In Vivo Study of the Effect of Valpromide and Valnoctamide in the Pilocarpine Rat Model of Focal Epilepsy

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Purpose. We evaluated the effectiveness of the commonly used antiepileptic drug sodium valproate (400 mg/kg) and two of its amide derivatives, valpromide and valnoctamide (both 100 mg/kg), in an *in vivo* rat model of focal epilepsy. Our main interest was to get insight into possible changes in extracellular amino acid neurotransmitter levels following administration of the drugs, both in control and in epileptic conditions.

Methods. Seizures were evoked in freely moving rats by intrahippocampal administration of pilocarpine via a microdialysis probe (10 mM for 40 min at 2 μl/min). Microdialysis was also used as *in vivo* sampling technique and alterations in extracellular hippocampal glutamate and GABA levels were monitored. Electrophysiological evidence for the presence or absence of seizures was simultaneously recorded with electrocorticography.

Results. The focally evoked pilocarpine-induced seizures were completely prevented by acute intraperitoneal pretreatment with each of the three drugs in the respective doses. Effective protection was reflected in the electrocorticographic recordings and in the lack of sustained elevations of the extracellular glutamate levels after pilocarpine perfusion. Little effects were seen on the basal extracellular amino acid levels after systemic administration of each of the compounds, nor after the intrahippocampal administration of sodium valproate.

Conclusions. Valnoctamide and valpromide (100 mg/kg) proved to be at least as effective as their parent compound sodium valproate (400 mg/kg) against pilocarpine-induced seizures. All three compounds however failed to induce significant initial alterations in extracellular hippocampal GABA release. This questions the enhancement of GABA-mediated inhibition as being one of their mechanisms of action.

KEY WORDS: sodium valproate; valnoctamide; valpromide; GABA; glutamate; pilocarpine.

INTRODUCTION

Despite the progress in antiepileptic therapy in the last years, still about 25% of the epileptic population is not seizure free. This means that the search for better therapies keeps going on and one of the approaches is to develop improved derivatives of the existing antiepileptic drugs (1).

Valproic acid is one of the major anticonvulsants clinically used. Several hypotheses have been proposed to explain the antiepileptic activity of valproate. The first proposes that valproate acts by enhancing GABA levels in the brain and thus by GABA-mediated inhibition; the second suggests a phenytoin like effect on voltage-dependent Na^+ channels; furthermore, valproate seems to attenuate neuronal excitation induced by the NMDA type of glutamate receptors (2–4). It is likely to accept that the drug's clinical activity relates to a combination of mechanisms, considering its broad spectrum of anticonvulsant activity. However, in animal models, valproate showed lower anticonvulsant potency than the other three established antiepileptic drugs: phenobarbital, phenytoin and carbamazepine. In addition, two major side effects, teratogenicity and hepatotoxicity, have been associated with valproate therapy. This has led to a substantial need to develop improved derivatives of valproate (5). Two of these derivatives are valpromide, used as antiepileptic and antipsychotic agent in several European countries, and valnoctamide, used as anxiolytic and occasionally as anticonvulsant drug. These amide derivatives were screened on antiepileptic activity in mice using the maximal electroshock seizure test, the threshold for maximal electroconvulsions and the subcutaneous pentylenetetrazole test, indicating activity against generalized tonic-clonic seizures and absences. In these tests, the amide derivatives showed to be more potent than sodium valproate (6). Valpromide and valnoctamide distribute better into the brain than sodium valproate, a fact that may contribute to their better anticonvulsant activity (7).

In the present study, we evaluated the effectiveness of the compounds in the focal pilocarpine rat model for psychomotor epilepsy. Turski *et al.* (1987) already examined the protective effects of sodium valproate against systemically evoked pilocarpine-induced seizures by investigating morphological brain damage, behavioural and electroencephalographic alterations (8). The efficacy of valpromide and valnoctamide has never been tested in an *in vivo* model for partial seizures with motor symptoms. Our additional, special interest was to monitor possible changes in transmitter levels induced by administration of the compounds *per se.* We also wanted to know whether systemic pretreatment with sodium valproate, valpromide or valnoctamide was able to suppress the pilocarpine-induced and seizure-related elevations of the neurotransmitters glutamate and GABA (9).

The pilocarpine model has been characterized earlier using the muscarinic receptor antagonist atropine, the voltage-dependent Na^+ channel blocker tetrodotoxin and the NMDA receptor antagonist MK-801. Briefly, we showed that pilocarpine-induced seizures are indeed initiated via muscarinic receptors and further mediated via NMDA receptors (9). The pathological features of pilocarpine-induced seizures in rats resemble those of human complex partial seizures (10). This is one of the most common forms of epilepsy in man and it is this type of seizures that represents a major therapeutic problem.

MATERIALS AND METHODS

Chemicals and Reagents

GABA, L-glutamate and pilocarpine were supplied by Sigma (St. Louis, MO, USA), sodium valproate by Labaz-

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Sanofi (Paris, France). Valpromide and valnoctamide were provided by Prof. Meir Bialer (Department of Pharmaceutics, School of Pharmacy, Faculty of Medicine, The Hebrew University, POB 12065, Jerusalem 91120, Israel). All other chemicals were analytical reagent grade or better and were supplied by Merck (Darmstadt, Germany). Aqueous solutions were made with purified water (Seralpur pro 90 CN, Belgolabo, Overijse, Belgium) and filtered through a 0.2 μm membrane filter. The perfusion fluid for microdialysis, the so-called modified Ringer's solution, contained 147 mM NaCl, 2.3 mM $CaCl_2$ and 4 mM KCl. Pilocarpine and sodium valproate were dissolved in Ringer's solution. Valpromide and valnoctamide were dissolved in a mixture of propyleneglycol:ethanol:saline (6:3:1).

Microdialysis

The protocols are in accordance with national rules on animal experiments and were approved by the Ethics Committee on Animal Experiments of the Faculty of Medicine and Pharmacy of the Free University of Brussels, Belgium. Male albino Wistar rats, weighing 270 to 300 g, were anaesthetized with a mixture of ketamine:diazepam (25:5 mg/kg) and mounted on the stereotaxic frame. Intracranial guides (CMA/Microdialysis, Stockholm, Sweden) were implanted in dorsal hippocampus. Coordinates towards bregma were L + 4.6, A − 5.6, V + 4.6 (11). Immediately after surgery, rats received an intraperitoneal injection of ketoprofen (4 mg/kg) to provide with post-operative analgesia and guide cannula obturators were replaced by CMA/12 microdialysis probes (3 mm membrane length, CMA/Microdialysis, Stockholm, Sweden). Probes were continuously perfused with Ringer's solution at a flow rate of 2 μl/min (CMA/100 microdialysis pump, CMA/Microdialysis, Stockholm, Sweden). The animals were allowed to recover from surgery overnight in their experimental microdialysis cages and received food and water ad libitum. During the experiment, the next day, dialysates were collected every 20 min from the freely moving animals (6 treatment groups).

Group 1 (n = 6): Control Group

The hippocampus was perfused with modified Ringer's solution during the collection of the first 8 dialysates. During collection 8, the animals received a sham IP injection (300 μl) of the mixture of propyleneglycol:ethanol:saline (6:3:1). Then, epilepsy was evoked by an intrahippocampal perfusion with 10 mM pilocarpine for 40 min (2 collections), after which the perfusion fluid was switched back to Ringer's solution for another 10 sampling intervals.

Group 2: Control Experiments with an Intraperitoneal (IP) Injection of Respectively 400 mg/kg Sodium Valproate (VPA) (n = 2), 100 mg/kg Valpromide (VPD) (n = 2), 100 mg/kg Valnoctamide (VCD) (n = 2)

The hippocampus was perfused with modified Ringer's solution throughout the entire experiment. During collection 8, the animals received an IP injection of respectively VPA, VPD or VCD in order to study the effect of the compounds on the baseline neurotransmitter levels.

Groups 3 (n = 6), 4 (n = 5), 5 (n = 5): Acute Intraperitoneally (IP) Pretreated Animals with Respectively 400 mg/kg Sodium Valproate (VPA), 100 mg/kg Valpromide (VPD), 100 mg/kg Valnoctamide (VCD)

The same protocol was followed as for control group 1 except that those animals received an IP injection of respectively VPA, VPD or VCD 15 min prior to the start of the pilocarpine perfusion (i.e. during collection 8).

Group 6 (n = 2): Intrahippocampal Pretreatment with 20 mM Sodium Valproate (VPA)

After 6 basal dialysate samples, 20 mM VPA dissolved in modified Ringer's solution was administered intrahippocampally via the probe for 120 min (6 collections). The perfusion fluid was then switched to a solution containing 20 mM VPA and 10 mM pilocarpine for 2 collection intervals. Finally, the perfusion fluid was switched back to the initial VPA solution for another 8 collection intervals.

Electrocorticography (ECoG)

Combined microdialysis-electrocorticography was performed in at least one animal of each experimental group. Two parasagittal grooves were drilled in the skull of the anaesthetized rats (ketamine:diazepam, 25:5 mg/kg). Electrodes for ECoG recordings were implanted as described previously (9) and fixed with dental cement so that the electrode tips touch the dura mater. Monopolar recordings towards a prefrontal reference electrode were polygraphically amplified and recorded with a time constant of 0.15 s, a high cut-off filter of 70 Hz, and a sensitivity of 500 μV/cm.

Chromatographic Assays

Chromatographic conditions and precolumn derivatisation procedures for the amino acids have been previously described in detail (12). For GABA determinations isocratic, reversed-phase, microbore LC with amperometric detection was used. Precolumn derivatization was performed with *o*-phthalaldehyde/*tert*-butylthiol and iodoacetamide. Glutamate determinations were carried out after precolumn derivatization with *o*-phthalaldehyde/β-mercaptoethanol, by gradient, reversed-phase, microbore LC with fluorescence detection.

Statistical Analysis

All results presented in the figures are expressed as the mean amino acid concentrations in μM with S.E.M. These dialysate concentrations were not corrected for the recovery across the dialysis membrane. Recovery normally varies between 10–15%. Basal values represent the mean neurotransmitter concentrations of the first 7 collection periods sampled under baseline conditions, i.e. before pilocarpine or drug administration. Statistical analysis of alterations of neurotransmitter concentrations with time compared to basal levels was performed by analysis of variance (ANOVA) for repeated measurements and Fisher's PLSD post hoc test ($\alpha = 0.05$).

RESULTS

Baseline Glutamate and GABA Levels in Hippocampus of Conscious Animals.

Basal hippocampal dialysate levels (mean ± S.E.M.) (n = 22) were 0,393 ± 0,049 μM for glutamate and 0,035 ± 0,006 μM for GABA.

Group 1: Control Group

All rats showed a pre-convulsive behaviour, characterized by tremor, scratching and wet dog shakes, starting about 30 min after the initiation of pilocarpine perfusion. On the ECoG recordings a slowing of the rhythmic activity resulting in theta and delta waves was observed during the pilocarpine perfusion. The behaviour evolved progressively into intermittent motor seizures characterized by tonic-clonic limb movements, rearing, salivation, intense masticatory jaw movements and occassional loss of balance. Approximately 1 hour after the start of pilocarpine perfusion, ECoG recordings showed clear patterns of tonic-clonic epileptic activity that lasted until the end of the experiment (Fig. 1a). The seizures were focally evoked but became secondarily generalized as evidenced by seizure activity at both the ipsi-and contralateral side of the focus. After an initial reduction of the baseline hippocampal overflow of glutamate to minimum 29% ($P = 0{,}0001$) and GABA to 57% ($P = 0{,}0002$) during the collections 9 and 10, the extracellular glutamate and GABA levels increased and remained elevated until the end of the experiments, respectively to maximum 200% ($P = 0{,}0001$) (Fig. 2a) and 194% ($P = 0{,}0002$) (Fig. 2b).

Group 2: Intraperitoneal (IP) Injection of Respectively 400 mg/kg Sodium Valproate (VPA), 100 mg/kg Valpromide (VPD), 100 mg/kg Valnoctamide (VCD)

After the IP injection of VPA, the extracellular glutamate levels increased. This correlated with the observed wet dog shakes at the same time. After 10 min the wet dog shakes stopped and the extracellular glutamate levels returned to baseline levels. No alterations in extracellular glutamate or GABA levels compared to baseline levels were observed after the IP injection of both VPD and VCD (data not shown).

Group 3: Acute Intraperitoneal (IP) Pretreated Rats with 400 mg/kg Sodium Valproate (VPA)

Extracellular amino acid levels increased, although not significantly, immediately after the IP injection of VPA (collection period 8), which correlated well with the observed wet dog shakes at that time (Fig. 2). This behaviour lasted for about 10 min, after which the animals became again very quiet. During the pilocarpine administration, a decrease in the extracellular levels of both amino acids was observed, as described for the control group. Acute IP pretreatment with 400 mg/kg VPA protected the rats from developing pilocarpine-induced seizures, which was confirmed by the ECoG recordings. No further significant alterations in extracellular glutamate or GABA levels compared to baseline levels were observed, with exception of the enhanced glutamate levels in the last collection period. These levels were however comparable with the glutamate levels obtained during collection period 8 (Fig. 2a).

Groups 4 and 5: Acute Intraperitoneal (IP) Pretreated Animals with Respectively 100 mg/kg Valpromide (VPD) and 100 mg/kg Valnoctamide (VCD)

VPD and VCD, both insoluble amide derivatives of sodium valproate, were dissolved in a mixture of propyleneglycol:ethanol:saline (6:3:1). Sham injections with this vehicle induced no alterations, neither in the extracellular hippocampal concentrations of glutamate and GABA (Fig. 2 control), nor in the behaviour of the animals. Acute IP pretreatment with VPD or VCD protected all animals from pilocarpine-induced convulsions. This was clearly demonstrated by the ECoG tracings (Fig. 1b) and the behavioural observation. The rats did not demonstrate any signs of pre-epileptic or epileptic behaviour, in fact they rather seemed to be sedated. The IP injection of VPD and VCD did not alter the hippocampal overflow of glutamate and GABA (Fig. 2). During pilocarpine administration, the extracellular amino acid levels significantly decreased, as described for control rats. However, this decrease in extracellular hippocampal glutamate and GABA overflow was not followed by an increase after the pilocarpine perfusion but returned to baseline levels.

Group 6: Intrahippocampal Pretreatment with 20 mM Sodium Valproate (VPA)

Local administration of 20 mM VPA in the hippocampus for 120 min via the microdialysis probe did not have any effect on the extracellular glutamate and GABA levels. It could neither protect the animals from developing seizures after the intrahippocampal co-administration of pilocarpine. The changes in extracellular hippocampal neurotransmitter concentrations after pilocarpine were similar to the transmitter changes observed in the control group (data not shown).

DISCUSSION

The present study confirmed the effectiveness of sodium valproate against pilocarpine-induced seizures (8,10). More interesting both valproate derivatives, valpromide and valnoctamide, proved to be at least as effective as their parent compound.

The pilocarpine rat model is an attractive model for human psychomotor epilepsy since it produces morphological changes and sprouting, altered membrane properties and altered synaptic responses of hippocampal neurones (13,14). The ECoG recordings of our control group experiments showed clear patterns of epileptic seizures. Behavioural changes of the animals also indicated epilepsy. The extracellular glutamate and GABA levels decreased during pilocarpine administration. The inhibition of glutamate release confirmed previous *in vitro* studies in which it was suggested that the activation of presynaptic muscarinic receptors, present on hippocampal glutamatergic nerve terminals, might directly inhibit glutamate release (15). Another *in vitro* study with intra- and extracellular single cell recordings demonstrated that acetylcholine exerted a muscarinic inhibitory effect upon both excitatory and inhibitory afferents to hippocampal neurons (16). This explains why the amino acid transmitters, GABA as well as glutamate, decreased during pilocarpine perfusion. This decrease was followed by a sustained increase to about 200% of both neurotransmitters. Increased extracellular glutamate levels can lead to endogenous excitotoxicity, which has been suggested as an underlying mechanism of chronic epilepsy. Increased extracellular GABA levels may act initially to suppress the firing of the glutamatergic neurons. This confirms earlier published results (9,17,18).

The increase in glutamate dialysate levels, observed immediately after the IP injection of 400 mg/kg valproate, was

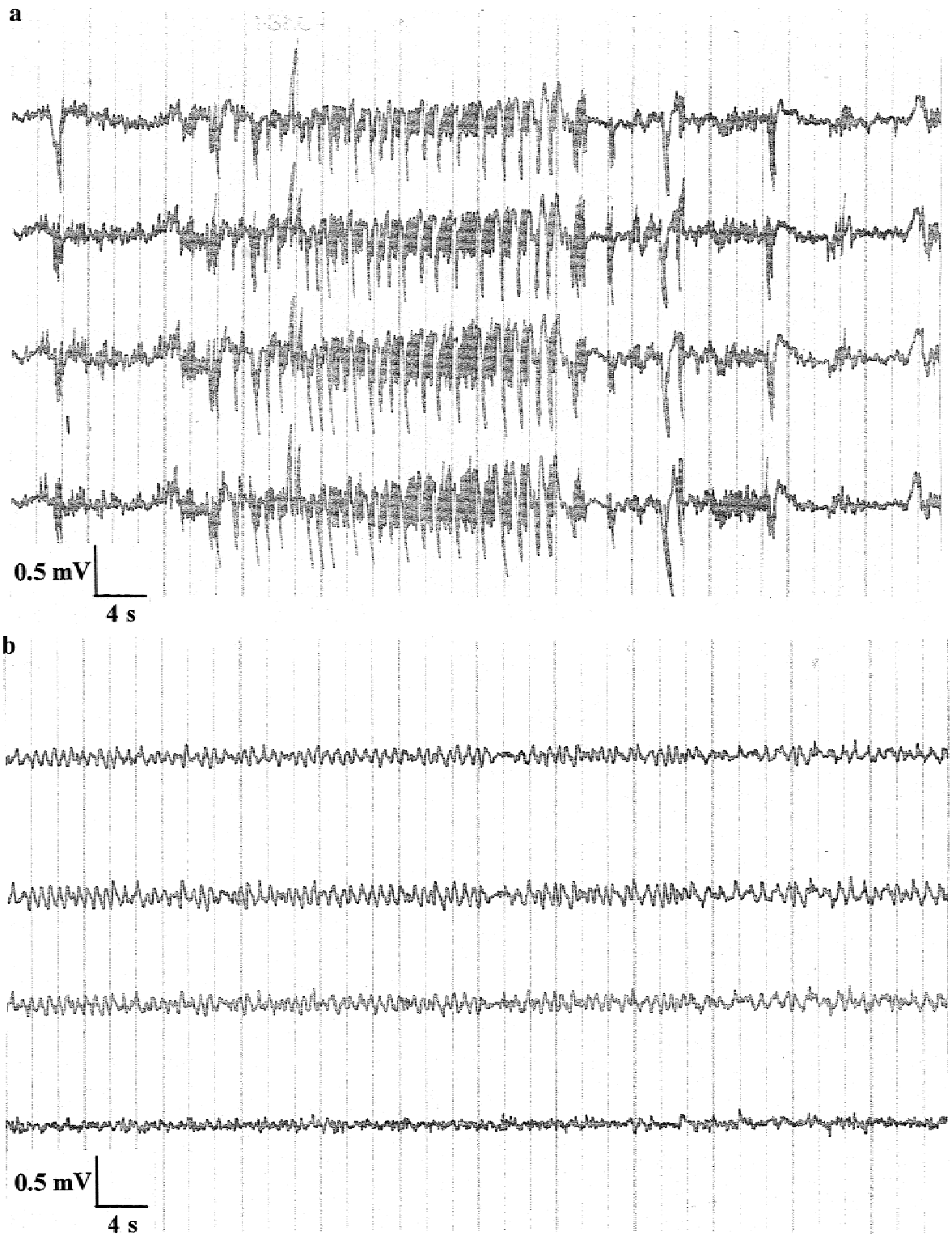

Fig. 1. ECoG recordings obtained from (a) a control rat after administration of 10 mM pilocarpine and during the limbic convulsions and (b) from a rat that received an IP injection of 100 mg/kg valpromide before intrahippocampal pilocarpine administration.

also seen by Biggs *et al.* (19). They studied the effects of IP administration of different doses of valproate on basal extracellular concentrations of various amino acids in the rat ventral hippocampus and found elevated glutamate levels following 400 mg/kg valproate. Lower doses of valproate did not alter hippocampal glutamate. In our study, the increases of glutamate induced by 400 mg/kg valproate were accompanied by a 10 min lasting period of wet dog shakes, after which the animals became very quiet again. We also observed these wet dog shakes in control experiments after pilocarpine perfusion as a pre-convulsive sign and when glutamate levels started to increase. Nevertheless, a dose of 400 mg/kg was needed to

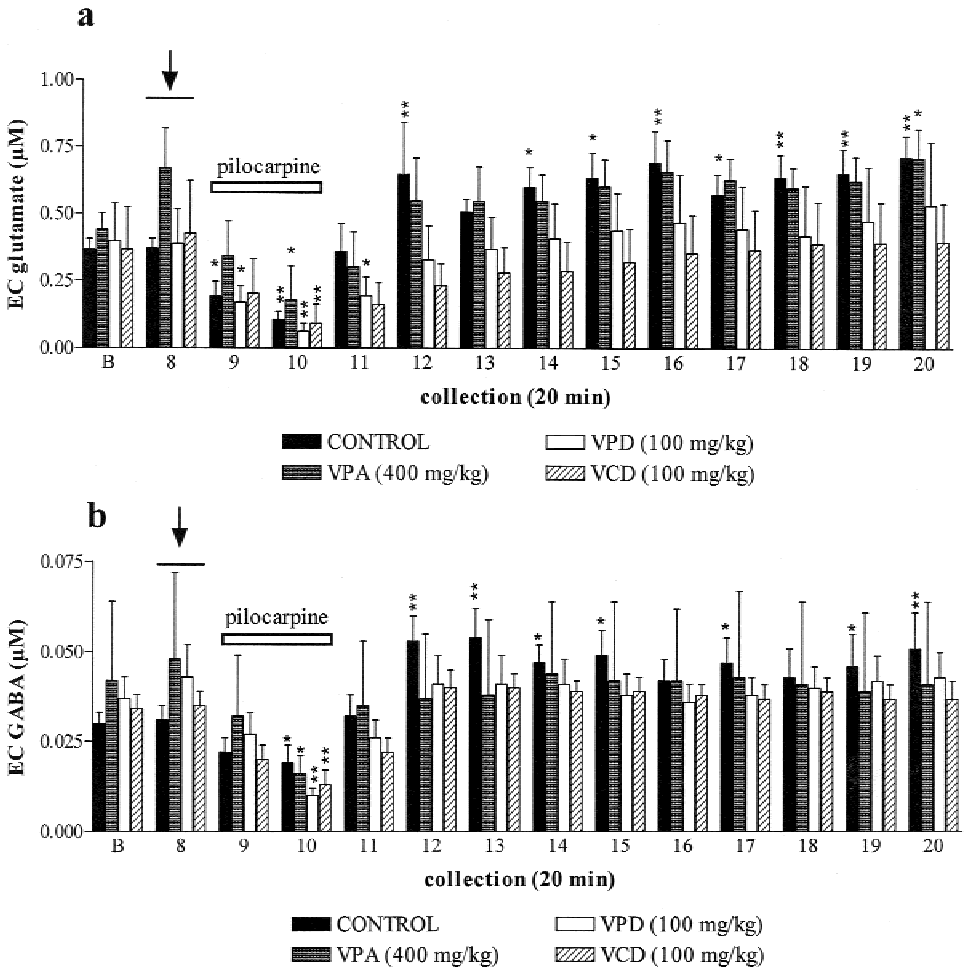

Fig. 2. Extracellular (EC) hippocampal microdialysate concentrations of (a) glutamate and (b) GABA in μM (mean ± S.E.M) in animals receiving an IP sham injection with the solvent mixture (CONTROL) (n = 6), an IP injection of 400 mg/kg sodium valproate (VPA) (n = 6), 100 mg/kg valpromide (VPD) (n = 5) or 100 mg/kg valnoctamide (VCD) (n = 5) in collection period 8 (indicated by arrow), followed by the intrahippocampal administration of 10 mM pilocarpine via the microdialysis probe in collection periods 9–10 (indicated by the open horizontal bar). Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc test [p = 0.05 (*) or p = 0.01 (**)].

protect the rats from pilocarpine-induced seizures, since lower doses did not have anticonvulsive effects.

IP injection of valproate did not alter baseline GABA levels. This observation was surprising since one of the proposed mechanisms of action of valproate is that it enhances GABA-mediated inhibition in the brain. We performed additional experiments in which valproate was given intrahippocampally. In the latter experiments, we also failed to see any effect of valproate on baseline glutamate and GABA levels. Moreover, these rats developed epilepsy and showed the same changes in amino acid dialysate concentrations as observed in control experiments. Administration of 100 mg/kg valpromide or valnoctamide did not alter the extracellular GABA and glutamate levels either. This questions the enhancement of GABA-mediated inhibition as being one of the mechanisms of action of valproate and its amide derivatives and/or the hippocampus as being one of their sites of action. In contrast to our data sampled from dorsal hippocampus, Biggs *et al.* (19) observed a biphasic, dose-dependent effect of valproate on basal GABA levels in ventral hippocampus. At 100 mg/kg, valproate reduced GABA concentrations by 50% compared to baseline levels. In a dose of 200 mg/kg, valproate had virtually no effect, whilst 400 mg/kg valproate raised extracellular GABA levels to 200% of basal values. It is possible that positioning of the probe in dorsal *versus* ventral hippocampus might explain this discrepancy of results.

Epileptic seizure activity following pilocarpine perfusion was not observed on the ECoG tracings from animals who received an acute IP injection of valproate, valpromide or valnoctamide, nor did we see changes in the rats' behaviour. This indicated protective and antiepileptogenic effects of the three drugs against focally evoked pilocarpine-induced seizures. After pilocarpine perfusion, glutamate and GABA levels in these rats pretreated with valproate, valpromide and valnoctamide were not significantly elevated. The attenuation of the extracellular glutamate levels was more pronounced in animals pretreated with the valproate derivatives than with the parent compound. The lack of an increase in extracellular glutamate after pilocarpine can be correlated to the abscence of seizures and may protect the rats from excitotoxic brain damage. Our results seem to confirm the earlier described higher potency of valpromide and valnoctamide compared to valproate (6,20). Valpromide and valnoctamide therapy may be a more efficient, alternative strategy for the chronic drug

treatment of patients with psychomotor seizures who are currently using sodium valproate.

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