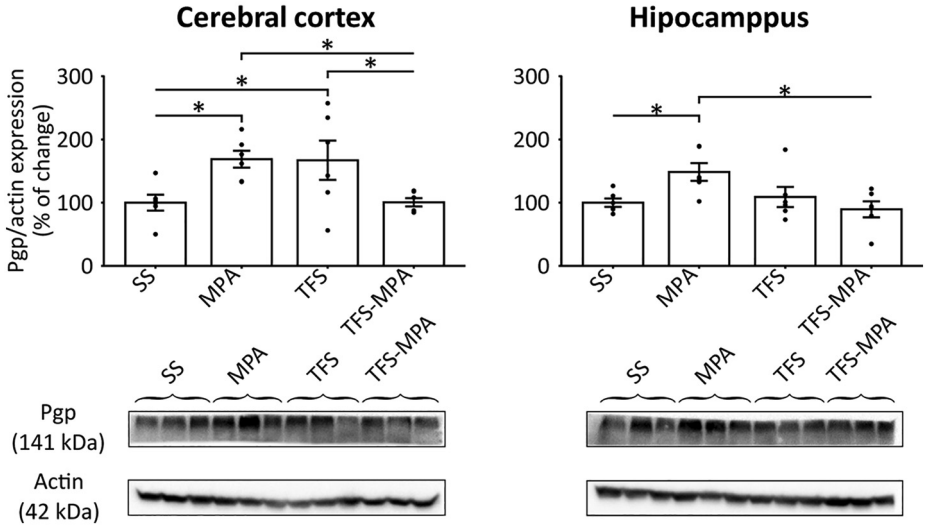

Fig. 3. Representation of the P-glycoprotein (Pgp) expression in the cerebral cortex and the hippocampus under different experimental conditions: after vehicle (SS) or TFS (TFS) administration, and after the induction of a single 3-mercaptopropionic acid (MPA)-induced seizure alone (MPA) and associated with TFS (TFS-MPA). Graphs represent the percentage of change of the Pgp/actin expression, considering the values of the SS group as a control condition (100%). The values are presented as mean ± S.E. and each dot represents an independent value. Values were analyzed with a one-way ANOVA followed by pairwise t test. Lower panels show representative blots of the Pgp expression under different experimental conditions. * $p < 0.05$.

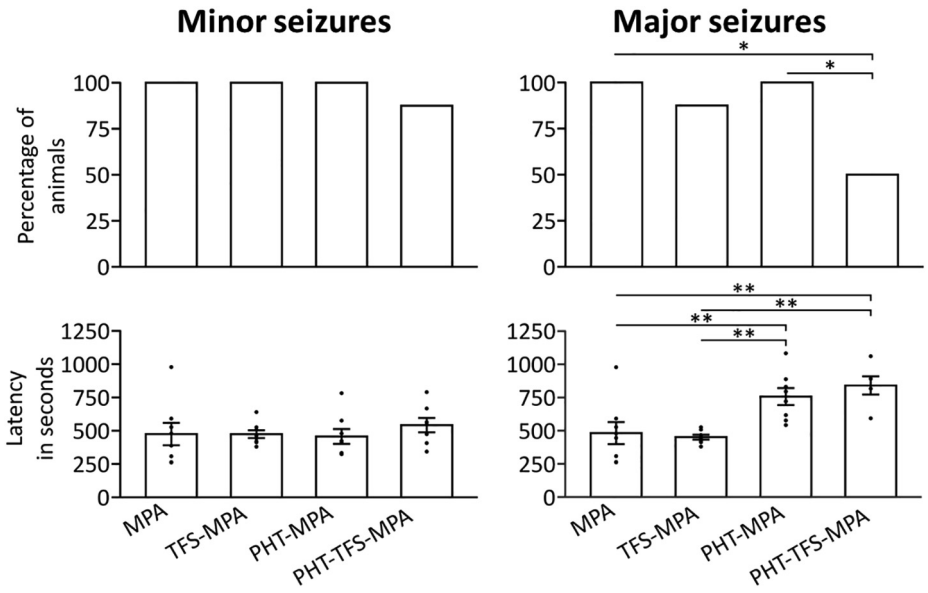

Fig. 4. Prevalence (percentage of animals) and latency (seconds) of the minor and major seizures of rats with drug-resistant seizures and under different treatments: 3-mercaptopropionic acid (MPA) alone; Transcranial Focal Stimulation (TFS) plus MPA; phenytoin plus MPA (PHT-MPA) and phenytoin plus Transcranial Focal Stimulation (TFS) plus MPA (PHT-TFS-MPA). Prevalence values were analyzed with a one-sided Fisher's exact test. Latency values are presented as mean ± S.E. and each dot represents an independent value. They were analyzed with a one-way ANOVA followed by pairwise t test. $^{*}p < 0.05$; $^{**}p < 0.01$.

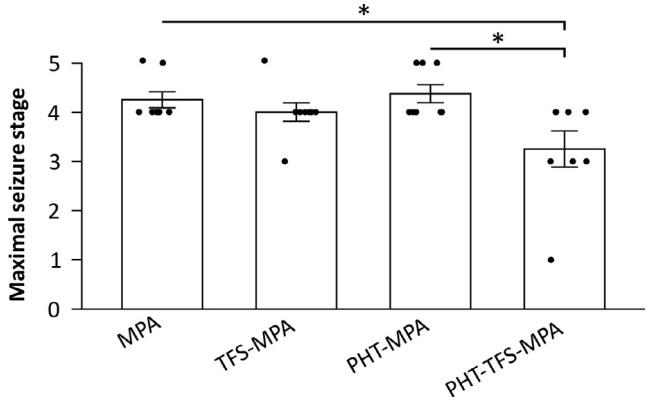

Fig. 5. Representation of the maximal seizure stage achieved by rats with drug-resistant seizures under different treatments (see legend of Fig. 3). Values are presented as mean ± S.E. and each dot represents an independent value. They were analyzed with Kruskal–Wallis test plus Connover test. $^{*}p < 0.05$.

zures can increase the brain excitability probably as a consequence of changes in local network susceptibility [17,38]. It is possible that the overexpression of Pgp as a consequence of a single convulsive seizure is involved in this phenomenon. We also found that TFS avoids the Pgp overexpression induced by an acute MPA-induced convulsive seizure. This effect can be explained since TFS lessens the seizure-induced augmented glutamate release [8], a condition essential for the Pgp overexpression [11,12,31].

On the other hand, the results obtained indicate that TFS augments the Pgp expression in the cortex of sham animals. This finding can result as a consequence of an excitatory effect induced by TFS in the cortex of nonconvulsive animals. Similarly, transcranial magnetic stimulation enhances cortical excitability in healthy subjects [39,40], an effect that depends on the brain excitability state [41,42]. Previous studies support that TFS does not induce brain damage [7] or changes in the short- and long-term memory of healthy rats [43]. However, further studies are necessary to determine the consequences of TFS in healthy subjects.

Despite these effects, we found that TFS applied before the MPA administration did not significantly modify the expression of the convulsive seizures in naive animals (TFS-MPA group of experiment 1). These findings are in contrast with the anticonvulsant effects of TFS in other experimental models of seizures (penicillin, pentylenetetrazol, and pilocarpine) [1,2,4]. The MPA-induced convulsive seizures are a consequence of lower GABAergic neurotransmission due to the reversible inhibition of glutamate decarboxylase (GAD) and activation of GABA-α-oxoglutarate aminotransferase (GABA-T) [25]. The results obtained indicate that TFS alone is not enough to block the MPA-induced effects on GAD and GABA-T. In contrast with other proconvulsant drugs, the MPA-induced seizures present a very sudden onset with violent running fits followed by clonic-tonic seizures, suggesting a higher seizure intensity [25]. It is possible that MPA enhances the excitability of brain areas in which TFS does not induce significant changes. Further experiments using Fos staining, an indirect procedure of neuronal activity, may allow us to investigate this issue.

Our experiments revealed that TFS alone did not revert the drug-resistant phenotype of the animals (TFS-MPA treatment of experiment 2). However, TFS combined with PHT (PHT-TFS-MPA treatment of experiment 2) was able to reduce the expression of the MPA-induced major seizures. This finding is in agreement with our previous study in which TFS augmented the effects of subeffective doses of diazepam when applied in rats during pilocarpine-induced *status epilepticus* [6].

For the present study, we applied PHT at 75 mg/kg i.p. It is known that PHT at 100 mg/kg i.p. has a half-life of 2.6 hours [44]. If 5 half-life periods of a drug are necessary for complete wash out [45], then, 13 hours are the period necessary to wash out the PHT. Therefore, a one-day interval between crossover maneuvers is proper to avoid the effects of previous treatments. However, studies suggest accumulation kinetics of PHT during its chronic administration [44]. On the other hand, additional experiments are necessary to determine the rate at which the TFS effects are washed out after stimulation has finished. These conditions may represent a limitation of the results obtained in the crossover protocol.

It is known that minor seizures (myoclonus as well as atypical and typical clonus) are induced by the activation of forebrain structures. They may progress to major seizure components (generalized clonic and tonic-clonic seizures) when the neuronal hyperactivity involves thalamus [46,47] and/or brain stem [48]. The results obtained from the present study revealed that TFS combined with PHT reduced the prevalence of major, but not minor, seizure components induced by MPA in animals with drug-resistant seizures. Future experiments are essential to determine how TFS alone and in combination with anti-seizure drugs modifies the neuronal activity of these brain areas, reducing the behavioral manifestations of the major seizure components. It is indicated that a high seizure severity is a relevant condition to develop drug resistance in epilepsy [49,50]. According to this notion, the reduced seizure severity induced by TFS in combination with pharmacotherapy can represent a novel strategy to avoid the drug-resistant phenotype.

In drug-resistant epilepsy, Pgp overexpression limits the brain penetration of anti-seizure drugs from blood to cerebral parenchyma [51]. The blockage of Pgp expression and/or function is considered a therapeutic strategy to control drug-resistant seizures [52]. The administration of Pgp inhibitors such as tariquidar or nimodipine reverses the drug-resistant phenotype [19,20,22,23]. However, these drugs induce side effects and their clinical use is inconclusive [53–56]. The results obtained from the present study support that TFS represents a novel therapeutic strategy to reduce Pgp expression and function.

Electrical modulation of the brain maybe applied in combination with anti-seizure drugs to get good control of seizures. However, the potential advantages of such combination may depend on the mechanisms underlying the inhibitory effects. Studies indicate that anti-seizure drugs enhancing GABAergic neurotransmission augment the effects induced by deep-brain stimulation, whereas Na^+ channel blockers avoid its protective effects [57]. We previously reported that TFS augmented the effects of subeffective doses of diazepam when applied in rats during pilocarpine-induced *status epilepticus* [6]. The present study revealed that TFS combined with PHT (PHT-TFS-MPA treatment of experiment 2) was able to reduce the expression of the MPA-induced major seizures. This group of evidence suggests that TFS can be combined with different anti-seizure drugs with diverse mechanisms. This situation represents a potential condition of TFS over other types of electrical modulation of the brain, especially in drug-resistant epilepsy.

Our previous studies support that short TFS is effective in controlling acute seizures [1–4] and status epilepticus when combined with a subeffective dose of diazepam [6,8]. In the present study, we found that 5min of TFS was effective to reduce drug-resistant seizures. However, 5min may be too short a period of TFS to abort seizures in subjects with drug-resistant epilepsy. Further studies are essential to determine if TFS is effective in experimental models of drug-resistant epilepsy with short, on and off, stimulation protocols such as vagal nerve stimulation and deep-brain stimulation [58,59].

5. Conclusion

The overall effects of TFS make it a valuable Pgp modulator. Further, TFS in combination with anti-seizure drugs can be advantageous as a therapeutic alternative for drug-resistant epilepsy over pharmacologic Pgp-inhibitors since it is noninvasive, nonpharmacologic, and does not appear to have side effects. Nevertheless, more research is needed to elucidate the most effective clinical indications.

Declaration of interest

Dr. Besio is the CEO of CREmedical which develops concentric ring technologies.

Acknowledgments

This study was partially supported by the National Council of Science and Technology of Mexico (CONACYT, scholarship, 622940 given to DPP), the National Autonomous University of México (UNAM) and the Plan of Combined Studies in Medicine (PECEM).

References

[1] Besio WG, Koka K, Cole AJ. Effects of noninvasive transcutaneous electrical stimulation via concentric ring electrodes on pilocarpine-induced status epilepticus in rats. Epilepsia 2007;48:070725162428001-??? https://doi.org/10.1111/j.1528-1167.2007.01202.x.

[2] Besio WG, Gale KN, Medvedev AV. Possible therapeutic effects of transcutaneous electrical stimulation via concentric ring electrodes. Epilepsia, 2010;51:85–7. https://doi.org/10.1111/j.1528-1167.2010.02617.x

[3] Besio WG, Liu X, Wang L, Medvedev AV, Koka K. Transcutaneous focal electrical stimulation via concentric ring electrodes reduces synchrony induced by pentylenetetrazole in beta and gamma bands in rats. Int J Neural Syst 2011;21:139–49. https://doi.org/10.1142/S0129065711002729.

[4] Makeyev O, Luna-Munguia H, Rogel-Salazar G, Liu X, Besio WG. Noninvasive transcranial focal stimulation via tripolar concentric ring electrodes lessens behavioral seizure activity of recurrent pentylenetetrazole administrations in rats. IEEE Trans Neural Syst Rehabil Eng 2013;21:383–90. https://doi.org/10.1109/TNSRE.2012.2198244.

[5] Valdés-Cruz A, Villasana-Salazar B, Williams B, Martínez-Vargas D, Magdaleno-Madrigal VM, Almazán-Alvarado S, et al. Transcranial focal electrical stimulation via concentric ring electrodes in freely moving cats: antiepileptogenic and postictal effects. Exp Neurol 2019;320:113012. https://doi.org/10.1016/j.expneurol.2019.113012.

[6] Besio W, Cuellar-Herrera M, Luna-Munguia H, Orozco-Suárez S, Rocha L. 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. Epilepsy Behav 2013;28:432–6. https://doi.org/10.1016/j.yebeh.2013.06.021.

[7] Mucio-Ramírez S, Makeyev O. Safety of the transcranial focal electrical stimulation via tripolar concentric ring electrodes for hippocampal CA3 subregion neurons in rats. J Healthcare Eng 2017;2017:1–7. https://doi.org/10.1155/2017/4302810.

[8] Santana-Gómez CE, Alcántara-González D, Luna-Munguía H, Bañuelos-Cabrera I, Magdaleno-Madrigal V, Fernández-Mas R, et al. Transcranial focal electrical stimulation reduces the convulsive expression and amino acid release in the hippocampus during pilocarpine-induced status epilepticus in rats. Epilepsy Behav 2015;49:33–9. https://doi.org/10.1016/j.yebeh.2015.04.037.

[9] During D. Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. The Lancet 1993;341:1607–10. https://doi.org/10.1016/0140-6736(93)90754-5.

[10] Luna-Munguia H, Orozco-Suarez S, Rocha L. Effects of high frequency electrical stimulation and R-verapamil on seizure susceptibility and glutamate and GABA release in a model of phenytoin-resistant seizures. Neuropharmacology 2011;61:807–14. https://doi.org/10.1016/j.neuropharm.2011.05.027.

[11] Bankstahl JP, Hoffmann K, Bethmann K, Löscher W. Glutamate is critically involved in seizure-induced overexpression of P-glycoprotein in the brain. Neuropharmacology 2008;54:1006–16. https://doi.org/10.1016/j.neuropharm.2008.02.008.

[12] Bauer B, Hartz AMS, Pekcec A, Toellner K, Miller DS, Potschka H. Seizure-induced up-regulation of P-glycoprotein at the blood-brain barrier through glutamate and cyclooxygenase-2 signaling. Mol Pharmacol 2008;73:1444–53. https://doi.org/10.1124/mol.107.041210.

[13] van Vliet EA, Zibell G, Pekcec A, Schlichtiger J, Edelbroek PM, Holtman L, et al. COX-2 inhibition controls P-glycoprotein expression and promotes brain delivery of phenytoin in chronic epileptic rats. Neuropharmacology 2010;58:404–12. https://doi.org/10.1016/j.neuropharm.2009.09.012.

[14] Lazarowski A, Czornyj L, Lubienieki F, Girardi E, Vazquez S, D'Giano C. ABC transporters during epilepsy and mechanisms underlying multidrug resistance in refractory epilepsy. Epilepsia 2007;48:140–9. https://doi.org/10.1111/j.1528-1167.2007.01302.x.

[15] Luna-Tortós C, Fedrowitz M, Löscher W. Several major antiepileptic drugs are substrates for human P-glycoprotein. Neuropharmacology 2008;55:1364–75. https://doi.org/10.1016/j.neuropharm.2008.08.032.

[16] Zhang C, Kwan P, Zuo Z, Baum L. The transport of antiepileptic drugs by P-glycoprotein. Adv Drug Deliv Rev 2012;64:930–42. https://doi.org/10.1016/j.addr.2011.12.003.

[17] Auzmendi JA, Orozco-Suárez S, Bañuelos-Cabrera I, González-Trujano ME, Calixto González E, Rocha L, et al. P-glycoprotein contributes to cell membrane depolarization of hippocampus and neocortex in a model of repetitive seizures induced by pentylenetetrazole in rats. Curr Pharm Des 2013;19:6732–8. https://doi.org/10.2174/1381612811319380006.
[18] Enrique A, Goicoechea S, Castaño R, Taborda F, Rocha L, Orozco S, et al. New model of pharmacoresistant seizures induced by 3-mercaptopropionic acid in mice. Epilepsy Res 2017;129:8–16. https://doi.org/10.1016/j.eplepsyres.2016.10.012.
[19] Höcht C, Lazarowski A, Gonzalez NN, Mayer MA, Opezzo JAW, Taira CA, et al. Differential hippocampal pharmacokinetics of phenobarbital and carbamazepine in repetitive seizures induced by 3-mercaptopropionic acid. Neurosci Lett 2009;453:54–7. https://doi.org/10.1016/j.neulet.2009.01.079.
[20] Höcht C, Lazarowski A, Gonzalez NN, Auzmendi J, Opezzo JAW, Bramuglia GF, et al. Nimodipine restores the altered hippocampal phenytoin pharmacokinetics in a refractory epileptic model. Neurosci Lett 2007;413:168–72. https://doi.org/10.1016/j.neulet.2006.11.075.
[21] Rocha L, Briones M, Ackermann RF, Anton B, Maidment NT, Evans CJ, et al. Pentylenetetrazol-induced kindling: early involvement of excitatory and inhibitory systems. Epilepsy Res 1996;26:105–13. https://doi.org/10.1016/S0920-1211(96)00046-0.
[22] Brandt C, Bethmann K, Gastens AM, Löscher W. The multidrug transporter hypothesis of drug resistance in epilepsy: Proof-of-principle in a rat model of temporal lobe epilepsy. Neurobiol Disease 2006;24:202–11. https://doi.org/10.1016/j.nbd.2006.06.014.
[23] van Vliet EA, van Schaik R, Edelbroek PM, Redeker S, Aronica E, Wadman WJ, et al. Inhibition of the multidrug transporter P-glycoprotein improves seizure control in phenytoin-treated chronic epileptic rats. Epilepsia 2006;47:672–80. https://doi.org/10.1111/j.1528-1167.2006.00496.x.
[24] Janigro D, Perju C, Fazio V, Hallene K, Dini G, Agarwal MK, et al. Alternating current electrical stimulation enhanced chemotherapy: a novel strategy to bypass multidrug resistance in tumor cells. BMC Cancer 2006;6. https://doi.org/10.1186/1471-2407-6-72.
[25] Löscher W. 3-mercaptopropionic acid: convulsant properties, effects on enzymes of the γ-aminobutyrate system in mouse brain and antagonism by certain anticonvulsant drugs, aminooxyacetic acid and gabaculine. Biochem Pharmacol 1979;28:1397–407. https://doi.org/10.1016/0006-2952(79)90443-X.
[26] Lazarowski A, Ramos AJ, García-Rivello H, Brusco A, Girardi E. Neuronal and glial expression of the multidrug resistance gene product in an experimental epilepsy model. Cell Mol Neurobiol 2004;24:77–85. https://doi.org/10.1023/B:CEMN.0000012726.43842.d2.
[27] Rosillo-de la Torre A, Zurita-Olvera L, Orozco-Suárez S, Garcia Casillas PE, Salgado-Ceballos H, Luna-Bárcenas G, et al. Phenytoin carried by silica core iron oxide nanoparticles reduces the expression of pharmacoresistant seizures in rats. Nanomedicine 2015;10:3563–77. https://doi.org/10.2217/nnm.15.173.
[28] Velíšková J, Velíšek L, Mareš P, Rokyta R. Ketamine suppresses both bicuculline- and picrotoxin-induced generalized tonic-clonic seizures during ontogenesis. Pharmacol Biochem Behav 1990;37:667–74. https://doi.org/10.1016/0091-3057(90)90544-R.
[29] Grabenstatter HL, Dudek FE. Effect of carbamazepine on spontaneous recurrent seizures recorded from the dentate gyrus in rats with kainate-induced epilepsy. Epilepsia 2019;60:636–47. https://doi.org/10.1111/epi.14680.
[30] Allgoewer A, Mayer B. Sample size estimation for pilot animal experiments by using a Markov chain Monte Carlo approach. Altern Lab Anim 2017;45:83–90. https://doi.org/10.1177/026119291704500201.
[31] Hartz AMS, Pekcec A, Soldner ELB, Zhong Y, Schlichtiger J, Bauer B. P-gp protein expression and transport activity in rodent seizure models and human epilepsy. Mol Pharmaceutics 2017;14:999–1011. https://doi.org/10.1021/acs.molpharmaceut.6b00770.s001.
[32] Merelli A, Ramos AJ, Lazarowski A, Auzmendi J. Convulsive stress mimics brain hypoxia and promotes the P-glycoprotein (P-gp) and erythropoietin receptor overexpression. recombinant human erythropoietin effect on P-gp activity. Front Neurosci 2019;13:750. https://doi.org/10.3389/fnins.2019.00750
[33] Enrique AV, Di Ianni ME, Goicoechea S, Lazarowski A, Valle-Dorado MG, Costa JJL, et al. New anticonvulsant candidates prevent P-glycoprotein (P-gp) overexpression in a pharmacoresistant seizure model in mice. Epilepsy Behav 2019:106451. https://doi.org/10.1016/j.yebeh.2019.106451.
[34] Farrell JS, Gaxiola-Valdez I, Wolff MD, David LS, Dika HI, Geeraert BL, et al. Postictal behavioural impairments are due to a severe prolonged hypoperfusion/hypoxia event that is COX-2 dependent. Elife 2016;5. https://doi.org/10.7554/eLife.19352
[35] Ristori C, Cammalleri M, Martini D, Pavan B, Casini G, Cervia D, et al. The cyclooxygenase-2/prostaglandin E2 pathway is involved in the somatostatin-induced decrease of epileptiform bursting in the mouse hippocampus. Neuropharmacology 2008;54:874–84. https://doi.org/10.1016/j.neuropharm.2008.01.008.
[36] Attia GM, Elmansy RA, Elsaed WM. Neuroprotective effect of nilotinib on pentylenetetrazol-induced epilepsy in adult rat hippocampus: involvement of oxidative stress, autophagy, inflammation, and apoptosis. Folia Neuropathol 2019;57:146–60. https://doi.org/10.5114/fn.2019.84423.
[37] Rauca C, Zerbe R, Jantze H. Formation of free hydroxyl radicals after pentylenetetrazol-induced seizure and kindling. Brain Res 1999;847:347–51. https://doi.org/10.1016/S0006-8993(99)02084-3.
[38] Borbély S, Czégé D, Molnár E, Dobó E, Mihály A, Világi I. Repeated application of 4-aminopyridine provoke an increase in entorhinal cortex excitability and rearrange AMPA and kainate receptors. Neurotox Res 2015;27:441–52. https://doi.org/10.1007/s12640-014-9515-7.
[39] Cui C, Song Y, Fan X, Guo Q, Wang J, Liu W. Excitability of the masseter inhibitory reflex after high frequency rTMS over the motor cortex: a study in healthy humans. Arch Oral Biol 2017;82:241–6. https://doi.org/10.1016/j.archoralbio.2017.06.014.
[40] Tang Z-M, Xuan C-Y, Li X, Dou Z-L, Lan Y-J, Wen H-M. Effect of different pulse numbers of transcranial magnetic stimulation on motor cortex excitability: single-blind, randomized cross-over design. CNS Neurosci Ther 2019;25:1277–81. https://doi.org/10.1111/cns.13248.
[41] Albuquerque PL, Campêlo M, Mendonça T, Mendes Fontes LA, Brito R de M, Monte-Silva K. Effects of repetitive transcranial magnetic stimulation and trans-spinal direct current stimulation associated with treadmill exercise in spinal cord and cortical excitability of healthy subjects: a triple-blind, randomized and sham-controlled study. PLoS One 2018;13. https://doi.org/10.1371/journal.pone.0195276
[42] Zrenner C, Desideri D, Belardinelli P, Ziemann U. Real-time EEG-defined excitability states determine efficacy of TMS-induced plasticity in human motor cortex. Brain Stimulation 2018;11:374–89. https://doi.org/10.1016/j.brs.2017.11.016.
[43] Rogel-Salazar G, Luna-Munguía H, Stevens KE, Besio WG. Transcranial focal electrical stimulation via tripolar concentric ring electrodes does not modify the short- and long-term memory formation in rats evaluated in the novel object recognition test. Epilepsy Behav 2013;27:154–8. https://doi.org/10.1016/j.yebeh.2013.01.006.
[44] Lolin YI, Ratnaraj N, Hjelm M, Patsalos PN. Antiepileptic drug pharmacokinetics and neuropharmacokinetics in individual rats by repetitive withdrawal of blood and cerebrospinal fluid: phenytoin. Epilepsy Res 1994;19:99–110. https://doi.org/10.1016/0920-1211(94)90020-5.
[45] Evans SR. Clinical trial structures. J Exp Stroke Transl Med 2010;3:8–18. https://doi.org/10.6030/1939-067X-3.1.8.
[46] Chen C, Li H, Ding F, Yang L, Huang P, Wang S, et al. Alterations in the hippocampal-thalamic pathway underlying secondarily generalized tonic-clonic seizures in mesial temporal lobe epilepsy: a diffusion tensor imaging study. Epilepsia 2019;60:121–30. https://doi.org/10.1111/epi.14614.
[47] Yang L, Li H, Zhu L, Yu X, Jin B, Chen C, et al. Localized shape abnormalities in the thalamus and pallidum are associated with secondarily generalized seizures in mesial temporal lobe epilepsy. Epilepsy Behav 2017;70:259–64. https://doi.org/10.1016/j.yebeh.2017.02.011.
[48] Browning RA, Nelson DK. Modification of electroshock and pentylenetetrazol seizure patterns in rats affer precollicular transections. Exp Neurol 1986;93:546–56. https://doi.org/10.1016/0014-4886(86)90174-3.
[49] Rogawski MA, Johnson MR. Intrinsic severity as a determinant of antiepileptic drug refractoriness. Epilepsy Currents 2008;8:127–30. https://doi.org/10.1111/j.1535-7511.2008.00272.x.
[50] Rogawski MA. The intrinsic severity hypothesis of pharmacoresistance to antiepileptic drugs. Epilepsia 2013;54:33–40. https://doi.org/10.1111/epi.12182.
[51] Tang F, Hartz AMS, Bauer B. Drug-resistant epilepsy: multiple hypotheses, Few Answers. Front Neurol 2017;8. https://doi.org/10.3389/fneur.2017.00301
[52] Langeh U, Chawla P, Gupta GD, Singh S. A novel approach to refractory epilepsy by targeting Pgp peripherally and centrally: therapeutic targets and Future perspectives. CNSNDDT 2020;19. https://doi.org/10.2174/1871527319999200819093109.
[53] Asadi-Pooya AA, Razavizadegan SMA, Abdi-Ardekani A, Sperling MR. Adjunctive use of verapamil in patients with refractory temporal lobe epilepsy: a pilot study. Epilepsy Behav 2013;29:150–4. https://doi.org/10.1016/j.yebeh.2013.07.006.
[54] Borlot F, Wither RG, Ali A, Wu N, Verocai F, Andrade DM. A pilot double-blind trial using verapamil as adjuvant therapy for refractory seizures. Epilepsy Res 2014;108:1642–51. https://doi.org/10.1016/j.eplepsyres.2014.08.009.
[55] Elkhayat HA, Aly RH, Elagouza IA, El-Kabarity RH, Galal YI. Role of P-glycoprotein inhibitors in children with drug-resistant epilepsy. Acta Neurol Scand 2017;136:639–44. https://doi.org/10.1111/ane.12778.
[56] Narayanan J, Frech R, Walters S, Patel V, Frigerio R, Maraganore DM. Low dose verapamil as an adjunct therapy for medically refractory epilepsy – an open label pilot study. Epilepsy Res 2016;126:197–200. https://doi.org/https://doi.org/10.1016/j.eplepsyres.2016.07.004
[57] Rocha L. Interaction between electrical modulation of the brain and pharmacotherapy to control pharmacoresistant epilepsy. Pharmacol Ther 2013;138:211–28. https://doi.org/10.1016/j.pharmthera.2013.01.009.
[58] Pérez-Carbonell L, Faulkner H, Higgins S, Koutroumanidis M, Leschziner G. Vagus nerve stimulation for drug-resistant epilepsy. Pract Neurol 2020;20:189–98. https://doi.org/10.1136/practneurol-2019-002210.
[59] Li MCH, Cook MJ. Deep brain stimulation for drug-resistant epilepsy. Epilepsia 2018;59:273–90. https://doi.org/10.1111/epi.13964.