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BIOCHEMICAL AND THERMODYNAMIC ASPECTS OF THE BINDING OF [^{3}H]GLYCINE TO ITS STRYCHNINE-INSENSITIVE RECOGNITION SITE ASSOCIATED WITH THE *N*-METHYL-D-ASPARTATE RECEPTOR COMPLEX

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Abstract—The molecular mechanism of interaction between glycine and its strychnine-insensitive binding site linked to the *N*-methyl-D-aspartate receptor was investigated by examining on the one hand the thermodynamic properties of glycine binding, and, on the other hand, the effects of various functional group modifying agents on ligand binding. Raising the incubation temperature from 0° to 37° resulted in a consistent decrease of glycine binding affinity. Calculation of thermodynamic parameters from the corresponding Van't Hoff plot showed that the binding of glycine was mainly entropy-driven, the change in enthalpy contributing only little (25–30%) to the change in Gibbs free energy. Chemical modification with the sulfhydryl-directed agents *p*-hydroxy-mercuribenzoate and *N*-ethyl-maleimide showed free -SH groups to be critical for ligand binding to the receptor site. Furthermore, guanidino groups on arginyl residues, sensitive to 2,3-butanedione, were also found to participate in glycine binding. Both the -SH and the guanidino groups could be protected against their inactivation by co-incubation with glycine, indicating a direct involvement of these functional groups in the binding process. Dithiothreitol, a disulfide-reducing agent, likewise prevented [^{3}H]glycine binding, suggesting that the glycine recognition site is stabilized by at least one disulfide bridge. It is concluded that the binding of glycine probably involves a strong ion–ion interaction between its carboxyl group and a positively charged guanidino group at the receptor site, resulting in a thermodynamically favorable increase in entropy by displacement of water molecules from the latter and a concomitant decrease in enthalpy. Furthermore, at least one free sulfhydryl group seems to participate in the binding process.

Amongst the various sub-types of excitatory amino acid receptors in the mammalian central nervous system [1], the *N*-methyl-D-aspartate (NMDA†) receptor has attracted considerable interest because it is believed to be involved in key physiological functions (e.g. long-term potentiation (LTP), learning and memory, developmental processes) on the one hand and in a variety of disease states such as epilepsy, neurodegenerative diseases and the sequels of ischaemic/hypoxic events (stroke) on the other hand. Therefore, in recent years, the NMDA receptor has been well characterized regarding its pharmacological and biophysical properties. The NMDA receptor complex comprises various functional domains interacting with each other, thus allowing a fine initiation and regulation of the receptor activity (for reviews, see Refs 2–5). In electrophysiological studies using the patch-clamp technique, Johnson and Ascher [6] showed that glycine enhances NMDA-evoked responses in cortical neurons from primary cell culture. Later studies using frog oocytes [7] demonstrated that the presence of glycine is an absolute requirement for channel opening by NMDA agonists, suggesting that glycine plays the role of a real co-agonist rather than a modulator at the NMDA receptor [8]. These results conferred a functional correlate to the strychnine-insensitive glycine binding site in the brain, which had been described by Kishimoto *et al.* [9] and Bristow *et al.* [10], before its functional relationship to the NMDA receptor complex was known. The strychnine insensitivity distinguishes this recognition site from the well-known receptor at which glycine acts as an inhibitory neurotransmitter and which is blocked by strychnine. Autoradiographic experiments [10, 11] showed a different distribution of the two binding sites in the rat CNS: the site which is affected by strychnine is sparse in higher brain areas and mainly found in the spinal cord and brainstem, whereas the presence of the strychnine-insensitive site parallels that of the NMDA receptor (with highest densities in the cortex, hippocampus and striatum), further corroborating the functional linkage of the two.

Although the NMDA receptor-coupled glycine binding site has been intensely investigated in pharmacological and physiological terms, little information is available about the biochemical nature of the interaction between glycine and its receptor. Therefore, our aim was on the one hand to get information about thermodynamic features of glycine binding using [^{3}H]glycine as ligand and on the other hand to identify functional groups which are essential

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† Abbreviations: DTT, dithiothreitol; LTP, long-term potentiation; NEM, *N*-ethyl-maleimide; NMDA, *N*-methyl-D-aspartate; PHMB, *p*-hydroxy-mercuribenzoate.

for ligand–receptor interaction, using various agents that modify such functional groups irreversibly.

MATERIALS AND METHODS

Chemicals. [^{3}H]Glycine (45–51 Ci/mmol) and tissue solubilizers (Protosol/Solvable) were purchased from Du Pont NEN. Unlabelled glycine and 2,3-butanedione were obtained from Fluka (Buchs, Switzerland). DL-Dithiothreitol (DTT), *N*-ethylmaleimide (NEM) and *p*-hydroxy-mercuribenzoate (PHMB) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The liquid scintillation fluid used was SafeFluor (Lumac, Landgraaf, The Netherlands). Other chemicals were purchased from several commercial sources.

Membrane preparation. Membrane preparation was based on the procedure of Enna and Snyder [12], as modified by Murphy *et al.* [13]. Male Sprague–Dawley rats (180–220 g) were decapitated and the brains removed rapidly. The cortex was dissected out and homogenized in 20 volumes of ice-cold 0.32 M sucrose using a Teflon–glass homogenizer with a motor driven pestle (800 rpm, six up and down strokes). The homogenate was centrifuged at 1000 *g* for 10 min at 4° and then the supernatant decanted and re-centrifuged at 20,000 *g* for 20 min at 4°. The supernatant was discarded and the pellet re-suspended in 20 volumes of ice-cold distilled water, using a Polytron tissue homogenizer (setting 5–6). After a centrifugation at 8000 *g* for 20 min at 4°, the supernatant and the buffy upper coat of the resulting pellet were carefully removed and centrifuged at 48,000 *g* for 10 min at 4°. The final pellets were resuspended in 20 volumes of ice-cold buffer (50 mM Tris–HCl, pH 8.0) and centrifuged as above, after which the pellets were frozen in a dry ice/ethanol bath and stored at −80° up to a month.

On the day of the experiment, the frozen pellets were left at room temperature for thawing, and then re-suspended in 20 volumes of 0.04% (w/v) Triton X-100 (prepared in the assay buffer) and incubated for 15 min at 37°. After a first centrifugation at 48,000 *g* for 10 min at 4°, the membranes were washed another three times by re-suspension in 20 volumes of ice-cold buffer and re-centrifugation as above to remove endogenous glycine and to wash out the remaining detergent.

Binding assay. The final pellet was re-suspended in ice-cold buffer (50 mM Tris–HCl, pH 8.0 at 0°) to give a final concentration of approximately 20 mg of original tissue weight per sample (≈50–200 μg of protein, determined with the Bradford assay [14]). The binding experiment was performed by incubating a 0.9 mL aliquot of these membranes with 10 nM [^{3}H]glycine at 0° (unless otherwise stated) in a final volume of 1.2 mL 50 mM Tris–HCl buffer, pH 8.0 (at 0°). Magnesium (10 mM $MgSO_4$) was also added to the assay to potentiate the binding of glycine [15]. Non-specific binding was determined by including 1 mM unlabelled glycine and amounted to 20–50% of the total binding. The incubation time was 60 min (equilibrium conditions). For thermodynamic studies, displacement experiments using concentrations of competing unlabelled glycine ranging from 15 nM to 1 μM were carried out at 0, 5, 15, 25, 30 and 37°. All samples were run at least in triplicates. To isolate the receptor–ligand complex, the incubation was terminated by centrifugation for 4 min at 12,000 *g*, on a Beckman Microfuge 12, in the cold, after which the pellets were superficially and rapidly rinsed with 100 μL of ice-cold incubation buffer. The pelleted membranes were given some time to dry, before the tips of the tubes were cut off, put in scintillation vials and the pellets digested in 350 μL of tissue solubilizer in a final volume of 7.5 mL scintillation fluid. After solubilization for at least 6 hr at 50°, the vials were shaken for 1 hr and the radioactivity determined by liquid scintillation spectrometry.

Treatment with group-specific reagents. These treatments were inserted during the preparation of the membranes as described above. After the incubation at 37° with Triton X-100 and one further washing step, the membranes were incubated with the indicated concentrations of the reagents for 30 min (DTT) or 45 min (NEM, PHMB and butanedione) at 37° in a 20-fold volume of buffer. Excess reagent was then removed by three further centrifugation/re-suspension steps.

For protection experiments, the membrane samples were pre-incubated with the concentrations of glycine given in the results section (Fig. 3) for 30 min at 37°, before they were exposed to 100 μM PHMB or 10 mM butanedione (also in the presence of glycine), as described above. Because high concentrations of glycine had to be removed, subsequent washing steps were carried out in a 45-fold volume of buffer and included an initial incubation for 15 min at 37°, followed by centrifugation and a further four washing steps in ice-cold buffer. The influence of any remaining glycine was taken into account by including control samples which were processed similarly in the presence of glycine at each protecting concentration used, but without the addition of the group modifying agents.

Calculation and expression of results. Displacement experiments were analysed by non-linear curve fitting to the logistic equation. The IC_{50} values obtained from such glycine inhibition curves were transformed into K_i values with the equation $K_i = IC_{50} - [L]$ [16], which is derived from the Cheng–Prusoff relation.

Thermodynamic experiments were analysed as follows [17, 18]: the change in Gibbs free energy ($\Delta G°$) occurring upon binding of a ligand consists of the contributions of changes in enthalpy ($\Delta H°$) and entropy ($\Delta S°$) (equation 1) and is related to the equilibrium association constant K_a according to equation 2, where R is the gas constant (1.99 cal/mol/°K), T is the temperature in °K and K_a corresponds to $1/K_i$.

$$\Delta G° = \Delta H° - T\Delta S°. \quad (1)$$

$$\Delta G° = -RT \ln K_a. \quad (2)$$

The enthalpy change is obtained from the integrated Van't Hoff equation 3:

$$\ln K_a = -\Delta H°/RT + \Delta S°/R. \quad (3)$$

The slope of the Van't Hoff plot (Fig. 1) is

Table 1. Effect of temperature on the binding of [^{3}H]-glycine to rat cortical membranes

Temperature (°C)	K_i value (nM)	N
0	118 ± 9	13
5	165 ± 44	5
15	160 ± 33	6
25	199 ± 49	6
30	215 ± 29	6
37	252 ± 43*	6

The K_i values were obtained from displacement experiments measuring the inhibition of specific [^{3}H]-glycine binding by competing concentrations of unlabelled glycine as described under methods.

Values are the means ± SEM from N experiments.

* Significantly different from the value at 0° (ANOVA, d.f. 5/36, F = 2.79, P = 0.03; Bonferroni-test).

$-\Delta H°/R$; the changes in Gibbs free energy and entropy can then be calculated from equations 1 and 2.

RESULTS

Identification of the [^{3}H]*glycine binding site under examination*

In separate experiments not related to this study, it was found that a great number of compounds had, under our experimental conditions, potencies as inhibitors of [^{3}H]glycine binding which were in agreement with the pharmacological profile which has been described in the literature (for reviews, see Refs 2 and 8) for the NMDA receptor-coupled glycine recognition site (data not shown). In particular, to give two examples of compounds which distinguish this site from the inhibitory glycine receptor: strychnine was inactive as a displacing agent up to a concentration of 200 μM, whereas D-serine inhibited glycine binding with an IC_{50} of *ca.* 300 nM.

Thermodynamic analysis of [^{3}H]*glycine binding*

The effect of temperature on glycine binding to the strychnine-insensitive, NMDA receptor-coupled binding site was investigated by performing inhibition experiments with [^{3}H]glycine and competing concentrations of unlabelled glycine. Nonlinear fitting of the curves indicated the interaction of glycine with one homogeneous class of sites at all temperatures tested (data not shown). The corresponding K_i values are listed in Table 1. Raising the temperature resulted in a steady and consistent decrease in affinity of glycine for its recognition site, although only the K_i values at the highest and lowest temperatures tested (37° and 0°) were significantly different from each other. An estimation of the B_{max} values [16] showed a tendency towards an increase of B_{max} with temperature, although this was not significant (data not shown). This parameter is, however, not relevant for the thermodynamic analysis of glycine binding. Figure 1 shows the Van't Hoff plot derived from the data given in Table 1. The thermodynamic parameters of glycine binding, calculated from the Van't Hoff plot, are given in Table 2. It can be seen that the change in enthalpy contributed only little (25–30%) to the Gibbs free energy change of glycine binding, the major component being an increase in entropy.

Chemical modification of the [^{3}H]*glycine binding site*

In order to explore the chemical nature of the glycine binding site, rat cortical membranes were pre-treated with several chemical agents known to react with distinct functional groups. The incubation time was chosen on the basis of pilot experiments

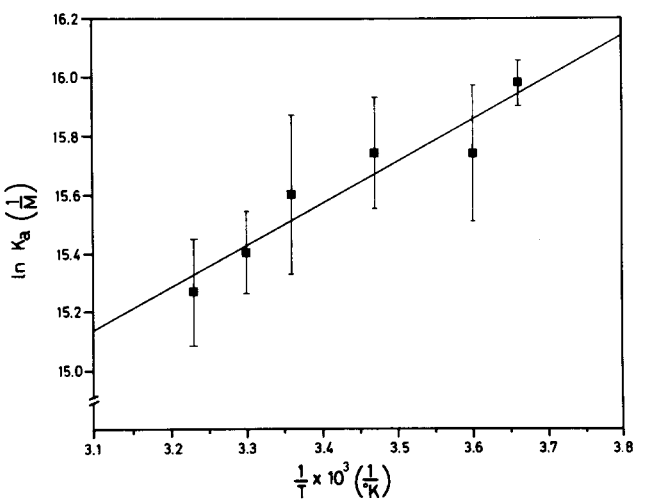

Fig. 1. Van't Hoff plot of [^{3}H]glycine binding. K_a values were determined from the equilibrium inhibition constants ($K_a = 1/K_i$) obtained from displacement experiments using unlabelled glycine as a competing agent. The data shown represent the means ± SEM of 5–13 independent determinations at each temperature; they are derived from the results shown in Table 1.

Table 2. Thermodynamic parameters of [^{3}H]glycine binding to rat cortical membranes

Temperature (°C)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol·°K)
		−2.4 ± 0.35	
0	−8.6 ± 0.07		22.7 ± 1.52
5	−8.7 ± 0.13		22.8 ± 1.74
15	−9.0 ± 0.11		22.9 ± 1.35
25	−9.2 ± 0.16		22.9 ± 1.65
30	−9.3 ± 0.09		22.6 ± 1.37
37	−9.4 ± 0.11		22.5 ± 1.46

Calculations of thermodynamic parameters were based on displacement experiments using [^{3}H]glycine and unlabelled glycine as a competing agent under the conditions given in Materials and Methods. $\Delta H°$ was determined from the slopes in the Van't Hoff plots (equation 3) of six independent experiments, and $\Delta G°$ and $\Delta S°$ were then calculated from equations 1 and 2 given in Materials and Methods.

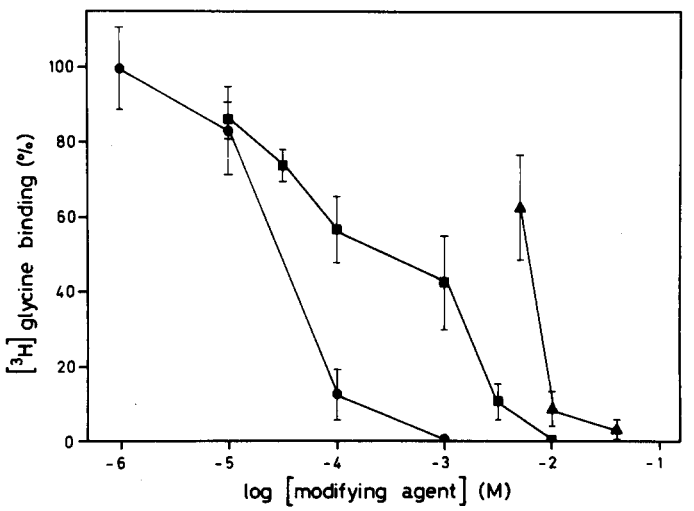

Fig. 2. Effects of functional group modifying agents on [^{3}H]glycine binding. Rat cortical membranes were pre-incubated with different concentrations of PHMB (●), DTT (■) or butanedione (▲) and then used for measuring [^{3}H]glycine binding as described in Materials and Methods. The data shown represent the means ± SEM of 2–6 independent experiments and are expressed in per cent of the binding obtained with control membranes which were not exposed to the modifying agents.

showing the effects of these agents on [^{3}H]glycine binding to be time dependent (data not shown). As can be seen in Fig. 2, the ligand binding was sensitive to all agents tested in a concentration-dependent manner. To inactivate the specific binding of [^{3}H]-glycine, sub-millimolar concentrations of PHMB, a reagent which forms mercuri-thio bonds, were needed (Fig. 2). A similar blockade could be obtained by NEM, another sulfhydryl reagent, which alkylates -SH groups of amino acids (78 ± 6% inhibition at 10 mM, N = 3). Furthermore, we found a marked sensitivity of this receptor site to treatment with 2,3-butanedione, a reagent of which the vicinal carbonyl groups form covalent bonds with the guanidino groups of arginine residues [19] (Fig. 2). Treatment of membranes with DTT (Cleland's reagent), a disulfide reducing reagent, likewise resulted in an almost total blockade of the binding site at the highest concentrations used (Fig. 2).

Protection experiments were performed with 0.1 mM PHMB or 10 mM 2,3-butanedione, concentrations at which both agents exerted a near maximal receptor blockade (see Fig. 2). Various concentrations of glycine were present prior to (30 min at 37°) and during the incubations with PHMB (Fig. 3A) and butanedione (Fig. 3B). In both cases the reduction of the ligand binding could be prevented by glycine in a concentration-related manner, although no full protection was obtained even at the highest glycine concentrations used.

DISCUSSION

The results of our thermodynamic experiments

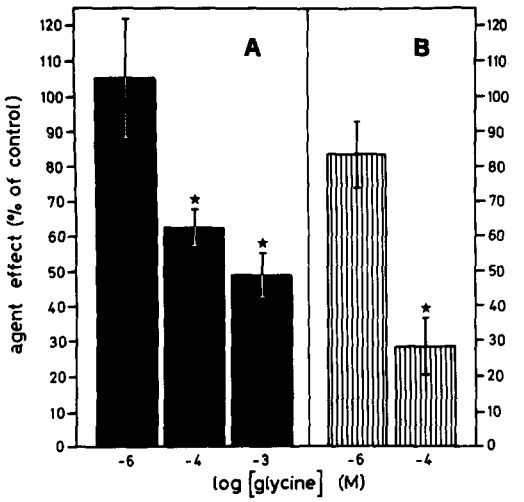

Fig. 3. Protection of [^{3}H]glycine binding against the effects of PHMB (panel A) or 2,3-butanedione (panel B) treatment. Samples of membranes were pre-incubated with the concentrations of unlabelled glycine indicated for 30 min at 37°. PHMB (100 μM; A) or butanedione (10 mM; B) were then added and the incubation continued for a further 45 min at 37°. Control samples contained similar concentrations of glycine without the modifying agents. Subsequent washing steps and the [^{3}H]glycine binding assay were performed as described in Materials and Methods. The results shown are the means ± SEM from 3–4 independent experiments and are expressed in per cent of the inhibition (agent effect) obtained in the absence of protecting glycine. Asterisks indicate a statistically significant protection by glycine ($P < 0.01$, Student's t-test).

demonstrate the binding of glycine to its strychnine-insensitive, NMDA receptor-coupled recognition site to be mainly entropy-driven, with a much smaller contribution of the enthalpy change. Due to the relatively large experimental variation normally occurring in centrifugation assays with low affinity ligands, the changes in K_i values found with changing temperatures (Table 1) were only statistically significant when the values at the two outer ends (0° and 37°) of the temperature range tested were compared. Nevertheless, they were consistently observed in our experiments. They are also in agreement with the finding of Kessler *et al.* [20] that the K_d for glycine was about twice as high at 30° compared to 0°, and similar observations [11, 21] that glycine binding was reduced with increasing temperature. The Van't Hoff plot (Fig. 1) was linear over the temperature range examined, suggesting that the binding pathway does not vary with temperature and is compatible with simple bimolecular kinetics.

The observed changes in enthalpy (decrease) and entropy (increase) were both thermodynamically favorable. Entropy increases upon ligand binding have been attributed [18] either to hydrophobic interactions or to displacement of water molecules ordered around the receptor site. Since glycine is a small, hydrophilic compound, the former cannot be the explanation for our findings. Therefore, we consider a displacement of water molecules (possibly from the strongly hydrated guanidino group found to be essential for glycine binding, see below) to be the most probable explanation for the entropy increase observed in our experiments. Conformational changes involved in information transfer and induced upon agonist binding might of course also be included in the final outcome of observed entropy changes. In this context, it should also be noted that thermodynamic parameters are not directly linked to the agonistic or antagonistic nature of a ligand, since structural and not pharmacological features seem to be relevant for the thermodynamic characteristics of the binding process [18].

We were further interested in exploring which functional chemical groups are of critical importance in the binding of glycine to its strychnine-insensitive, NMDA receptor-coupled binding site. This was achieved by chemical modification procedures using agents which specifically interact with certain functional groups. Firstly, we found at least one sulfhydryl group to be essential for glycine binding, since the receptor site was irreversibly blocked by the two applied -SH group directed reagents PHMB (Fig. 2) and NEM. Besides this -SH group, we found a cationic guanidino group of an arginine residue also to be important for ligand binding. This was shown by the chemical modification with butanedione [19], which blocked [^{3}H]glycine binding in a concentration-dependent manner. We were able to protect these two sites against the chemical modification by PHMB and butanedione, using glycine as a protecting compound (Fig. 3). No full protection was obtained, however, even at high glycine concentrations. This may well be due to the kinetic nature of the interaction of the compounds with the receptor site: whereas PHMB and NEM react irreversibly with their respective partner groups, glycine displays an extremely rapid exchange rate at its binding site. In fact, association and dissociation kinetics were found to be very rapid even at 0° in our own pilot experiments (not shown) as well as in the literature [9, 21]. Moreover, glycine, being hydrophilic, will be washed out of the membranes more rapidly than the more hydrophobic modifying agents used. Therefore, although an indirect protection due to changes in receptor conformation induced by glycine cannot be fully ruled out, our findings are in agreement with the plausible explanation that these -SH and guanidino groups are located at or in the close vicinity of the ligand binding site. In that case, the identified guanidino group of an arginyl residue is likely to interact, being positively charged, with the ionized carboxyl group of glycine. Such an ion–ion interaction would also contribute to the negative enthalpy change found in our thermodynamic experiments. As for the -SH group, it is not clear whether it directly interacts with the glycine molecule. Hydrogen-bonding with the amino group of glycine is possible, but not very likely, since the latter is protonated at physiological pH (thus -SH cannot act as a hydrogen donor), and since the sulfhydryl group is only a weak hydrogen bond acceptor. On the other hand, it is conceivable that such a group is

involved in the information transfer occurring subsequent to glycine binding. For example, it is known from other receptor systems that in certain cases sulfhydryl groups are involved in receptor–G-protein coupling. In fact, alkylation with NEM has been frequently used to uncouple G-proteins from their respective receptors. Of special interest in this context is the recent report [22] showing that -SH groups are critically involved in allosteric interactions at the strychnine-sensitive, inhibitory glycine receptor.

The tertiary structure of the strychnine-insensitive glycine binding site seems to be stabilized by at least one disulfide bridge, since the disulfide reducing agent DTT abolished glycine binding (Fig. 2). This result is interesting in the light of recent findings [23–25] showing that DTT enhanced NMDA-elicited responses which, on the other hand, were depressed by the application of an oxidizing reagent [23]. These studies suggest that the reducing potential of the synaptic cleft might, among other factors, play an important role in NMDA receptor modulation. In fact, the redox state of the NMDA receptor complex has been reported to vary widely among neurons, whereby the normal state seems to be a more oxidized and hence a more depressed one [23, 25]. The finding that DTT potentiates NMDA receptor-mediated responses seems, at first, to be contradictory to our observation that this agent inhibits the binding of the positive NMDA modulator glycine. It should be noted, however, that much milder conditions (shorter incubation times, lower concentrations) of DTT exposure were used in the studies mentioned above compared to our experiments. Thus, the disulfide bridge which, when reduced, potentiates NMDA responses is probably not the same as the one which stabilized glycine binding in our experiments. The findings that the oxidized and reduced forms of the receptor were similarly modulated by glycine [23, 25] and that glycine synergistically potentiated the effect of DTT on NMDA-induced LTP [24] are in line with this interpretation. It is clear that these complex mechanisms of NMDA receptor regulation will need further investigation.

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