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Pharmacology, Biochemistry and Behavior 78 (2004) 781–786

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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# Pregnenolone sulfate potentiates the effects of NMDA on hippocampal alanine and dopamine

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Received 19 March 2004; accepted 17 May 2004
Available online 28 July 2004

**Abstract**

The aim of the present study was to analyze biochemical effects of a neurosteroid, pregnenolone sulfate (PS), which accompany changes in the threshold of seizures, and to establish the contribution of local, hippocampal monoaminergic and amino acid systems, to the control of convulsive activity. Pretreatment of mice with PS (intracerebroventricularly) selectively enhanced the potency of peripherally (intraperitoneally) administered NMDA at the $LD_{16}$ (88.0 mg/kg) to induce clonic–tonic convulsions (PS, $LD_{84}$ = 184.7 nM; 95% CL = 181.4–188.1). The proconvulsive actions of picrotoxin and bicuculline, the GABA-A receptor antagonists, were not modified by pretreatment of mice with PS. Administration of PS alone (up to 240 nM icv) did not show any seizure-like activity. PS given at $LD_{84}$, together with NMDA (at the $LD_{16}$), increased the hippocampal concentration of alanine, and enhanced local metabolism of dopamine in a period immediately preceding the onset of seizures significantly stronger than did NMDA alone. These and other data indicate that the enhancement by PS of hippocampal levels of alanine may contribute to the seizures development as this amino acid is a precursor of glutamate, and a co-agonist of the NMDA receptors. On the other hand, simultaneously occurring stimulation of hippocampal dopaminergic system may be considered a compensatory phenomenon, limiting seizures propagation through the limbic forebrain. Summarizing, our results show that PS-induced potentiation of NMDA seizures is accompanied by selective changes in hippocampal dopamine turnover and alanine concentration.


*Keywords:* Mice; Seizures; Hippocampus; Pregnenolone sulfate; NMDA; Alanine; Dopamine

## 1. Introduction

Dehydroepiandrosterone sulfate (DHEAS) and pregnenolone sulfate (PS) are sulfated endogenous neurosteroids known to antagonize GABA-A receptor-mediated inhibitory responses, and to potentiate NMDA receptor-mediated excitatory responses, in vitro (Ceccon et al., 2001; Czlonkowska et al., 2003; Weaver et al., 1998). Both drugs are also potent memory-enhancers (Darnaudery et al., 1999), suggesting a clinical application in the management of amnesia or dementia. However, PS can also decrease seizures threshold (Kokate et al., 1999), and has been found to exacerbate NMDA-induced death of hippocampal neurons (Weaver et al., 1998). The in vivo effects of PS along with other sulfated steroids are much less recognized.

Given the suggested clinical role of sulfated steroids, and the paucity of in vivo data, we have decided to further examine the interaction between PS and brain monoaminergic and amino acid neurotransmitter systems, using behavioural and biochemical approach. For that purpose, we have studied the effects of a pretreatment

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doi:10.1016/j.pbb.2004.05.009

of mice with PS on the picrotoxin-, bicuculline-, and NMDA-induced seizures. Moreover, the influence of a joint pretreatment of mice with PS and NMDA on hippocampal amino acids and monoamines concentration was analyzed in the mouse hippocampus, in vitro. This brain structure was selected for the biochemical part of the study for its well recognized role in the development and propagation of seizure activity. Biochemical tests were performed just before the onset of seizures, in the period of prodromal behavioural symptoms of convulsions, to analyze the local biochemical changes immediately preceding the appearance of seizures. This focused the study on the intrinsic processes directly linked to the onset of convulsions, thus omitting the confounding, nonspecific effects of seizures, causing a general depletion of endogenously stored transmitters and modulators. There were two goals of the study: (i) to analyze the possible neurochemical substrates for PS-induced changes in the seizures threshold, and (ii) to establish the contribution of local, hippocampal monoaminergic and amino acid systems, to the control of convulsive activity.

## 2. Materials and methods

### 2.1. Animals

The experiments were carried out on adult male albino Swiss mice weighing 20–25 g. All animals were acclimatized to their cages for 5 days before testing. They were housed under a 12 h light–dark cycle, at a controlled temperature (20 °C), with water and food ad libitum. All experiments were conducted between 11 a.m. and 4 p.m. The experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC). All experimental procedures using animal subjects were approved by the Local Committee for Animal Care and Use at the Medical University in Warsaw.

### 2.2. Convulsant test

The chemoconvulsants: picrotoxin (PTX), bicuculline (BIC), and *N*-methyl-D-aspartic acid (NMDA) were administered intraperitoneally. PTX and NMDA were dissolved in 0.9% NaCl and water, respectively, and BIC was suspended in 2% Tween solution. The mice were placed singly in Plexiglas cages (20 × 25 × 15 cm) immediately after convulsant injection and observed for 30 min for the occurrence of the following signs: wild running and jumping, posturing (Straub tail), clonic convulsions (repetitive movements involving all limbs simultaneously). The seizures increased in severity and frequency and eventually progressed to status epilepticus, loss of righting response, tonic hindlimb extension and death. The proconvulsive potency of chemoconvulsants was defined as the percentage of animals showing consistent seizures leading to death within 30 min after the administration. For subsequent experiments, the dose of chemoconvulsants was chosen to be within its $LD_{16}$ limits (effective lethal dose in 16% of mice), as determined during preliminary experiments. The same procedure was repeated using $LD_{16}$ doses of the three examined neurotoxins to determine the profile of action of PS. PS was administered ICV 10 min before chemoconvulsant injection, and the dose response of its action on seizures was subsequently determined.

### 2.3. Surgical procedure and microinjections

The intracerebroventricular injections of PS were performed according to the procedure described previously (Czlonkowska et al., 2000; Herman, 1975). Mice were anaesthetized with ketamine (50 mg/kg/10 ml ip) and a sagittal incision was made along the midline of the skull. The bones were cleaned of connective tissue and the superior and transverse venous sinuses were identified. A small hole was made 2 mm caudal to the bregma and 2 mm lateral to the sagittal suture using a sharp needle. The hole was made by rotating the needle. The animals were tested further after a minimum of 4 days of recovery. Microinjections were given unilaterally using a Hamilton microsyringe through a 3-mm-long injection needle. PS was injected in a volume of 5 μl/50 s. The injection needle was removed after 30 s, and 10 min later chemoconvulsants were administered intraperitoneally. The injection site was checked by injection of methylene blue solution (5 μl/50 s) according to the above procedure on the last day of the experiment, and 10 min prior to the decapitation of animals.

### 2.4. Drugs

The following drugs were used: 3β-hydroxy-5-pregnen-20-one sulfate (pregnenolone sulfate, PS) (Sigma-Aldrich, Poland), *N*-methyl-D-aspartate (NMDA) (Sigma-Aldrich, Poland), picrotoxin (PTX) (Sigma-Aldrich, Poland), (+)-bicuculline (BIC) (Sigma-Aldrich, Poland). PS was suspended in 45% 2-hydroxypropyl-β-cyclodextrin (CDX) (RBI, USA), and was sonificated for 30 min before intracerebroventricular administration.

### 2.5. Administration regimen

In the biochemical part of the experiment, PS (at $ED_{84}$, i.e. the dose inducing clonic–tonic convulsions in 84% of mice pretreated with $LD_{16}$ of NMDA) was given alone and with NMDA (at $LD_{16}$). PS was administered intracerebroventricularly 10 min before NMDA injection. The solvents: CDX (icv) and water (ip) were administered 20 and 10 min before decapitation, respectively. Mice were decapitated 5–10 min after NMDA administration, just before the commencement of seizures, when the prodro-

mal symptoms of seizures such as body tremor, exophthalmus and piloerection could be observed.

### 2.6. Biochemical analysis of monoamines and amino acids concentration

The mouse brains were rapidly removed, and the hippocampus was dissected bilaterally and frozen at −70 °C. Dopamine (DA), 3,4-dihydroxyindolacetic acid (DOPAC), homovanilic acid (HVA), serotonin (5-HT), 5-hydroxyindolacetic acid (5-HIAA) and glutamate (GLU), GABA, alanine (ALA), arginine (ARG) and glycine (GLY) were assayed using a fully automated high-pressure liquid chromatography system with electrochemical detection and standard biochemical methods as reported by us previously (Stefański et al., 1993).

In the case of amino acids concentration analysis, HPLC experiments were performed using a Luna $C_{18}$, 25 cm, 5 μm reverse-phase column. Compounds were eluted isocratically with mobile phase delivered at 0.75 ml/min using a Shimadzu Class VP LC 10AD pump. An Antec elctrochemical detector (“Intro”) with a flow-through cell was used linked to a Shimadzu Class VP Integrator SCL-10 Avp. A high-density glassy carbon working electrode (Antec) was operated at +0.85 V. A Rheodyne injection valve with a 20-μl sample loop was used to manually inject the samples.

Preparation of the mobile phase and the derivatising agents were based on the methods according to the Rowley et al. (1995). The mobile phase consisted of 0.1 M monosodium phosphate and 0.5 mM EDTA with 25% methanol (v/v) water adjusted to pH 4.5 with 1 M phosphoric acid. Then it was filtered through 0.45 μm filters and degassed for 15 min. Stock solutions (0.01 M) of amino acids standards were prepared in double deionised water and kept at 4 °C for 5 days. To prevent adhesion to the glass, GABA standards were prepared in polyethylene vials. Working solutions were prepared daily by dilutions of the stock solution. To obtain agents for derivatisation, OPA (22 mg, Fluka) was dissolved in 0.5 ml of absolute ethanol and 0.9 ml of sodium tetraborate buffer (0.1 M) adjusted to the pH 10.4 with 5 M sodium hydroxide. The reaction of derivatisation was performed at room temperature. Derivatising agent (20 μl) was reacted with 1 ml of amino acid standard for 5 min in polyethylene vial before injection onto the column.

### 2.7. Data analysis

The $LD_{16}$ and $LD_{84}$ values with 95% confidence limits (CL) were determined using a computerized version of the Litchfield and Wilcoxon procedure. Fisher’s exact probability test was used for specific comparisons between treatments. In the biochemical assay, the data are shown as means ± S.E.M., and were checked statistically by one-way analysis of variance followed by the Newman–Keuls test. Statistical package Statistica for Windows, Release 6 (StatSoft, USA), was used for statistical calculations.

## 3. Results

The dose–response experiments with chemoconvulsants revealed the following $LD_{16}$, subsequently used in the part of the study with PS: PTX = 6.0 mg/kg, BIC = 9.0 mg/kg, NMDA = 88.0 mg/kg. Central administration of PS (4–240 nM), 10 min before PTX and BIC, did not change the convulsant potency of both neurotoxins (Fig. 1). However, administration of PS (icv) dose dependently increased the convulsant potency of NMDA (PS, $LD_{16}$ = 137.17; CL = 134.7–139.9; $LD_{50}$ = 159.16; CL = 142.6–177.7; $LD_{84}$ = 184.7; CL = 181.4–188.1). The joint administration of PS and NMDA progressively induced body tremor, Straub tail, wild running, and at the highest dose, tonic–clonic seizures and death. PS alone (up to 240 nM icv) did not induce seizures in mice, but it caused hyperexcitability and hyperactivity in some animals.

In the biochemical part of the experiment, analysis of variance showed significant differences among groups in the concentrations of DA, DOPAC, HVA, HVA/DA (monoamine turnover rates), and alanine in the hippocampus [DA, $F(4,44) = 2.83$, $P < .05$; DOPAC, $F(4,44) = 8.25$, $P < .001$; HVA, $F(4,44) = 5.01$, $P < .01$; HVA/DA, $F(4,43) = 4.91$, $P < .01$; alanine, $F(4,40) = 8.30$, $P < .001$] (Figs. 2 and 3). None of the parameters related to the activity of hippocampal serotonergic activity was changed [5-HT, $F(4,44) = 1.42$, $P > .05$; 5-HIAA, $F(4,44) = 1.61$, $P > .05$; 5-HIAA/5-HT, $F(4,44) = 1.72$, $P > .05$].

The activity of hippocampal DA-system was enhanced by a joint PS + NMDA administration (Fig. 2). The local concentrations of DA ($P < .05$), DOPAC ($P < .05$) and HVA ($P < .01$), as well as the metabolic ratio (HVA/DA; $P < .05$), were significantly higher in comparison with control water group. In this group, the levels of HVA ($P < .05$), and the metabolic ratio (HVA/DA, $P < .05$), were higher also in comparison to control CDX-treated animals. PS + NMDA group showed an enhanced concentration of DOPAC ($P < .01$), HVA ($P < .01$) and HVA/DA ratio ($P < .05$), compared to rats, which were given NMDA only. Pretreatment with PS alone increased the hippocampal level of DOPAC ($P < .05$, vs. CDX-control; and $P < .01$, vs. NMDA group), as well as the HVA/DA ratio vs. CDX control group ($P < .05$), whereas NMDA alone did not cause any changes in the concentrations of monoamines and their metabolites.

Post hoc tests revealed that the hippocampal alanine concentration was higher in the PS + NMDA pretreated animals, in comparison with other groups (by 80.2% vs. control water, $P < .01$; by 61.9% vs. control CDX, $P < .01$;

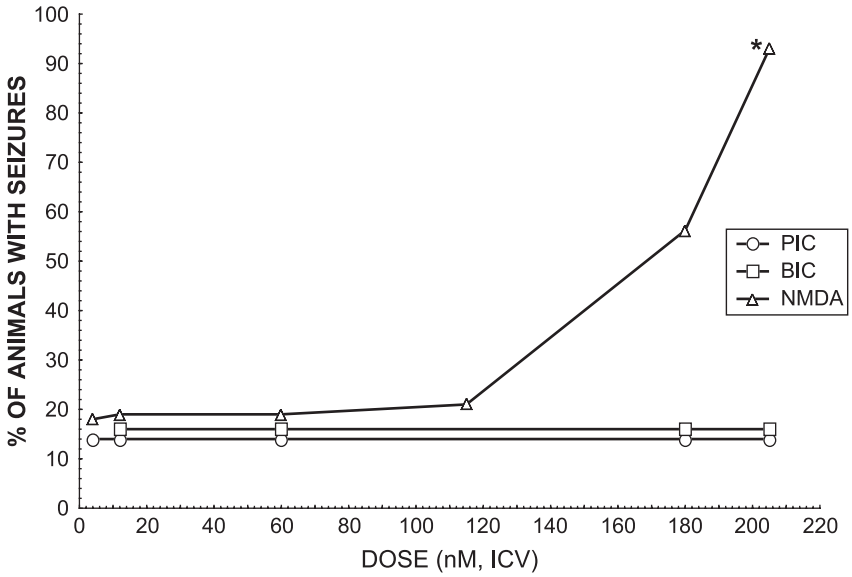


Fig. 1. Potentiation by PS of PTX-, BIC- and NMDA-induced seizures. The doses of neurotoxins were within the $LD_{16}$ limits (see Materials and methods). Each data point indicates the percentage of animals with seizures. The *x*-axis indicates the dosage of PS. PS was injected intracerebroventricularly 10 min before the convulsant administration. $n = 6–8$ mice per one data point. * $P < .05$ compared to the control group with an appropriate solvent (Fisher's exact probability test).

by 26.3% vs. NMDA, $P < .05$) (Fig. 3). NMDA alone also increased local alanine (by 42.7% vs. control water, $P < .05$), but this effect was less pronounced than after a joint pretreatment of mice with NMDA and PS ($P < .05$ vs. NMDA-alone group).

## 4. Discussion

Pretreatment of mice with PS (icv) significantly and selectively enhanced the potency of peripherally administered NMDA to induce generalized clonic–tonic convul-

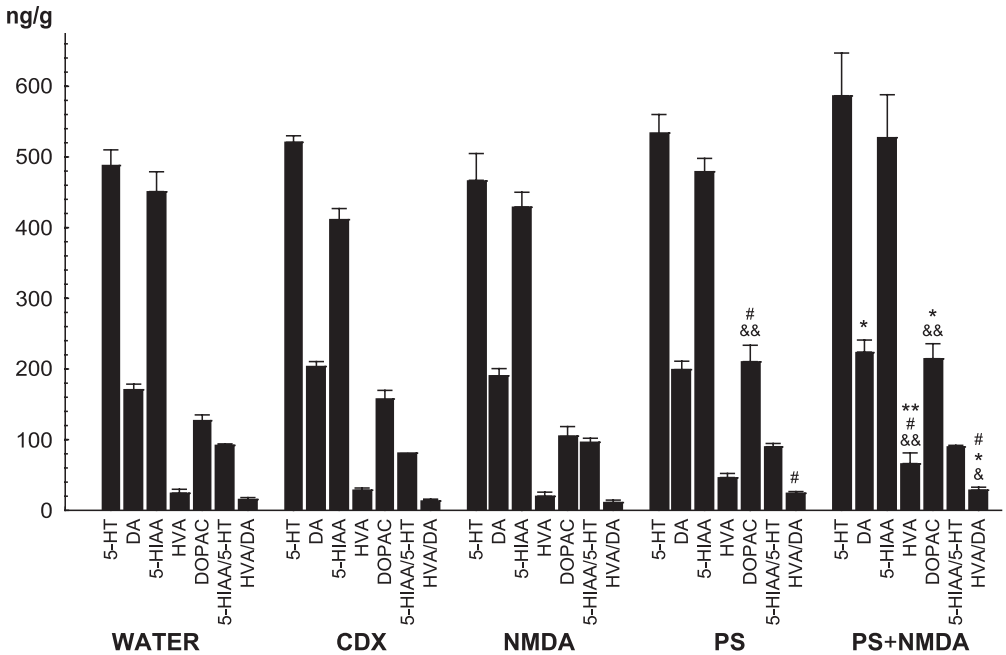


Fig. 2. Effects of NMDA ($LD_{16}$), PS (at $ED_{84}$, i.e. the dose inducing clonic–tonic convulsions in 84% of mice pretreated with $LD_{16}$ of NMDA) and NMDA + PS on the concentration of dopamine, serotonin and their metabolites, as well as the monoamines' turnover rate [(HVA/DA) × 100; (5-HIAA/5-HT) × 100] in the mouse hippocampus. The drugs were administered acutely at the doses established in the behavioural part of the experiment. The data are shown as means ± S.E.M. (ng/g of tissue). The number of mice in the groups varied from 9 to 10. * Differs from the control water group, # Differs from the control CDX group, & Differs from the NMDA group (Newman–Keuls test). $*^{,\#,\&}P < .05$; $**^{,\#\#,\&\&}P < .01$.

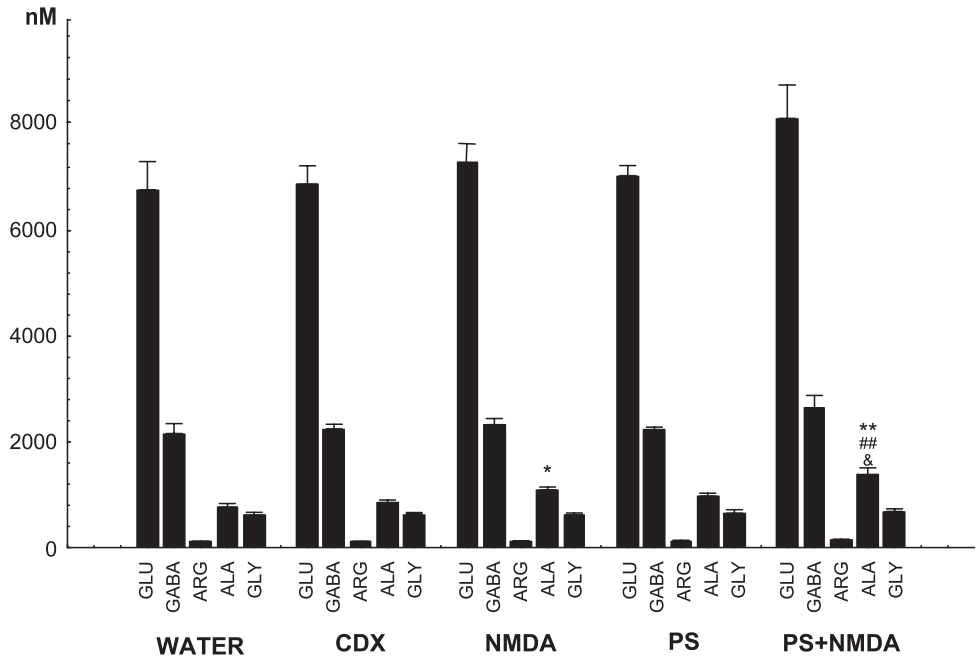


Fig. 3. Effects of NMDA ($LD_{16}$), PS (at $ED_{84}$, i.e. the dose inducing clonic–tonic convulsions in 84% of mice pretreated with $LD_{16}$ of NMDA) and NMDA + PS on the concentration of glutamate (GLU), GABA, alanine (ALA), arginine (Arg) and glycine (Gly) in the mouse hippocampus. The drugs were administered acutely at the doses established in the behavioural part of the experiment. The data are shown as means ± S.E.M. (nM). The number of mice in the groups varied from 9 to 10. * Differs from the control water group, $^{\#}$Differs from the control CDX group, $^{\&}$Differs from the NMDA group (Newman–Keuls test). $^{*,\&}P<.05$; $^{**,\#\#}P<.01$.

sions in mice. The proconvulsive action of picrotoxin and bicuculline, the GABA-A receptor antagonists, was not modified. This finding indicates, that PS is a positive modulator of NMDA receptors. Such conclusion is consistent with the other authors' results on the enhancement by PS of NMDA-induced death of hippocampal neurons (Weaver et al., 1998), and prolongation of NMDA receptor-mediated evoked synaptic currents (Ceccon et al., 2001). It has been suggested that the NR2 subunit of NMDA receptor is critical to the receptor modulation by PS (Malayev et al., 2002).

It is noteworthy that among all amino acids analyzed, only alanine concentration was significantly increased in the mouse hippocampus, in the time immediately preceding the onset of seizures, in the group of NMDA and PS pretreated animals. It should be emphasized that the majority of experimental data in this field has been focused on the effects of a convulsive agent-induced seizures on neurotransmitters concentration, indicating a general increase in amino acids and monoamines (Alam and Starr, 1993; Smolders et al., 1997). A different approach, i.e. analysis of changes in neurotransmitter level in the prodromal period of seizure development, before the onset of convulsions, has attracted much less attention. Such studies could help to characterize more deeply the nature of seizure disorders, by analyzing neurotransmitter activity in a moment just before culmination of longer-lasting central processes evoked by a convulsant administration.

In this context, the changes in alanine concentration deserve special attention, because they were the only ones preceding the onset of seizures. However, the meaning of potentiation by PS of NMDA-induced increase in hippocampal alanine is not clear enough. Both glutamine and alanine are important precursors of glutamate (Armano et al., 2002), especially during recovery from ischemia and hypoxia, when the alanine concentration rises, and that of glutamate falls (Erecinska et al., 1994). Accordingly, it was found that when glutamate stores are depleted, alanine production rises under such conditions, replenishing the stores of glutamate (Erecinska et al., 1994; Griffin et al., 1998). Furthermore, it has been recently reported that alanine can also act as a carrier of ammonia nitrogen between glutamatergic neurons and neighbouring astrocytes, contributing to neurotoxicity (Waagepetersen et al., 2000).

It was also suggested, that similarly to glycine, D-serine and other receptor co-agonists, D-alanine potentiates glutamate neurotransmission via selective stimulation of strychnine-insensitive glycine site at the NMDA receptor (Nishikawa et al., 2000). The presence of D-alanine in the hippocampal tissue of rodents has been established (Morikawa et al., 2001), and although our measurements reflect the changes in total alanine concentration, it cannot be excluded that both isoforms of this amino acid contributed to these changes, which is a point deserving further investigation.

It is also important to note that folate seizures after focal injection of folic acid into the amygdala in the rabbit were found to be accompanied by a pronounced elevation of extracellular alanine in the hippocampus (Lehmann, 1987),

whereas the tissue concentration of glutamate remained at control levels during the seizures (Lehmann, 1987). These findings correspond very well with the present data. Thus, the increase in hippocampal alanine, most probably accompanying ischemia and hypoxia at the early stage of seizure activity, finally leads to seizures enhancement. The lack of changes in hippocampal concentration of glutamate indicates that at the time of seizures development, the endogenous tissue stores of this amino acid are not yet significantly depleted.

Simultaneously, the hippocampal concentrations of dopamine and its metabolites were significantly increased in the PS and NMDA pretreated animals. The tissue concentrations of serotonin and 5-HIAA remained unchanged. In the literature, there is a great number of experimental data indicating that the dopaminergic input to the hippocampus is tonically active, and functions to prevent epileptogenesis (Alam and Starr, 1993, 1994; Cavalheiro et al., 1994; Ferraz et al., 2002; Smolders et al., 1997). For example, activation of the dopaminergic system, via D2 receptors in the dorsal hippocampus, is capable of protecting the animals against limbic motor seizures, arising from excessive muscarinic stimulation of the hippocampus (Alam and Starr, 1993, 1994). The changes in the hippocampal dopamine most probably reflect the enhancement of the local monoamine release and metabolism. Thus, activation of hippocampal dopaminergic system may be considered a compensatory mechanism activated to limit seizure propagation through the limbic forebrain.

To sum up, the present study demonstrated selective biochemical changes in the hippocampal dopamine turnover and alanine concentration, accompanying the enhancement by PS of NMDA-induced seizures. These changes point at the particular and most probably opposite roles of hippocampal alanine and dopamine in the initiation and propagation of seizure activity, in the period immediately preceding the onset of clonic–tonic convulsions. However, it remains to be elucidated how these findings can be generalized to other models of epilepsy.

## Acknowledgements

The study was supported by Grant No. 59/2003 from the Institute of Psychiatry and Neurology in Warsaw.

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