

Seizures induced by microperfusion of glutamate and glycine in the hippocampus of rats pretreated with latrunculin A

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Abstract

Changes in the membrane distribution of *N*-methyl-D-aspartate (NMDA) glutamate receptors seem to produce dramatic modifications in neuronal excitability and other properties of the neuron. In order to determine *in vivo* if these effects are due to the binding of extracellular glutamate and glycine to NMDA extrasynaptic receptors, we perfused the hippocampus of freely moving rats with the actin depolymerizant agent latrunculin A (4 μ M) through microdialysis probes. One month later, continuous microperfusion of glutamate (1 mM) or glycine (1 mM) was used to induce epileptic seizures in the animals pretreated with latrunculin A. Glutamate microperfusion induced seizures in 50% of the animals studied, and glycine induced seizures in 75% of the rats. However, no effect was observed on control rats, or on those animals previously treated with picrotoxin. Simultaneous microperfusion of 100 μ M MK-801 significantly reduced the number and duration of seizures induced by both glutamate and glycine. This study demonstrates that the application of latrunculin A results in long-term changes in susceptibility to the epileptogenic action of glutamate and glycine.

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Much recent research has focused on the role of ion channels and receptor localization and mobility. The localization of NMDARs at synaptic sites is achieved through interactions between their intracellular domains, cytoskeletal elements [2,4] and other cytoplasmically located submembrane proteins in the postsynaptic density, many of them scaffolding proteins [20]. F-actin seems to be responsible for targeting NMDARs to synaptic sites because treatment with actin-depolymerizing agents such as latrunculin A selectively reduces the numbers of synaptic NMDAR clusters without affecting nonsynaptic clusters [1,2,14].

Glutamate receptors are mainly concentrated in the postsynaptic complex of central synapses. This implies a highly organized and stable postsynaptic membrane with tightly anchored receptors. The identification of many anchoring proteins such as rapsyn, gephyrin and PSD-95, and the association of postsynaptic density proteins with a molecular

scaffold have strengthened this view [20]. Nevertheless, recent evidence suggests that AMPA and NMDA receptors at synapses are highly dynamic [8,21]. It is becoming evident that scaffolding proteins have much more dynamic properties than simply acting as inert scaffolds for other molecules [20]. Both the rapid incorporation of AMPA and NMDA receptors into synapses and their removal by endocytosis are activity dependent, suggesting that this exchange may underlie several forms of synaptic plasticity. Tovar and Westbrook [21] demonstrated that NMDA receptors move laterally between synaptic and extrasynaptic pools, providing an alternate mechanism for altering synaptic strength on the time course of minutes. Synaptic and extrasynaptic NMDA receptors have been shown to play a different role in excitotoxicity [13,19], and NMDA receptors expressed in a single neuron can be differentially regulated based on subcellular localization. Li et al. [7] proposed that distinct regulation of synaptic versus extrasynaptic NMDA receptors provides a mechanism for receptor adaptation in response to a variety of stimuli. Furthermore, Hardingham et al. [6] provided

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evidence that a key determinant of the nature of NMDA receptor signalling is the location of the activated receptor.

These previous data suggest a sequence of events, which may be involved in the long-term response of a postsynaptic neuron. These are increased excitatory activity in the presynaptic neuron, calcium influx, phosphatases and/or protein kinase activation, phosphorylation/dephosphorylation of receptors, ion channels and other proteins, cytoskeletal changes, receptor and ion channels mobilization, and finally, long-term changes in postsynaptic excitability. If this reflects the physiological sequence leading to biochemical and morphological changes involved in the normal function of neuronal networks, it is possible that biochemical and/or morphological alterations in any of these steps might produce pathological consequences. For instance, deregulated mobility caused by alterations in cytoskeletal proteins is likely to be involved in hyperexcitability leading to epileptic seizures.

We have previously reported [16] that latrunculin A induces long-term changes in neuronal excitability leading to the onset of sporadic spontaneous seizures from 1 to 6 months after latrunculin A treatment. One hypothesis which might explain the action of latrunculin A is the displacement of synaptic NMDA receptors to extrasynaptic locations, as observed *in vitro* [2], where they may be easily activated by intercellular glutamate of glial origin. In order to test this hypothesis, we perfused the rat hippocampus with latrunculin A and, after a resting period of 1 month, increased the extracellular concentrations of glutamate and glycine in the hippocampus. Rats pretreated with picrotoxin were used as controls to test the specificity of the effect of glutamate and glycine in rats pretreated with latrunculin A. Picrotoxin seizures do not induce long-term changes in excitability as measured by picrotoxin seizure thresholds [18]. However, a decrease in picrotoxin seizure thresholds is observed when glutamate and glycine concentrations in the rat hippocampus are increased [17].

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-h light:12 h 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. 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. L358/118/12/1986).

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 midline, 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 [11] 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, UK), and also with a Minihuit electroencephalograph (Alvar Electronic, Paris, France).

We used the 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 modified Ringer's solution (NaCl 147 mM, KCl 4.0 mM, CaCl₂ 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.

We used latrunculin A for a selective actin depolymerization at the dendrite level. Stock solutions of latrunculin A (Molecular Probes, Eugene, Oregon, USA) were prepared in ethanol (100 $\mu\text{g}/\mu\text{l}$) and kept at -20°C until used. Solutions in modified Ringer's fluid appropriate for microperfusion containing a maximum of 1:18750 ethanol were prepared weekly at a concentration 4 $\mu\text{g}/\text{ml}$. Control-modified Ringer's fluid and picrotoxin solutions containing 1:18750 ethanol were prepared weekly. For the control experiments, modified Ringer's solution containing 1:18750 ethanol was perfused at a constant flow rate of 2 $\mu\text{l}/\text{min}$ throughout the experiment.

Rats were divided into three groups: in the control group (eight rats), vehicle solution was perfused daily for 8 h/day with continuous EEG monitoring for three consecutive days. In the second group (eight rats), latrunculin A (4 μM) was dissolved in modified Ringer and perfused daily for 8 h/day with continuous EEG monitoring for three consecutive days. In the third group (eight rats), a convulsive dose of picrotoxin was perfused following the previously described protocol for picrotoxin threshold seizures [18]. After a resting period of 1 month, the animals from the three groups were perfused

Table 1

Experimental protocol for the microperfusion of drugs and amino acids in three groups of eight rats

	Weeks								
	1	2	3	4	5	6	7	8	9
Group 1	Vehicle	EEG controls	EEG controls	EEG controls	Glu or Gly	Gly or Glu	Glu or Gly + MK801	Gly or Glu + MK801	EEG controls
Group 2	PCT	EEG controls	EEG controls	EEG controls	Glu or Gly	Gly or Glu	Glu or Gly + MK801	Gly or Glu + MK801	EEG controls
Group 3	Lat A	EEG controls	EEG controls	EEG controls	Glu or Gly	Gly or Glu	Glu or Gly + MK801	Gly or Glu + MK801	EEG controls

EEG controls were performed both with and without probe introduction during control. On weeks 5–8, EEG controls were also performed the days following amino acid administration. PCT: picrotoxin; Lat A: latrunculin A.

during 3 h with 1 mM glutamate or 1 mM glycine dissolved in modified Ringer solution. These concentrations were selected because they induce a decrease in picrotoxin seizure threshold when perfused into the rat hippocampus [17]. All animals were perfused with one of both amino acids in a random order, with a resting period of at least 1 week between both experiments. The same animals were perfused with solutions containing the same amino acid concentrations plus 100 μ M MK-801, allowing the same resting periods between experiments. Frequent control EEGs were recorded in all the animals during all the experimental period (at least twice a week), and they were directly observed and videotaped in search for spontaneous seizures. The experimental protocol is summarized in Table 1.

EEG records were analyzed using the Medilog 9200 software, version 7.2. Spike and wave discharges duration and seizure duration were evaluated. Statistical significance of the difference in number of seizures, seizure duration and interictal discharges duration was determined by Student's paired *t*-test.

At the end of the experiments the 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 μ m coronal sections were cut and stained with cresyl violet, and the position of the probe was checked under light microscopy.

Our results on individual seizure thresholds and seizure types were consistent with those described previously [18]. Seizure threshold among animals varied between 100 and 300 μ M, although it remained unchanged in repeated between day experiments within individual rats.

No spontaneous seizures were observed in controls and animals pretreated with threshold doses of picrotoxin. Rats pretreated with picrotoxin did not show any seizures or EEG discharges when glutamate (1 mM) or glycine (1 mM) was perfused into the hippocampus. However, when pretreated with latrunculin A, seizures appeared randomly from 1 month after treatment. Most of these seizures were observed but not recorded, because they happened while the animals were out of the experimental unit. In two animals pretreated with latrunculin A seizures were recorded during controls, in one rat without simultaneous probe introduction and in the other one during vehicle microperfusion (Fig. 1A). Seizures were observed in 75% of the animals during glycine

(1 mM) hippocampal microperfusion, and in 50% of the animals during glutamate (1 mM) microperfusion (Fig. 1A). Mean seizure duration was 36.7 ± 7.4 s in controls showing spontaneous seizures (both with and without probe introduction), but it was significantly increased to 54.1 ± 12.3 s ($p < 0.05$) during glycine microperfusion and 72.3 ± 16.9 s ($p < 0.01$) during glutamate microperfusion (Fig. 1B). EEG paroxysmal discharges (EEG discharges without observable behavioral manifestations) (Fig. 2B) were observed in 100% of the rats during glutamate microperfusion and in 75% of the rats during glycine microperfusion. Mean paroxysmal discharges duration was 6.3 ± 1.2 s in controls, and it was significantly increased to 63.6 ± 16.2 s ($p < 0.01$) during glutamate microperfusion and 59.3 ± 13.6 s ($p < 0.01$) during glycine microperfusion (Fig. 2A). The simultaneous

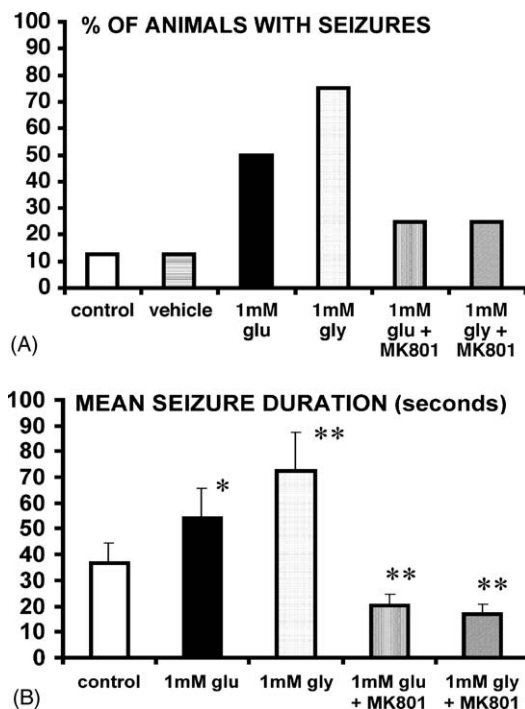


Fig. 1. Seizures induced as an effect of glutamate (1 mM) and glycine (1 mM) continuous microperfusion in the rat hippocampus. (A) Percentage of rats showing at least one seizure 1 month after latrunculin A (4 μ M) perfusion. (B) The significant increase in seizure duration induced by glutamate and glycine is prevented by the continuous microperfusion of MK-801 (100 μ M). Data are mean \pm S.E. ($n = 8$ from the latrunculin A-treated group) of the values obtained by the analysis of electroencephalographic recordings. * $P < 0.005$, ** $P < 0.01$ by Student's paired *t*-test.

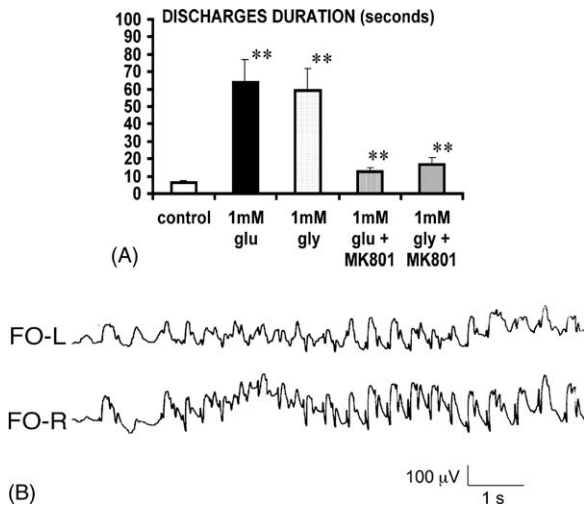


Fig. 2. Interictal discharges induced by glutamate (1 mM) and glycine (1 mM) continuous microperfusion in the rat hippocampus. (A) The significant increase in discharge frequency and duration induced by glutamate and glycine is prevented by the continuous microperfusion of MK-801 (100 μ M). Data are mean \pm S.E. ($n = 8$ from the latrunculin A-treated group) of the values obtained by the analysis of electroencephalographic recordings. Control bar represents the results during EEG recordings without probe insertion. Vehicle bar represents the results during EEG recordings during vehicle microperfusion. ** $P < 0.01$ by Student's paired t -test. (B) EEG recording of spike and wave interictal discharges during glutamate microperfusion in a rat previously treated with latrunculin A. Control bar represents the mean \pm S.E. of seizure duration in animals with spontaneous seizures both with and without probe introduction ($n = 2$). FO-L: fronto occipital left; FO-R: fronto occipital right.

perfusion of MK-801 together with the amino acids significantly reduced the number of seizures in animals perfused with glutamate and glycine (Fig. 1A), as well as the mean duration of seizures induced by glutamate (to 20.4 ± 4.2 ; $p < 0.001$) and glycine (to 16.98 ± 5.3 ; $p < 0.001$) (Fig. 1B) and the mean duration of paroxysmal discharges (12.4 ± 4.9 for glutamate; 16.9 ± 6.5 for glycine; $p < 0.001$) (Fig. 2A).

This study reports on the previously uninvestigated *in vivo* effect of actin depolymerization on brain susceptibility to high glutamate and glycine concentrations. The comparison between the results in picrotoxin- and latrunculin A-treated rats shows that increased susceptibility to high glutamate and glycine concentrations is not a consequence of acute seizures, but a specific effect of latrunculin A action in the rat hippocampus.

One of the effects of latrunculin A in neuronal cultures is the displacement of NMDA and AMPA synaptic receptors to extrasynaptic locations [2]. The role of nonsynaptic receptors in mass sustained convulsive activity has been postulated [3,15]. Recently, Fellin et al. [5] have shown that neuronal synchrony can be achieved by activation of extrasynaptic NMDA receptors with astrocytic glutamate.

These results, taken together with previous data on the absence of effect of increased extracellular glutamate and glycine in normal rats [9,17], support the proposal that exogenous glutamate never reaches postsynaptic space, and its

action is only produced under special conditions by activating extrasynaptic receptors [14,19].

Several studies suggest that NMDA receptor compartmentalization and distribution, as well as the NMDA receptor interaction with other proteins, are essential for excitotoxic effects [6,14]. It is possible that relationships between NMDA receptors and the intracellular processes, which lead to increased excitability or cellular death, might be different in extrasynaptic locations [6,7,19]. This suggests that glutamate action may differ with the increase in the number of extrasynaptic receptors.

The evidence suggests that AMPA and NMDA receptors at synapses are highly dynamic and able to move laterally between synaptic and extrasynaptic pools [8,21]. Also, *in vitro* studies on the action of latrunculin A show that actin filament depolymerization induces a displacement of AMPA and NMDA receptors to extrasynaptic locations [1,2,14], which indicates that the epileptogenic action of intercellular glutamate and glycine occurs only when the structure or localization of glutamate ionotropic receptors is altered. The protective effect of MK-801 suggests that glutamate and glycine perfused through the microdialysis probes most probably act by binding to NMDA receptors.

Our results do not support the idea that signals from extrasynaptic NMDA receptors necessarily trigger cell death pathways as reported [6], but the differences between the intercellular signals reaching a neuron in a living brain compared to those reaching a cultured neuron should be taken into account. For instance, the cultures are usually not enriched with astrocytes [6], which provide living brain neurons with many antiapoptotic signals [13].

Furthermore, recent evidence on the role of astrocytic glutamate in mediating neuronal synchrony [5] strongly supports the role of extrasynaptic NMDA receptors in epileptic neuronal synchronization.

Although most papers [2,21] refer to the effect of latrunculin A as "mobilization" of synaptic receptors to extrasynaptic locations, it is not easy to demonstrate that those receptors found in extrasynaptic locations are the same as those which appeared previously in the postsynaptic space. It seems probable that actin filament depolymerization might lead to receptor internalization and proteolysis [23], while new receptors, probably with a different subunit composition, would be carried to the cell membrane without the precise location control of physiological conditions. Those receptors may be activated more easily by the glutamate perfused through microdialysis probes producing biochemical modifications leading to epileptic seizures. Actin filament depolymerization by latrunculin A might also alter protein anchoring to NMDA receptors, modifying the intracellular signalling induced by both synaptic and extrasynaptic receptors.

The greater effect of glycine as compared with glutamate is intriguing. It may be due to the fact that we have perfused the same concentration for both amino acids, while physiological intercellular levels of glycine are usually lower

than glutamate levels. Furthermore, both glial and vascular transport mechanisms are much more active for glutamate clearance [10].

Our results, taken together with previous research discussed above, favor the hypothesis that extrasynaptic NMDA glutamate receptors might be involved in diverse pathological consequences linked to this neurotransmitter. A possible interesting outcome is that selective inhibition of extrasynaptic NMDA receptors might have interesting therapeutic consequences in the treatment of epilepsy, as well as in the prevention of epileptogenesis in patients with brain lesions, cerebral ischemia or stroke. During these injuries, it seems probable that the excess of extracellular glutamate from any source (including blood–brain barrier alterations) might activate extrasynaptic NMDA receptors [12] contributing to epileptogenesis. Further research is needed in order to establish the role of extrasynaptic NMDA receptors in epilepsy.

Generalized inhibition of NMDA receptors is highly problematic, because receptor function is required for normal synaptic transmission. The use of NMDA inhibitors, both competitive and non-competitive, with a strong antiepileptic effect in experimental models of epilepsy, has not as yet proved to be useful in clinical trials [22]. One of the future proposals in the search for new antiepileptic drugs might involve the development of selective methods for blocking extrasynaptic NMDA receptors without inactivating synaptic receptors.

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