

Effect of ionotropic glutamate receptors antagonists on the modifications in extracellular glutamate and aspartate levels during picrotoxin seizures: a microdialysis study in freely moving rats

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Abstract

Our previous studies have shown a local decrease in glutamate and aspartate levels during seizures, induced by picrotoxin microdialysis in the hippocampus of chronic freely moving rats. In this paper, we study the effect of continuous hippocampal microperfusion of the NMDA, AMPA and kainate glutamate receptor inhibitors 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801); 6,7-dinitroquinoxaline-2,3-dione (DNQX), and 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466). We also examine the action of L(-)-threo-3-hydroxyaspartic acid (THA), a glutamate and aspartate reuptake blocker, on the modification of extracellular glutamate and aspartate levels induced by picrotoxin, using the microdialysis method in freely moving rats. We found that changes in extracellular hippocampal concentrations in both amino acids are prevented by NMDA, AMPA and kainate receptor inhibitors. Seizures elicited under DNQX also induce a transient increase in aspartate extracellular levels coincident with seizure time. L(-)-threo-3-hydroxyaspartic acid increased the basal extracellular concentrations of both amino acids, but did not prevent the seizure-related decrease. Our results suggest that glutamate, the major neurotransmitter at the synaptic level, may also play an important role in non-synaptic transmission during seizures. © 2000 Elsevier Science Ltd. All rights reserved.

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In vivo microdialysis technique has been used to quantify, in a defined brain region, a presumed increase in extracellular glutamate release involved in the onset of epileptic seizures in several animal models and in humans. Recent reviews can be found elsewhere (Rogawski, 1995; Chapman, 1997). The relevance of these studies lies in the assumption that excessive exocytosis of glutamate at the pre-synaptic level may

escape from the uptake in the synaptic cleft and diffuse to the neuronal extracellular milieu. However, experimental data suggest that no link exists between seizure activity and the increase in extracellular glutamate concentrations (Miece et al., 1996; Obrenovitch et al., 1996).

Using the microdialysis method with a new experimental design on a new chronic whole-animal model (Sierra-Paredes and Sierra-Marcuño, 1996a, 1996b) we have shown that during the interictal discharges, no changes in the extracellular levels of glutamate, aspartate, g-aminobutyric acid (GABA), glycine or taurine occur. However, during ictal discharges a significant

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decrease in the levels of extracellular glutamate and aspartate was found (Sierra-Paredes et al., 1998). These data suggest that modifications in extracellular glutamate and aspartate may be related to seizures rather than to paroxysmal activity, supporting the neurophysiological differences between ictal and non-ictal activity (Matsumoto and Ajmone-Marsans, 1964a, 1964b; Wyler, 1982)

Evidence supporting the role of AMPA and NMDA receptors in the development and expression of epileptic seizures has been documented (Rogawski, 1995; Chapman, 1997). On these grounds, the modifications of extracellular glutamate and aspartate levels may be related to ionotropic glutamate receptors activity or it may depend on the interplay between both AMPA and NMDA receptors. On the other hand, there is a little doubt that both neurons and glia release substances in the brain extracellular microenvironment (Martin, 1992). Some transmitter amino acids (glutamate, aspartate, glycine and taurine) are present at high concentrations in astrocytes (Levi and Patrizio, 1992), which have the enzymatic machinery for their synthesis (Hertz, 1982). Release of neuroactive amino acids from glia can be induced by receptor stimulation (Lehmann and Hansson, 1988; Barnes, 1991; Martin, 1992). It is also well known that astroglia expresses ion channels and membrane receptors for most of the known neurotransmitters and neuromodulators (Murphy and Pearce, 1987; Seifert and Steinhäuser, 1995; Farb et al., 1995; Steinhäuser and Gallo, 1996). These data suggest that astrocytes play some role in the regulation of the concentration of amino acids, ions and other neuroactive substances in the extracellular space. It is possible, also, that glutamate in the neuronal microenvironment may be released by axon terminals mismatched from the receptor localization. However, such a type of axon terminal has not yet been found in the hippocampus (Herkenham, 1991)

On these grounds, the decrease of glutamate and aspartate in the brain extracellular microenvironment related to seizures (Sierra-Paredes et al., 1998) may be the result of either a reduced release or an increased reuptake by neurons or glia.

To test both hypotheses we have used our whole-animal model in which partial seizures can be elicited repeatedly on different days without concomitant changes in threshold or seizure patterns by intrahippocampal microperfusion of picrotoxin. The method involves the use of freely-moving rats with the microdialysis technique, and in chronic experiments carried out on the same animal, it enables the study of seizure patterns and thresholds as well as the concomitant modifications of extracellular amino acid levels and the effects of the glutamate receptor antagonists over a period up to six months (Sierra-Paredes and Sierra-Marcuño, 1996a, 1996b; Sierra-Paredes et al., 1998)

In this experimental model, we have studied the effects of picrotoxin seizures on the levels of extracellular glutamate and aspartate under the influence of: 1) The intrahippocampal microperfusion of the NMDA receptor antagonist 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801); 2) the intrahippocampal microperfusion of 6,7-dinitroquinoxaline-2,3-dione (DNQX); 3) the intrahippocampal microperfusion of 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466); and 4) the intrahippocampal microperfusion of L(-)-threo-3-hydroxyaspartic acid (THA), a potent inhibitor of glutamate and aspartate reuptake. All experiments were performed on each animal with one week intervals. Picrotoxin, receptor antagonists and transporter inhibitor were dialyzed through the probe to avoid possible dynamic effects imposed by the blood-brain barrier to some systemic administered drugs (Aguilar-Veiga et al., 1991).

A preliminary report of some of the findings has been published in abstract form (Sierra-Paredes et al., 1997b)

1. Experimental procedure

1.1. Materials

Pentobarbital, picrotoxin, amino acids, *N*-acetyl-L-cysteine and *o*-phthalaldehyde were purchased from Sigma (St Louis, MO, USA). HPLC grade methanol was obtained from Merck (Darmstadt, Germany). MK-801, DNQX, GYKI 52466 and THA were obtained from RBI (London, UK). Ultrapure water from Milli-RO/Q system (Millipore, Bedford, MA, USA) was used for the study. All other chemicals used were of analytical grade.

Twenty adult male Sprague-Dawley rats, initially weighing 250–300 g were used. They were housed in groups of three under controlled environmental conditions (ambient temperature $21 \pm 1.8^\circ\text{C}$, humidity 50–60%, 12:12 h light/dark cycle) with free access to food and water except during testing. Rats were obtained from the animalary of the University of Santiago. All experiments were performed in a laboratory with controlled environmental conditions and at the same time in the morning to avoid circadian variations. All efforts were made to minimize animal suffering, and our chronic animal protocols were designed to reduce the number of animals used (Sierra-Paredes and Sierra-Marcuño, 1996a). Animal care followed Spanish legislation on Protection of Animals Used in Experimental and Other Scientific Purposes, in agreement with the European Union regulations (O.J. of E.C. L358/1 18/12/1986)

The rats were anaesthetized with pentobarbital (40

mg/kg) and placed in a stereotaxic instrument (D. Kopf, Tujunga, CA, USA). Under aseptic conditions, two stainless steel microscrews (to be used as electrodes for EEG recording) were positioned in the skull above the frontal and occipital areas of each hemisphere; one screw, used as a reference electrode, was anchored in the mid-line, 7–9 mm rostrally to the coronal suture. The intracerebral guide for the microdialysis probe (CMA/12, CMA/Microdialysis AB, Stockholm, Sweden) was implanted vertically into the ventral hippocampus. Stereotaxis coordinates, derived from the atlas of Paxinos and Watson (1986), were 5 mm posterior, 4.8 mm lateral and 4 mm ventral for the tip of the cannula relative to bregma and dural surface.

Wires from the microscrews were soldered to a miniature plug (Cannon MD1-9SL1, ITT Cannon, Santa Ana, USA) and fixed firmly to the skull with dental cement. After surgery, the rats were housed in individual cages and received antibiotic therapy for 4–5 days.

The experiments were carried out on conscious, freely moving rats 10 days after surgery. From the fourth day, the animals were placed daily for 3 h in the experimental unit for habituation. Bipolar cortical EEGs were recorded on magnetic tapes using a Holter-EEG system (Oxford-Medilog 9200, Oxford, U.K.), and also with a Minihuit electroencephalograph (Alvar Electronic, Paris, France).

During an experimental session, recording time was distributed as follows: (a) A 15 min reference EEG was recorded before every probe introduction; (b) 120 min basal EEG; (c) 75 min post picrotoxin microperfusion control.

All habituation and experimental sessions were recorded on videotape using a standard camera in order to relate behaviorally observed seizures with the EEG recordings.

In our experiments, seizure threshold was defined as the lowest picrotoxin concentration which produced a specific pattern of EEG and/or behavioral seizures after a 5 min perfusion through the rat hippocampus. Only one picrotoxin dose was perfused in each experimental session. The lowest picrotoxin concentration used was 100 μM , and the dose was slowly increased (+25 μM each step) in each animal in successive experimental sessions at 7 day intervals until an EEG-behavioral seizure was induced. This seizure was defined as the threshold seizure. Each animal was randomly perfused four times with the same picrotoxin concentrations, which had induced seizures to ensure that these doses would produce the same type of seizure and extracellular amino acid concentration on different days. Seizure types and rest periods between experimental sessions were described previously in detail (Sierra-Paredes and Sierra-Marcuño, 1996a).

In order to test the effect of MK-801, DNQX and

GYKI 52466, they were dissolved in Ringer's solution at a concentration of 100 μM and perfused continuously throughout the experiment following the same protocol as that for Ringer's solution in the control experiments. As these substances prevent picrotoxin-induced seizures at threshold levels, picrotoxin doses were increased in the same fashion until a new seizure was obtained.

THA was also dissolved in Ringer's solution at a concentration of 500 μM and continuously perfused after 120 min of control sampling, in order to test their effect on basal amino acid concentrations. Picrotoxin was administered 60 min after THA, and sample collection followed the same protocol described above.

We used CMA/120 system for freely moving animals (CMA/Microdialysis AB, Stockholm, Sweden) and CMA/12 microdialysis probes with 4 mm of membrane length. The probe was connected via polyethylene tubing to a syringe selector (CMA/111), and to 1 ml syringes mounted on a microinjection pump (CMA/100). Before starting each experiment, the probe was perfused with ethanol and distilled water. After checking the integrity of the probe under a light microscope, it was perfused with a sterile Ringer's solution (NaCl 147 mM, KCl 4.0 mM, CaCl_2 2.4 mM) for 10 min, tested routinely for in vitro recovery before every experiment and then introduced into the rat hippocampus through the chronically implanted intracerebral guide. Samples were collected in a fraction collector (CMA/140) every 15 min starting 60 min after the beginning of perfusion. Ringer's solution was perfused at a constant flow rate of 2 $\mu\text{l}/\text{min}$ throughout the experiment. Picrotoxin dissolved in Ringer's solution was perfused at the same rate for 5 min 60 min after the start of sample collection. Five samples were collected after picrotoxin administration.

Quantitative analyses of amino acids in brain microdialysates were performed by a slightly modified HPLC method previously reported for brain tissue (Soto-Otero et al., 1994). The assay involves a pre-column derivatization of amino acids with *o*-phthalaldehyde and *N*-acetyl-L-cysteine, which leads to the formation of very stable isoindol derivatives, followed by fluorimetric detection. Briefly, sample preparation involved the addition of 90 μl of 0.1 M sodium borate buffer (pH 9.5) and 20 μl of derivatization agent (*o*-phthalaldehyde and *N*-acetyl-L-cysteine 1:1 molar in methanol) to 10 μl of brain dialysate. After 4 min of reaction, 20 μl of the resulting mixture were injected into the chromatograph. Then, the amino acid derivatives were eluted through a Spherisorb ODS column (220 \times 4.6 mm i.d., 5 μm), using the following gradient of solvents A (50 mM sodium acetate buffer, pH 5.5) and B (methanol): the initial composition of solvent B was 5%, from time 0 to 40 min a linear gradient proceeded to

25% B, and from time 25 to 45 min the gradient achieved 100% B. The flow rate was 1.2 ml/min and the amino acid derivatives were fluorimetrically monitored with $\lambda_{\text{ex}} = 344$ nm and $\lambda_{\text{em}} = 443$ nm. Calibrations were made using microdialysates from different solutions of the tested amino acids in Ringer solution with concentrations ranging from 1 to 100 μM .

The 210 min EEG was recorded once the animal was habituated to the experimental conditions and before starting picrotoxin perfusion. The recording was analyzed and compared with the control EEG recorded after all experiments were finished.

EEG records were analyzed using the Medilog 9200 software, version 7.2. Spike and wave discharges duration, seizure duration, and seizure latencies were evaluated after picrotoxin administration.

At the end of the experiments, rats were anaesthetized with Nembutal and killed by decapitation. A probe was introduced and perfused with Sudan black to help locate the position of the probe. Then, the brain was removed and placed in 4% phosphate buffered formaldehyde solution. A week later, 50 μm coronal sections were cut and stained with cresyl violet, and the position of the probe was checked under light microscopy.

Amino acid concentrations are expressed as estimated extracellular concentrations calculated from *in vitro* calibration. Statistical analysis of microdialysis data was performed using ANOVA for repeated

measures. Differences between the groups were regarded as significant if $p < 0.05$. Data are expressed as mean \pm SEM values.

2. Results

2.1. Seizure thresholds and basal glutamate concentrations

Our results on individual seizure thresholds and seizure types are consistent with those described previously (Sierra-Paredes and Sierra-Marcuño, 1996a). Seizure threshold among animals varied between 100 and 500 μM , but remained unchanged in repeated between day experiments within individual rats.

Basal amino acid concentrations showed no significant modifications in repeated between day experiments within individual rats over a period up to six months.

Picrotoxin seizures produced a significant decrease ($p < 0.01$) in glutamate (from 17.4 ± 1.7 to 11.3 ± 0.8 μM , Fig. 1) and aspartate (from 7.3 ± 2.1 to 3.2 ± 0.6 μM , Fig. 2) extracellular concentrations which lasted for more than 1 h. These changes in glutamate and aspartate concentrations were observed in all the animals and are consistent with those previously described in detail (Sierra-Paredes et al., 1998). The neurochemical pattern is related to the timing of seizures, but not to the seizure intensity, frequency duration.

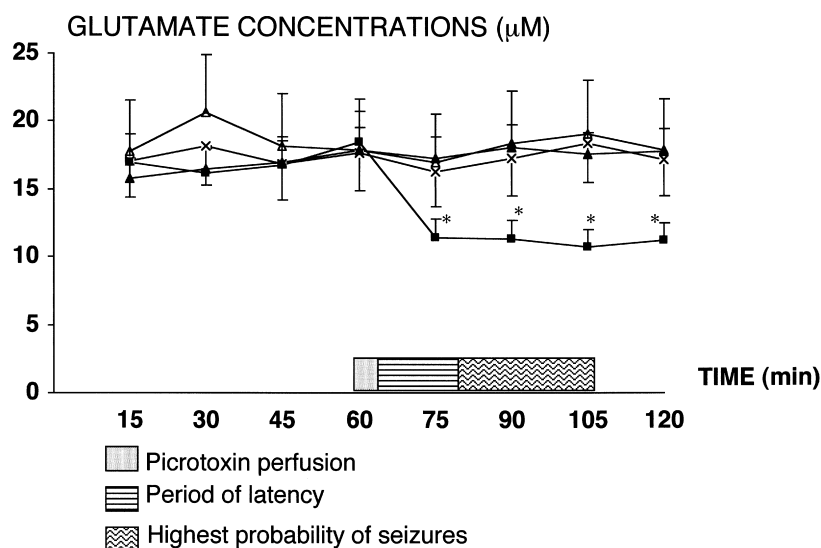


Fig. 1. Effect of continuous perfusion of PCT alone (■), MK-801 (▲), DNQX (-Δ) and GYKI 52466 (-x) on glutamate extracellular concentrations. Data values are mean \pm SEM in repeated experiments in 10 animals. Glutamate levels decreased significantly ($* p < 0.01$) when the PCT concentration reached the seizure threshold. After MK-801, DNQX or GYKI 52466 continuous perfusion, glutamate concentrations did not show statistically significant modifications when compared to controls (first 4 samples) by two-way ANOVA.

2.2. Effect of seizures under continuous perfusion of MK-801

The perfusion of MK-801 (100 μM) alone induced continuous ipsilateral slow waves on the EEG in 65% of the waking animals with no significant modifications in the hippocampal concentrations of the amino acids studied. (Figs. 1 and 2).

Continuous perfusion of MK-801 prevented the decrease in glutamate (Fig. 1) and aspartate (Fig. 2) hippocampal levels observed when picrotoxin alone induced seizure activity. Mean estimated extracellular concentrations during seizures under MK-801 continuous perfusion were $17.1 \pm 2.1 \mu\text{M}$ for glutamate (Fig. 1) and $6.6 \pm 1.6 \mu\text{M}$ for aspartate (Fig. 2). GABA, glycine and taurine levels remained unchanged.

2.3. Effect of seizures under continuous perfusion of DNQX

Continuous perfusion of DNQX (100 μM) prevented the decrease in glutamate (mean estimated extracellular concentration: $17.9 \pm 3.2 \mu\text{M}$; Fig. 1) and increased transiently aspartate (from $6.4 \pm 0.9 \mu\text{M}$ to $13.8 \pm 4.1 \mu\text{M}$, $p < 0.01$; Fig. 2) hippocampal levels observed when picrotoxin induced seizure activity. No changes were observed in other amino acids studied or when DNQX was perfused alone.

2.4. Effect of seizures under continuous perfusion of GYKI 52466

Continuous perfusion of GYKI 52466 (100 μM) prevented the decrease in glutamate (mean estimated extracellular concentration: $17.9 \pm 2.7 \mu\text{M}$; Fig. 1) and aspartate (mean estimated extracellular concentration: $7.1 \pm 1.2 \mu\text{M}$; Fig. 2) hippocampal levels observed when picrotoxin induced seizure activity. No changes were observed in other amino acids studied or when GYKI 52466 was perfused alone.

2.5. Effect of seizures under continuous perfusion of THA

Continuous microperfusion of THA (500 μM) produced a significant increase ($p < 0.01$) on aspartate ($11.9 \pm 1.3 \mu\text{M}$) and glutamate ($28.5 \pm 4.9 \mu\text{M}$) basal concentrations with no modifications on basal EEG or in seizure thresholds or seizure types after picrotoxin administration. THA did not prevent the decrease of glutamate and aspartate extracellular concentrations (to $18.2 \pm 2.2 \mu\text{M}$ and $8.6 \pm 0.9 \mu\text{M}$ respectively) during seizures (Fig. 3)

3. Discussion

The decrease of extracellular glutamate and aspartate in relation to hippocampal induced seizures were

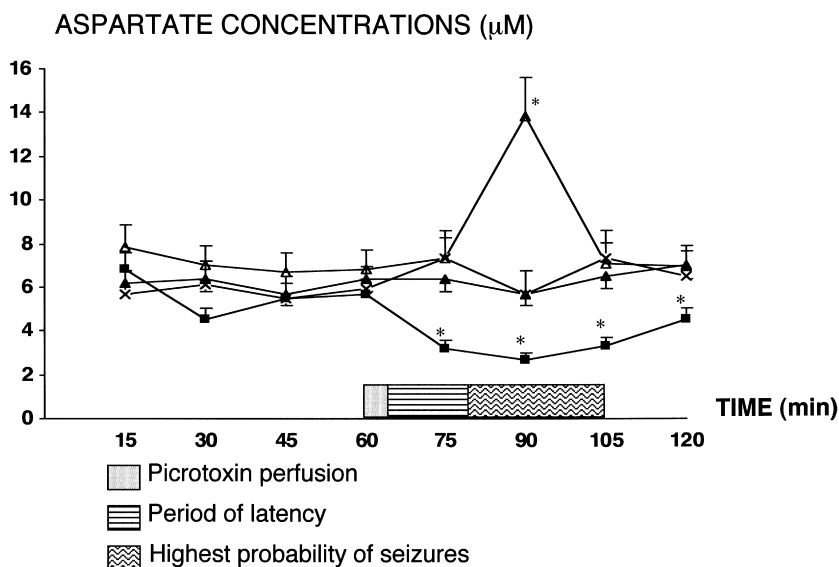


Fig. 2. Effect of continuous perfusion of PCT alone (■), MK-801 (-▲-), DNQX (-Δ-) and GYKI 52466 (-x-) on aspartate extracellular concentrations. Data values are mean \pm SEM in repeated experiments in 10 animals. Aspartate levels decreased significantly ($* p < 0.01$) when the PCT concentration reached the seizure threshold. After MK-801 or GYKI 52466 continuous perfusion, aspartate concentrations did not show statistically significant modifications when compared to controls (first 4 samples) by two-way ANOVA. After DNQX microperfusion, a significant ($* p < 0.01$) increase in aspartate concentration was observed 30 min after picrotoxin administration.

not prevented by long-term microperfusion of THA in our animals. These results differ from those described in previous papers where an increase in extracellular glutamate preceded the onset of seizure activity (Rogawski, 1995), and under glutamate uptake inhibitors not significant changes during seizure activity (Millan et al., 1991) were reported.

These differences may be explained by our methodological approach: chronic experiments; a period of 10 days recovery after surgery; threshold seizures; intracerebral microperfusion of picrotoxin and long-term intracerebral microperfusion of receptor antagonists.

The analysis of the modifications in the extracellular glutamate and aspartate levels in relation to the hippocampal seizures under long-term perfusion of receptor antagonists was hampered by difficulty in determining the cellular origin of the extracellular amino acids.

Recent research suggests that the brain extracellular environment appears to be a dynamic entity which maintains a steady intracellular milieu with regard to ions and small molecules, critical for the neuronal function, under the control of neurons and glial cells (Nicholson, 1995).

While we have no clear picture of the role of neuronal and non-neuronal cells in keeping the levels of neuroactive amino acids in the extracellular milieu *in vivo*, these problems have been approached *in vitro* using neuronal and astroglial cultures, and also brain slice preparations (Lehmann and Hanson, 1988; Nakanishi, 1992; Steinhäuser et al., 1994).

However, it is difficult to interpret the data obtained in experiments *in vivo* through *in vitro* research

because the tools for this comparison have not yet been developed. Occasionally, some of the results from *in vivo* experiments are contrary to those obtained in brain slice preparations (Sierra-Paredes et al., 1999). An interesting approach to this problem was that used by Rothstein et al. (1996), which compared the levels of extracellular glutamate between knockout animals for neuronal or glial glutamate transporters.

Bearing in mind all these problems, the analysis of our results is simplified if we take also into account the concept of non-synaptic transmission (Fuxe and Agnati, 1991; Bach-y-Rita, 1991; Vizzi and Kiss, 1998) as a complementary hypothesis to the glutamate action on synaptic transmission.

At the synaptic level, there is much experimental data to support the role of glutamate and glutamate receptors in the genesis of epileptiform discharges in the hippocampus (Rogawski, 1995; Wheal et al., 1991).

The excitatory post-synaptic potentials mediated by glutamate receptors may be increased by recurrent stimulation through a mechanism of glutamate exocytosis positive feedback (Herrero et al., 1992a, 1992b), which is mediated by post-synaptically generated arachidonic acid (Lynch et al., 1989) and which induces an enhancement in the amount of glutamate release by synaptic vesicles. The effects of the increased release of glutamate is also potentiated by blocking the GABAergic inhibition with picrotoxin. Both NMDA and non-NMDA receptors participate in this enhanced synaptic stimulation (Andreasen et al., 1989).

The epileptiform synaptic potentials in the hippocampus are clearly dependent on the vesicular glutamate

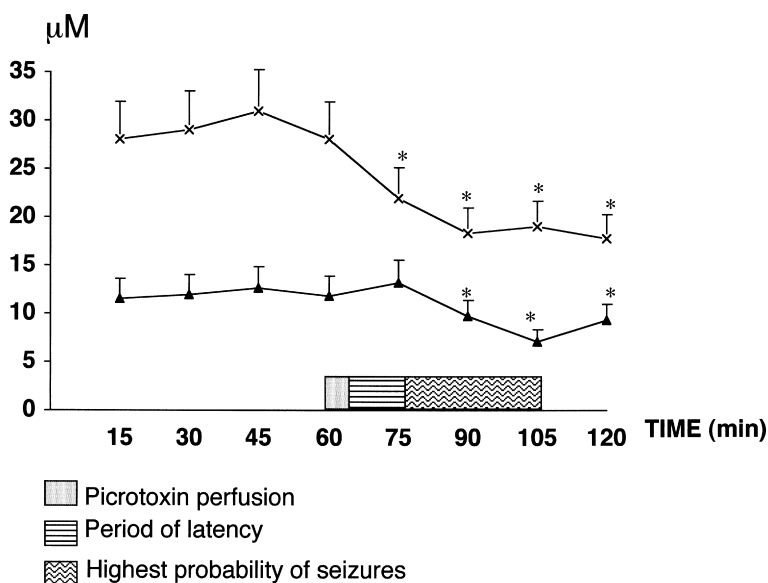


Fig. 3. Effect of continuous perfusion of THA on aspartate (▲) and glutamate (x) extracellular concentrations. Data values are mean \pm SEM in repeated experiments in 10 animals. Basal aspartate and glutamate levels approximately doubled when compared with control experiments. However, a significant decrease ($* p < 0.01$) in both concentrations was observed during seizures.

mate pool. However, it is difficult to understand the role of extracellular glutamate and aspartate concentrations based only on its synaptic effect.

Astroglial cells encapsulate synapses, and astroglial glutamate carriers have sufficient capacity to remove all glutamate released from the presynaptic site (Hertz, 1979). Glial cells respond to synaptically released glutamate by activation of electrogenic transporters, which generate a current that is directly proportional to the amount of glutamate released (Diamond et al., 1998). The high capacity and affinity of glutamate uptake by astrocytes in the synaptic region maintains synaptic transmission possible for long periods of time with a high signal-to-noise ratio (Rönnback and Hansson, 1997).

The need for astroglial cells working at the synaptic region as effective system for clearance of the presynaptically released glutamate may be clearly understood by taking into account the role of the glutamate-glutamine cycle in both the presynaptic region and the glial cells (Schousboe, 1981). Upon release into the synaptic cleft, the glutamate diffuses away, and the glial glutamate transporters internalize the extracellular glutamate. Inside the glial cells, glutamate is metabolized into glutamine, which is transported out of the glial cells and into the synaptic terminal for subsequent resynthesis of glutamate. Since glutamine does not act on the glutamate receptor, this release does not interfere with synaptic transmission (Schousboe et al., 1977). Also, using knockout rats (Rothstein et al., 1996), it was shown that extracellular glutamate levels were unmodified in the animals which had lost neuronal glutamate transport, while the animals which had lost the glutamate transport in glia showed an increase in extracellular glutamate concentrations, suggesting a major role for glial glutamate transporters in the clearance of extracellular glutamate.

This special role of the astrocytes at the synaptic region may be explained by the astroglial heterogeneity (Wilkin et al., 1990). We also need to take into account the morphological (de Robertis, 1965) and the pharmacological (Curtis and Eccles, 1958) evidences of synaptic barriers, and also the fact that during the period in which picrotoxin induced only inter-ictal discharges without concomitant seizures, no modifications in the extracellular glutamate and aspartate were observed (Sierra-Paredes et al., 1998).

Taken together, all these data suggest that the epileptiform synaptic potential is generated by the glutamate synaptic pool without the participation of the extracellular glutamate pool.

It also explains why increase in the extracellular glutamate concentration does not produce electrophysiological changes indicative of excessive excitation (Obrenovitch et al., 1996), and appears insufficient to induce neuronal degeneration (Massieu et al., 1995).

The increase in extracellular glutamate observed during seizures was related to neuronal damage (Liu et al., 1997; Peña and Tapia, 1999).

A different picture is observed on the role of glutamate and aspartate in non-synaptic transmission.

Fagni et al. (1983) suggested that the L-glutamate added to the bath stimulated different glutamate receptors to those activated by the endogenous neurotransmitter, and postulated the existence of extrasynaptic receptors.

Evidence of extrasynaptic glutamate receptors has been reported (Aoki et al., 1994; Kullman et al., 1996), and a comparison of some properties of synaptic and non-synaptic NMDA receptors has been published (Clark et al., 1997). Some data suggest that the extracellular glutamate and aspartate may act on neuronal and glial NMDA non-synaptic receptors (Farb et al., 1995; Seifert and Steinhäser, 1995).

The decrease in extracellular glutamate and aspartate levels related to seizures was not prevented by THA, a potent inhibitor of glutamate and aspartate reuptake. This fact may have two explanations. The first is that recurrent paroxysmal depolarization induced by glutamate at the synaptic level may block the glutamate release by glial cells. The ability of the glial cells to change the extracellular environment has been postulated as mechanism which simultaneously modulates the excitability of many neurons (Olsson et al., 1997). Thus, the decrease in extracellular glutamate and aspartate may be a way to reduce the neuronal cluster excitability.

It has been stated that seizures are always the consequence of an abnormal synchronizations of millions of neuronal discharges (Traub and Wong, 1982). A second possibility would be that the paroxysmal depolarization generated at the synapses induces a massive binding of extracellular glutamate and aspartate at non-synaptic glutamate receptors and it is this which induces abnormal neuronal synchronization. Repolarization or hyperpolarization of the mass of neurons by diazepam stops the seizures and produces a rapid return to basal glutamate and aspartate levels (Sierra-Paredes et al., 1997). Furthermore, these mechanisms explain the effect of MK-801 preventing the seizures.

The role of non-synaptic receptors in mass sustained convulsive activity has been postulated (Bach-y-Rita, 1991). An increase in glutamate binding in surgically removed brain tissue from patients with intractable seizures has also been reported (Hosford et al., 1991).

Taking into account that in our animals the decrease of extracellular glutamate was observed before the start of the seizures, it is possible that both processes may in a temporal sequence: firstly, extracellular glutamate may bind massively to extrasynaptic NMDA receptors to induce an abnormal synchrony, and then, a decrease of glial release may be due to decrease in neuronal activity in order to stop the seizures.

In all possibilities suggested, it is possible to understand the parallel behavior of extracellular glutamate and aspartate, despite the fact that aspartate is not released by synaptic vesicles.

Although we focus primarily on the possible role of extracellular glutamate and aspartate to explain the neuronal synchrony in the development of seizures, it is not possible to rule out the involvement of other neuronal mechanisms. However, the hypothesis described above seems the best suited to integrate our data with the majority of previously published experimental research.

The fact that MK-801 prevented the seizures and also stopped the decrease of extracellular glutamate and aspartate may be due to its effect as antagonist on synaptic and non-synaptic NMDA receptors. However, under continuous perfusion of MK-801, and increasing the picrotoxin concentration, the seizures induced do not result in a decrease of both amino acids. Under these experimental conditions, the required neuronal synchronization to induce the seizures is not achieved through the participation of non-synaptic NMDA receptors, but is induced by local excitatory circuits through axon collaterals (Dichter and Ayala, 1987), or by synchronous neural after discharges without active chemical synapses (Taylor and Dudek, 1982).

During seizures induced under long-term perfusion of DNQX, a transient increase in aspartate was observed. Aspartate is not released synaptically, but is released by glial cells (Martin, 1992). However, no data were reported on differential effects of GYKI 52466 and DNQX on glial cells. At the neuronal level, the difference between the effect of GYKI 52466 and BNQX on kainate-evoked currents was reported (Vizi et al., 1996). With the present data, it is difficult to explain the mechanisms by which aspartate increases in the extracellular fluid.

GYKI 52466 and DNQX are more effective against seizures induced by electroshock or pentylenetetrazol (Löscher and Hönack, 1994; Vizi et al., 1996) than in protecting against focal seizures induced by intrahippocampal microperfusion of picrotoxin. This may be due to methodological differences. The systemic administration of the AMPA receptor antagonists have effect in brain areas other than the hippocampus, which may play a significant role in the control or diffusion of epileptic seizures. Also, the cellular mechanisms implied in generalized epilepsy differ from those of focal epilepsy (Gloor and Fariello, 1988). Diazepam remains the drug of first choice for the treatment of status epilepticus (Schab, 1967), and it is the best drug to stop experimentally the induced status in rats (Sierra-Paredes and Sierra-Marcuño, 1996; Sierra-Paredes et al., 1998), however, diazepam induces only a slight increase in the threshold for intracerebral elec-

trical stimulation responses in the limbic system (Sierra et al., 1969).

Seizures may have different causes and there are likely to be multiple ones underlying cellular and molecular mechanisms responsible for the different epileptic phenomena. Our data suggest that glutamate and aspartate in the extracellular milieu modulate the excitability of many neurons simultaneously. On these grounds, microdialysis in freely-moving rats and chronic experiments as described above may be a useful approach to study information traffic between neuron cells.

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References

- Aguilar-Veiga, E., Sierra-Paredes, G., Galán-Valiente, J., Soto-Otero, R., Méndez-Alvarez, E., Sierra-Marcuño, G., 1991. Correlation between ethosuximide brain levels measured by high performance liquid chromatography and its antiepileptic potential. *Res. Com. Chem. Pathol. Pharmacol.* 71 (3), 351–364.
- Andreasen, M., Lambert, J.D.C., Jensen, M.S., 1989. Effects of new non-N-methyl-D-aspartate antagonists on synaptic transmission in the in vitro rat hippocampus. *J. Physiol (Lond.)* 414, 317–336.
- Aoki, C., Venkatesan, C., Go, C.G., Mong, J.A., Dawson, T.M., 1994. Cellular and subcellular localization of NMDA-R1 subunit immunoreactivity in the visual cortex of adult and neonatal rats. *J. Neurosci.* 14, 5202–5222.
- Bach-y-Rita, P., 1991. Thoughts on the role of volume transmission in normal and abnormal mass sustained functions. In: Fuxe, K., Agnati, L.F. (Eds.), *Volume Transmission in the Brain*. Raven Press, New York, pp. 489–496.
- Barnes, B.A., 1991. New roles for glia. *J. Neurosci.* 11, 3685–3694.
- Chapman, A.G., 1997. Microdialysis. In: Engel Jr, J., Pedley, T.A. (Eds.), *Epilepsy: A Comprehensive Textbook*. Lippincott-Raven Publishers, Philadelphia, pp. 1067–1072.
- Clark, B.A., Farrant, M., Cull-Candy, S.G., 1997. A direct comparison of the single-channel properties of synaptic and extrasynaptic NMDA receptors. *J. Neurosci.* 17, 107–116.
- Curtis, D.R., Eccles, R.M., 1958. The effect of diffusional barriers upon the pharmacology of cells within the central nervous system. *J. Physiol (Lond.)* 141, 446–463.
- de Robertis, E., 1965. Some new electron microscopical contribution to the biology of neuroglia. In: De Robertis, E., Carrera, R. (Eds.), *Progress in Brain Research*, vol 15: *Biology of Neuroglia*. Elsevier, New York, pp. 1–11.
- Diamond, J.S., Bergles, D.E., Jahr, C.E., 1998. Glutamate release monitored with astrocyte transporter currents during LTP. *Neuron* 21, 425–433.

- Dichter, M., Ayala, G.F., 1987. Cellular mechanisms of epilepsy: a status report. *Science* 237, 157–164.
- Fagni, L., Baudry, M., Lynch, G., 1983. Classification and properties of excitatory amino acid receptors in the hippocampus. Part I: Electrophysiological studies of an apparent desensitization and interactions with drugs which block transmission. *J. Neurosci.* 3, 1538–1546.
- Farb, C.R., Aoki, T., LeDoux, J.E., 1995. Differential localization of NMDA and AMPA receptor subunits in the lateral and basal nuclei of amygdala: a light and electron microscope study. *J. Comp. Neurol.* 362, 86–108.
- Fuxe, K., Agnati, L.F., 1991. Two principal modes of electrochemical communications in the brain: volume versus wiring transmission. *Adv. Neurosci.* 1, 1–9.
- Gloor, P., Fariello, R.G., 1988. Generalized epilepsy: some of its cellular mechanisms differ from those of focal epilepsy. *Trends Neurosci.* 11, 63–68.
- Herkenham, M., 1991. Mismatches between neurotransmitter and receptor localizations: Implications for endocrine functions in the brain. In: Fuxe, K., Agnati, L.F. (Eds.), *Advances in Neuroscience, vol 1: Volume Transmission in the Brain. Novel Mechanisms for Neural Transmission*. Raven Press, New York, pp. 63–87.
- Herrero, I., Miras-Portugal, M.T., Sanchez-Prieto, J., 1992a. Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation. *Nature*. 360, 163–166.
- Herrero, I., Miras-Portugal, M.T., Sanchez-Prieto, J., 1992b. Activation of protein kinase C by phorbol esters and arachidonic acid required for the optimal potentiation of glutamate exocytosis. *J. Neurochem.* 59, 1574–1577.
- Hertz, I., 1979. Functional interactions between neurons and astrocytes I. Turnover and metabolism of putative amino acid transmitters. *Prog. Neurobiol.* 13, 277–323.
- Hertz, I., 1982. Astrocytes. In: Lajtha, A. (Ed.), *Handbook of Neurochemistry*. Plenum Press, New York, pp. 319–355.
- Hosford, D.A., Crain, B.J., Cao, Z., Bonhaus, D.W., Friedman, A.H., Okazaki, M.M., Nadler, J.V., McNamara, J.O., 1991. Increased AMPA-sensitive quisqualate receptor binding and reduced NMDA receptor binding in epileptic human hippocampus. *J. Neurosci.* 11, 428–434.
- Kullman, D.M., Erdemli, G., Asztély, F., 1996. LTP of AMPA and NMDA receptor-mediated signals: evidence for presynaptic expression and extrasynaptic glutamate spill-over. *Neuron* 17, 461–474.
- Lehmann, A., Hansson, E., 1988. Kainate-induced stimulation of amino acids release from primary astroglial cultures of the rat hippocampus. *Neurochem. Int.* 13, 557–561.
- Levi, G., Patrizio, M., 1992. Astrocytes heterogeneity: endogenous amino acid levels and release evoked by non-N-methyl-aspartate receptor agonists and by potassium-induced swelling in type-1 and type-2 astrocytes. *J. Neurochem.* 58, 1943–1952.
- Liu, Z., Stafstrom, C.E., Sarkisian, M.R., Yang, Y., Hori, A., Tandon, P., Holmes, G.L., 1997. Seizure-induced glutamate release in mature and immature animals: in vivo microdialysis. *Neuroreport* 8, 2019–2023.
- Löscher, W., Hönack, D., 1994. Effects of non-NMDA antagonist NBQX and the 2,3-benzodiazepine GYKI 52466 on different seizure types in mice: comparison with diazepam and flumazenil. *Br. J. Pharmacol.* 113, 1349–1357.
- Lynch, M.A., Errington, M.L., Bliss, T.V.P., 1989. Nordihydroguaiaretic acid blocks the synaptic component of long-term potentiation and the associated increase in release of glutamate and arachidonate: an in vivo study in the dentate gyrus in the rat. *Neuroscience* 30, 693–701.
- Martin, D.L., 1992. Synthesis and release of neuroactive substances by glial cells. *Glia* 5, 81–94.
- Massieu, L., Morales-Villagrán, A., Tapia, R., 1995. Accumulation of extracellular glutamate by inhibition of its uptake is not sufficient for inducing neuronal damage: an in vivo microdialysis study. *J. Neurochem.* 64, 2262–2272.
- Matsumoto, H., Ajmone-Marsan, C., 1964a. Cortical cellular phenomena in experimental epilepsy: inter-ictal manifestations. *Exp. Neurol.* 9, 286–304.
- Matsumoto, H., Ajmone-Marsan, C., 1964b. Cortical cellular phenomena in experimental epilepsy: ictal manifestations. *Exp. Neurol.* 9, 305–326.
- Millan, M.H., Obrenovitch, T.P., Sarna, G.S., Lok, S.Y., Symon, L., Meldrum, B.S., 1991. Changes in rat brain extracellular glutamate concentration during seizures: an in vivo dialysis study with on line enzymatic detection. *Epilepsy Res.* 9, 86–91.
- Miege, M., Boutelle, M.G., and Fillenz, M., 1996. The source of physiologically stimulated glutamate efflux from the striatum of conscious rats. *J. Physiol. (Lond.)* 497, 745–751.
- Murphy, S., Pearce, B., 1987. Functional receptors for neurotransmitters on astroglial cells. *Neuroscience* 2, 381–394.
- Nakanishi, S., 1992. Molecular diversity of glutamate receptors and implications for brain function. *Science* 258, 597–603.
- Nicholson, C.H., 1995. Extracellular space as the pathway for neuron-glia cell interactions. In: Kettenmann, H., Ransom, B.R. (Eds.), *Neuroglia*. Oxford University Press, New York, Oxford, pp. 387–397.
- Obrenovitch, T.P., Urenjak, J., Zilkha, E., 1996. Evidence disputing the link between seizure activity and high extracellular glutamate. *J. Neurochem.* 66, 2446–2454.
- Olsson, T., Rönnback, L., Hansson, E., 1997. Astroglia and brain function. In: Hansson, E., Olsson, T., Rönnback, L. (Eds.), *On Astroglia and Glutamate Neurotransmission*. Springer- R.G. Landes, New York, pp. 1–14.
- Paxinos, G., Watson, C., 1986. In: *The Rat Brain in Stereotaxic Coordinates*, 2nd ed. Academic Press, London.
- Peña, F., Tapia, R., 1999. Relation among seizures, extracellular amino acid changes and neurodegeneration induced by 4-aminopyridine in rat hippocampus: a microdialysis and electroencephalographic study. *J. Neurochem.* 72, 2006–2014.
- Rogawski, M.A., 1995. Excitatory amino acids and seizures. In: Stone, T.W. (Ed.), *CNS Neurotransmitters and Neuro-modulators: Glutamate*. CRC Press, New York, pp. 219–237.
- Rönnback, L., Hansson, E., 1997. Does astroglial network perform qualitative modifications of neuronal messages? In: Hansson, E., Olsson, T., Rönnback, L. (Eds.), *On Astroglia and Glutamate Neurotransmission*. Springer-R.G. Landes, New York, pp. 155–187.
- Rothstein, J.D., Dykes-Hobert, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A., Wang, Y.F., Schtelke, J.P., Welty, D.F., 1996. Knockout of glutamate transporters reveals a major role from astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16, 675–686.
- Seifert, G., Steinhäuser, C., 1995. Glial cells in the mouse hippocampus express AMPA receptors with an intermediate Ca^{2+} permeability. *Eur. J. Neurosci.* 7, 1872–1881.
- Schab, R.S., 1967. Intravenous diazepam in the treatment of prolonged seizure activity. *N. Engl. J. Med.* 276, 779–784.
- Schousboe, A., 1981. Transport and metabolism of glutamate and GABA in neurons and glial cells. *Int. Rev. Neurobiol.* 22, 1–45.
- Schousboe, A., Svenneby, G., Hertz, L., 1977. Uptake and metabolism of glutamate in astrocytes cultured from dissociated mouse brain hemispheres. *J. Neurochem.* 29, 999–1005.
- Sierra, G., Acuña, C., Otero, J., Docampo, G., 1969. Experimental study of a new psychosedative drug: HS-2314. *Int. J. Neuropharmac.* 8, 153–160.
- Sierra-Paredes, G., Cornes, J.M., Sierra-Marcuño, G., 1999. Calpain inhibitor I retards seizure offset in the hippocampus of freely moving rats. *Neurosci. Lett.* 263, 165–168.
- Sierra-Paredes, G., Galán-Valiente, J., Vazquez-Illanes, M.D.,

- Aguilar-Veiga, E., Soto-Otero, R., Méndez-Alvarez, E., Sierra-Marcuño, G., 1997. Effects of Dizocilpine and Diazepam on hippocampal amino acid levels during picrotoxin-induced seizures. *Epilepsia* 38 (suppl. 3), 179.
- Sierra-Paredes, G., Galán-Valiente, J., Vazquez-Illanes, M.D., Aguilar-Veiga, E., Soto-Otero, R., Méndez-Alvarez, E., Sierra-Marcuño, G., 1998. Extracellular amino acids in the rat hippocampus during picrotoxin threshold seizures in chronic microdialysis experiments. *Neurosci. Lett.* 248, 53–56.
- Sierra-Paredes, G., Sierra-Marcuño, G., 1996a. Microperfusion of picrotoxin in the hippocampus of freely moving rats through microdialysis probes: a new method of induce partial and secondary generalized seizures. *J. Neurosci. Methods* 67, 113–120.
- Sierra-Paredes, G., Sierra-Marcuño, G., 1996b. Effects of NMDA antagonists on seizure thresholds induced by intrahippocampal microdialysis of picrotoxin in freely moving rats. *Neurosci. Lett.* 218, 62–66.
- Soto-Otero, R., Méndez-Alvarez, E., Galán-Valiente, J., Aguilar-Veiga, E., Sierra-Marcuño, G., 1994. Quantitative analysis of neuroactive amino acids in brain tissue by liquid chromatography using fluorescent pre-column labelling with *o*-phthalaldehyde and *N*-acetyl-L-cysteine. *Biomed. Chromatogr.* 8, 114–118.
- Steinhäuser, C., Gallo, V., 1996. News on glutamate receptors in glial cells. *Trend. Neurosci.* 19, 339–345.
- Steinhäuser, C., Jabs, R., Kettenmann, H., 1994. Glutamate activates a cationic conductance and blocks potassium currents in identified glial cells of the mouse hippocampal slice. *Hippocampus* 4, 19–36.
- Taylor, C.P., Dudek, F.E., 1982. Synchronous neural afterdischarges in rat hippocampal slices without active chemical synapsis. *Science* 218, 810–812.
- Traub, R.D., Wong, R.K.S., 1982. Cellular mechanisms of neuronal synchronization in epilepsy. *Science* 216, 745–747.
- Vizi, E.S., Kiss, J.P., 1998. Neurochemistry and pharmacology of the major hippocampal transmitter systems: Synaptic and nonsynaptic interactions. *Hippocampus* 8, 566–607.
- Vizi, E.S., Mike, A., Tarnawa, I., 1996. 2,3-Benzodiazepines (GYKI 52466 and analogs): negative allosteric modulators of AMPA receptors. *CNS Drug Reviews* 2, 91–126.
- Wheal, H.V., Simpson, L., Phelps, S., Stockley, E., 1991. Excitatory amino acid receptor subtypes and their roles in epileptiform synaptic potentiation in the hippocampus. In: Wheal, H.V., Thomson, A. (Eds.), *Excitatory Amino Acids and Synaptic Transmission*. Academic Press, New York, pp. 239–264.
- Wilkin, G.P., Marriot, D.R., Cholewinsky, A.J., 1990. Astrocyte heterogeneity. *Trends Neurosci.* 13, 43–46.
- Wyler, A.R., 1982. Neuronal activity during seizures in monkeys. *Exp. Neurol.* 76, 574–585.