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BENEFICIAL EFFECTS OF DELTA SLEEP INDUCING PEPTIDE ON METAPHIT SEIZURES

STANOJLOVIĆ OLIVERA*, HRNČIĆ D*, RAŠIĆ ALEKSANDRA*, ŠUŠIĆ VESELINKA** and ŽIVANOVIĆ DRAGANA*

*School of Medicine, Belgrade
**Serbian Academy of Sciences and Arts, Belgrade

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The effects of delta sleep inducing peptide on metaphit (1-[1(3-isothiocyanatophenyl)-cyclohexyl]piperidine) provoked seizures in adult rats depending on whether it was administered prior or upon metaphit were studied.

Animals were administered: 1. saline; 2. metaphit 3. DSIP and 30 min later metaphit (protected group); 4. metaphit and 8 h later DSIP (blocked group) or 5. DSIP alone. Upon the treatment, the rats were exposed to sound stimulation at hourly intervals and the incidence and behavioral severity (running, clonus and tonus) and EEG correlates of seizures were analyzed .

Metaphit produced EEG abnormalities as spikes and spike-wave complexes and increased power spectra. Time-course studies revealed the peak of convulsive activity 7-12 h after metaphit administration. DSIP significantly increased power spectra of δ band no matter whether it was injected prior or after metaphit.

Treatment with DSIP prolonged latency and reduced all convulsive components for a long period, as well as the mean seizure grade, but it was more pronounced in the protected than in the blocked group.

Key words: metaphit, seizures, protection, DSIP, blockade, rat

INTRODUCTION

The reasons for sleep duality remained unclear although the existence of REM sleep elapses was recorded almost five decades ago (Gamundi *et al.,* 2003). During 1970s numerous sleep neuropeptides were identified and Monnier *et al.,* (1977) described a delta sleep-inducing nonapeptide (DSIP). Beside its controversial sleep promoting-effect, DSIP was described as a neuromodulatory peptide with a broad spectrum of physiological actions, but the exact mechanism underlying its action is still far from being completely understood. Epilepsy and sleep have a significant and intimate inter-relationship (Dinner, 2002). The anticonvulsive properties of DSIP were demonstrated in generalized forms of epilepsy induced by substances that act as blocking agents of GABA receptors.

e.g. bicuculline, picrotoxin and corazol (Shandra *et al.,* 1993), as well as in models with epileptic foci in the cerebral cortex induced by penicillin and strychnine (Prudchenko *et al.,* 1993).

Metaphit (1-[1(3-isothiocyanatophenyl)-cyclohexyl]piperidine), a phencyclidine (PCP) analog is known by its long-term binding to the ionic channel of the NMDA receptor complex (Rafferty *et al.*, 1985). It was shown to increase general brain excitability and to induce audiogenic seizures in small rodents, as judged by changes in EEG recordings of paroxysmal activity (Debler *et al.,* 1989; Šušić *et al.,* 1991). Metaphit-induced audiogenic seizures could be blocked by competitive (Živanović *et al.,* 1998) and noncompetitive NMDA receptor antagonists (Šušić *et al.,*1993 a).

The above results prompted us to study the effects of DSIP injected before or after metaphit administration on metaphit-induced generalized audiogenic reflex epilepsy convulsions and abnormal EEG in adult Wistar rat males.

MATERIAL AND METHODS

Adult Wistar rat males (170-200 g), bred at the Military Medical Academy Breeding Laboratories, Belgrade were used. The animals were maintained under controlled enviromental conditions (22-24 ^{o}C, 50-60% humidity, 12 h/12 h light/dark cycle, light on at 9 a.m.) with free access to standard laboratory chow and tap water. They were housed individually in transparent plastic cages (55x35x15 cm).

In order to determine seizure activity, audiogenic stimulation (AGS) was applied for 60 s using an electric bell (on the top of the cage) generating 100±3 dB and frequency of 5-8 kHz. None of the untreated animals screened for audiogenic susceptibility expressed seizure activity. The first stimulation was applied 60 min after metaphit administration and repeated thereafter at hourly intervals during the experiment for about 30 h. Audiogenic seizure behavior was assessed by incidence of motor seizures and seizure severity grade using a descriptive rating scale from 0-3 (0 = no response; 1 = wild running only; 2 = wild running followed by clonic seizures; 3 = wild running progressing to generalized clonic convulsions followed by tonic extension of limbs and tail). All experimental procedures were performed in accordance with the European Council Directive (86/609/EEC), and were approved by the Animal Care Committee of the University of Belgrade.

The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), positioned in a stereotaxic apparatus and three gold-plated recording electrodes were implanted over the frontal, parietal and occipital cortices. An EEG apparatus (Alvar, France) with a modified output degree enabling to transfer output signals to the input circuit of 8-channel, 12-byte AD card PCL-711B (Advantech Co. Ltd.) installed into a computer and the corresponding software were used.

Length of epochs for EEG analyses depended on characteristic EEG changes and varied broadly from 7-25 s. Selected EEG power spectra were analyzed visually and by Matlab software. Such analyses provided absolute and

relative numerical values of the individual EEG components. The power spectra were plotted and the integrated energy signals expressed as μV^2/Hz.

Subsequenly, 52 rats were divided into the following groups: 1.control, saline-injected (n=6); 2. metaphit administered (MP; 10 mg/kg; n=12); 3. DSIP (1.0 mg/kg; n =14) injected 30 min prior to metaphit (10 mg/kg), referred to as the *protected* group *(P)*. This group served to evaluate the ability of DSIP to inhibit metaphit from eliciting audiogenic seizure susceptibility in rats; 4. metaphit (10 mg/kg) and 8 h later DSIP (1.0 mg/kg; n =14, *blocked* group, *B*) This group was used to evaluate the ability of DSIP to block the occurrence of audiogenic seizures in rats made susceptible by metaphit. Only metaphit-treated animals displaying seizures in 8 previous tests received DSIP and AGS, and were followed at hourly intervals in order to investigate their effects on fully developed seizures, as well as the last formed group i.e. 5. DSIP injected (1.0 mg/kg, n= 6). The injecting solutions given i.p. in a total volume of 0.1 mL were prepared in sterile physiological saline.

The significance of differences was assessed between P vs. MP and B vs. MP group. To evaluate the differences in the incidence of convulsive components Fisher's exact probability test was used. Kruskal-Wallis one-way ANOVA and Mann Whitney U-test were applied to evaluate the differences of the mean seizure grade. Significance of the differences in mean duration (means ± S.E.M.) of convulsive components was estimated by Kruskal Wallis ANOVA test (*$p<0.05$, **$p<0.01$).

RESULTS

Behavior and EEGs of control animals challenged for AGS were unchanged and epileptic signs were not recorded in none of the rats during the entire experiment.

Metaphit in the dose of 10 mg/kg, produced initial EEG epileptic signs and first motor changes within the first hour after the administration. The incidence and severity of convulsive responses to audiogenic stimulation were the highest 8-12 h upon metaphit injection (10 out of 12 animals and 2.25±0.32 mean seizure grade in 10 h) to be gradually decreasing thereafter. No response to the AGS was recorded 31 h following metaphit injection.

DSIP injected 30 min before metaphit completely prevented clonic and tonic seizure activity already during 1- 3 h after administration (Table 1; Figs. 1 and 2). Running was not observed during the first hour, while later on, a statistically significant reduction in the number of running convulsions was registered in the P group comparing to the metaphit group.

AGS-induced clonic convulsions were absent in group P for the initial period up to 4 h and later on. Their incidence was significantly reduced in comparison with the metaphit group. A complete absence of tonic seizures was observed during the first 3 h of AGS tests in the P group, however the severity of tonic seizures was significantly reduced (Table 1; Fig. 1).

Also, from Fig. 2 it can be seen that the mean seizure grades in group P were significantly reduced in comparison with the metaphit group.

Table 1. Time course of seizure incidence in metaphit-treated adult Wistar rats induced by audiogenic stimulation and modulation with DSIP

t(h)	running MP n=12	P n=14	B n=14	clonus MP n=12	P n=14	B n=14	tonus MP n=12	P N=14	B n=14
1	2	0		1	0		1	0	
2	3	1		2	0		2	0	
3	4	1		3	0		3	0	
4	4	1		4	0^{a*}		3	1	
5	9	3^{a**}		7	1^{a**}		6	1a	
6	8	3^{a*}		8	3^{a*}		7	3	
7	9	4^{a*}		9	3^{a**}		9	2^{a**}	
8	9	6	14	9	5	14	8	4	14
9	9	4^{a*}	14	9	4^{a*}	14	7	4	11
10	10	4^{a**}	13	10	3^{a**}	12	7	2^{a**}	10
11	9	6	12	9	2^{a**}	10	5	1^{a*}	9
12	9	4^{a**}	8	8	4	8	7	2^{a*}	5
13	8	4	8	6	3	6	5	3	3
14	7	4	8	7	4	4	7	4	3
15	8	4	8	5	4	5	5	4	5
16	6	3	6	6	3	3	5	0^{a*}	3
17	5	3	4	5	3	4	2	2	1
18	4	2	4	4	2	4	4	1	2
19	5	2	3	5	2	3	5	2	1b
20	5	2	3	4	2	3	4	2	2
21	3	1	3	2	1	3	2	1	2
22	3	0^{a*}	2	3	0	2	3	0	2
23	4	0^{a*}	0^{b*}	3	0	0	3	0	0
24	4	0^{a*}	0^{b*}	4	0^{a*}	0b*	4	0^{a*}	0^{b*}
25	5	0^{a*}	0^{b*}	4	0^{a*}	0b*	4	0^{a*}	0^{b*}
26	3			3		0	3		0
27	2			2			2		
28	2			2			2		
29 30	3 1			3 1			3 1		

n – number of animals; t – time after metaphit administration; mp – metaphit (10 mg/kg); P – Protected group to whom DSIP (1.0 mg/kg) was injected 30 min prior to metaphit; B – Blocked group in which rats received metaphit and 8h later DSIP (1.0 mg/kg). P and B were subjected to an intense audio stimulation (100±3 dB, 60s) on hourly basis after the injection. Significance of the differences in the number of convulsions was evaluated as P vs. MP (a) and B vs. MP (b) by Fisher's exact probability test (*$p<0.05$ and **$p<0.01$).

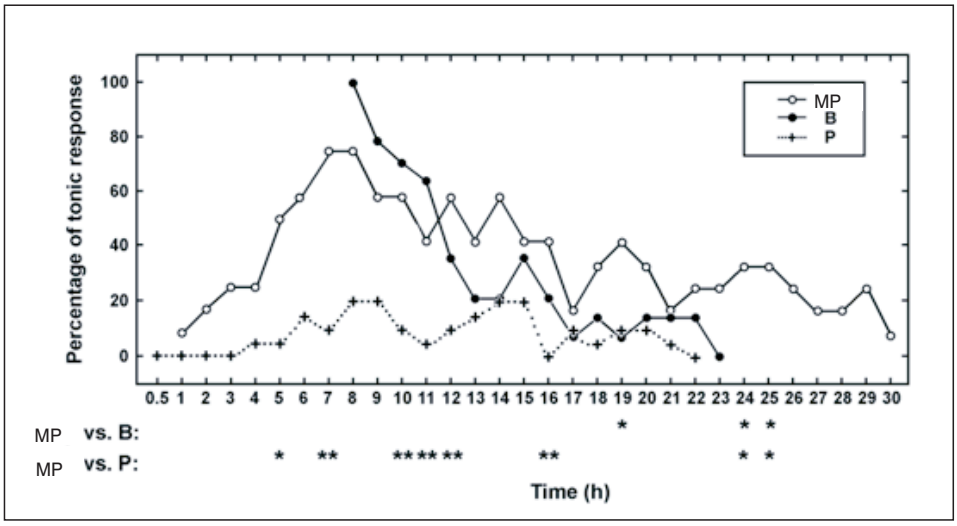

Figure 1. Incidence of the tonic component of metaphit-induced seizures followed by DSIP (1.0 mg/kg, i.p.). All animals were exposed to an intense AGS at hourly intervals after the injection.
Significance of the differences between the experimental groups: P *vs.* MP (†) and B *vs.* MP (‡), (*p<0.05) was evaluated by Fisher's exact probability test. For details see the caption to Table 1.

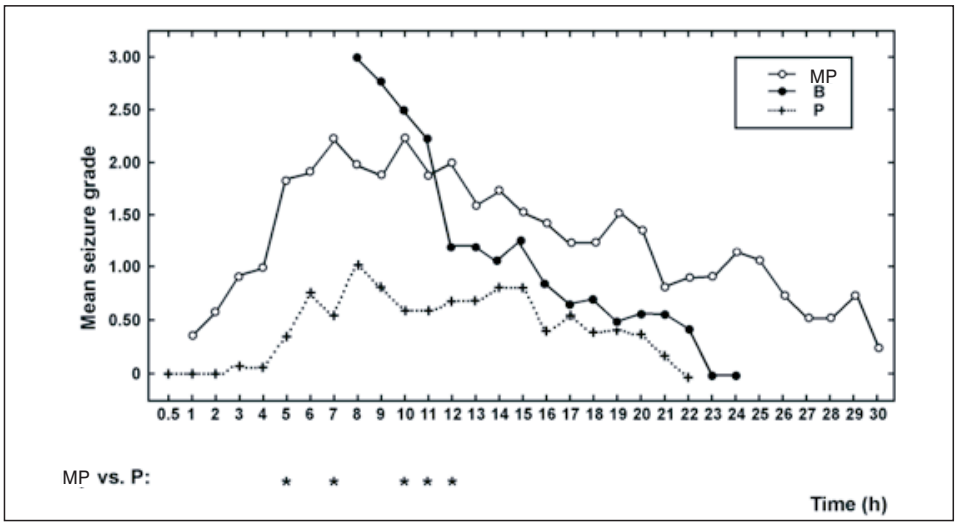

Figure 2. Time course of seizure grade in metaphit-induced convulsive activity followed by DSIP administration. Severity of seizures (expressed as seizure score) was determined by a descriptive rating scale ranging from 0 to 3. Significance of the differences in mean seizure grades between the groups was estimated by Kruskal-Wallis one–way ANOVA and Man Whitney U-test (*p<0..05).

These two groups also differed in the duration of seizure components. Four hours after metaphit administration numerous changes have been observed in group P. *The latent period* (period from sound onset to the first seizure component i.e. running) was prolonged in both DSIP-treated groups of animals (P and B). From the data presented in Fig. 3 it is obvious that the application of DSIP prior to metaphit was very efficient even during the first four hours after metaphit injection. Also, the mean duration of running+clonic+tonic seizure components in group P was significantly shorter when compared to the group treated with metaphit only during the same period, i.e. 1 h [F (2.23)=265.0, 42.0 and 95.5 for running, clonus and tonus, respectively]; 2 h [F (2.24)=87.0, 37.33 and 9.39 for running, clonus and tonus, respectively]; 3 h [F (2.25)=32.0, 7.80 and 85.3 for running, clonus and tonus, respectively] and 4 h [F (2.25)=9.55, 7.80 and 10.28 for running, clonus and tonus, respectively]; $p<0.01$ for all points.

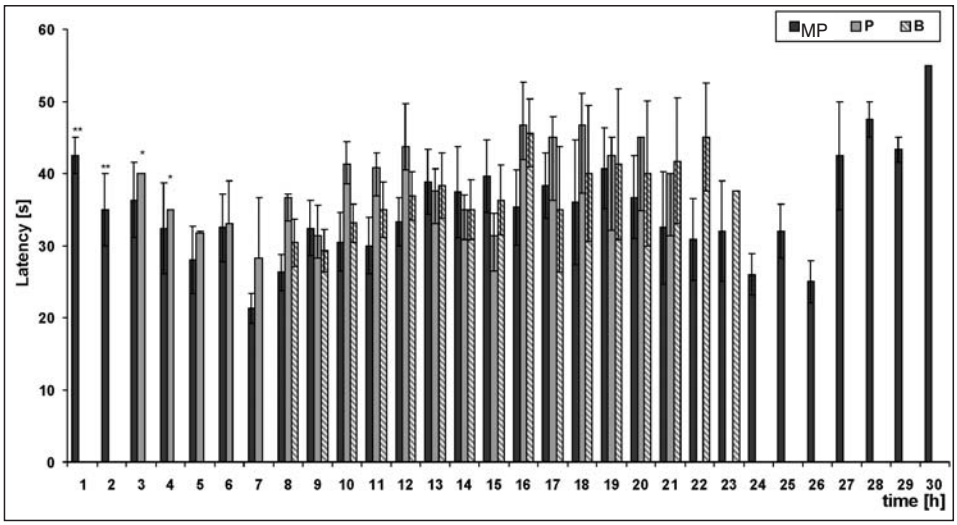

Figure 3. Latency elapsed until the onset of audiogenic seizure. Latency –time from the onset of sound stimulation to the onset of running. Values expressed as means ± SD stated in seconds. Significance of the differences in mean duration of latency between P and MP groups was evaluated by Kruskal – Wallis ANOVA test (*$p<0.05$ and $p<0.01$). For group treatment see Table 1 caption

The same holds true for the mean duration of running 11 h [F (8.53)=21.05] ($p<0.01$); clonic 5 h [F (2.28)=6.76; $p<0.05$] and tonic convulsive component 8 h [F (8.62) =14.65; $p<0.01$]; 12 h and 13 h [F(8.29)=10.90 and 6.90, respectively; $p<0.01$] after metaphit administration.

For the blocked group (B), metaphit-treated rats that developed convulsions with maximal seizure activity during the first 8 h were used and treated with DSIP. Animals in this group displayed mildly reduced seizures expressed as running

accompanied by clonic convulsions 4 h after DSIP injection (the 12th AGS) with the incidence of 8 out of 14 animals and tonic convulsions with the incidence of 5 out of 14 rats (Table 1).

In fully developed convulsions, DSIP acted by decreasing seizure severity and significantly reducing running+clonic+tonic seizures activity in comparison with the metaphit group as shown in Table 1 and Fig. 1. In group B, the mean seizure grades were reduced, the reduction being most prominent 3 h - 5 h after DSIP injection (Fig. 2).

Mean duration of running component in the blocked group was significantly shorter 11 h [F (8.53)=21.05; $p<0.01$] and 14 h [F (8.40)=4.51; $p<0.05$] after metaphit administration when compared to the group treated with metaphit only. The same holds true for the duration of the tonic component in metaphit+DSIP group 13 h after metaphit injection, in comparison with the group that received metaphit only [F(8.29)=6.90; $p<0.01$].

EEG manifestations of metaphit-induced seizures started with separated spikes without clinical seizure activity. About 8 h after metaphit administration the power spectra increased and EEG progressed into polyspikes, paroxysmal synchronous spiked-wave discharges (predominant frequency in the 1-4 Hz range) was observed especially in the period of sound onset and seizure events (Figs. 4, 5, 6).

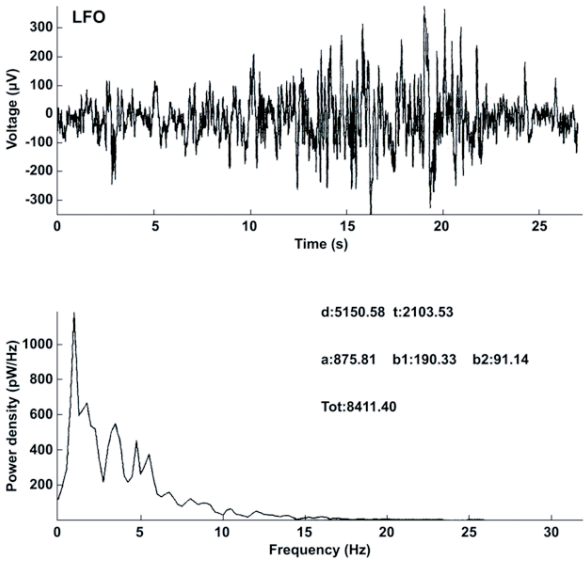

Figure 4. Electroencephalogram records (upper tracings) and the corresponding power spectra (bottom) of the typical electrographic seizure discharges in metaphit-treated adult Wistar rat males. Spiking activity was observed during 1.5 h upon metaphit administration (clonic-tonic convulsions). Numbers in the lower parts of the figure refer to the absolute power (μV^2) of the δ, θ, α, β_1, β_2 bands and of the total power spectra (Tot). LFO: left - fronto-occipital cortex. Time calibration 5 s; amplitude calibration 100 μV.

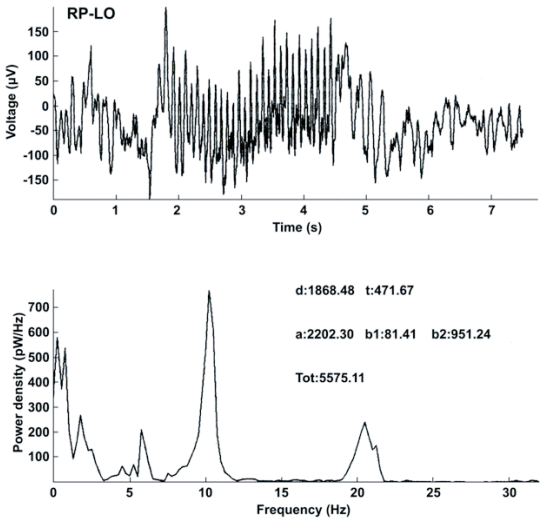

Figure 5. EEG record of maximal motor seizure response (grade 3) induced by audiogenic stimulation (100±3 dB, 60 s) in a metaphit-treated rat during sound stimulation. Note EEG postictal depression and very intense power spectrum that was temporary decreased after the sound onset. RP-LO: right parietal - left occipital cortex. For details see caption to Fig. 3. Time calibration 1 s; amplitude calibration 100 μV

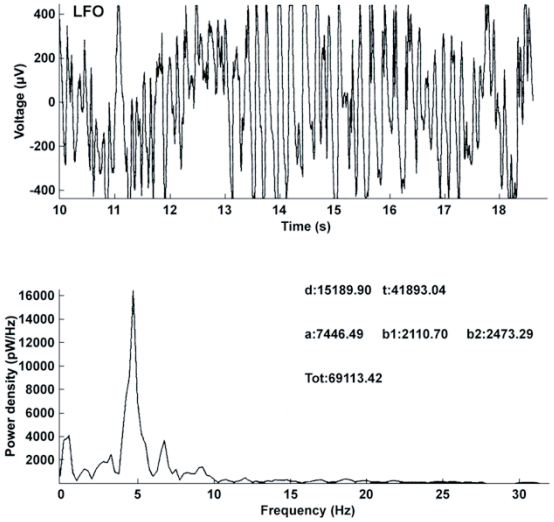

Figure 6. EEG record of tonic extension in a metaphit-treated rat (grade 3). Note high amplitude low-frequency synchronized spikes (1-5 Hz) and the corresponding intensive power spectra (16 000 μV^2). RP-LO: right parietal - left occipital cortex. For details see caption to Fig. 3. Time calibration 1 s; amplitude calibration 100 μV

In order to determine whether DSIP in the applied dose really reduced and suppressed metaphit-induced brain excitability, including both motor seizure activity and paroxysmal EEG changes, the animals were continuously EEG waves monitored for about 25 h after DSIP (P group).EEG characteristics of metaphit epileptic action were overlapped with bursts of high amplitude EEG dominantly in the delta range (Fig. 7)

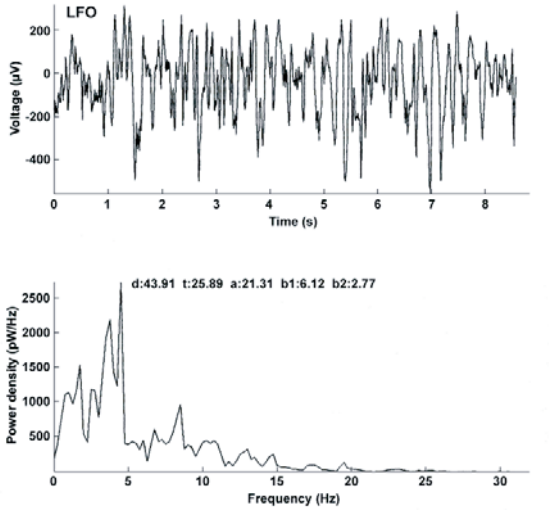

Figure 7. EEG recording in a rat treated with DSIP (1 mg/kg) 30 min following metaphit administration. High amplitude waves in 1-5 Hz range (delta waves, making 44% of the spectrum) and a very intense power spectrum (μV^2) changed metaphit-induced EEG spiking. LFO: left - fronto-occipital cortex. Time calibration 1 s; amplitude calibration 100 μV

DISCUSSION

In the present study we assessed the influence of DSIP injected prior or upon metaphit on metaphit-induced seizure activity.

The data summarized in Table 1 and depicted in Figs.1, 2 and 3 clearly demonstrate DSIP-related reduction in the number of seizure manifestations both in P and B groups. This DSIP effect was more prominent in group P and reduced metaphit effects were visible in almost all experimental time points in comparison with metaphit group. Prolongation of latent period was observed in both DSIP treated groups in comparison with the group treated with metaphit alone, but it was more pronounced in goup P than group B. It should be pointed out that the number of metaphit-induced seizure components and their duration were mildly reduced in group B, while some seizure components were completely absent in group P for a long period of time after DSIP application. The results presented here are in accordance with the data reported previously on DSIP tetra peptide

analogue effects and the ability of DSIP to potentiate valproate anticonvulsive activity in metaphit seizures (Stanojlović *et al.,* 2004, Hrnčić *et al.,* 2006).

The information on ionotropic and metabotropic NMDA antagonists and drug combinations efficient as epilepsy suppressors was very valuable (Loscher, 1998) and resulted in the design and synthesis of a new generation of antiepileptic drugs acting on NMDA/AMPA receptors. However, about a third of patients were found to be resistant to current pharmacotherapies and the seizures remained uncontrolled (Loscher, 2002). The discovery of pharmacoresistance and epileptogenesis could be more successful by developing experimental models relevant to the epileptic conditions in humans and a number of experimental animal models have been proposed so far in order, to help to elucidate the pathophysiology of this severe disease condition (Brandt *et al.,* 2003; Stable *et al*., 2002).

Injection of metaphit to intact rats was reported to occupy the NMDA receptors and to induce behavioral and EEG changes (Šušić *et al.,* 1991, 1993a, 1993b). This could be connected to the results of Meldrum (1994) who reported that following the activation of NMDA receptors by glutamate, cytosolic Ca^{2+} concentration increases and overstimulates excitatory amino acid receptors to the convulsive threshold.

CNS structures involved in the control of electrocortical activity use glutamate as a transmitter, but the excitations up to epileptogenesis are also glutamate-mediated. On the other hand, DSIP, a nonapeptide, was imunohistochemically identified in the pineal gland, hypothalamus, septum, hippocampus and other regions of rat brain (Skagerberg *et al.,* 1991).

There are several proposed mechanisms by which exposure to excess DSIP could reduce seizures. Shandra *et al.* (1998) suggested that antiepileptic DSIP activity was based on the reduction of excitatory amino acid system and decreased glutamic acid release from presynaptic terminals, or its direct action on neuronal membranes leading to changes in the activity of the NMDA receptors. The number of neurons activated by glutamate was shown to be significantly decreased after preliminary microiontophoretic DSIP application, i.e. DSIP acted blocking the excitatory effect of glutamate (Umriukhin, 2002). Recently, Lipovac *et al.* (2003) hypothesized that metaphit could inhibit not only the influx and over-accumulation of glutamate in the brain but also the glia uptake of glutamate, thereby increasing the extra cellular glutamate levels that may be the reason for an increased brain excitability.

It is worth mentioning that pretreatment with DSIP prior to experimental hypoxia was reported to have pronounced antistress and antioxidant potency (Khvatova *et al.,* 1995, 2003). Our results demonstrating the delay in the onset of generalized convulsive activity in group P and alleviation of metaphit effects in group B could be connected to data of Mendzeritskii *et al.,* (1996) who showed that DSIP applied before exposure of animals to increased pressure prevented the disbalance between inhibitory and excitatory neuromediators, supported inhibitory processes, and reduced glutamate and aspartate levels. Besides, DSIP was shown to prevent the development of spontaneous experimental audiogenic

epilepsy due to optimal ratio between monoamines in brain structures under hypokinetic stress condition (Mendzeritskii *et al.,* 1997).

Anticonvulsive activity of DSIP is manifested as reduced movement activity, increased latent period and decreased mean intensity of convulsions (Shandra, *et al.,* 1993, 1996, 1998). DSIP given intranigrally in picrotoxin-induced convulsions in rats expressed a strong antiepileptic effect with substantial decrease in the severity of the convulsions (Shandra *et al.*, 1996) and prolongation of the latent period (Prudchenko *et al.,* 1993).

Given the promising results obtained with native nonapeptide DSIP in metaphit-provoked generalized epilepsy induced by audiogenic stimulus in rats, gives the hope of designing and synthesizing a potentially useful glutamate antagonist in the near future that could be used as an efficient antiepileptic.

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Address for correspondence:
Dr Olivera Stanojlović, M.D. Ph.D.
Laboratory for Neurophysiology
Department of Physiology, School of Medicine,
University of Belgrade,
Višegradska 26/II, 11000 Belgrade, Serbia
e-mail: solja@afrodita.rcub.bg.ac.yu

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KORISTAN UČINAK DELTA PEPTIDA SPAVANJA NA EPILEPSIJU IZAZVANU METAFITOM

STANOJLOVIĆ OLIVERA, HRNČIĆ D, RAŠIĆ ALEKSANDRA, ŠUŠIĆ VESELINKA
i ŽIVANOVIĆ DRAGANA

SADRŽAJ

Efekti delta peptida spavanja (DSIP) na metafitom (1-[1(3-isothiocyanatophenyl)-cyclohexyl] piperidine) izazvanu epilepsiju u pacova opservirani su u zavisnosti od momenta njegove aplikacije: pre ili posle metafita.

Životinjama je aplikovan: 1. fiziološki rastvor; 2. metafit; 3. DSIP i 30 minuta kasnije metafit (protektivna grupa); 4. metafit i 8 h kasnije DSIP blokirajuća grupa) i 5. samo DSIP. Posle tretmana životinje su izlagane zvučnoj stimulaciji u jednočasovnim intervalima pri čemu su analizirani incidenca i intenzitet napada (trčanje, klonus i tonus), kao i EEG korelati napada.

Metafit je uzrokovao EEG abnormalnosti u vidu šiljaka i šiljak – talas kompleksa i povećavao spektralnu snagu. Analiza vremenskog toka konvulzivne aktivnosti ukazala je da se njen maksimum dostiže 7-12 h po aplikaciji metafita. DSIP je značajno povećao spektralnu snagu u ä talasnom opsegu bez obzira na vreme davanja (pre ili posle metafita).

Tretman DSIP-om produžava latentni period, redukuje sve konvulzivne komponente, kao i srednji intenzitet napada za duži period, mnogo izražajnije u protektivnoj nego u blokirajućoj grupi.