Modulatory effects of delta sleep-inducing peptide in a lindane model of generalized seizures

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Abstract: Delta sleep-inducing peptide (DSIP) is an endogenous peptide that is constantly present in several different brain regions. Lindane is used as a pesticide and scabicide, but it also induces seizures refractory to conventional antiepileptics. The aim of this paper was to determine whether DSIP modulates lindane-induced seizures in rats in a behavioral and electroencephalographic (EEG) study. DSIP (1 mg/kg, i.p.) or dimethyl-sulfoxide (DMSO, 0.5 ml/kg, intraperitoneally (i.p.)) were injected 30 min before lindane (8 mg/kg, i.p.) to adult male rats with previously implanted electrodes for EEG registration. During the following 30 min, the EEG was registered, and the following behavioral characteristics of seizures were observed: incidence, latency and intensity. A descriptive scale with grades from 0 to 4 provided an estimate of seizure intensity. In the EEG, the number and duration of ictal periods were analyzed using NeuroSciLaBG (Belgrade, Serbia) software. The lethality rate was also analyzed. DSIP-treated animals showed significantly modified characteristics of lindane-induced seizures when compared to the group without DSIP pretreatment (i.e. a reduced seizure intensity and a prolonged seizure latency period). However, no significant effects of DSIP on seizure incidence and lindane-induced lethality were observed. EEG analyses showed a significantly decreased number of lindane-induced EEG ictal periods in DSIP-treated animals, but with unaltered duration. These results show that DSIP favorably modulates lindane-induced seizures in rats, showing a potential to be an adjuvant component of antiepileptic treatment strategy for refractory seizures.

Key words: behavior; delta sleep-inducing peptide (DSIP); EEG; epilepsy; refractory seizures lindane

INTRODUCTION

Epilepsy is one of the most common neurological disorders [1,2] with an estimated incidence of 50 cases per 100000 persons annually [3]. The total annual costs of epilepsy treatment is about 15.5 billion euros in Europe [4,5]. The majority of these costs are due to antiepileptic drug treatment, especially in cases with refractory forms requiring polytherapy. Epilepsy is caused by a sudden and excessive hyperactivity of neurons, which is the consequence of excitatory over inhibitory phenomena in the central nervous system (CNS). Usually, it is characterized by distinctive motor manifestations appearing as seizures [1]. Major excitatory phenomena are related to glutamate and its receptors, while major inhibitory phenomena are related to γ-aminobutyric acid (GABA) and its receptors. Nonetheless, it has been proven that a variety of

alternative mechanisms are also involved in the pathophysiology of epileptic seizures, such as mutations of ion channel genes, events related to glial cells, other neurotransmitters and neuromodulator disarrangements [2].

Due to its significance, the relation between the basic function of the CNS (sleep and its dysfunction) epilepsy has been the subject of many studies for some time now. In fact, the lack of sleep can change the appearance and the course of seizures, as well as their frequency [6]. Numerous antiepileptic drugs can change the architecture of sleep by as yet unclear mechanisms, while on the other hand, antiepileptic therapy can also have somnogenic effects [7-10]. Sleep and epilepsy, especially in terms of sleep spindles and ictal discharges, share many common mechanisms. These interactions include shared neuronal circuits

for sleep and epileptiform activity, increased synchronization during slow-wave sleep (SWS), and more complex interactions that depend on the intrinsic physiological and pathophysiological characteristics [11,12]. The observed global decrease in excitability across sleep appears to be differentially regulated by SWS and REM sleep. As reviewed elsewhere [13,14], it has been shown that neocortical and hippocampal neurons decrease firing activity during REM sleep. Corticothalamic projections from the major extrinsic input to the GABAergic reticular thalamic nucleus determine the generation of synchronized activities in the thalamus, which has a pacemaker role in the electrogenesis of thalamic oscillations [15]. When $GABA_A$-receptor-mediated inhibition is reduced, spindle-like oscillations are replaced by slower spike- and slow-wave types of discharges [11], showing that sleep and epilepsy, especially in terms of sleep spindles and ictal phenomena, are correlated by the dysfunction of excitatory and inhibitory mechanisms.

Delta sleep-inducing peptide (DSIP) is an endogenous and somnogenic nonapeptide (Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu) [16]. DSIP was discovered by Monnier et al. [16] who found that it increases delta activity in an EEG. Shandra et al. [17,18] showed that a neuroprotective mechanism of DSIP probably functions through the reduction of excitatory amino acids and the blockage of calcium channels on the postsynaptic membrane. The beneficial effects of DSIP have been presented in different studies dealing with epilepsy, stress and arterial hypertension [19-21].

Lindane (γ-hexachlorocyclohexane) is an organochlorine pesticide and scabicide still widely used in agriculture, but also in human and veterinary medicine, despite the fact that it was prohibited by the Stockholm Convention because of its neurotoxicity [22]. Lindane is used in underdeveloped and developing countries [23]. Symptoms of toxicity with lindane range from headaches and vertigo to pronounced seizures and death [24]. These symptoms are considered to be the result of GABA type A ($GABA_A$) receptor blockage, which reverses the inhibitory effect of GABA on local discharge and paroxysmal discharge spread [25]. Apart from CNS effects, lindane has a strong toxic impact on numerous other organ systems: the cardiovascular, gastrointestinal, reproductive systems, as well as on endocrine glands [26]. In order to examine its

mechanisms of action, and the possibility of antiepileptic therapy, a model of lindane-induced seizures in rats has been developed [25]. Due to the well-known limitations of clinical studies of epilepsy, the results obtained from animal-based experiments can help in the selection of promising combinations of antiepileptic drugs, which beside anticonvulsive activity also have minimal side effects [27]. The lindane model is refractory to numerous classical antiepileptic drugs, like carbamazepine, phenytoin and felbamate [28].

Lindane-induced seizures are predominantly the result of impaired GABAergic neurotransmission, while DSIP is capable of acting neuroprotectively by enhancing GABAergic neurotransmission and reducing excitatory amino acids. Bearing this in mind, it is reasonable to examine whether DSIP is capable of beneficially modulating lindane-induced seizures. To this end, we performed the current study with the aim of studying the effects of acute systemic application of DSIP on behavioral signs and on EEG manifestations of lindane-induced seizures in rats, an animal model resembling refractory seizures.

MATERIALS AND METHODS

Animals

All experimental procedures were in full compliance with the European Council Directive (2010/63/EU) and approved by the Ethical Committee of the University of Belgrade (Permission No 298/5-2). Adult male Wistar albino rats (2 months old, 200-230 g body weight (b.w.)) were used in the study (obtained from the Military Medical Academy Breeding Laboratory, Belgrade, Serbia). The animals were housed in transparent plastic cages with *ad libitum* access to food (Purina rat chow) and water. They were kept in a sound-attenuated chamber under controlled ambient conditions (22-23°C, 50-60% relative humidity, 12/12 h light/dark cycle with light switched on at 8 a.m.) and habituated to handling. The acclimatization period lasted for 7 days.

Drugs

All drugs were purchased from Sigma-Aldrich Chemical Co., U.S.A. and were of analytical purity.

Experimental groups

The following experimental groups were formed based on our previous results [25] and preliminary experiments: (i) control (group C; DMSO, n=6); (ii) DSIP 1 mg/kg (DSIP, n=8); (iii) lindane 8 mg/kg (L, n=10); (iv) DSIP 1 mg/kg 30 min prior to lindane administration 8 mg/kg (DSIP+L, n=8). All drugs were freshly dissolved in saline and administered (i.p.) in a volume of 0.1 ml/100 g rat b.w.

Behavioral recordings

Rats that were placed in separate transparent plastic wired-covered cages were observed for 30 min for behavioral manifestations of lindane-induced seizures. These were assessed by the incidence of motor seizures and their intensity, as well as duration of latency. Seizure intensity was qualified by a modified descriptive rating scale [29] with grades defined as: grade 1 – head nodding, lower jaw twitching; grade 2 – myoclonic body jerks (hot-plate reaction), bilateral forelimb clonus with full rearing (Kangaroo position); grade 3 – progression to generalized clonic convulsions followed by tonic extension of fore and hind limbs and tail; grade 4 – prolonged severe tonic-clonic convulsions lasting over 10 s (*status epilepticus*) or frequent repeated episodes of clonic convulsions for an extended period of time (over 5 min). Latency to seizure was defined as the time from the lindane injection to the first seizure response and was also recorded. For rats without seizures, a 30-min latency time was scored. Lethality was recorded at the end of the observation period.

Surgery

The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Three gold-plated recording electrodes were implanted over the frontal, parietal and occipital cortices in a stereotaxic apparatus. Dental acrylic cement was used to fix this system to the skull. Animals had a recovery period of one week prior to further experiments. A 24-h-long habituation to the recording situation was also applied.

EEG recording

An 8-channel EEG apparatus (RIZ, Zagreb, Croatia) was used. The signals were digitized using a SCB-68 data acquisition card (National Instruments Co, Austin, Texas, USA). A sampling frequency of 512 Hz/channel and 16-bit A/D conversion were used for the EEG signals. The cutoff frequencies for EEG recordings were set at 0.3 Hz and 100 Hz for the high-pass and low-pass filters, respectively. Ambient noise was eliminated using a 50-Hz notch filter. Data acquisition and signal processing were performed with LabVIEW platform software developed in the Laboratory (NeuroSciLaBG [29]). EEGs were recorded in freely moving rats during a 30-min recording session. EEG traces were visually inspected and analyzed during subsequent offline analysis. The power spectra density (obtained by the Fast Fourier transformation method) of the characteristic epochs was plotted and the integrated energy signals expressed as $\mu V^2/Hz$. Ictal periods in EEG were defined as follow: (i) spontaneous and generalized spiking activity; (ii) lasting>1 s; (iii) amplitude of at least twice the background EEG activity [29]. The number and duration of ictal periods were calculated during a 30-min period after lindane administration.

Data analysis

Significance of the differences in the seizure incidence and lethality were evaluated by Fisher's exact probability test. Since the normal distribution of the data on seizure latency, intensity of seizure, as well as the number and duration of ictal periods in EEG were not estimated by Kolmogorov-Smirnov test, nonparametric analysis (Mann Whitney U test) was used to determine the statistical significance of the differences between the groups (*p<0.05, **p<0.01). The results were expressed as medians with 25th and 75th percentiles.

RESULTS

Behavior assessment

All vehicle-treated rats (group C) and DSIP-treated rats (group DSIP) showed normal behavior without

any signs of seizures. The incidence of seizures in the group of rats treated with lindane at a convulsive dose (8 mg/kg, i.p., group L) was 80%. In the group of rats which received DSIP (1 mg/kg, i.p.) prior to lindane administration (group DSIP+L), the seizure incidence was lowered to 62.5%. However, according to Fisher's exact probably test, this difference between DSIP+L and L groups regarding seizure incidence did not attain statistical significance (p>0.05, Fig. 1A).

Beside seizure incidence, we analyzed the duration of the latency period to the first sign of seizure and seizure intensity as parameters of convulsive behavior. DSIP administered prior to lindane (group DSIP+L) led to a significant prolongation of the latency period to the first sign of seizure when compared to group L (DSIP+L *vs* L, p<0.05 as assessed by the Mann Whitney U test; Fig. 2A). The same holds true regarding seizure intensity. Seizure intensity was significantly lower in the DSIP+L group compared to the L group (DSIP+L *vs* L, p<0.05 as assessed by the Mann Whitney U test, Fig. 2B). The maximal seizure intensity in group of rats treated by lindane (group L) was grade 4, while there were no seizures of grade 4 in the group of rats treated with DSIP prior to lindane (group DSIP+L, maximal seizure intensity was grade 3).

A lethal outcome was observed in 30% of animals from group L, and in 25% of animals in group DSIP+L at the end of the observation period. This difference in lethality between groups DSIP+L and L was not statistically significant (p>0.05, Fisher's exact probability test, Fig. 1B).

EEG analysis

Bioelectrical brain activity registered by EEG in all vehicle-treated rats (group C), as well as in DSIP-treated rats (group DSIP) were without any signs of ictal activity. During the registration procedure the rats were quiet but awake (Fig. 3A). Spontaneous, generalized and sporadic ictal activity (defined as ictal periods, a typical one is presented on Fig. 3B) was recorded in rats treated by lindane alone (group L) and those treated with DSIP prior to lindane (group DSIP+L). Offline analysis of the EEG recordings included an assessment of the number and duration of these manually identified ictal periods defined according to criteria described in the Materials and Methods
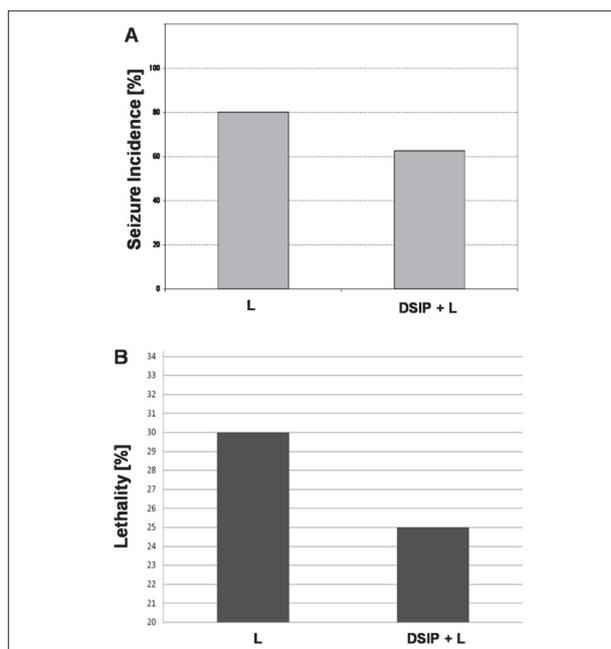

Fig. 1. The effects of DSIP on the incidence of seizures (**A**) and lethality (**B**) induced by lindane in rats. Lindane was administered in a dose of 8 mg/kg (L group). DSIP (1 mg/kg) was administered 30 min prior to lindane (DSIP+L). Seizure incidence: the number of convulsing animals in the group out of the total number of animals in the group, expressed in percentages. Lethality was registered at the end of the observation period. Significance of the difference was determined by Fisher's exact probability test.
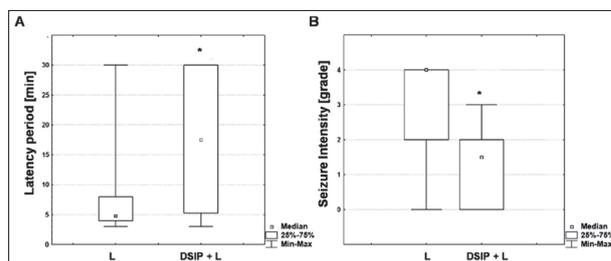

Fig. 2. DSIP modulated seizure intensity (**A**) and the latency time (**B**) of lindane-induced seizures. Seizure intensity was assessed by a descriptive rating scale from 0-4. Latency period: the time from lindane administration to the first sign of a seizure. Significance of the difference was determined by Mann Whitney U test (*p<0.05). For details see the caption to Fig. 1.

section. The number of ictal periods in rats treated with lindane alone (group L) ranged from 9 to 14 per rat, while this number ranged from 6 to 8 per rat in the group of rats treated with DSIP prior to lindane (group DSIP+L). Moreover, the median number of ictal periods was significantly lower in the DSIP+L group when compared to the L group according to the Mann Whitney U test (p<0.05, Fig 4A). On the other
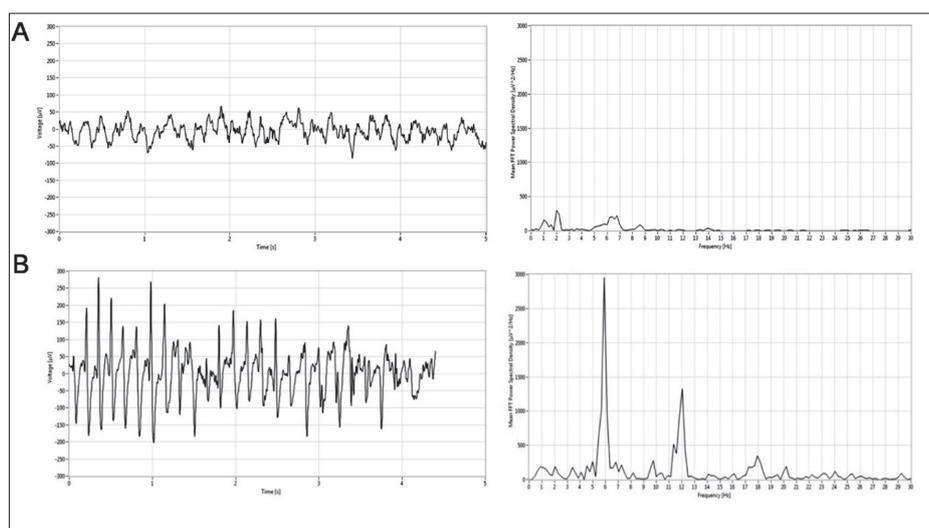
Fig. 3. Representative EEG traces (left panels) with corresponding power spectra diagrams (right panels) in control (**A**) and lindane-treated rat (**B**). EEGs were recorded in freely moving rats. Corresponding power spectra densities were obtained by fast Fourier transformation. Note the burst of spiking activity in the B trace representing a characteristic ictal period induced by lindane in rats. Lead: left frontal, right parietal. Amplitude calibration: 50 µV, time calibration: 1 s. For details see caption to Fig. 1.
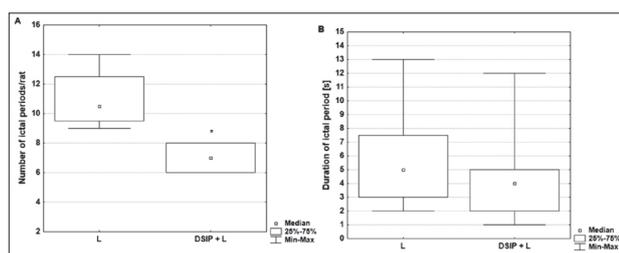

Fig. 4. The effects of DSIP on the number (**A**) and duration (**B**) of EEG ictal periods induced by lindane. The EEG ictal period denotes a spontaneous and generalized spiking activity lasting>1 s, with an amplitude of at least twice the background EEG activity. Significance of the difference was determined by Mann Whitney U test (*p<0.05). For details see the caption to Fig. 1.

hand, the minimal duration of one ictal period was 2 s in the L group and 1 s in the DSIP+L group, while the maximal duration of one ictal period was 13 s in the L group and 12 s in the DSIP+L group. Median duration of ictal periods was not significantly different between these two groups (DSIP+L *vs* L, p>0.05, Mann Whitney U test, Fig 4B).

DISCUSSION

DSIP is a natural and ubiquitous somnogenic nonapeptide, which has no side effects even at very high doses. DSIP takes part in numerous non-sleep related physiological functions; its antiepileptic role [30,31] was of most interest to us. In our laboratory, we examined DSIP as an anticonvulsive neuropeptide in metaphit (1-(1(3-isothiocyanatophenyl)-cyclohexyl

(piperidine)audiogenic epilepsy [30]. In the current study, we addressed the ability of DSIP to act against lindane-induced seizures, a seizure model which is refractory to numerous classical antiepileptic drugs [28].

The present study showed that there was a significant interaction between the effect of lindane and delta sleep-inducing peptide on the CNS of experimental animals. Actually, DSIP administrated 30 min prior to lindane exhibited a tendency to reduce the incidence of lindane-induced seizures, but the achieved reduction was not statistically significant. Furthermore, DSIP significantly reduced seizure intensity and prolonged the latency period of lindane-induced seizures. Moreover, EEG analyses showed significant reduction of the number of ictal periods after DSIP administration, without significant alteration in ictal period duration. Thereby the behavioral and electrical parameters of hyperexcitability caused by lindane were reduced by the protective effects of neuropeptide DSIP.

Epileptic seizures arise from an excessively synchronous and sustained discharge of a group of neurons, i.e. increased neuronal excitability. Hyperexcitability can be caused by alterations in the membrane and metabolic properties of neurons. Therefore, seizure initiation is characterized by high-frequency bursts of action potentials and hypersynchronization of a neuronal population with a paroxysmal depolarizing shift in its pathophysiological basis. These synchronized bursts originating simultaneously from numerous neurons, result in ictal activity in the EEG (reviewed in detail

elsewhere [2,32]). Herein we quantified and analyzed the number and duration of ictal periods in an EEG induced by lindane and modified by DSIP.

It was shown that lindane induces generalized epileptic seizures in rats in a dose-dependent manner, which is distinctive on EEG [25]. The characteristics of lindane-induced seizures are similar to those of kainate-induced seizures [31]. The mechanism by which lindane induces neuronal hyperexcitability and neurotransmitter concentration changes in the neural structures remains unclear, but it is believed that the main effect of lindane is manifested through blockage of $GABA_A$ chloride channels [34, 35]. Furthermore, the effect on calcium mobilization is certainly one of the contributing factors [36]. We have shown the involvement of NO-signaling in lindane proconvulsive effects [29]. However, the latest research has shown that lindane-induced seizures are refractory to numerous antiepileptic drugs (carbamazepine, phenytoin, felbamate) [28]. According to the results of the present research, DSIP affected the parameters of convulsive behavior, especially the latency period and the intensity of seizures, as well as the EEG manifestations of lindane proconvulsive effects. The frequency of defined ictal periods was lowered when DSIP preceded lindane, but the duration of these periods remained unaltered. Delta waves in the EEG are those with the smallest frequencies and indeed, DSIP-induced delta activity modulated the electrical events in neurons caused by lindane.

Numerous steps have been taken in order to clarify DSIP mechanisms of action. DSIP optimizes the excitation/inhibition relationship [36]. Namely, it potentiates GABA-activated currents in the hippocampal and cerebellar neurons in rats [37], which could be one of the major mechanisms of action in the model of lindane-induced seizures. On the other hand, Shandra et al. [18] believe that the neuroprotective role of DSIP on NMDA receptors functions through the reduction of excitatory amino-acids and decrease of Ca^{2+} influx. It is important to know that DSIP also affects the activity of transaminases [38].

Epileptogenesis is also a consequence of oxidative stress [39,40]. DSIP optimizes the prooxidant-antioxidant balance by increasing the activities of antioxidant enzymes and the levels of antioxidants [41,42]. These effects of DSIP on antioxidant capacities could be explained by increased expression of genes for superoxide dismutase 1 and glutathione peroxidase 1, which were evoked by prolonged DSIP treatment [43]. In conjunction with the role of DSIP in amelioration of oxidative stress is its role in the stabilization of cell membranes. DSIP increases membrane stability, changes its selective permeability and inhibits the accumulation of lipid peroxidation products by modulating the physicochemical characteristics of the membrane [44]. DSIP has been shown to adopt a compact conformation which permeates the lipid bilayer due to reduced charge, increased lipophilicity and intramolecular hydrogen-bond stabilization [45]. Its neuroprotective effects could be related to the role of DSIP in the stabilization of the structure and functioning of neuronal membranes [46]. It is also important to emphasize that DSIP has a tranquilizing effect on neurons, especially on glutaminergic hippocampal neurons and the neurons of the front hypothalamic nucleus [47].

Careful examination of convulsive behavior parameters suggests a beneficial modulatory role for DSIP in the lindane model of seizures. However, since the effect of DSIP on lindane seizure incidence was minor, it is not plausible to regard DSIP as a powerful antiepileptic drug for a monotherapy regime in refractory cases. On the other hand, DSIP can be regarded as a valuable add-on drug in the treatment of refractory epilepsies. The following findings additionally support this view: (i) the synergic effect of DSIP as a native neuropeptide together with valproate (a classical antiepileptic drug) in the treatment of the epileptic seizures; (ii) results of numerous studies where the antiepileptic role of DSIP was shown on models of generalized epilepsy induced by picrotoxin, corasol, kainate, NMDA and metaphit [18,30,46,47]; (iii) DSIP, unlike other anticonvulsants drugs, has no side effects, as even an overdose has no negative effect on the CNS [50].

When it comes to a potentially wider clinical application of DSIP, oral peptide delivery as a preferable way of administration should be considered. DSIP is capable of permeating the blood-brain barrier [51] and the intestinal mucosa of neonatal rats, but the adult intestinal mucosa appears to be a barrier for DSIP. Therefore, additional structural changes or coupling are necessary to ensure intestinal membrane permeability for DSIP [52]. A successful attempt in DSIP application was the creation of Deltaran in

which DSIP is combined with glycine for intranasal administration of an aqueous solution [53]. Subsequent studies demonstrated geroprotective, anticarcinogenic and neuroprotective effects of DSIP and Deltaran [53,54].

The results of this research show that DSIP can be considered as an integral constituent of treatments of generalized epilepsy. Among numerous efforts to find an effective add-on drug for alleviating epilepsy with minimal side effects, this research provides a step forward.

Author contributions: DH, ŽG, OS designed the experiment, performed the experiments and drafted the manuscript. NŠ and AA contributed to the behavioral studies and the draft of the manuscript. ARM contributed to the EEG studies and manuscript draft. All authors reviewed and approved the final manuscript text.

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