Original article

Effects of N(2-propylpentanoyl)urea on hippocampal amino acid neurotransmitters in spontaneous recurrent seizure rats

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Background: N(2-propylpentanoyl) urea (VPU) is a new valproic acid (VPA) analog with higher anticonvulsant activity than its parent compound in various animal models including seizure acutely induced by pilocarpine.
Objective: Investigate its effects on hippocampal amino acid neurotransmitters in spontaneous recurrent seizure (SRS) rats.
Methods: Pilocarpine hydrochloride was used to induce status epilepticus (SE). Animals were visually observed for two hours/day for an episode of SRS for six weeks. Microdialysis experiment was performed to detect hippocampal amino acid neurotransmitters on those rats that developed SRS.
Results: In comparison to normal rats, hippocampal glutamate, gamma-aminobutyric acid (GABA), and glycine, significantly increased in SRS rats. Occurrence of SRS in the faces of increased level of inhibitory neurotransmitters suggests the key role played by glutamate in the genesis and control of SRS. Based on the observation in pilocarpine-induced SE, the level of glutamate in SRS rats significantly decreased by a clinically effective anticonvulsant, VPA (300 and 600 mg/kg, i.p). Similar profile on hippocampal glutamate was also exhibited by VPU (50 and 100 mg/kg, *i.p.*).
Conclusion: The possible role of VPU in controlling seizure in SRS rats and subsequently human temporal lobe epilepsy as VPA was suggested.

Keywords: N-(2-propylpentanoyl)urea, valproic acid, pilocarpine, spontaneous recurrent seizure, amino acid neurotransmitters

Administration of a potent muscarinic agonist, pilocarpine, in rats produces sequential behavioral changes. These are divided into three distinct periods: an acute phase, in which a limbic status epilepticus is built up, followed by a seizure-free period that lasts for 4-44 days, and finally, a chronic period with spontaneous recurrent seizure (SRS), a phenomena similar to unprovoked seizure observed in epileptic patients [1, 2]. The brain damage induced by sustained seizure in acute phase could be considered as the initial precipitating injury event. This is commonly found in patients with human temporal lobe epilepsy (TLE), which usually demonstrates several pathological features of hippocampus such as neuronal cell loss or mossy fiber sprouting similar to the findings in SRS rats [3]. In addition, anticonvulsant profiles of some antiepileptic drugs in SRS rats were found to be similar to those of human. The complex partial seizure response profiles of acutely-induced seizure in naive rats were similar to human [4-7]. The pilocarpine model of epilepsy (PME) is particularly advantageous for uncovering mechanisms of epileptogenesis as well as seeking for new molecules with better efficacy against complex partial seizure, which occurs in patients with TLE [5, 8-11].

N-(2-propylpentanoyl)urea or valproyl urea (VPU) is a valproic acid (VPA) analogue firstly

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synthesized by Saisorn et al. [12]. It has been shown to possess higher anticonvulsant activity in acute seizure models in mice Maximal Electroshock (MES) and Pentylenetrazol (PTZ) tests [13]. Recently, VPU was found to protect and decrease severity of pilocarpine–induced seizure in an acute phase of status epilepticus in rats. Furthermore, by microdialysis study, VPU was found to significantly decrease the level of pilocarpine-induced increases in hippocampal excitatory neurotransmitters, glutamate, and aspartate. This was proposed to be a mechanism underlying anticonvulsant activity of VPU in the test model [14].

It is still unclear whether such effects of VPU could be observed in a more realistic epilepsy model. The present study was undertaken to evaluate the effects of VPU on the level of hippocampal amino acid neurotransmitters in freely-moving pilocarpine-induced SRS rats.

Materials and methods
Chemicals

VPA, scopolamine methylnitrate, carboxymethyl cellulose sodium salt (CMC), and pentobarbital sodium were purchased from Sigma Chemicals (St Louis, USA). Pilocarpine hydrochloride was purchased from Nacalai Tesque (Tokyo, Japan). VPU was a generous gift provided by Dr. C. Patarapanich (Faculty of Pharmaceutical Sciences, Chulalongkorn University). VPU and VPA were suspended in 0.5% carboxymethylcellulose (CMC) and were injected intraperitoneally.

Animals

Male Wistar rats weighing 220-270 g obtained from National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand were housed in groups of four to five rats under controlled environmental conditions of a 12 hours light/dark cycle at $25\pm1^{o}C$ with free access to food and tap water *ad libitum*. They were acclimatized for at least one week prior to the experiments. All animals care and handling were performed according to the Guide for the Care and Use of Laboratory Animals by the National Research Council of Thailand. All procedures were approved by the Committee on Animal Care at the Naresuan University.

Pilocarpione-induced seizure

Acute phase of status epilepticus was induced by an intraperitoneal injection of pilocarpine hydrochloride

(380 mg/kg) dissolved in saline solution (0.9%). Scopolamine methylnitrate (1 mg/kg) was injected subcutaneously 30 minutes before the administration of pilocarpine to counteract the peripheral cholinergic effects of pilocarpine. Control animals were injected with equal volume of normal saline. Diazepam (10 mg/kg, *i.p.*) was used to interrupt status epilepticus (SE) at 60 minutes after the beginning of SE. SE was defined as the onset of continuous or multiple generalized seizure of stage 4 or 5 of Racine scale [14-16]. The animals were visually observed for the occurrence of SRS for two hours/day, seven days a week, for six weeks. The animal was considered epileptic if it presented at least one episode of SRS ($\geq$ stage 4, according to the Racine scale) during the whole observation period of six weeks. The epileptic rats were then randomly assigned to vehicle-, VPU-, or VPA-treated groups (five rats in each group) for the microdialysis experiments.

One group of normal rats and five groups of SRS rats were used in microdialysis experiments. Normal rats (receiving normal saline solution) treated with 0.5% CMC served as control group (n=4).

One group of SRS rats (n=5) were treated with 0.5% CMC. The other four SRS groups were injected with VPU [50 (n=5) or 100 (n=5) mg/kg] or VPA [300 (n=5) or 600 (n=5) mg/kg] intraperitoneally.

Microdialysis experiments

The animals were anesthetized with Nembutal (50 mg/kg i.p.) and placed in a stereotaxic frame. Microdialysis tube was inserted transversely into the hippocampus. The stereotaxic coordinate (AP -3.6 mm and H -3.3 mm) [17] for hippocampus was used for the transverse implantation of the microdialysis tubing. All coordinates were referred to bregma. After microdialysis probe implantation, the rat was allowed 48 hours for recovery prior to the experiment. After recovery period, the rat was placed in the collecting sample instrument, which allowed free movement. The artificial cerebrospinal fluid (aCSF, pH 7.3) was perfused into the probe by a perfusion pump at a constant flow rate of 2 μL/min). Two hours after stabilization period, three basal dialysate samples were collected every 30 minutes. VPU, VPA, or vehicle (0.5% CMC) was intraperitoneally injected, and six dialysate samples were collected every 30 minutes from the free-moving animals. The hippocampal amino acid neurotransmitters were assayed after derivatization with *o*-phthaldiadehyde (OPA) by high-

performance liquid chromatography with a gradient pump and a fluorimetric detector as described by Lindorth and Mopper [18]. The rate of mobile phase was 1 mL/min. The solution of OPA was maintained by an addition of 4 μL 2-mercaptoethanol every four days.

At the end of each experiment, the rat brain was removed to confirm the appropriate position of microdialysis probed by coronal section (50 μm thick) and staining with 1% Cresyl violet for the microscopic observation. The data was valid only when the right positioning of microdialysis probe in hippocampus was confirmed.

Statistical analysis

The experimental data was presented as mean ± SEM. Alteration of hippocampal amino acid neurotransmitters after the administration of test substances was expressed as percentage of change from their respective basal value determined from three consecutive dialysate samples before the administration of the test substance. Statistical analysis of the changes of amino acid dialysate among different groups was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test. A p-value <0.05 was considered significant.

Results

Intraperitoneal administration of pilocarpine produced SE in 35 out of 38 rats (92.11%). Among these pilocarpine-treated rats, 28 animals survived and developed SRS, however, three of them died during the course of experiment. Therefore, microdialysis experiment was successfully carried out in 25 of these SRS rats. After the occurrence of SRS, they were randomly assigned to five groups of vehicle-, VPU-(50 and 100 mg/kg) and VPA-(300 and 600 mg/ kg). No statistical difference was noted among these five different treatment groups in terms of onset to develop SE, Racine score of SE, latency to develop SRS and the severity of the SRS firstly observed.
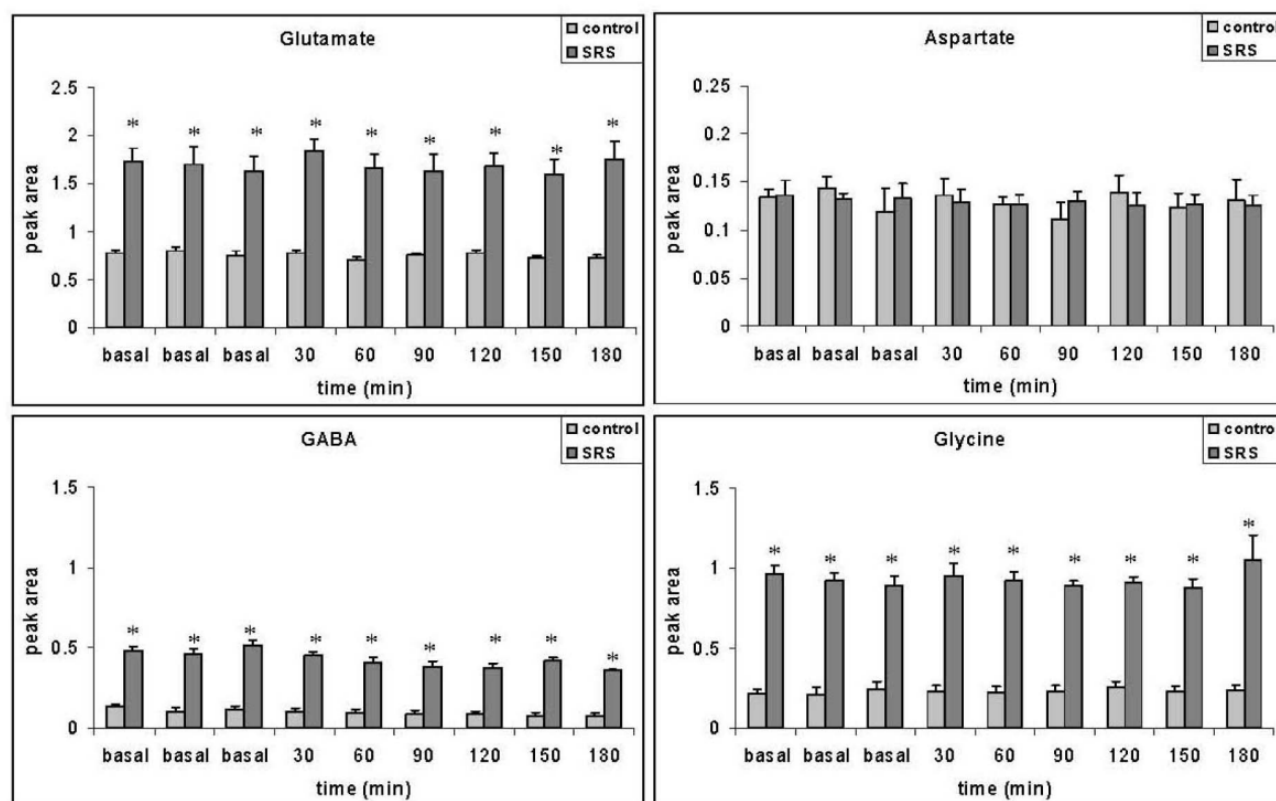

Fig. 1 The extracellular levels of hippocampal glutamate, aspartate, glycine and GABA between control (n=4) and spontaneous recurrent seizure (SRS) (n=5) rats (mean±SEM) expressed as the peak area. The asterisks denote the values significantly different from control group (p <0.01).

Body weight as indicative of the gross consequences of the SE on the general condition of rats was followed for six weeks after pilocarpine treatment. During the first four weeks period of SRS group, weight gain was significantly lower than that of the saline-treated group, but the body weight of these two groups was not statistically different at the end of experiments. In contrast, microdialysis experiments demonstrated that basal level of hippocampal glutamate, glycine, and gamma-aminobutyric acid (GABA) in SRS rats was significantly higher than their respective values in normal rats. On the other hand, hippocampal aspartate level showed no difference throughout the course of microdialysis experiments of 180 minutes (**Fig.1**).

In comparison to SRS rats receiving 0.5% CMC, the administration of VPU at the doses of 50 and 100 mg/kg or VPA at the doses of 300 and 600 mg/kg significantly decreased the levels of hippocampal glutamate. As illustrated in **Fig. 2**, the effects of both VPU and VPA were rather prominent. About 20-30% reduction of extracellular glutamate was noted at 30 min after the administration of the test substances and sustained or further depressed throughout the observation period of 180 minutes.

As demonstrated in **Fig. 3**, except for a transient and minute decrease of aspartate exclusively noted at 60 min after the administration of VPU (50 or 100 mg/kg), no significant effect of VPU on the level of hippocampal aspartate was observed at any time points. In contrast, a significant and sustained decrease of aspartate level was observed after the administration of VPA in the dose of 300 or 600 mg/kg. The extracellular aspartate was reduced by approximately 50% at 30 minutes after the administration of VPA 600 mg/kg and still depressed to the same extent until the end of experimental period.
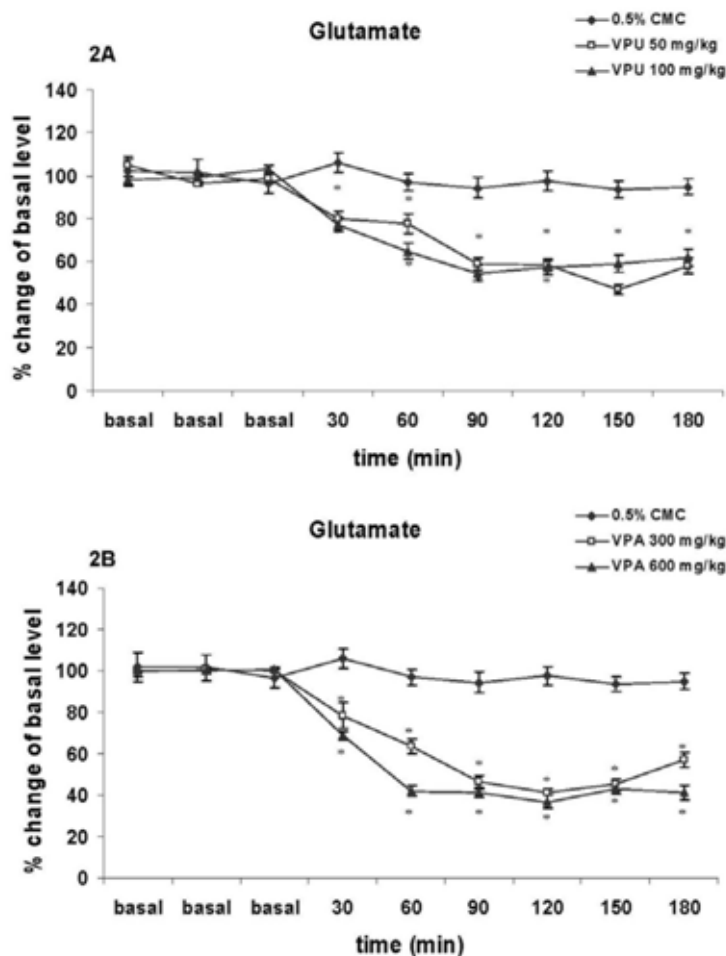

Fig. 2 Effects of intraperitoneal injection of VPU (50, 100 mg/kg, n=5) (**2A**) and VPA (300, 600 mg/kg, n=5) (**2B**) on the extracellular levels of hippocamapal glutamate (in percentage of the basal level) (mean±SEM) in SRS rats. The asterisks denote the values significantly different from SRS group (p <0.05)
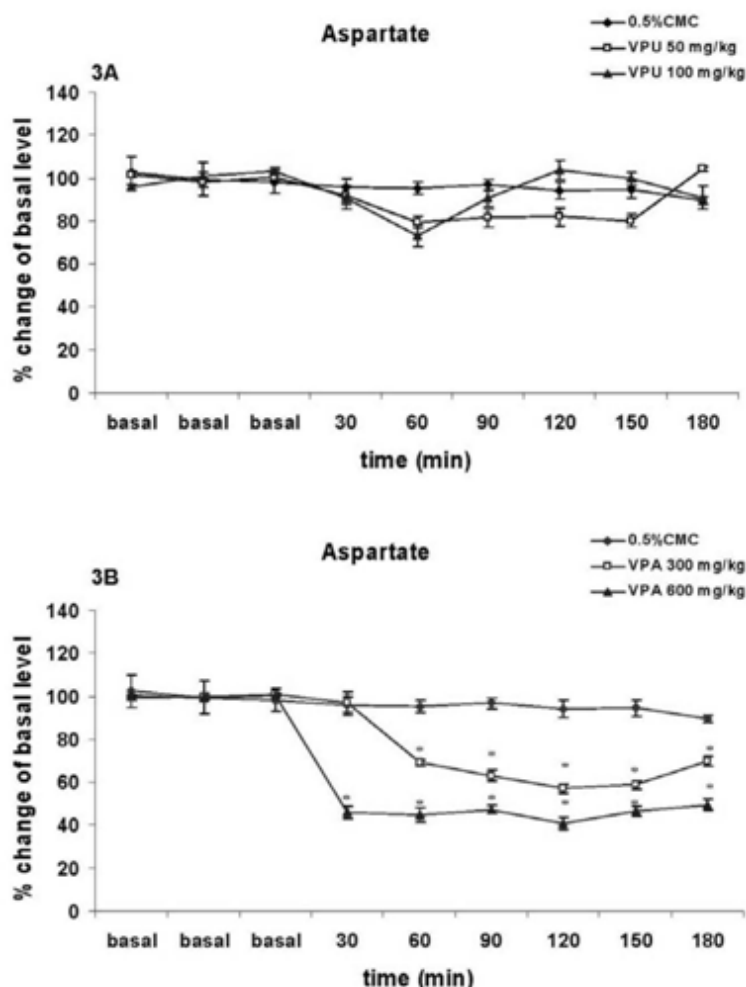
Fig. 3 Effects of intraperitoneal injection of VPU (50, 100 mg/kg, n=5) (**3A**) and VPA (300, 600 mg/kg, n=5) (**3B**) on the extracellular levels of hippocampal aspartate (in percentage of the basal level) (mean±SEM) in SRS rats. The asterisks denote the values significantly different from SRS group (p <0.05).

Extracellular level of glycine (**Fig. 4**) was found to be significantly decreased (about 20-30%) by both VPU and VPA, however, a difference in time profile was observed. The same extent of glycine reduction was elicited by 50 and 100 mg/kg of VPU. The effects were noted at 30 min after the administration of VPU, persistent until 150 minutes and no longer observed at 180 minutes. On the other hand, depressant effect of VPA on glycine was initially observed at 120 min and persistent to the end of observation period.

As shown in **Fig.5**, approximately the same decreases on the level of extracellular GABA was obtained by the administration of 50 and 100 mg/kg of VPU during the observation period of 30 or 60 to 150 minutes. However, the depressant effect of VPU was no longer significant at 180 minutes. No significant effect except a short-lived decrease of GABA level (60-90 min) was obtained by VPA in the dose of 300

mg/kg. The administration of VPA 600 mg/kg showed no significant effect on the level of hippocampal GABA except a transient and minute increase of GABA seen only at 30 minutes.

Discussion

The pilocarpine model of chronic epilepsy provides a useful animal model for studying mechanisms and therapeutic approaches to temporal lobe epilepsy. In this model, excessive and sustained stimulation of cholinergic receptors can lead to status epilepticus and seizure-related brain damage in rodents [8, 19]. In the present study, about 74% of the experimental animals receiving a single injection of 380 mg/kg of pilocarpine survived and developed SRS after the latency period. The results observed agree well with previous reports by Cavalheiro et al. [2].
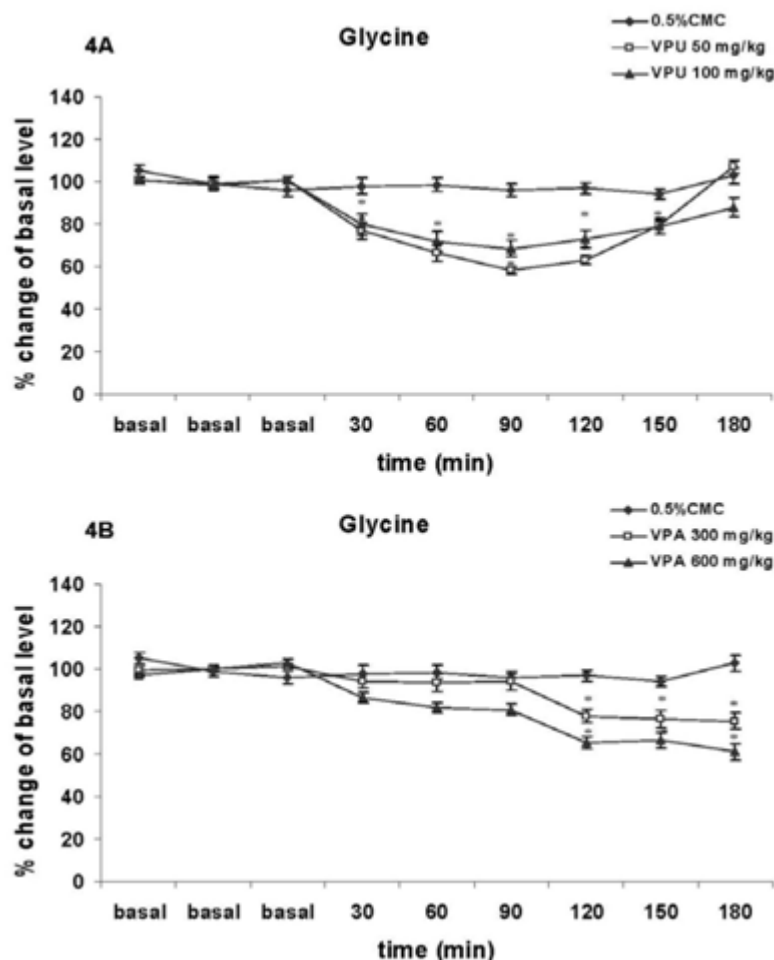
Fig. 4 Effects of intraperitoneal injection of VPU (50, 100 mg/kg, n=5) (**4A**) and VPA (300, 600 mg/kg, n=5) (**4B**) on the extracellular levels of hippocampal glycine (in percentage of the basal level) (mean±SEM) in SRS rats. The asterisks denote the values significantly different from SRS group (p <0.05).

The features of the SRSs observed in the chronic phase of pilocarpine model resemble human epilepsy that seizures evolve spontaneously. However, drug trial in SRS rats is rather rare because development of SRS animals is time and resource consuming. Thus, it should be reserved for those compounds previously demonstrating anticonvulsant activity in standard screening models [2, 5, 20]. Accordingly, very few anticonvulsant studies were conducted in SRS animals. Conventional antiepileptic drugs, including phenobarbital, phenytoin, and carbamazepine but not ethosuximide, were found to reduce the number of seizures in chronic phase of pilocarpine-induced seizures. Such anticonvulsant profiles were different from those observed in acute experiment of pilocarpine-induced seizure but correlated to human complex partial seizure [5]. Acute anticonvulsant effect of VPU has been shown in MES and PTZ

tests [13] and later on in pilocarpine-induced seizure [14]. Reduction of pilocarpine-induced increment of hippocampal glutamate was suggested to be the mechanism underlying the anticonvulsant observed.

In comparison to normal rats, the SRS rats in the present study demonstrated the higher basal levels of hippocampal glutamate, glycine, and GABA whereas the level of hippocampal aspartate remained comparable. Increases of all amino acid neurotransmitters concentration (aspartate, glutamate, GABA, and glycine) in hippocampal homogenates of PME rats were previously demonstrated [21]. Difference in methods used might explain the discrepancy noted on aspartate. It is noteworthy that alteration of hippocampal aspartate and glycine, which exist in comparatively lower level than those of glutamate and GABA in temporal lobe of SRS rats and epileptic patients seemed to be variable whereas
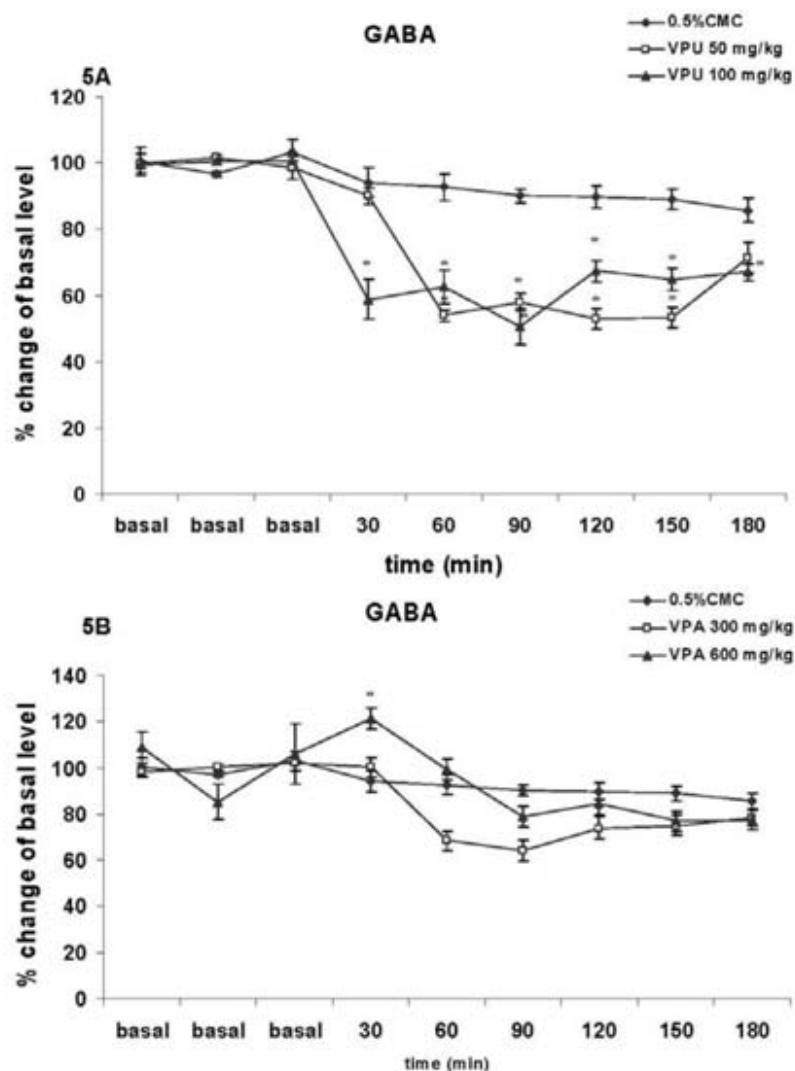
Fig. 5 Effects of intraperitoneal injection of VPU (50, 100 mg/kg, n=5) (**5A**) and VPA (300, 600 mg/kg, n=5) (**5B**) on the extracellular levels of hippocampal GABA (in percentage of the basal level) (mean±SEM) in SRS rats. The asterisks denote the values significantly different from SRS group (p < 0.05).

increases in glutamate and GABA were consistently found by different investigators [21-23]. We previously reported that acute administration of pilocarpine exclusively induced a dramatic increase of glutamate but not aspartate, GABA, or glycine [14]. Thus, increases of hippocampal GABA and glycine seen in the SRS hereby could be a result of an attempt to antagonize the effect of glutamate, which significantly increased in silent and chronic periods [21]. Furthermore, significant involvement of hippocampal glutamate in spontaneous recurrent seizure has been identified in both human and animals [24, 25]. Therefore, the occurrence of pilocarpine-induced SRS in the present study is most likely to be

explained by the increased hippocampal glutamate.

Interestingly, in accordance with the reduction of hippocampal glutamate and aspartate observed in the present study, VPA in the dose of 600 mg/kg/day has been found to exert its anticonvulsant activity in chronic period of PME whereas a minimal reduction of seizure frequency is observed in SRS rats treated with VPA in the dose of 450 mg/kg/day [5]. Therefore, the notion in our previous report that reduction of hippocampal glutamate and aspartate seemed to underlie the anticonvulsant activity of VPA in pilocarpine-induced SE [14], should be applicable in PME as well. Similarity observed could be due to the increment of hippocampal glutamate, which was commonly seen after the acute

administration of pilocarpine as well as in the process of pilocarpine-induced epileptogenesis turning the normal rats into epileptic rats [14, 21, 22].

In conclusion, the occurrence of SRS was mostly ascribed to increased hippocampal glutamate, and SRS occurred in the faces of rising level of inhibitory amino acid neurotransmitters (glycine and GABA) in conjunction with the inconsistency alteration of hippocampal aspartate in PME rats previously reported by other investigators [14, 21-23]. It is suggestive that hippocampal glutamate may play a key role in the genesis and control of spontaneously recurrent seizure. Like VPA, VPU had suppressing effect on hippocampal glutamate of PME rats. Therefore, it is likely that VPU should be able to exert clinical profile similar to those of VPA. However, investigation on some other underlying anticonvulsant mechanisms should be further carried out.

Acknowledgements

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