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Glycine enhances extracellular $^{45}Ca^{2+}$ response to NMDA application investigated with microdialysis of rabbit hippocampus *in vivo*

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Abstract. This study evaluates the role of glycine in *in vivo* modulation of the activity of excitatory amino acid receptors sensitive to N-methyl-D-aspartate (NMDA) in the rabbit hippocampus. Changes of extracellular calcium concentration were studied by the microdialysis technique combined with ^{45}Ca-utilizing clearance method. The steady state level of amino acids in the dialysate was analyzed with HPLC. Pharmacologically active substances were applied directly to the hippocampus *via* the dialysis medium. Glycine concentration in the extracellular fluid of the hippocampus was in the range of 10^{-5} M. Application of NMDA induced a drop of calcium concentration in the extracellular compartment of the hippocampus. This effect was inhibited by 7-Cl-kynurenic acid, an antagonist of glycine binding site on the NMDA receptors. Application of glycine and D-serine to the dialysis medium did not interfere with the basal level of extracellular calcium in the hippocampus, but resulted in an enhancement of the NMDA-induced decrease of calcium concentration. These results indicate that in the hippocampus the NMDA receptors are under constant positive modulation by endogenous glycine. Since glycine binding sites on the NMDA receptors are not saturated by an endogenous ligand, glycine may play a role in regulation of the NMDA receptor activity in the hippocampus *in vivo*.

Key words: rabbit hippocampus, *in vivo* microdialysis, NMDA receptors, glycine, extracellular $^{45}Ca^{2+}$

INTRODUCTION

A subclass of metabotropic, qisqualate-sensitive excitatory amino acid receptors and several subclasses of ionotropic glutamate receptors have been recognized (Costa et al. 1988, Cotman et al. 1988). Kainate- and AMPA-specific receptors operate channels permeable to monovalent cations and are involved in fast excitatory neurotransmission (MacDermott and Dale 1987). The subclass of N-methyl-D-aspartate (NMDA)-specific receptors is in the focus of most extensive electrophysiological and biochemical investigations. These receptors operate ionic channels permeable to calcium (Ascher and Nowak 1987), and are responsible for various physiological and pathological processes in the brain (Wieloch 1985, Rothman and Olney 1986, Cotman et al. 1988). The complex structure of the NMDA receptor includes, apart from the binding site for agonists, and proteins constituting the ion channel permeable to Ca^{2+}, Na^{+}, and K^{+}, also numerous ligand binding sites for Mg^{2+}, Zn^{2+}, glycine and polyamines, that modify the activity of receptors by positive or negative allosteric modulation (Costa et al. 1988, Costa 1989, Wróblewski and Danysz 1989).

A positive modulatory site on the NMDA receptor complex for glycine, that is strychnine-insensitive, has been recognized in ligand binding experiments (Reynolds et al. 1987, Pullan and Cler 1988). Many *in vitro* studies definitively revealed that occupation of the strychnine-insensitive glycine binding site on the NMDA receptor is a prerequisite for its response to NMDA. Electrophysiological investigations demonstrated that glycine is required for full activation of NMDA receptors (Johnson and Ascher 1987, Kleckner and Dingledine 1988). Functional studies have indicated that occupation of the positive modulatory site by glycine or other specific ligands reduces NMDA receptor desensitization (Thomson 1989), and attenuates Mg^{2+}-evoked inhibition of calcium uptake to the cultured neurons stimulated with NMDA (Wroblewski et al. 1989). In turn 7-Cl-kynurenic acid, an antagonist of the glycine binding site, potently inhibits the activity of NMDA receptors (Kemp et al. 1988).

Glycine concentration in the brain extracellular fluid reaches 10^{-5} M (Reynolds 1990) and increases to a much higher level in some pathological conditions including brain ischemia (Globus et al. 1991). In turn, the affinity of the glycine binding sites is within the range of 0.1 - 10 μM (Cotman et al. 1988). Thus, it is not clear how glycine may play any regulatory role in the brain, since its binding sites may be completely saturated *in vivo* by endogenous glycine (Kleckner and Dingledine 1988, Reynolds 1990). According to recent *in vitro* data, glycine potentiates the NMDA-evoked dopamine release in brain slices and synaptosomes (Werling et al. 1990, Fink et al. 1990, Krebs et al. 1991). *In vivo* experiments also demonstrated potentiation by glycine of the effect of NMDA agonists on cGMP formation in the cerebellum (Danysz et al. 1989, Wood et al. 1989, Rao et al. 1990).

The hippocampus, and particularly its CA1 subfield is a structure rich in NMDA receptors (Greeamyre et al. 1985). They are involved in long-term potentiation, learning and memory formation as well as ischemic nerve cell injury (Wieloch 1985, Rothman and Olney 1986, Cotman et al. 1988). It is, therefore, important to understand the regulation of NMDA receptors in the hippocampus *in vivo*, including involvement of glycine in this process. The aim of this work was to study the ability of agonists and antagonists of the glycine binding sites to modulate NMDA receptor-mediated changes in extracellular calcium concentration in the rabbit hippocampus *in vivo*.

METHODS

Albino rabbits (n=16), weighing 2.5-3.5 kg, were implanted under pentobarbital anesthesia into the dorsal part of the hippocampus, 4 mm behind and 18 mm lateral to the Bregma, with a transhippocampal probe, made up of a 10 mm long, 0.3 o.d. dialysis tubing (Cuprofan, Gambro, m.w. cut-off 10,000), glued between polyethylene tubes, as de-

scribed in detail elsewhere (Łazarewicz et al. 1986). All experiments were performed 24 h after surgery. At the end of each experiment the rabbits were sacrificed and the position of the probes was determined by visual examination.

For dialysis-perfusion experiments the animals were unanesthetized and unrestrained. The extracellular fluid was dialyzed against Krebs-Ringer bicarbonate buffer containing 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 0.4 mM KH_2PO_4 and 25 mM $NaHCO_3$ at a rate of 2.5μ l/min.

To determine changes in extracellular Ca^{2+} concentration in the rabbit hippocampus *in vivo*, the rabbits were perfused for 1 h *via* the dialysis probe with medium containing $^{45}CaCl_2$ (total dose 15 μCi) ($5.5x10^5$Bq). This labeling with ^{45}Ca was followed by 1.5 h equilibration with standard medium, then the desired medium (control or containing inhibitors or modulators: 1 mM 2-amino-5-phosphonovaleric acid (APV), 10 μM MK-801, 1 mM 7-Cl-kynurenic acid, 2.5 mM glycine or 1 mM D-serine) was introduced and dialysis was continued for the next 30 min. After this period samples of dialysate were collected in 5- or 10-min fractions for radioactivity measurements. The basal $^{45}Ca^{2+}$ efflux for 60 min was followed for 20 min by the medium containing 1 mM NMDA, after which the basal medium was reintroduced, and the perfusion continued for 2 h. Inhibitors or modulators were present in the dialysis medium to the end of the experiments. The radioactivity in samples of dialysate was determined by liquid scintillation counting. The radioactivities of samples collected during the first and last 30 min of the experiment were used to extrapolate the control values for each time point by computer-aided semilogarithmic regression analysis and all the results were expressed as a percentage of this extrapolated basal value, as described previously (Łazarewicz et al. 1986).

Endogenous amino acids in the perfusates were detected by reverse phase high-performance liquid chromatography and fluorescence detection, following precolumn derivatization with o-phthaldialdehyde (Lindroth and Mopper 1979, Jones et al. 1981).

N-methyl-D-aspartic acid (NMDA), glycine, D-serine, 7-Cl-kynurenic acid and 2-amino-5-phosphonovaleric acid (APV) were purchased from Sigma Chemicals (St. Louis, MO). MK-801 was obtained from Merck, Sharp and Dohme (Germany).

Statistical analyses were performed using nonparametric Mann-Whitney U test. For graphical reason all values were presented as means ± SEM.

RESULTS

Effect of NMDA on extracellular calcium concentration in the rabbit hippocampus *in vivo*

Application of NMDA for 20 min directly to the hippocampus *via* the dialysis medium resulted in a decrease of extracellular calcium concentration. This effect of NMDA was dose-dependent and sensitive to administration to the dialysis medium of competitive and non-competitive antagonists of the NMDA receptors, 1 mM 2-amino-5-phosphonovaleric acid (APV) and 10 μM MK-801, respectively (results not shown). This effect evoked by 1 mM NMDA appeared after 20-min application of the agonist, whereas normalization of extracellular calcium concentration after cessation of 1 mM NMDA infusion was reached after 40 min (Fig. 1).

Effects of glycine and D-serine on basal and modified by NMDA extracellular levels of calcium in the rabbit hippocampus *in vivo*

Application of 2.5 mM glycine and 1 mM D-serine did not disturb the basal level of extracellular calcium in the hippocampus (results not shown). However, these amino acids potentiated NMDA-induced decrease of calcium concentration. In the presence of 2.5 mM glycine the drop of calcium level evoked by 1 mM NMDA was more rapid and more pronounced than in the medium containing 1 mM NMDA alone (Fig. 1). The effect of higher

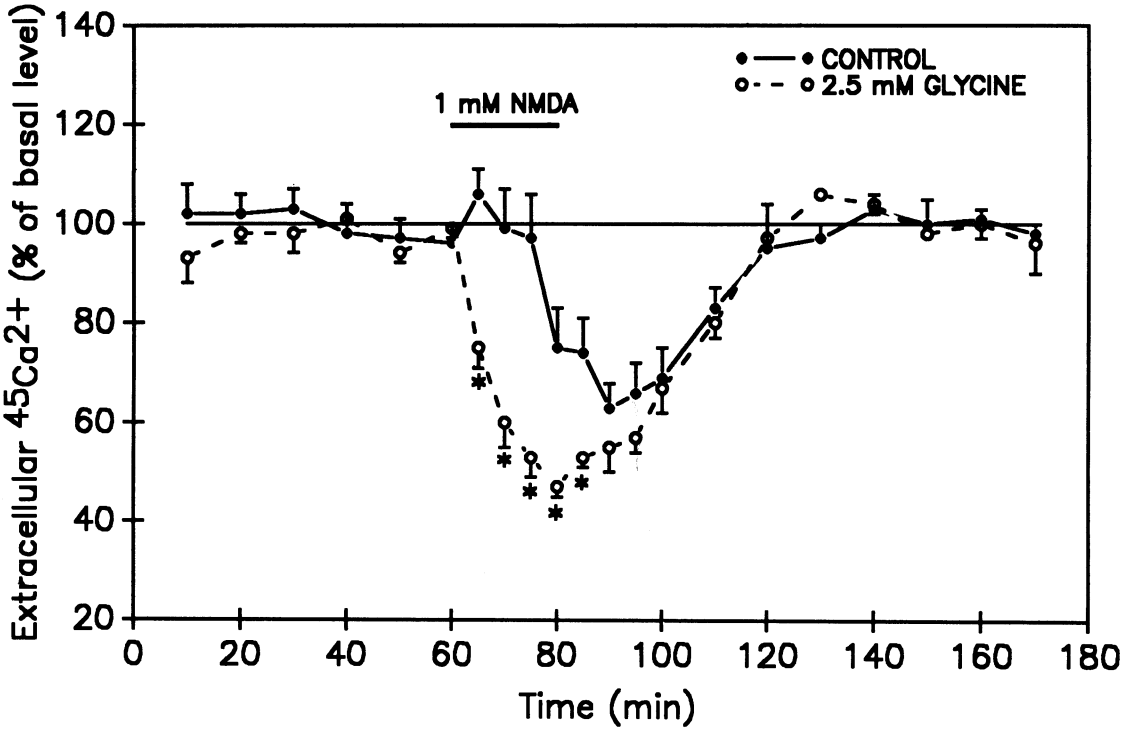

Fig. 1. Effect of glycine on NMDA-evoked decrease of extracellular calcium concentration in the rabbit hippocampus *in vivo*. Glycine was present in the dialysis medium to the end of the experiment. NMDA was applied as indicated by bar. Results of four experiments, presented as means ±SEM. *Means statistically different from control ($P<0.05$).

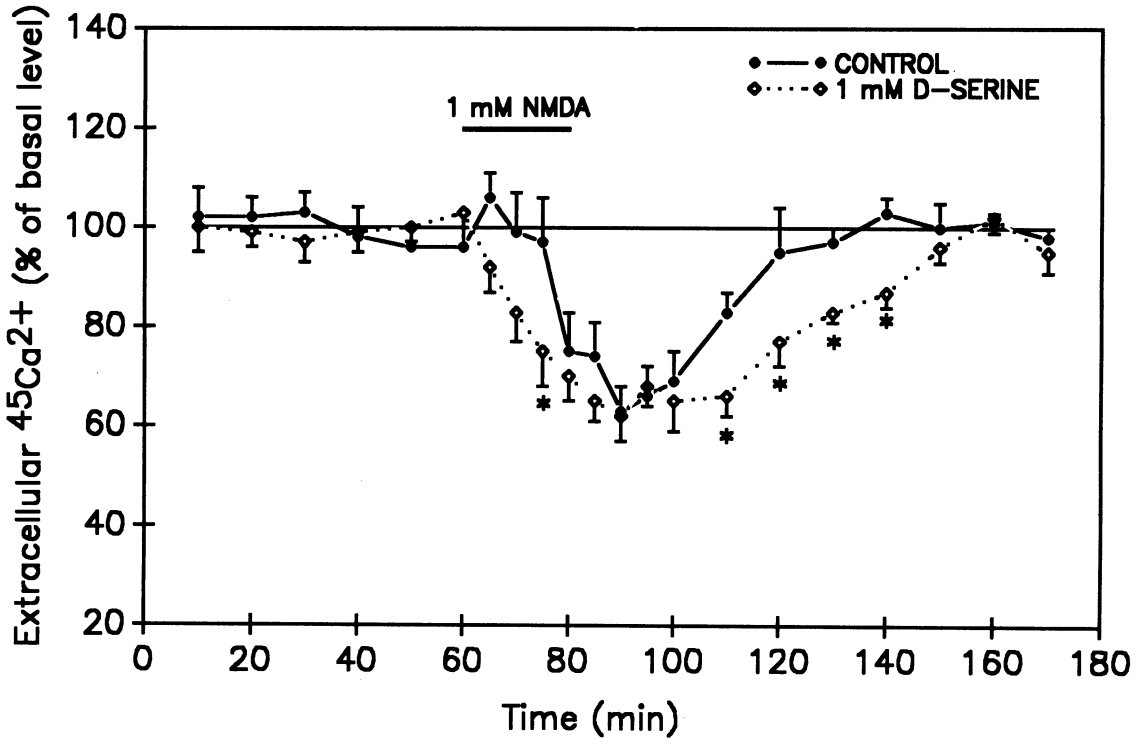

Fig. 2. Effect of D-serine on NMDA-evoked decrease of extracellular calcium concentration in the rabbit hippocampus *in vivo*. D-serine was present in the dialysis medium to the end of the experiment. NMDA was applied as indicated by bar. Results of four experiments, presented as means ±SEM. *Means statistically different from control ($P<0.05$).

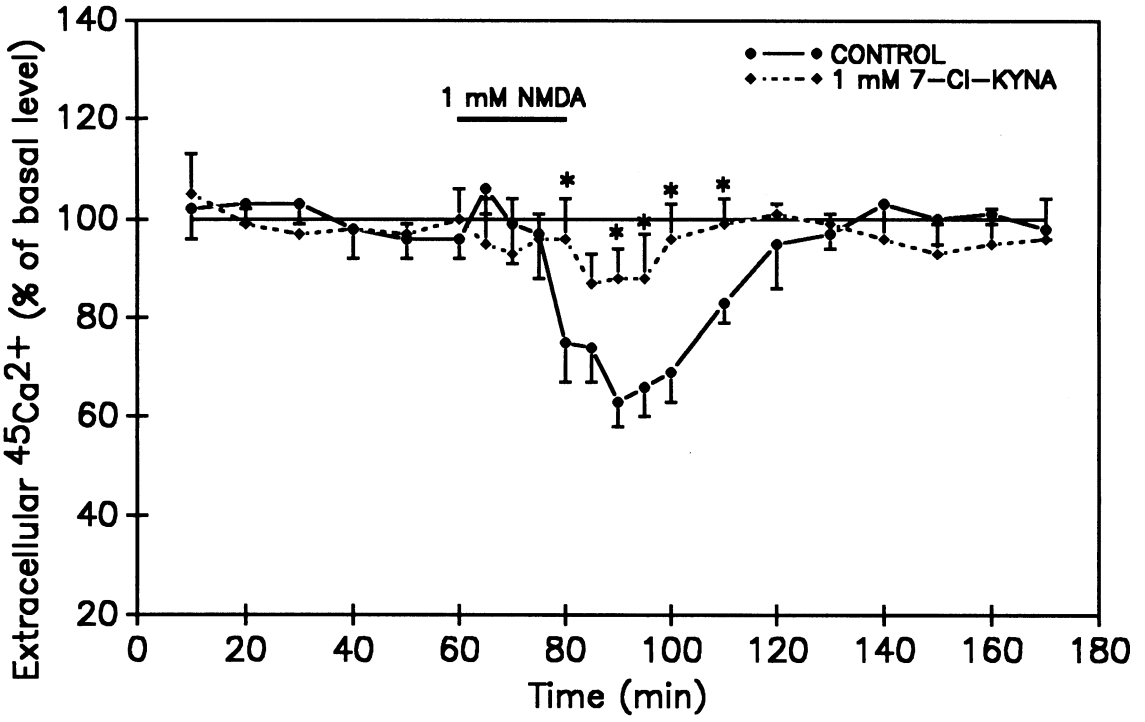

Fig. 3. Effect of 7-Cl-kynurenic acid on NMDA-induced decrease of extracellular calcium concentration in the rabbit hippocampus *in vivo*. 7-Cl-kynurenic acid (7-Cl-KYNA) was present in the dialysis medium to the end of the experiment. NMDA was applied as indicated by bar. Results of four experiments, presented as means ±SEM. *Means statistically different from control ($P<0.05$).

concentrations of NMDA (2.5 mM and 5 mM) was not potentiated by 2.5 mM glycine (results not shown). D-serine, that is a selective ligand of glycine regulatory sites on the NMDA receptors also potentiated the effect of NMDA. In the presence of 1 mM D-serine the decrease of extracellular calcium concentration evoked by 1 mM NMDA was more rapid and normalization of extracellular calcium concentration was reached later than in the medium containing 1 mM NMDA alone, whereas the maximal drop of extracellular calcium concentration was not altered (Fig. 2).

Effect of 7-chlorokynurenate on NMDA evoked decrease of extracellular calcium concentration in the rabbit hippocampus

The effect of 1 mM NMDA, applied without exogenous glycine or D-serine, was almost completely inhibited in the presence of 1 mM 7-chlorokynurenic acid, an antagonist of glycine binding sites on the NMDA receptors (Fig. 3).

Concentrations of amino acids in the perfusion medium

Table 1 shows concentrations of several endogenous amino acids found in four consecutive 10-min fractions of perfusate collected before administration of NMDA to the rabbit hippocampus. The dominating amino acid in the perfusate was glutamine, whereas the levels of neuroactive amino acids including glutamate, aspartate, taurine and phosphoethanolamine were low. The level of glycine in the perfusate was within the range of 4.17-4.52 μM, whereas the basal concentration of GABA was below detection limits of our analytical system. Concentrations of all amino acids tested were stable during the 40-min period of dialysis. The average recovery factor for amino

TABLE I

Concentrations of some amino acids in the dialysate of rabbit hippocampus *in vivo* during 1-h perfusion with standard medium

Amino acid	Concentration in samples collected at			
	0 min	20 min	40 min	60 min
	(μM)			
Aspartate	0.16±0.05	0.11±0.03	0.14±0.05	0.18±0.05
Glutamate	0.43±0.09	0.37±0.08	0.41±0.08	0.44±0.21
Glutamine	62.4±6.0	64.4±3.9	62.0±6.5	60.6±5.4
Glycine	4.20±0.12	4.17±0.14	4.26±0.28	4.52±0.16
Taurine	1.14±0.12	1.17±0.10	1.16±0.04	1.23±0.08
Phosphoethanolamine	1.26±0.16	1.27±0.16	1.37±0.19	1.52±0.15

Before collecting the samples of dialysate, perfusion was carried out for 90 min in order to equilibrate the system. Data represent means ±SEM from 5 experiments.

acids in the dialysis probe used in our study under our experimental conditions equals 40% (Hamberger and Nystrom 1984). Using this factor and concentrations of amino acids found in the dialysate, we estimated the extracellular concentration of glycine at the level of 10.5 μM.

DISCUSSION

The response of the hippocampal NMDA receptors was studied by measuring the NMDA-evoked decrease in the extracellular calcium concentration, which reflects activation of the ionic channels gated by these receptors. The agonist was introduced in standard Mg^{2+}-containing medium. Although Mg^{2+} ions are known from *in vitro* experiments to block the activity of NMDA channels in a voltage-dependent manner (Ascher and Nowak 1988), in our experimental conditions application of NMDA to the rabbit hippocampus *via* the dialysis probe induced a large drop of the extracellular Ca^{2+} concentration, indicating a massive activation of the NMDA receptor-channel complex. In these conditions the Mg^{2+} block may be released by spontaneous depolarization of the electrically active neurons in the hippocampus. It is known that blockade of the NMDA channels by magnesium may be also attenuated in the presence of endogenous glycine (Wróblewski et al. 1989). The NMDA-evoked decrease of extracellular calcium concentration in the hippocampus observed in this study is dose-dependent and sensitive to selective antagonists of the NMDA receptors, thus it is a specific, receptor-mediated process.

The effect of NMDA was potently inhibited by 7-Cl-kynurenate, an antagonist of the glycine binding site on NMDA receptors (Kemp et al. 1988). This finding, in agreement with other data (Johnson and Ascher 1987, Kleckner and Dingledine 1988), illustrates the requirement for endogenous glycine to achieve the NMDA receptor activation. Our results indicate that NMDA receptors in the rabbit hippocampus *in situ* are under a constant positive modulatory influence of endogenous extracellular glycine. 7-Cl-kynurenate competes with endogenous glycine and effectively blocks its access to the binding sites on the NMDA receptors. A relatively high 10 μM concentration of endogenous

glycine in the extracellular fluid of the rabbit hippocampus was detected in our experiments, in agreement with previous data (Reynolds 1990).

It was found in this study that, although administration of glycine and D-serine into the hippocampus does not influence the basal level of extracellular calcium, it enhances, however, the NMDA-evoked decrease of extracellular calcium concentration. A similar effect was demonstrated in *in vitro* studies on the NMDA-evoked release of dopamine from brain slices and synaptosomes (Fink et al. 1990, Werling et al 1990, Krebs et al. 1991), and in *in vivo* experiments on the NMDA receptor-induced cGMP production in the cerebellum (Danysz et al. 1989, Wood et al. 1989, Rao et al. 1990). Moreover, intracerebellar injection of D-serine alone was shown to result in an increase of the cGMP level (Wood et al. 1989, Rao et al. 1990). This effect reflects potentiation of the ongoing NMDA-mediated neuronal activity in the cerebellum. A similar effect was not found in our studies. We cannot exclude the possibility that the sensitivity of our measurements may not be sufficient to monitor small changes in extracellular calcium concentration, evoked by potentiation of spontaneous NMDA neurotransmission. However, the ineffectiveness of glycine and D-serine given alone to the hippocampus may as well indicate that the endogenous glycine concentration is sufficient to support physiological NMDA transmission. During application of exogenous NMDA, that leads to abnormal activation of the NMDA receptors, additional doses of glycine (or D-serine) are required to potentiate the effect of NMDA.

Our results indicate that in the given experimental conditions, under excessive glutamatergic stimulation, the glycine binding sites on the NMDA receptors in the rabbit hippocampus are not completely saturated with the endogenous ligand. Considering this point, we should keep in mind that in our experiments the application of NMDA to the hippocampus was preceded by prolonged dialysis-perfusion which could drain the tissue of endogenous glycine. However, this does not seem to be the case since the estimated concentration of glycine in the dialysate was still within the range of 10 μM. This result agrees well with the data of other authors (Reynolds 1990). Although this value exceeds or at least equals the *in vitro* affinity of binding sites for glycine in the NMDA receptors, estimated within the range of 0.1-10 μM (Cotman et al. 1988), our data indicate that *in vivo* glycine may play a regulatory role in the NMDA receptor activation in the hippocampus, especially during excessive stimulation of these receptors.

It has been demonstrated that brain ischemia leads not only to the release of glutamate and aspartate, but also to a significant increase of extracellular glycine concentration in vulnerable brain regions including the hippocampus (Globus et al. 1991). This elevated glycine concentration persisted in the hippocampus during the recirculation period (Globus et al. 1991). Interestingly, hypothermia, which is known to provide protection against ischemic neuronal injury, was shown to prevent also the ischemia-evoked increase in hippocampal concentrations of glycine (Baker et al. 1991).

To summ up, in this study we monitored the NMDA-evoked changes in the extracellular calcium concentration in the rabbit hippocampus *in vivo* while interfering with glycine binding sites in the NMDA receptors. It was found that blockade of these sites completely prevents the drop of extracellular calcium concentration evoked by NMDA, whereas agonists of the glycine binding sites potentiate the effect of NMDA. These results indicate that extracellular glycine provides an adequate support to normal NMDA receptor neurotransmission in the hippocampus, whereas the increase of glycine concentration may potentiate excessive stimulation of the NMDA receptors. This may occur in some pathological conditions including brain ischemia.

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