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# Glycine Regulation of the *N*-Methyl-D-aspartate Receptor-Gated Ion Channel in Hippocampal Membranes

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## SUMMARY

The *N*-methyl-D-aspartate receptor-gated ion channel (NMDA channel) is regulated by glycine. To examine the interaction of glycine and NMDA receptor ligands on NMDA channel function, we used a biochemical marker of channel opening, [$^3$H]*N*-(1-[thienyl]cyclohexyl)piperidine (TCP). We quantified [$^3$H]glycine, L-[$^3$H]glutamate, and TCP binding in an identical membrane preparation. This allowed direct comparison of NMDA and glycine receptor occupancy and channel activation. Glycine increased the association and dissociation rates of NMDA-dependent TCP binding to hippocampal membranes, without altering the $K_d$ or $B_{max}$ for TCP binding. Structurally similar amino acids mimicked the action of glycine, with D-isomers being more potent than L-isomers. The potency of glycine in regulating TCP binding matched that for displacing [$^3$H]glycine. Glycine stimulation of TCP binding required the presence of NMDA agonists and was inhibited by the NMDA antagonist D-2-amino-5-phosphonovaleric acid. Glycine stimulation of NMDA-dependent TCP binding was not associated with an increase in agonist binding to the NMDA receptor. Likewise, NMDA stimulation of glycine-dependent TCP binding was not associated with an increase in the binding of glycine to the glycine receptor. These findings permit the following conclusions: 1) glycine stimulates TCP binding solely by increasing the access of TCP to its site in the NMDA channel; 2) TCP binding can be used to quantify glycine regulation of the NMDA channel; 3) a stereospecific glycine receptor, as part of the NMDA receptor-channel complex, regulates NMDA-evoked channel opening by a mechanism not involving increased agonist binding to the NMDA receptor. Thus, it appears that the mechanism of glycine and NMDA receptor regulation of the NMDA channel is analogous to that of a two-key lock; both receptors, by independent and mutually required mechanisms, alter channel conformation to allow ion passage.

NMDA receptor-mediated neurotransmission has been implicated in numerous physiologic and pathologic neuronal processes. These include synaptogenesis (1), spatial learning (2), long term potentiation (2–4), kindling development (5, 6), and ischemic neuronal injury (7, 8). NMDA receptor-mediated regulation of these neuronal processes likely involves activation of a receptor-gated ion channel (9–11).

Recent findings have demonstrated that the NMDA channel is regulated by the neutral amino acid glycine. Glycine increases NMDA-evoked currents in *Xenopus* oocytes expressing rat NMDA receptors (12) and in embryonic cultured neurons (13). Glycine increases NMDA-evoked currents by increasing the opening frequency of the NMDA channel (13). However, the mechanism by which glycine interacts with the NMDA receptor-channel complex to increase channel activation remains unclear.

Investigation of the mechanisms by which NMDA and glycine receptor ligands interact to regulate channel activation would be facilitated by the use of a biochemical marker of channel opening. Recent findings suggest that binding of the NMDA UCA TCP may serve as such a marker. Electrophysiologic studies, demonstrating that UCAs block NMDA-evoked currents by a use-dependent mechanism (14–16), initially raised the possibility that UCAs block NMDA-evoked currents by lodging in the channel and that this lodging required prior activation of the channel. Subsequent biochemical studies (17, 18), demonstrating that NMDA receptor ligands regulate TCP binding by altering the accessibility of the ligand to its binding site, further support this idea. Together, these findings provide the initial validation for the use of TCP binding as a marker of NMDA channel activation in a membrane preparation.

Previous studies, demonstrating that glycine can increase the binding of UCAs (18–25), raise the possibility that glycine regulates NMDA channel function in membranes as well as in intact cell preparations. The objectives of the work presented here are to 1) determine the mechanism of glycine potentiation of TCP binding, 2) characterize the pharmacology of the glycine receptor regulating TCP binding, and 3) determine whether the

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**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; APV, amino-phosphono-valeric acid; TCP, [$^3$H]*N*-(1-[thienyl]cyclohexyl)piperidine; UCA, uncompetitive antagonist.

mechanism of glycine and NMDA potentiation of TCP binding involves reciprocal allosteric interactions (i.e., does glycine increase channel activation by increasing agonist occupancy of the NMDA receptor and/or does NMDA increase channel activation by increasing agonist occupancy of the glycine receptor?).

## Materials and Methods

**Membrane preparation.** Hippocampal membranes were prepared from male Sprague-Dawley rats (300–375 g) that were killed by decapitation. Each pair of hippocampi was homogenized (Polytron setting 6, 20 sec) in 10 ml of ice-cold buffer (50 mM Tris acetate, pH 7.7, containing 1.0 mM EDTA) and centrifuged (23,000 × *g*, 20 min, 4°). The membranes were then washed by eight additional cycles of homogenization, resuspension in fresh buffer, and centrifugation. Before the third and fourth centrifugation the membranes were frozen in a dry ice and methanol bath. Before the fifth centrifugation, the membranes were incubated at 37° for 15 min. The fourth through ninth washes used 5.0 mM Tris acetate buffer (pH 7.2, no added EDTA). Membranes were stored frozen (24 hr to 2 weeks) after the third centrifugation, until the day of the binding experiment. This protocol for membrane preparation is an extensive modification of our previously published method (17). The modifications in the preparation were necessary to reduce endogenous glycine levels to the point where they did not interfere with the measurement of glycine dose-response curves.

**TCP binding assay.** TCP binding was measured in 1 ml of a 5 mM Tris acetate buffer (pH 7.2 at 25°). Reactions were terminated by vacuum filtration using a Skatron cell harvester and Skatron Filtermats (catalog no. 7034). TCP binding to the filters was reduced by pretreatment with 5 ml of 0.075% polyethylenimine (26). After vacuum filtration, the filters were rinsed for 10 sec with ice-cold buffer. All TCP binding experiments were carried out in the presence of 10 μM NMDA unless otherwise specified.

TCP association experiments were conducted by incubating hippocampal membranes with 2.5 nM TCP for varying periods of time before filtration. TCP dissociation experiments were conducted after first incubating the membranes for 500 min with TCP. Dissociation of TCP commenced with the addition of 0.1 ml of buffer containing 12.5 μM nonradioactive TCP. Specific binding (TCP binding in the presence of 1.25 μM nonradioactive TCP) was linear with protein concentration (0.08–0.15 mg/ml) and accounted for more than 80% of the total binding (in 10-min incubations in the presence of optimal concentrations of NMDA and glycine). Nonspecific binding did not vary as a function of time.

Equilibrium analysis of TCP binding was undertaken by incubating membranes with TCP until steady state conditions were achieved (500-min incubation). Saturation binding isotherms were generated by incubating the membranes with concentrations of that ranged from 0 to 105 nM. Nonspecific binding was determined by incubating the membranes with a 500 times excess concentration of nonradioactive TCP or by curve-fitting analysis of total binding, as described below.

**Glutamate and glycine binding assays.** L-[$^3$H]Glutamate and [$^3$H]glycine binding was quantified under conditions identical to those used to measure TCP binding (including 2.5 nM nonradioactive TCP in the incubation medium).

[$^3$H]Glycine binding (100 nM) was determined after a 40-min incubation (equilibrium) at 25°. Nonspecific binding was determined in the presence of 1 mM nonradioactive glycine. Reactions were terminated by centrifugation (Microfuge for 15 min). The pelleted membranes were rinsed three times with ice-cold buffer (3 × 2 ml) and then solubilized with 0.15 ml of Protosol (New England Nuclear, Boston, MA). Under these conditions, specific binding was linear with protein concentration and accounted for 45–55% of the total binding.

L-[$^3$H]Glutamate binding (10 nM) was determined after a 10-min incubation at 25°. Nonspecific binding was determined by incubation with 100 μM nonradioactive L-glutamate. The NMDA-displaceable component of glutamate binding was determined by incubation with 100 μM NMDA. Reactions were terminated by filtration using the Skatron cell harvester. Filters were wetted and cooled with ice-cold buffer immediately before filtration. Under these conditions, L-[$^3$H]glutamate binding was linear with protein concentration and greater than 80% of total binding was displaceable with nonradioactive glutamate. NMDA displaced approximately 25% of the total L-[$^3$H]glutamate binding. Equivalent amounts of NMDA receptor binding were obtained using NMDA (100 μM) or D-APV (100 μM) to displace L-[$^3$H]glutamate binding.

**Analysis of binding data.** Kinetic binding parameters were determined using the iterating curve-fitting program KINETIC (27). Observed association ($K_{obs}$) and dissociation ($K_2$) rate constants were obtained from the least squares fit of the data. The association rate constant ($K_1$) was determined using the equation $([K_{obs} - K_2]/[TCP])$ (27). These rate constants carry the prefix *F* to denote that the fraction of time the binding site is accessible to the ligand contributes to the calculated rate constant (17, 28, 29). Equilibrium binding parameters were determined using the curve-fitting program LIGAND (27). Analysis of data was undertaken using both experimentally determined and calculated values for nonspecific binding. Both procedures gave similar values for binding parameters ($K_d$ and $B_{max}$); however, using the computer-generated estimate for nonspecific binding resulted in a lower variance. Thus, the $K_d$ and $B_{max}$ for TCP binding were determined using this procedure. Utilizing these kinetic and equilibrium approaches, it is possible to determine whether glycine alters TCP binding by altering the affinity or number of sites or by altering the accessibility of the ligand to the site (Table 1).

For both kinetic and equilibrium binding experiments, the data were systematically examined using single- and multiple-site models. Data were first fit to single-site curves. Subsequent analysis was undertaken using progressively more complex models. The relative goodness with which the data fit single- and multiple-site models was then compared statistically. For the purpose of comparing the effect of glycine on TCP association with that on TCP dissociation, single-site analysis was used. We used this approach for comparing association and dissociation curves because it eliminated the ambiguity that arises when multiple sites for association are compared with multiple sites for dissociation. That is, in the case of multiple-site analysis, it is unclear which kinetic process of association corresponds to a specific kinetic process of dissociation. Rate constants for association and dissociation calculated in this manner reflect the overall or average rate constants for the population of channels being examined and are, therefore, designated as overall rate constants.

In certain experiments, the dose-response properties of different

TABLE 1

**Relationship between possible means of glycine stimulation of TCP binding and binding parameters**

The terms $FK_1$ and $FK_2$ refer to the apparent association and dissociation rate constants, respectively. The factor *F* in these terms refers to the fraction of time that the receptor is in an accessible conformation (17, 28, 29).

| Binding parameter | Mechanism of increased TCP binding | | |
|---|---|---|---|
| | Affinity | Number | Access |
| Association rate constant ($FK_1$) | (Increased)[a] | No change | Increased |
| Dissociation rate constant ($FK_2$) | (Decreased)[a] | No change | Increased |
| Kinetic $K_d$ ($FK_2/FK_1$) | Decreased | No change | (No change)[b] |
| Equilibrium $K_d$ | Decreased | No change | No change |
| $B_{max}$ | No change | Increased | No change |

[a] Increased affinity can be the consequence of an increase in the association rate constant, a decrease in the dissociation rate constant, or both.

[b] If *F* is increased equally for the association and dissociation reactions, then there will be no change in the apparent $K_d$ ($FK_2/FK_1$) and no change in binding at steady state. However, if either accessibility (*F* of association) or egressibility (*F* of dissociation) alone is modified then this will be detected as a change in apparent affinity, even though the true affinity of the binding site is not altered (29).

glycine analogs on TCP binding were determined at a single nonequilibrium measurement of binding. A 10- or 40-min incubation was chosen because these times allowed a high degree of specific binding while still reflecting changes in association rate. Dose-response curves were analyzed using a curve-fitting program designed for analysis of dose-response data (30).

## Results

**Effect of glycine on the kinetics of TCP binding.** To investigate the mechanism by which glycine increases TCP binding, the kinetics of TCP association and dissociation were examined. Regardless of glycine concentration tested, TCP binding association (Fig. 1) and dissociation (not shown) curves fit a two-site model better than a one-site model ($p < 0.01$). Moreover, there was a trend for a three-site model to be an even better fit (two-site versus three-site, $p < 0.1$). Glycine, in a dose-dependent manner, increased the rate of TCP association but had no effect on the amount of binding as steady state was reached.

To examine the action of glycine on association and dissociation rates for TCP, $FK_{obs}$, $FK_1$, and $FK_2$ rate constants were determined. These analyses were conducted using a single-site model (see above). Increasing concentrations of glycine increased overall $FK_{obs}$, $FK_2$, and $FK_1$ rate constants for TCP binding (Table 2). The action of glycine on the overall association and dissociation rates was quantitatively similar.

Glycine stimulation of TCP association and dissociation was dependent upon NMDA receptor activation, because it could be blocked by 100 μM APV (Table 2).

**Effect of glycine on TCP binding at steady state.** To determine whether the multiple kinetic processes detected in the TCP association and dissociation curves reflect TCP binding to multiple sites with differing affinities, binding isotherms were generated. The $K_d$ and $B_{max}$ for TCP binding were determined under equilibrium conditions in the presence of 0.3 and 3.0 μM glycine. These concentrations were selected because they represented approximately $EC_{50}$ and $EC_{100}$ concentrations (based on the action of glycine on TCP association rate constants). Under these conditions, TCP binding best fit a single-site model (Fig. 2). Increasing the concentration of glycine had no effect on the $K_d$, or the $B_{max}$ (0.3 μM glycine: TCP $K_d$ = 9.5 ± 0.5 nM, $B_{max}$ = 1.95 ± 0.05 pmol/mg; 3.0 μM glycine: TCP $K_d$ = 8.6 ± 0.9 nM, $B_{max}$ = 1.98 ± 0.06 pmol/mg). The finding that glycine had no effect on the TCP binding isotherm was not the consequence of endogenous glycine obscuring the action of added glycine because dissociation rates, after a 500-min incubation, were still dependent upon the amount of added glycine. Equilibrium analysis of the effect of lower concentrations of glycine on TCP binding is precluded by the very long times required to reach equilibrium.

**Effect of glycine analogs on TCP binding.** The structural requirements and stereospecificity of glycine receptor regulation of TCP association rate were examined using a

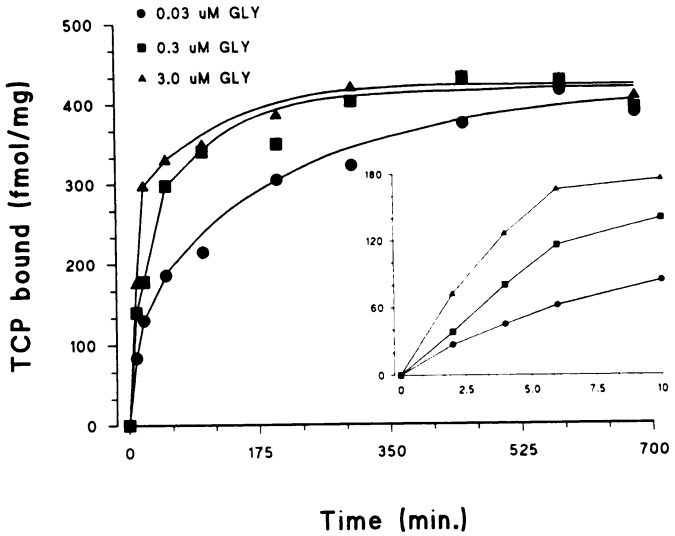


**Fig. 1.** Association of TCP binding in the presence of 30 μM NMDA and varying concentrations of glycine. *Inset*, the first 10 min of association. Each *point* is the mean of duplicate measurements. A total of 17 different time points, generated in two separate experiments, were used to construct each curve (not all points are shown). For each concentration of glycine, the data fit a two-site model better than a one-site model ($p < 0.01$). *Lines* are computer-generated best fit of a two-site model.

TABLE 2

**Effect of glycine on the kinetic binding parameters of TCP**

Glycine produced a dose-dependent increase in the overall observed association rate ($FK_{obs}$), the association rate ($FK_1$), and the dissociation rate ($FK_2$) constants of TCP binding ($p < 0.02$ by regression analysis) but no change in the ratio of the two constants. It should be noted that because these overall rate constants reflect multiple kinetic processes, they cannot be used to calculate a $K_d$. It should also be noted that these values, based on the entire association curve (including both fast and slow components), are lower than values obtained using only the initial (predominately fast) components of the reactions (18).

| Glycine | $F_K$obs | $FK_2$ | $FK_1$ | $FK_2/FK_1$ |
|---|---|---|---|---|
| nM | $min^{-1}$ | $min^{-1}$ | $10^6\ M^{-1}\ M^{-1}$ | |
| 20 | 0.019 ± 0.004 | 0.007 ± 0.002 | 4.8 | 1.4 |
| 50 | 0.021 ± 0.002 | 0.006 ± 0.002 | 6.0 | 1.0 |
| 100 | 0.040 ± 0.008 | 0.008 ± 0.003 | 12.7 | 0.6 |
| 200 | 0.045 ± 0.003 | 0.013 ± 0.003 | 12.8 | 1.0 |
| 500 | 0.059 ± 0.005 | 0.017 ± 0.006 | 16.8 | 1.0 |
| 1000 | 0.087 ± 0.013 | 0.018 ± 0.006 | 27.6 | 0.7 |
| 1000 + APV | 0.002 ± 0.002 | 0.002 ± 0.002 | | |

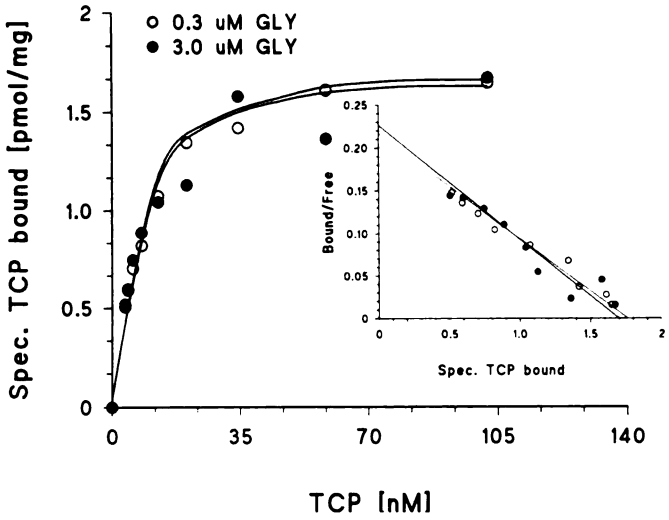


**Fig. 2.** Binding isotherms for TCP in the presence of 30 μM NMDA and either 0.3 or 3.0 μM glycine. Each value is the mean of duplicate measurements. *Inset*, a Scatchard-type transformation of the binding isotherms. The concentrations of TCP ranged from 2.0 to 105 nM. The use of higher concentrations of TCP was precluded by the presence of displaceable TCP binding to the filters. The use of lower concentrations of TCP was precluded by the time required to reach equilibrium under these conditions. Similar results have been obtained in three separate experiments.

nonequilibrium measure of binding. D-Serine was nearly as potent as glycine in stimulating NMDA-dependent TCP binding (Figs. 3 and 5). D-Alanine was slightly less potent. L-Isomers of serine and alanine were less potent than their respective D-isomers by at least 1 order of magnitude but were equally efficacious (Fig. 3). The potency of these amino acids in stimulating NMDA-dependent TCP binding closely matched their potency in stimulating NMDA-evoked currents in frog oocytes expressing rat NMDA receptors (Fig. 4). The ability of strychnine and kynurenic acid to inhibit glycine-dependent TCP binding was tested. Strychnine (10 μM or less) failed to inhibit glycine-dependent TCP binding. Kynurenic acid, on the other hand, reduced glycine-stimulated TCP binding in a dose-dependent manner and produced a parallel rightward shift in the glycine dose-response curve (Table 3; Fig. 5).

**Effect of glycine analogs on [$^3$H]glycine binding.** The relationship between the affinity of different glycine analogs for a hippocampal binding site and their potency in stimulating TCP binding was examined. D- and L-serine, D- and L-alanine, B-alanine (Fig. 6), and kynurenic acid (not shown) all displaced [$^3$H]glycine in a dose-dependent manner. The potency of these amino acids in displacing [$^3$H]glycine closely matched their

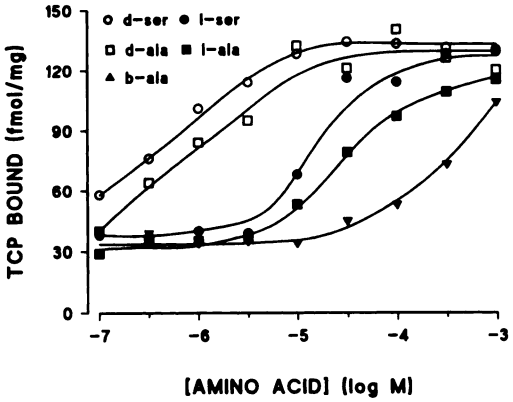


**Fig. 3.** Effect of different glycine analogs on TCP binding in the presence of 10 μM NMDA. Each value is the mean of triplicate measurements. These values were obtained under nonequilibrium conditions (10-min incubation). Comparable results have been obtained in three separate experiments.

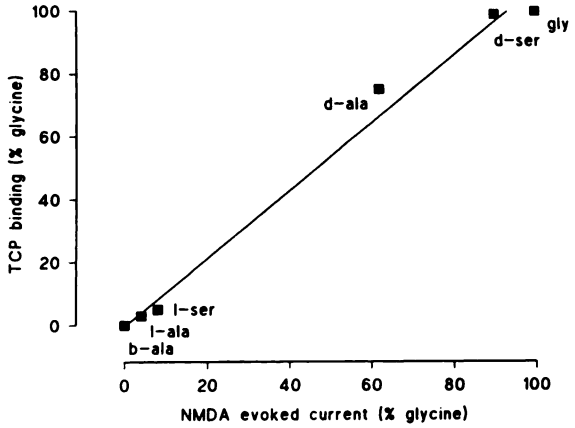


**Fig. 4.** Correlation of the effect of glycine agonists (3 μM) on NMDA-evoked current and on NMDA-dependent TCP binding. TCP binding was measured in a 10-min incubation. Values are normalized to glycine = 100% response. Values for NMDA-evoked currents were taken from experiments using frog oocytes that were injected with rat MRNA (12). The linear regression was statistically significant ($p < 0.001$).

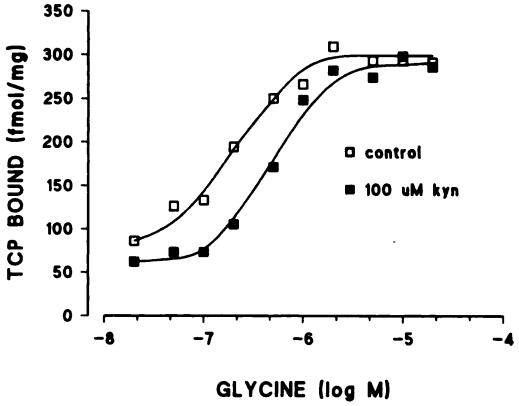


**Fig. 5.** Kynurenic acid (100 μM) inhibition of glycine-stimulated TCP binding (40-min incubation, 100 μM NMDA). Values are the means of triplicates measurements. Similar results have been obtained in two separate experiments. Note that 100 μM NMDA was used in this experiment to reduce kynurenic acid displacement of NMDA.

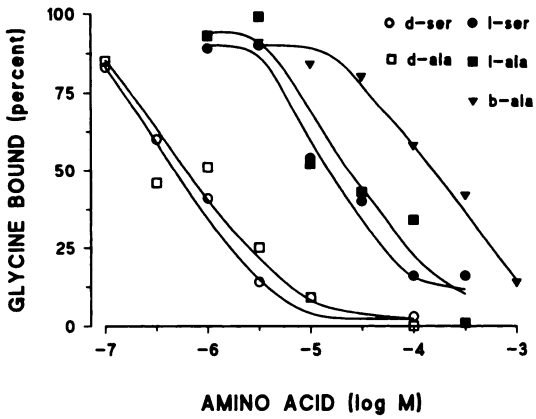


**Fig. 6.** Effect of different glycine analogs on [$^3$H]glycine binding (40-min incubation, 10 μM NMDA and 0.1 μM [$^3$H]glycine). Values are the means of two or three separate experiments.

TABLE 3

**Comparison of the potencies of glycine agonists and antagonists on [$^3$H]glycine and TCP binding**

The $EC_{50}$ of different amino acids in potentiating or, in the case of kynurenic acid, inhibiting TCP binding in the presence of 10 μM NMDA were calculated using nonlinear regression analysis. The $IC_{50}$ values for different amino acids in displacing [$^3$H]glycine were determined by analysis of competition curves. $K_i$ values were determined using the equation $K_i = IC_{50}/(1 + [\text{glycine}]/K_d)$. Values for glycine binding are the means of three separate experiments. Values for TCP binding are the means of two separate experiments. There was a statistically significant correlation between the potencies of these amino acids in displacing [$^3$H]glycine and in regulating TCP binding ($p < 0.05$).

| Amino acid | Glycine binding $K_i$ | TCP binding $EC_{50}$ |
|---|---|---|
| | μM | |
| Agonists | | |
| Glycine | 0.23 ± 0.02[a] | 0.31 ± 0.02 |
| D-Serine | 0.64 ± 0.05 | 0.67 ± 0.12 |
| D-Alanine | 0.48 ± 0.08 | 1.34 ± 0.06 |
| L-Serine | 14.5 ± 2.4 | 15.18 ± 3.10 |
| L-Alanine | 37.7 ± 16.7 | 40.31 ± 0.92 |
| B-Alanine | 148.0 ± 54.3 | 583.0 ± 246.5 |
| Antagonists | | |
| Kynurenic acid | 117.0 ± 37 | 64.5 ± 14.7 |
| Strychnine | >100 | >10 |

[a] The value for glycine is a $K_D$, as determined by curve fitting of a binding isotherm (mean ± standard error of five separate binding isotherms)

TABLE 4

**Effect of glycine on [$^3$H]glutamate binding**

Values are the mean ± standard error of triplicate measurements of L-[$^3$H]glutamate binding. Glycine had no dose-dependent effect on total, glutamate-displaceable, or NMDA-displaceable L-[$^3$H]glutamate binding (ANOVA). Similar results have been obtained in three separate experiments.

| Glycine | Total glutamate binding | Glutamate-displaceable binding | NMDA-displaceable binding |
|---|---|---|---|
| *nM* | | *fmol/mg* | |
| 0 | 159.7 ± 7.5 | 139.7 ± 4.8 | 66.3 ± 0.4 |
| 30 | 134.3 ± 6.2 | 114.3 ± 6.4 | 41.0 ± 5.7 |
| 100 | 142.2 ± 5.0 | 122.3 ± 6.4 | 49.0 ± 11.6 |
| 300 | 148.0 ± 13.0 | 128.3 ± 10.8 | 55.0 ± 5.5 |
| 1000 | 147.3 ± 9.1 | 127.0 ± 6.6 | 53.7 ± 1.3 |

TABLE 5

**Effect of NMDA on [$^3$H]glycine and [$^3$H]TCP binding**

NMDA had no dose-dependent effect on glycine binding under conditions in which NMDA markedly potentiated TCP binding. Values are the mean ± standard error from three separate experiments.

| NMDA | Specific glycine binding | Specific TCP binding |
|---|---|---|
| *μM* | *pmol/mg* | *fmol/mg* |
| 0 | 0.91 ± 0.22 | 24.1 ± 8.1 |
| 0.1 | 1.01 ± 0.11 | 32.8 ± 3.3 |
| 1.0 | 0.83 ± 0.19 | 121.6 ± 14.4 |
| 3.0 | 0.76 ± 0.32 | 153.0 ± 1.6 |
| 10.0 | 0.83 ± 0.25 | 243.2 ± 24.9 |
| 30.0 | 1.12 ± 0.31 | 260.8 ± 12.9 |

potency in stimulating or, in the case of kynurenic acid, inhibiting TCP binding (Table 3). Strychnine had no effect on glycine binding at concentrations as great as 100 μM.

**Glycine and glutamate binding: allosteric interactions.** To determine whether glycine increased TCP binding by increasing agonist binding to the NMDA receptor, the effect of glycine on NMDA-displaceable and non-NMDA-displaceable glutamate binding was determined. Glycine, under conditions that markedly potentiated TCP binding, had no dose-dependent effect on either the total or NMDA-displaceable component of L-[$^3$H]glutamate binding (Table 4). To determine whether NMDA increased TCP binding by increasing agonist binding to the glycine receptor, the effects of NMDA on [$^3$H] glycine binding were examined. NMDA, at concentrations that markedly potentiated TCP binding, had no effect on [$^3$H] glycine binding (Table 5).

## Discussion

The principal findings of this study are that 1) glycine increased the overall association and dissociation rate constants for TCP binding but had no effect on the $K_d$ or the $B_{max}$ of TCP binding, 2) the pharmacology of glycine analogs in potentiating or inhibiting TCP binding matched that for potentiating or inhibiting NMDA-evoked currents and for displacing [$^3$H]glycine binding, and 3) glycine and NMDA receptor agonists had no effect on each other's binding under conditions that markedly stimulated TCP binding.

Glycine has been reported to potentiate the binding of TCP and other UCAs (19–25, 31). Conceivably this potentiation could be accomplished by increasing the affinity of the TCP binding site, by increasing the number of binding sites, or by increasing the accessibility of TCP to a channel "guarded" binding site. Kinetic and equilibrium analyses of TCP binding allowed distinction among these possibilities. The hypothesis that glycine increased the affinity or the number of TCP binding sites (e.g., Refs. 20, 21, 24, 25, and 31) is not supported by the data, because glycine had no effect on the level of steady state binding in the kinetic experiments and did not decrease the $K_d$ or increase the $B_{max}$ for TCP binding when determined under equilibrium conditions. On the other hand, the hypothesis that glycine increased the access of TCP to its binding site is supported by the data, because glycine dose dependently increased both the overall association and dissociation rate constants (also see Refs. 17, 18, 32, and 33).

The simplest model of channel-gated TCP binding predicts that the association and dissociation reactions should approximate first-order reactions (17, 28). The finding that both the association and dissociation curves fit a two-site model better than a one-site model suggests a more complex situation. One explanation for the multiple kinetic processes is the presence of multiple TCP binding sites with differing affinities. However, equilibrium analysis of TCP binding (here and Refs. 18 and 33) suggests that only a single TCP binding site is present. One explanation for these apparently discordant findings is that, although TCP binds to a single site, there is heterogeneity in the rate at which TCP gains access to this site.

There are several possible explanations for heterogeneity in the accessibility of TCP to its binding site. One is that there are multiple routes of entry of TCP into the channel. If this is the case then, because APV can prevent virtually all TCP binding, each of these routes requires channel activation (Table 2 and Ref. 17). A second explanation is "run down" (34). Run down is a phenomenon observed in electrophysiologic recordings in which a loss of agonist-activated currents can be prevented by inclusion of an energy source. If this were the case then the occurrence of run down during the first 500 min of the association reaction should result, contrary to the data, in only a single (slow) site being detected in the dissociation reactions. A third possibility is a heterogeneity in the fraction of time that different subpopulations of NMDA channels spend in the open conformation. Heterogeneity in the fraction of time that different subpopulations of channels spend in an open conformation could be due to differences in the affinity state of the NMDA receptor by which the channels are gated or in the structure of the subunits of the channel itself. That is, differences in the stoichiometry of channel subunits could result in different degrees of channel activation. Regardless of the mechanisms underlying the complexity of the rate curves, the findings that NMDA and glycine increase the rate of TCP association and dissociation without altering the $K_d$ or $B_{max}$ for TCP binding are consistent with the contention that NMDA and glycine regulate TCP binding by regulating the channel-gated accessibility of the ligand to its site.

The idea that glycine regulates TCP binding by altering the accessibility of the ligand to the binding site, rather than altering the affinity or the number of binding sites, contrasts with results of a number of previous studies, using presumed equilibrium conditions (20, 21, 24, 25, 31). The discrepancy in the findings may result from differences in the preparation or the incubation buffer or from a failure of the previous investigations to reach equilibrium for all conditions tested. If Scatchard analysis is applied to TCP binding under nonequilibrium conditions, then the potentiating effect of glycine on TCP association will give the false appearance of increasing the affinity of the TCP binding site. As shown here, even in the

presence of saturating concentrations of NMDA and $EC_{50}$ concentrations of glycine, steady state TCP binding was reached only after approximately 500 min of incubation. Lower concentrations of agonist will result in even longer times to equilibrium.

The validity of using TCP binding to study glycine regulation of the NMDA channel is further supported by the experiments examining the pharmacology of glycine regulation of TCP binding. Both the rank order and the absolute potencies of stereoselective glycine agonists in stimulating TCP binding closely matched that for stimulating NMDA-evoked currents in *Xenopus* oocytes (12). This finding strongly suggests that the same receptor mediates both actions. Thus, both the pharmacology and the kinetics of glycine regulation of TCP binding support the contention that glycine regulation of NMDA channel activation, first characterized electrophysiologically, can be quantified in a membrane preparation.

One of the unique advantages of a biochemical investigation of channel activation, as compared with traditional electrophysiologic approaches, is the ability to quantify receptor-ligand interactions under conditions identical to those used to measure channel activation. We exploited this advantage to directly examine the relationship between the affinity of different glycine analogs at a hippocampal glycine binding site and their ability to regulate NMDA channel function. Using this approach, we and others (35) have found that the potency and stereoselectivity of glycine agonists in potentiating TCP binding closely matched that for displacing [$^3$H]glycine. We also found that the potency of the relatively nonselective glycine antagonist kynurenic acid (36) in inhibiting TCP binding matched that for displacing [$^3$H]glycine binding. Conversely, strychnine, a potent inhibitor of [$^3$H]glycine binding in spinal cord and medulla, had no effect on binding to hippocampal membranes. This is consistent with previous findings indicating that the cortical glycine binding site is insensitive to strychnine (37). This suggests that the glycine site characterized by radioligand binding studies is the receptor that mediates glycine potentiation of NMDA dependent TCP binding. Thus, the parallel measures of the actions of glycine analogs on glycine binding and on channel activation (measured with TCP binding) form a bridge between neuropharmacologic and electrophysiologic studies of the NMDA receptor-glycine receptor-ion channel complex.

Preliminary findings in other laboratories (33, 38–40) have shown that glycine and NMDA receptor ligands can reciprocally regulate the binding of one another through allosteric mechanisms. These findings raised the possibility that the mechanism by which glycine increases channel activation involves increased agonist occupancy of the NMDA receptor. However, glycine had no effect on glutamate binding to an NMDA-displaceable site under conditions identical to those in which glycine increased channel activation (as measured with TCP binding). This finding demonstrates that glycine potentiation of agonist binding to the NMDA receptor is not necessary for glycine activation of the NMDA channel. The possibility that NMDA and glutamate potentiation of channel activity requires increased glycine binding is also not supported by these data, because NMDA had no effect on glycine binding under conditions identical to those in which NMDA markedly potentiated TCP binding.

The idea that glycine does not increase channel activation by increasing the affinity of the NMDA binding site is further supported by previous electrophysiologic and biochemical findings. We and others have shown that glycine does not produce a leftward shift in the dose-response curve for NMDA stimulation of TCP binding, as would be expected if glycine were increasing the affinity of the NMDA receptor (17, 22). Moreover, Kleckner and Dingledine (12) have shown that glycine may be an absolute requirement for NMDA-evoked currents and does not simply produce a leftward shift in the NMDA dose-response curve. Thus, these data taken together support the idea that, although glycine and NMDA receptors must both be occupied for optimal channel activation, the mechanism of channel activation does not require allosteric changes in the NMDA or glycine binding sites themselves.

The reason for the absence of allosteric interactions between NMDA and glycine receptor binding reported here, as compared with previous reports, is unknown. One possible explanation is differences in the preparation of the membranes. Previous investigations of glycine and glutamate interactions have used membranes prepared in sucrose or detergents, whereas in these studies no such treatment was used. It is noteworthy that the amount of glutamate binding to glutamate- and NMDA-displaceable sites reported here is comparable to previously reported values for detergent-treated P2 preparations (38). Whether the reciprocal interactions of glutamate and glycine on the binding of one another detected in other preparations is of functional significance remains to be determined.

To summarize, these findings further confirm that the NMDA channel is regulated by at least two separate sites, each with a distinct pharmacology. Moreover, it seems likely that the mechanism by which these sites regulate channel activation involves independent and parallel, but mutually required, actions on channel conformation. This mechanism is analogous to that of a two-key lock in which both keys must be turned before opening. The question of just how many more regulatory sites are present on the receptor(s)-channel complex remains to be determined.

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