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EFECTS OF ETHANOL ON ELECTROENCEPHALOGRAPHIC AND BEHAVIORAL SIGNS OF METAPHIT-INDUCED AUDIOGENIC SEIZURE

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The goal of the experiment was to give an answer to the question whether the simultaneous action of metaphit and audiogenic stimulation, which together lead to generalized reflex seizure in rodents, could be modified by ethanol.

The rats divided in four groups received (i.p.): saline; metaphit (10 mg/kg); metaphit (10 mg/kg) + ethanol (2 g/kg); and ethanol (2 g/kg). Ethanol was administered to the metaphit-treated animals which had displayed seizures in the first eight tests. Audiogenic stimulation was applied at hourly intervals starting from the first hour after giving the metaphit injection throughout 16 hours of the experiment. For EEG recordings, three gold-plated electrodes were implanted into the rat skull.

Metaphit led to hypersynchrounus epileptiform activity which forms polyspikes and spike-wave complexes. Behavior was represented by established grades of motor seizures. It was noticed that ethanol significantly decreased EEG signs of seizure, reduced the frequency as the amplitude of the waves increased (dominant ones were δ and θ). Ethanol completely blocked all the manifestations of the convulsive behavior of metaphit-treated animals.

The results of this experiment suggest that ethanol inhibits behavioral and modifies EEG signs of the metaphit induced audiogenic generalized epilepsy.

Key words: metaphit, seizures, ethanol, EEG, rat

INTRODUCTION

Previous studies proposed the existence of a relationship between epilepsy and ethanol. Ethanol may have either proconvulsive or anticonvulsive effects on epileptic activity in different experimental epilepsy models (Kozan *et al*., 2006). The molecular mechanisms involving these interactions are still not well known since an ideal model for their study is currently unavailable. In addition, responses

to ethanol may vary due to factors such as genetic predisposition, ethanol concentration, and stimuli such as stress and socialization.

The effects of ethanol on the central nervous system (CNS) depends on its dose, as well as on the fact whether its intake is acute or chronic. In humans, plasma concentration of ethanol of aproximately 5-20 mM are found in the body during mild intoxications (i.e. mood changes, anxiolysis, excitation, impaired cognition), while sedation, anxiolysis and motor incordination are associated with 20-50 mM ethanol (Hilary *et al.*, 1999).

Ethanol exerts its behavioral effects largely by interacting with receptors of brain neurotransmitters (Tabakoff *et al*., 1993). In particular, the gamma-aminobutyric acid type A ($GABA_A$), N-methyl-D-aspartate (NMDA), glycine (Valenzuela *et al*., 1998), neuronal nicotinic (Cardoso *et al*., 1995) and 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors (Lovinger *et al*., 1999) are ligand gated ion chanels that have been shown to be directly modulated by ethanol. It is well documented that acute ethanol exposure potentiates ion currents at $GABA_A$ and glycine receptors (Mihic *et al*.,1999) but inhibits them at NMDA receptors (Hoffman *et al*., 1989).

The excessive stimulation of NMDA and non-NMDA receptors with excitatory amino acid agonists (glutamate and aspartate) was shown to lead to an increased transmembrane influx of Ca^{2+} and Na^{+} ions that depolarize critical populations of neurons (Lipovac *et al.,* 1993, 2003).

It has been shown perviously that metaphit, given either by intraperitoneal or intracerebroventricular route, facilitated the induction of audiogenic seizures in small rodents (i.e. acted as an epileptic agent) (Šušić *et al*., 1991, 1993; Stanojlović *et al*., 2000, 2004, 2007). According to Bluth *et al*. (1989), metaphit, a phencyclidine (PCP) analogue (Rafferty *et al*., 1985), causes a long-term blockade of PCP receptors and irreversibly binds to ionic channels of the NMDA/PCP receptor complexes.

There are still no data in the available literature on the behavioral and electrocortical ethanol-induced changes in the methaphit model of acute tonic-clonic audiogenic seizures. Beacuse of that, our efforts were focused on answering the question whether simultaneous action of metaphit and audiogenic stimulation (AGS) could be modified by ethanol.

MATERIAL AND METHODS

All experiments were performed at the Neurophysiological Laboratory, Department of Physiology, School of Medicine, University of Belgrade.

Animals

Adult (2-months-old) Wistar male rats, weighing 170-200 g, raised at the Military Medical Academy Breeding Laboratories, Belgrade, were used. The animals were kept under controlled environmental conditions during the experiments (ambient temperature 21 ± 2°C, 50% humidity and a 12 h light-dark cycle with lights switched on at 09:00 h). Rats were housed individually in

transparent plastic cages (55 x 35 x 15 cm) with free access to standard laboratry animal chow and tap water.

Audiogenic stimulation (AGS)

None of the untreated animals screened for audiogenic susceptibility expressed seizure activity. 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. The first stimulation was applied 60 min after metaphit administration and thereafter at hourly intervals during the experiment.

Behaviour

Audiogenic convulsive behavior was assessed by the incidence of motor seizures and seizure severity grade, determined as reported by Anlezark *et al.* (1976) and modified in audiogenic-resistant rats by Šušić and Marković (1993).

Descriptive rating scale ranging from 0 to 3 (0 = no response; 1 = wild ruing only; 2 = wild running followed by clonic seizures; 3 = wild running progressing to generalized clonic convulsion followed by tonic extension of fore-and hindlimbs and tail).

Surgery

For the EEG recordings, rats were anaesthetized with nembuthal (100 mg/kg, i.p.), positioned in a stereotaxic apparatus and three gold-plated recording electrodes were implanted over the frontal, parietal, and occipital cortices.

The experiments were performed in accordance with the Helsinki Declaration and animals were left to recover for 7 days after surgery.

Recordings and EEG

An EEG apparatus (RIZ, Zagreb, Croatia) with a modified output enabling transfer of output signals to the input circuit of an eigh-channel, 12-byte analogue-to-digital card (PCL-711B; Advantech Co.Ltd., Taiwan, ROC) installed into a computer and the corresponding software were used. Lenght of epochs for EEG analysis depended on characteristic EEG changes. Selected EEG power spectra were analysed visually and by the fast Fourier transformation method. Frequency range was defined by the time constant (0.3 s, lower and upper limit frequencies of 0.5 and 30 Hz, respectively). The power spectra were plotted and the integrated energy signals expressed as μV^2.

Experimenatal groups

A total of 31 animals was divided into four groups as follows: Group1: control, saline-injected (n = 6); Grup 2: metaphit administered (10 mg/kg; n = 12); Group 3: metaphit + ethanol administered (10 mg/kg metaphit + 2 g/kg ethanol; n = 7); and Group 4: ethanol alone (2 g/kg; n = 6).

Ethanol was injected 8h after metaphit administration, when metaphit audiogenic seizures were fully developed. Rats were used only once. Injected

solutions given i.p. in a total volume of 0.1 mL were freshly prepared in sterile physiological saline.

Data analysis

Statistical analysis included assessing the significance of the differences between experimental groups: Group 2 (metaphit) *vs.* Group 3 (metaphit + ethanol). Evaluation was done using Fisher's exact probability test for the incidence data and Mann-Whitney *U* test for the differences in mean seizure grade (**P*<.05, ***P*<.01).

Drugs

Metaphit methanesulphonate was purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Absolute ethanol was a product of Merck KGaA, Germany.

RESULTS

No EEG changes or behavioral epileptic signs were recorded in control animals, regardless of whether they were exposed to AGS.

Behavior

All metaphit-treated animals expressed normal gross behavioral activity. Behavioral observation showed that the incidence and severity of convulsive responses on AGS were the highest 7-12 h after metaphit injection, in seven of 10 rats (i.e. 70%), median seizure grade 3. About 25% of metaphit-treated animals never responded to AGS and behaved normally during this critical time period.

The time course study revealed a high incidence of reduction of all convulsive components occurring for 3 h after ethanol injection. Incidence of seizures in Group 3 was significantly reduced in comparison with Group 2 in the three defined time points 9h , 10h and 11h ($p<0.01$) (Figure 1).

The mean seizure grade was significantly lower in Group 3 *v.s.* Group 2 at the following time points: 9 h ($U = 15$, $p<0.01$), 10 h ($U = 10.9$, $p<0.01$) and 11 h ($U = 15$, $p<0.01$) when behaviour was observed to be grade 0 (Figure 2).

Electroencephalographic studies

Visual inspection of the present data recordings of metaphit-treated rats supports the view of synchronized EEG cortical sleep-like patterns approximately 40 min after metaphit injection. This EEG picture developed into seizure activity induced by AGS and reached a typical burst of spike-wave complexes at 2-4 Hz (Figure 3).

Our results revealed the differences in EEG frequency bands and power spectra between ethanol-treated rats and the corresponding controls. It was noticed that ethanol significantly decreased EEG signs of seizure, reduced the frequency and increased the amplitude of the waves, thus dominant ones were δ and θ (0-7 Hz). Additionally, a significant decrease in the highest frequency range was recorded 1, 2, and 3h after ethanol administration (Figure 4).

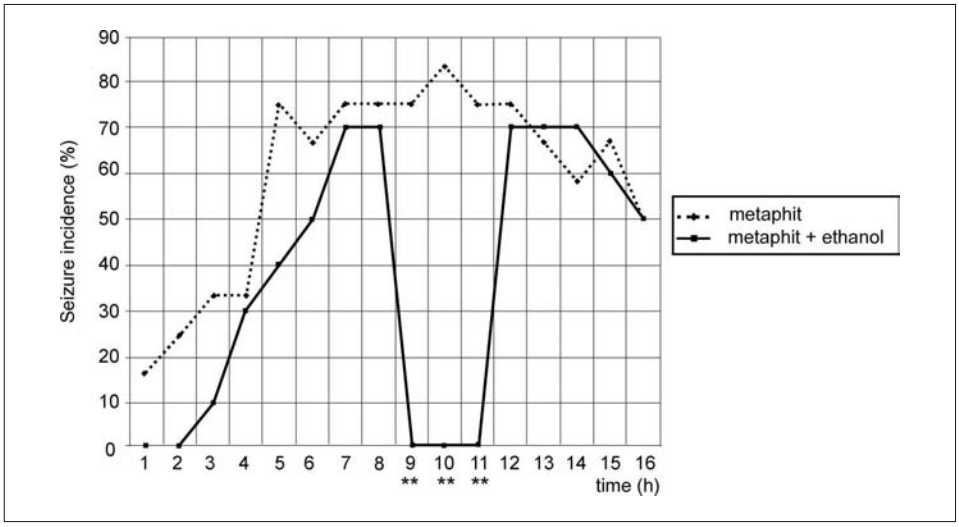

Figure 1. The incidence of metaphit (10 mg/kg, i.p.) induced seizures followed by ethanol injection after 8 h. All animals were exposed to an intense AGS (100 ± 3dB, 60 s) at hourly intervals after metaphit injection during the experiment.
Comparison of metaphit *vs.* metaphit + ethanol was done using Fisher's exact probability test (**P*<.05, ***P*<.01).

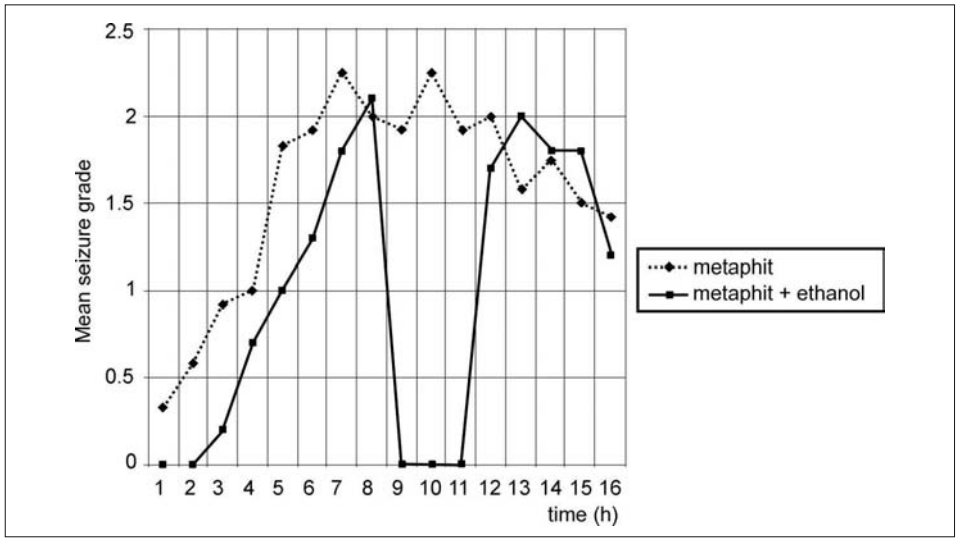

Figure 2. Time course of mean seizure grade upon metaphit injection followed by ethanol after 8 hours. Severity of seizures (expresed as a seizure score) was determined by a descriptive rating scale from 0 to 3 (0 = no response, 1 = wild running only, 2 = clonic seizure, 3 = tonic extension);
Statistical evaluation: metaphit *v.s.* metaphit + ethanol group was done using Mann-Whitney *U* test (**P*<.05, ***P*<.01).

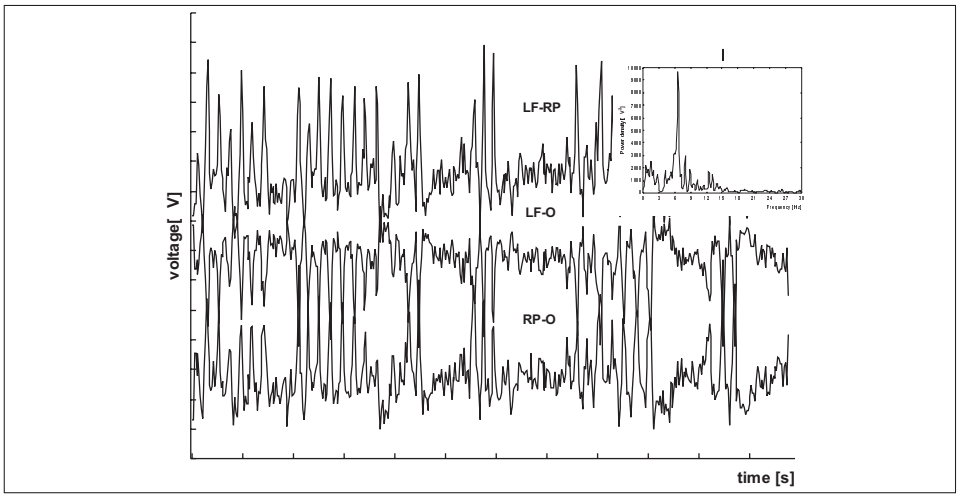

Figure 3. Representative electroencephalographic tracings of maximal severe (grade 3) audiogenic seizures elicited in metaphit treated rats. Synchronized EEG sleep-like patterns developed into seizure activity by AGS and reached a typical burst of spike-wave complexes. Power spectra started to increase in the course of metaphit epilepsy.
LF-RP: left fronto-right parietal cortex; LF-O: left frontooccipital cortex; RP-LO: right parietal-left occipital cortex.
Time calibration, 1 s; Amplitude calibration 50 μV.

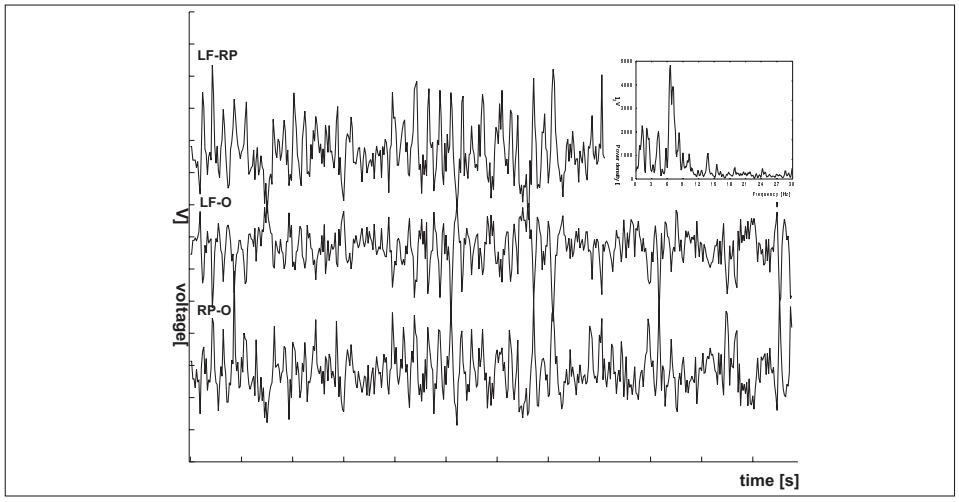

Figure 4. EEG recordings and a power spectrum after administration of ethanol (2 g/kg) to rats previously treated with metaphit. Spiking and sleep-like patterns without clinical seizure activity (Grade 0) during audiogenic stimulation occurred.
Power spectra of EEG epochs show reduced frequency and dominant ones were δ and θ (0-7 Hz).

DISCUSSION

It is highly likely that no single model could be useful for all types of epilepsy (Dziki *et al*., 1992). Stanojlovic *et al.* (2000, 2004, 2007) reported that the metaphit experimental model of generalized reflex audiogenic epilepsy is suitable, altought not ideal, for evaluating potential drugs for the prevention and/or inhibition of the progression of epilepsy.

Metaphit ireversibly binds to the ligand-gated ion channels of the NMDA receptor complex, opening it for Na^+ and Ca^{2+} influx and/or up regulating NMDA receptors and increasing receptor affinity for the binding of natural ligands, such as glutamate and aspartate (Debler *et al*., 1989). It is possible that metaphit inhibits the back-influx mechanism transport system for glutamate at the luminal side of the brain capillaries, resulting in an overaccumulation of glutamate in the brain, as suggested by Lipovac *et al*. (1993, 2003), who hipotesized a metaphit related inhibition of the glial uptake of glutamate resulting in an increase in extracellular glutamate levels in the brain. Similarly, metaphit affects numerous neurotransmitter systems and receptors, such as serotonergic (Nabeshima *et al*., 1989), dopaminergic, voltage-dependent sodium channels (Reith *et al*., 1991) and sigma receptors (Wang *et al*.,1987). Ishida *et al.* (2002) demonstrated that audiogenic seizures induced by high-intensity sound stimulation in genetically susceptible mice and rats expressed typical and characteristic signs of epilepsy (running, clonus and tonus). In this model, convulsions were triggered by creating an imbalance between excitatory and inhibitory brain activities, mainly by increasing excitatory influences related to glutamate activity.

In our present experiment, metaphit-treated adult Wistar male rats that developed a convulsive activity of the highest seizure score (Grade 3) with continuous generalized seizure activity, after 8 h were treated with ethanol. To our kowledge, this is the first study in which the effects of ethanol have been examined in the metaphit model of epilepsy. The results showed that 2 g/kg ethanol completely abolished clonic-tonic seizure activity for 3 h after administration ($p < 0.01$) and modifies EEG components of the metaphit-induced audiogenic seizure.

The ethanol doses used in the present experiment were similar to those applied in experiments of anticonvulsant effects of ethanol against NMDA-, kainic acid- and picrotoxin-induced convulsions in rats reported by other authors (Kulkarni *et al*., 1990). Ethanol (2 g/kg, i.p.) offered protection against these agents, and it was most effective against picrotoxin and least effective against kainic acid. MK_{801}, NMDA receptor antagonist, also provided protection against these agents. Ineffective doses of MK_{801} (0.1 mg/kg, i.p.) and ethanol (0.5 g/kg, i.p.), when administered concurrently, had a facilitatory anticonvulsant effect, thereby providing protection against mortality following severe convulsions induced by NMDA (Kulkarni *et al*., 1990).

Blockade of neuronal responses mediated by the NMDA receptor has been found to occur at lower concentrations of ethanol (Hilary *et al*., 1999). Lovinger *et al*. (1989) found an inhibitory action of ethanol on NMDA responses between 5 and 50 mM, while responses to kainate or quisqualate were affected only slightly by these concentrations. Concentrations of ethanol, as low as 10 mM, inhibited

NMDA-stimulated Ca^{2+} uptake by more than 30% while concentration of 50 mM inhibited NMDA-dependent Ca^{2+} influx by approximately 50% (Hoffman *et al*., 1989).

The results of Rabe *et al*. (1990) demonstrate that *in vivo* actions of ethanol on the NMDA systems may be dependent on glycine concentrations at these receptor sites. Hoffman *et al*. (1989) suggested in different studies using various concentrations of NMDA, as well as phencyclidine (PCP) and glycine, that ethanol affected the coagonist binding site of the NMDA receptor-channel complex, rather than the PCP recognition site. Yaka *et al*. (2003) results suggest that the interaction between tyrosine kinase Fyn and the NR2B subunit of the NMDA receptor mediates the acute sedative effects of ethanol.

GABA systems, also have been implicated as targets for ethanol at the cellular, molecular and behavioural level (Liang *et al*., 2006). Ethanol, acutely enhances Cl^- transport throught the $GABA_A$ receptor channel (Suzdak *et al*., 1986). While much efforts concentrated on the effects of ethanol on $GABA_A$ receptor-mediated synaptic transmission, $GABA_B$ receptors are acknowledged as targets for ethanol (Lewohl *et al*., 1999).

These findings suggest that the anticonvulsant actions of ethanol may be attributed to its ability to antagonize NMDA-mediated excitatory responses and thus to facilitate the GABAergic transmission.

Our results show that ethanol has a significant anticonvulsant activity in the metaphit model of seizures as it reversed metaphit-induced behavioral and motor changes. Ethanol expressed no effect on electrocortical activity which displayed no antiepileptic activity.

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UTICAJ ETANOLA NA ELEKTROENCEFALOGRAFSKE I BIHEJVIORALNE ZNAKE METAFITOM-INDUKOVANE AUDIOGENE EPILEPSIJE

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SADRŽAJ

Cilj ovog rada je bio da se utvrdi da li udružena aktivnost metafita i audiogene stimulacije, koja dovodi do generalizovane refleksne epilepsije u pacova, može biti modifikovana etanolom.

Pacovi su bili podeljeni u četri grupe koje su intraperitonealno (i.p) tretirane: fiziološkim rastvorom; metafitom (10 mg/kg); metafitom (10 mg/kg) + etanolom (2 g/kg) i etanolom (2 g/kg). Etanol je administriran životinjama koje su prethodno tretirane metafitom i koje su pokazivale epileptičke napade u prvih osam testiranja. Audiogena stimulacija je primenjivana u jednočasovnim intervalima počevši jedan sat nakon injekcije metafita i zatim, tokom svih 16 h trajanja eksperimenta. Za elektroencefalografsko (EEG) registrovanje u lobanje pacova su bile implantirane po tri pozlaćene elektrode.

U EEG zapisu je observirana metafitom indukovana hipersinhronizovana epileptiformna aktivnost u formi šiljak – talas kompleksa i grupisanih šiljaka dok je ponašanje definisano gradusima. Uočeni su efekti etanola koji značajno umanjuju EEG znake epilepsije, redukuju frekvenciju i povećavaju amplitudu talasa (dominiraju talasi δ i θ). Etanol kompletno blokira sve manifestacije konvulzivnog ponašanja metafitom tretiranih životinja.

Rezultati ovog eksperimenta ukazuju da etanol inhibira konvulzivno ponašanje i menja EEG znake metafitom indukovane audiogene generalizovane epilepsije.