ELSEVIER

On the True Affinity of Glycine for Its Binding Site at the NMDA Receptor Complex

Michael L. Berger

Institute of Biochemical Pharmacology, University of Vienna, Austria

To determine the exact potency of inhibitors acting at the glycine site of the NMDA receptor complex using [^{3}H]glycine as a radioligand, the true equilibrium constant (K_D) of the radioligand has to be known. To achieve this goal, (1) the contamination of water by glycine was studied, (2) the true affinity of glycine for the NMDA receptor was estimated by different methods, and (3) the inhibition constant of a number of antagonists was determined. HPLC analysis of the deionized water used for the preparation of the assay buffers and solutions resulted in glycine concentrations below 6 nmol/L in four out of seven samples analyzed. The observable equilibrium constant of [^{3}H]glycine binding (K_{obs}) was found to vary, in the presence of 10-μmol glutamate per liter, between 36 and 163 nmol/L (mean ± SD: 69 ± 21 nmol/L, 140 determinations). Based on the observation that the polyamine spermine increased the potency of glycine, but not that of glycine antagonists, an algorithm was developed to calculate K_D from K_{obs}- and IC_{50}-values obtained with and without 100-μmol spermine per liter. Using four different antagonists, K_D-values between 24 and 57 nmol/L were obtained. Computer analysis of glycine-stimulated [^{3}H]MK-801 binding resulted in EC_{50}-values between 22 and 35 nmol/L. Based on these results, a true affinity constant for [^{3}H]glycine of 40 nmol/L was assumed and used for the determination of K_i-values of 15 different inhibitors of [^{3}H]glycine binding.

Key Words: [^{3}H]glycine binding sites; NMDA receptor complex; Rat hippocampal membranes; Glycine contamination

Introduction

The mammalian brain's most important excitatory neurotransmitter, glutamic acid, interacts in neuronal tissue with several types of receptors. The most abundant of these is the NMDA receptor, named after the prototypical agonist N-methyl-D-aspartic acid. It is now well established that glutamate acts on this receptor as a coagonist with the small neutral amino acid glycine (Johnson and Ascher, 1987; see Kemp and Leeson, 1993, for review) and that the endogenous polyamines spermine and spermidine can potentiate their action (Ransom and Stec, 1988; see Williams et al., 1991, for review). Most binding studies using ^{3}H-labeled glycine and several types of membrane preparations demonstrated equilibrium dissociation constants for the ligand between 100 and 230 nmol/L (Kishimoto et al., 1981; Bristow et al., 1986; Jones et al., 1989; Kessler et al., 1989; Marvizón et al., 1989; Monahan et al., 1989; Thedinga et al., 1989; Hood et al., 1990; McNamara et al., 1990; Baron et al., 1991; Grimwood et al., 1992; Schneider and Urwyler, 1992). Some authors mentioned in particular the problem of microbial glycine contamination and the difficulty of preparing "glycine-free" assay buffers and solutions (e.g., Kleckner and Dingledine, 1988; Kessler et al., 1989). Due to various degrees of glycine contamination, the affinity constants observed (K_{obs}) can be expected to be considerably higher than the true affinity constant (K_D), which, at present, still seems to be unknown. Without a knowledge of the K_D of the radioligand used, however, IC_{50}-values published by different laboratories for inhibitors of [^{3}H]glycine binding cannot be compared to each other. In addition, knowledge about the true affinity of glycine to its regulatory site at the NMDA receptor complex is of importance in connection with a possible physiological role of glycine as a neuromodulator.

Address reprint requests to Dr. M. Berger, Institute of Biochemical Pharmacology, A-1090 Vienna, Borschkegasse 8A, Austria.

Received July 1994; revised and accepted February 1995.

Journal of Pharmacological and Toxicological Methods 34, 79–88 (1995)

655 Avenue of the Americas, New York, NY 10010

1056-8719/95/$9.50
SSDI 1056-8719(95)00028-G

JPM Vol. 34, No. 2
October 1995:79–88

The following study describes four different approaches to deal with the problem of environmental glycine contamination:

1. A sensitive HPLC technique was applied to measure directly the glycine concentration in the deionized water used for preparation of the assay buffer.
2. The results of 140 consecutive saturation analyses are presented.
3. The influence of spermine on the K_{obs} of [^{3}H]glycine and on the IC_{50}s of four glycine antagonists was studied, exploiting observations that spermine increased the affinity of glycine, but not that of glycine antagonists to the binding site (Sacaan and Johnson, 1989; Ransom and Deschenes, 1990). A possible explanation for this phenomenon is a difference in the sites for binding glycine agonists and antagonists at the NMDA receptor complex (Yoneda et al., 1994).
4. Stimulation of [^{3}H]MK-801 binding by various glycine concentrations was used to extrapolate to the background level of glycine.

The primary goal of this investigation was to establish a reliable method for the quantitative characterization of inhibitors of [^{3}H]glycine binding.

Methods

Preparation of Buffer and Solutions

Deionized water was used for the preparation of all aqueous solutions. Tap water was passed directly over the ion exchange resin (Seradest SF 2500, Seral, Alhäuser GmbH, 5412 Ransbach-Baumbach, Germany). All "post-column" parts (plastic tubes, fittings, Ohmmeter) were regularly (once a month) disassembled and brushed thoroughly in hot (60° C) tap water containing detergent (especially the inner wall of the tubes). The glass vessel used for the preparation of the homogenization and incubation buffer (50-mmol Tris-acetate per liter, pH 7.0 at room temperature) was cleaned by filling it completely with hot diluted detergent and by leaving it for at least 10 min in a sonifying water bath; thereafter, the vessel was rinsed several times with hot tap water and finally 3 times with deionized water; plastic centrifugation tubes and glass vials used for the preparation of [^{3}H]MK-801 aliquots were subjected to the same treatment. All other solutions were prepared in new plastic tubes kept free from dust; stock solutions were stored at −20° C, including aliquots of concentrated buffer. A pH electrode was used only to adjust the pH of the concentrated buffer (1.25-mol Tris-acetate per liter, pH 7.15 at room temperature); the pH of 1–10-mmol/L stock solutions of test substances in 50-mmol buffer per liter was adjusted, where necessary, using pH indicator paper. During pipetting, the experimenter wore gloves and a surgical mask. For pipetting larger volumes, and for the resuspension of membrane pellets, an eppendorf multipette with disposable 12.5-mL tip was used; no glass pipettes were used. Whenever possible, incubation solutions were covered with a sheet of paper.

HPLC Determination of Glycine

Glycine was determined fluorimetrically after precolumn derivatization with ortho-phthalaldehyde and mercaptoethanol (OPA-reagent), and isocratic separation on a C_{18}-reversed phase column (ESA HR-80, 80 × 4.6 mm, particle size 3 μm, eluent 0.1-mol Na_2HPO_4 per liter, 0.13-mmol EDTA per liter, 23% methanol, pH 6.0 with concentrated phosphoric acid, Donzanti and Yamamoto, 1988). The injection volume was 300 μL. Solutions with additional glycine concentrations from 10 to 300 nmol/L were prepared from the same water sample and analyzed as well. The concentrations of glycine in the water samples were calculated by linear extrapolation of the concentration/response curves to the negative abscissa (see Figure 1). In most of the samples, the concentrations of glutamic acid and glutamine were determined as well, in the same way.

Membrane Preparation

Male Wistar rats (300–400 g) were killed by stunning and decapitation; the brains were removed from the skull and chilled in a saline/ice mixture (−0.5° C) for 10–60 min. Hippocampi were dissected from the unfrozen brains and divided longitudinally in the CA3 part and the remaining part, consisting mainly of CA1 and dentate gyrus (Berger et al., 1986). This latter part contains the highest density of NMDA receptors in the rat brain and was used for membrane preparation. The tissue was weighed (90–100 mg per rat) and homogenized in 50-mmol Tris-acetate buffer per liter (pH 7.0) in a glass/Teflon Potter homogenizer (10 strokes, 7 turns per second, 0° C); the final volume of the suspension was approximately 100 times the tissue volume. Membranes were pelleted (10 min, 35,000*g*), resuspended in fresh buffer by repeated pipetting, and centrifuged again. After a further resuspension in fresh buffer, two different treatment protocols with Triton X-100 were followed. For [^{3}H]glycine binding assays, membranes were treated with 0.08% Triton X-100 at 0° C for 15 min, similar to the procedure described by Ogita et al. (1989), however without agitation, centrifuged and resuspended in fresh buffer. For [^{3}H]MK-801 binding assays, the final concentration of Triton X-100 was only 0.02%; suspensions were incubated in a 37° C water bath for 10 min, kept on ice for another 5 min, centrifuged, and resuspended in fresh buffer. The maximal number of

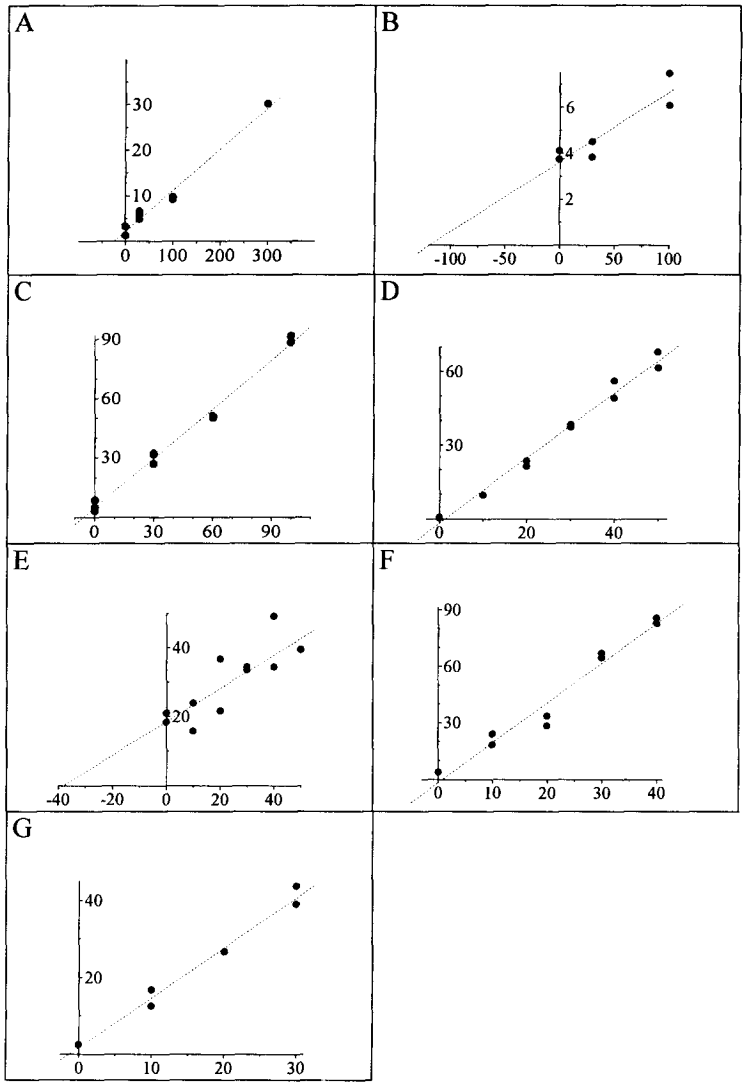

Figure 1. Quantification of glycine in deionized water, by linear extrapolation to peakheight zero of added glycine concentrations between 10 and 300 nmol/L. Ordinate: peakheight in mm; abscissa: nmol/L added glycine. The background concentration of glycine is obtained by reading the intercept of the correlation line with the negative abscissa (values in last column of Table 1). A–G represent the results obtained with seven samples of deionized water taken on different days. Adjustment of the fluorimeter geometry resulted in increased sensitivity in measurements C to G, as compared to A and B.

[^{3}H]MK-801 binding sites was higher with the latter protocol than with the former (unpublished observation). After the Triton treatment, membranes were washed 2 additional times and stored as aliquots at −80° C. They were washed once more after thawing, immediately before the binding assay.

[^{3}H]Glycine Binding Assay

To account for eventual irregularities due to environmental glycine contamination, a particular pipetting protocol was elaborated with a mixed grouping of vials, to determine concomitantly the actual equilibrium dissociation constant for glycine (K_{obs}) and the IC_{50}s of two test substances. Only by this strategy was it possible to compare inhibition potencies of numerous compounds to each other. One shortcoming of this strategy was the limited number of different glycine concentrations. The concentration of [^{3}H]glycine (42–47.5 Ci/mmol, NEN) was 10 nmol/L; additional concentrations (final concentrations 30 nmol/L, 100 nmol/L, and 300 nmol/L) were accomplished by adding nonradioactive glycine (Merck) and by correcting the specific activity. In most of the 140 experiments performed, Eadie-Hofstee plots were linear, and the following correlation coefficients (r) were obtained (number of experiments in parentheses): 1.00 (69), 0.99 (38), 0.98 (15), (6), 0.96 (6), 0.95 (1), 0.94 (1), 0.93 (1), 0.91 (2), and 0.88 (1). In general, [^{3}H]glycine binding assays were performed in the presence of 10-μmol glutamic acid per liter, to saturate the NMDA site of the receptor complex. Some of the compounds tested inhibited the NMDA-sensitive binding of [^{3}H]glutamate as well as that of [^{3}H]glycine. Because antagonists acting at the NMDA site have been described to modulate indirectly, in a glutamate reversible manner, the binding

JPM Vol. 34, No. 2
October 1995:79–88

of [^{3}H]glycine (Hood et al., 1990; Monahan et al., 1990), compounds acting at both sites had to be tested in excess glutamic acid to exclude allosteric influences on the glycine site.

For the determination of IC_{50} values, membranes were incubated with 10-nmol [^{3}H]glycine per liter and five different concentrations of the inhibitor, the highest and the lowest inhibitor concentration differing from each other by a factor of 100. Nonspecific binding was determined in presence of 100-μmol D-serine per liter (Merck) and contributed less than 10% to total binding. Incubations were run on ice in polypropylene scintillation vials (biovials, Beckmann) and started by the addition of membrane suspension corresponding to approximately 1-mg original tissue weight per vial, incubation volume 1 mL. After 20 min, vials were centrifuged at the highest tolerated speed (44,000*g*, Sorvall RC5, SS24 rotor) for 15 min, to guarantee firm adhesion of the pellets during the subsequent superficial rinsing with ice-cold buffer (2 times 4.6 mL, i.e., to the upper edge of the vial). Pellets were incubated with 100-μL water at 60° C for 30 min; this procedure was sufficient to set free the radioactivity. After addition of 2.5 mL of water-miscible scintillation cocktail (ultima gold, Packard), samples were quantified in a β-counter with automatic quench correction (Packard 1900 TR).

[^{3}H]MK-801 Binding Assay

Approximately 100-μL alcoholic stock solution of [^{3}H](+)MK-801 ([^{3}H]dizocilipine, NEN, 21–30 Ci/mmol) was transferred with a disposable glass micropipette into a cleaned (see above) glass vial and blown dry with nitrogen. After addition of 20 mL 50 mmol Tris-acetate buffer per liter, the radioactivity of two 20-μL aliquots was counted; according to the specific activity and the desired concentration of the radioligand in the binding assay, the solution was diluted and divided in glass vials into equal portions, each sufficient for one assay, and stored at −20° C for up to 4 weeks. Conditions for the [^{3}H]MK-801 binding assay were as follows: incubation volume 1 mL, disposable polypropylene vials (14 mL), 5-nmol [^{3}H]MK-801 per liter, 10-μmol glutamic acid per liter, 24–25° C (waterbath); nonspecific binding without glutamic acid, but with 10-μmol D-2-amino-5-phosphonovaleric acid per liter (Tocris Neuramin) and 1-μmol 5,7-dichlorokynurenic acid per liter (Tocris Neuramin). Incubations were started by addition of membrane suspension corresponding to approximately 1-mg original tissue per vial, in groups of 10 vials, with 5-min break between groups. After 2 hr, incubations were stopped by addition of 7-mL ice-cold buffer and vacuum filtration, using a filtration instrument of Hoefer Scientific Instruments (San Francisco, CA, USA) and glass fibre filters (GF/B 25 mm, Whatman), soaked immediately before in 0.5% polyethylene imine (Serva). Each filter was washed by sucking 2 times 7-mL ice-cold buffer through it. Filtering and washing of 10 samples took 5 min. Washed filters were placed in 20-mL scintillation vials. After addition of 8-mL toluene-based scintillation fluid (Rotiscint 11, Carl Roth, Karlsruhe, Germany), the firmly closed vials were warmed to 50° C, shaken for 1 hr (3 shakes per sec) and counted in a β-counter.

Data Analysis

Linear correlation analysis of Eadie-Hofstee plots yielded B_M and apparent K_D values (K_{obs}) for [^{3}H]glycine binding. Inhibition curves were computer fitted to the function

$$B(I) = B_o \cdot IC_{50}^{nH}/(IC_{50}^{nH} + I^{nH}) + NB \quad (1)$$

where I is the inhibitor's concentration, B_o the amount of [^{3}H]glycine bound in the absence of inhibitor, n_H the Hill coefficient, and NB the nonspecific binding. Because the Hill coefficient was close to 1 for all inhibitors tested (see Table 5), the Cheng–Prusoff correction in its simplest form (Cheng and Prusoff, 1973) was used to calculate inhibition constants:

$$K_i = IC_{50}/(1 + L/K_D) \quad (2)$$

where L is the ligand concentration. With an unknown extra-concentration of the (unlabeled) ligand present (L_E), correlation analysis of the Eadie-Hofstee plots will not result in true, but only in the observable, apparent dissociation constants (K_{obs}). Inhibition of binding of a radioligand by the ligand in its unlabeled form (i.e., the inhibition of [^{3}H]glycine binding by environmental glycine) will result in a K_i value identical with the K_D of the radioligand, and a simple relationship between true and observable dissociation constant (K_D and K_{obs}) is obtained from the Cheng–Prusoff correction, substituting in Equation 2 K_D for K_i and K_{obs} for IC_{50}:

$$K_{obs} = K_D + L_E. \quad (3)$$

Thus, Equation 2 can be written in the form

$$K_i = IC_{50}/[1 + (L + L_E)/(K_{obs} - L_E)] \quad (4)$$

The polyamine spermine increases the affinity of glycine to its binding site without influencing the K_i values of glycine antagonists (Sacaan and Johnson, 1989; Ransom and Deschenes, 1990); therefore, the following equation should be valid:

$$IC_{50}/[1 + (L + L_E)/(K_{obs} - L_E)] = \tilde{IC}_{50}/[1 + (L + L_E)/(\tilde{K}_{obs} - L_E)] \quad (5)$$

where $\tilde{IC}_{50}$ and $\tilde{K}_{obs}$ were determined in the presence of spermine. With the exception of L_E, all parameters in the equation are known or can be measured. Thus, an

expression for the excess glycine concentration is obtained by solving for L_E:

$$L_E = (\tilde{K}_{obs} - K_{obs}\, q)/(1 - q), \text{ with}$$
$$q = (IC_{50}/\tilde{IC}_{50}) \cdot (\tilde{K}_{obs} + L)/(K_{obs} + L) \quad (6)$$

Also binding of [^{3}H]MK-801 can be expressed as a function of nominally added glycine (L, just to remain within the formalism introduced above) and extra (background) glycine (L_E), simply by introducing L_E into the Michaelis-Menten equation:

$$B(L) = B_M \cdot (L + L_E)/(EC_{50} + L + L_E) \quad (7)$$

The same equation has been used in electrophysiological experiments by Johnson and Ascher (1992) to describe concentration-response curves in presence of added plus contaminating glycine. Specific binding of [^{3}H]MK-801 was determined in the presence of 0, 10, 30, 100, and 300 nmol added glycine per liter and computer fitted to Equation 7.

Results

Concentration of Glycine in Water

Glycine concentrations in seven different samples of deionized water were found to range between 0 and 120 nmol/L (Table 1). In four samples, a concentration below 6 nmol/L was found, and the concentration dependence of the peak heights exhibited an excellent linear correlation (see Figure 1C, D, F, and G). The two samples with the highest glycine content yielded more scattered correlations (B and E). In parallel with the glycine supplemented samples of deionized water, water samples of different origins were analyzed too. No significant differences were found in the glycine content of glass-distilled water, deionized water, deionized water standing for 3 months in a carefully cleaned plastic bottle, aqua fontis, and commercial water (Merck, Darmstadt) (1–6 nmol/L, 4–7 determinations, F = 1.2, analysis of variance). However, regression analysis with added glycine was performed only with samples of deionized water.

Table 1. Concentrations of Glutamic Acid, Glutamine, and Glycine (nmol/L) in Deionized Water, as Determined by Reversed Phase HPLC after Pre-Column Derivatization with Orthophthal-aldehyde and Mercaptoethanol

Experiment	glu	gln	gly
A	9.6 ± 2.0	ND	25 ± 8
B	7 ± 19	ND	120 ± 30
C	2.0 ± 0.4	0.1 ± 0.9	5.6 ± 2.0
D	5.5 ± 1.3	2.4 ± 1.0	0*
E	46 ± 6	32 ± 4	38 ± 6
F	0.7 ± 1.0	0.5 ± 2.0	0*
G	0*	0*	2.3 ± 0.6

Values were obtained by linear extrapolation to zero of nominal concentrations between 10 and 300 nmol/L (±SD, 4–9 degrees of freedom). Summary of 7 experiments (A–G, see Figure 1).

*The actual extrapolation result was slightly (and not significantly) smaller than zero; ND, not determined.

[^{3}H]Glycine Binding

Over a time period of almost 4 years, the concentration dependence of [^{3}H]glycine binding was determined altogether on 140 occasions, always under the same experimental conditions (including 10-μmol glutamic acid per liter). In Figure 2 the apparent dissociation constants resulting from these experiments (K_{obs}) are plotted against the date of the experiment. Most values were located between 40 and 80 nmol/L (mean 69 nmol/L, see Table 2), and they did not appear to be randomly scattered between these boundaries. Omission of glutamic acid resulted in slightly higher numbers (mean 93 nmol/L, see Table 2).

Stimulation by Spermine

The apparent dissociation constant of [^{3}H]glycine, as determined in the presence of 10-μmol glutamic acid per liter, was reduced by 100-μmol spermine per liter from 69 to 36 nmol/L (see Table 2); this effect of 100-μmol

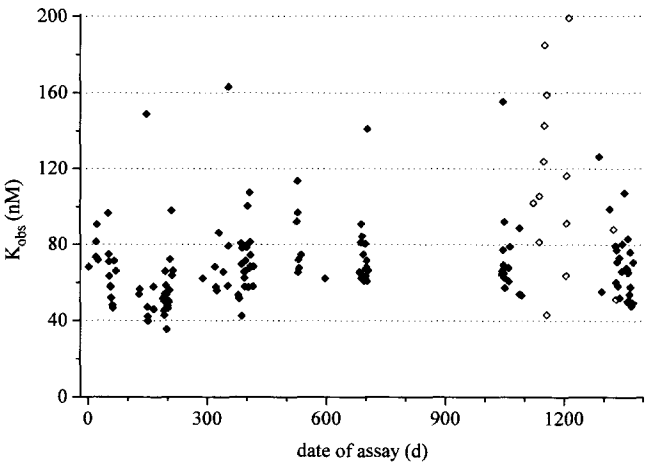

Figure 2. Apparent equilibrium dissociation constants of [^{3}H]glycine binding (K_{obs}) to rat hippocampal membranes, as determined in 140 experiments in Vienna over a time period of almost 4 years; open symbols refer to 14 additional values obtained by the author during a stay at the Institute of Pharmaceutical Chemistry, University Frankfurt/Main. Clusters of values obtained almost daily are separated by empty periods, when no assays were performed. Variability within each cluster may be explained by accidential glycine contamination varying from day to day, whereas obvious differences in the level of whole clusters point to additional influences, possibly the glycine contamination of buffer substances, of the ion exchange raisin, or of the radiolabeled compound.

JPM Vol. 34, No. 2
October 1995:79–88

Table 2. Effects of Glutamate and Spermine on [^{3}H]glycine Binding Parameters (mean ± SD)

Additions	K_{obs} (nmol/L)	B_M (fmol/mg tissue)	*n*
None	93 ± 22	134 ± 25	9
10-μmol/L glu	69 ± 21*	129 ± 27	140
10-μmol/L glu and 100-μmol/L spn	36 ± 14**†	165 ± 30*†	28

K_{obs}, observable dissociation constant; B_M, maximal number of binding sites; *n*, number of experiments; spn, spermine; *,**, significantly different from "no additions," with $p < .01, .001$, respectively; †, significantly different from "10-μmol/L glu," with $p < .01$ (analysis of variance).

spermine per liter was somewhat less pronounced than the effects reported in the literature for 300-μmol spermine per liter (Sacaan and Johnson, 1989) and for 1-mmol spermine per liter (Ransom and Deschenes, 1990). The inhibitory potency of four different glycine antagonists was reduced by 100-μmol spermine per liter, reflected by an increase of the IC_{50} values (Table 3). This increase in the IC_{50} values of competitive glycine antagonists in the presence of spermine does not justify the assumption that spermine directly influences the interaction of the inhibitors with the glycine site; the apparent weakening of the inhibitors' potency by spermine might solely be the consequence of the spermine-induced increase of [^{3}H]glycine's affinity. In fact, data presented by Sacaan and Johnson (1989) and by Ransom and Deschenes (1990) suggested that spermine increased the affinity of glycine agonists only, without any effect on the affinity of glycine antagonists. The impact of changes of a radioligand's affinity constant on the IC_{50} of a competing inhibitor is given by the Cheng–Prusoff correction:

$$IC_{50} = K_i \cdot (1 + L/K_D) \quad (2a)$$

IC_{50} values of glycine antagonists were increased, in the presence of 100-μmol spermine per liter, by factors between 1.69 and 5.19 (Table 3); the observed spermine-induced changes of the glycine dissociation constant, however, predicted only factors between 1.02 and 1.29. This discrepancy was easily resolved by assuming a certain concentration of additional unlabeled glycine (L_E), allowing the conversion of apparent dissociation constants (K_{obs} and $\tilde{K}_{obs}$) into true dissociation constants (K_D and $\tilde{K}_D$) via Equation 3. Extra glycine concentrations calculated from the results of 18 experi-

Table 3. Inhibition of [^{3}H]glycine Binding to Hippocampal Membranes by Four Different Glycine Antagonists in Presence of 10-μmol Glutamic Acid Per Liter, Influence of 100-μmol Spermine Per Liter

	Measured quantities					Calculated quantities			
Antagonist	L (nmol/L)	IC_{50} (μmol/L)	K_{obs} (nmol/L)	$\tilde{IC}_{50}$ (μmol/L)	$\tilde{K}_{obs}$ (nmol/L)	L_E (nmol/L)	K_D (nmol/L)	$\tilde{K}_D$ (nmol/L)	K_i (μmol/L)
CIAA:	9.99	12.6	55.7	27.5	24.7	14.8	40.9	9.9	7.9
	9.74	11.9	58.1	38.3	30.5	24.3	33.8	6.2	5.9
	9.81	25.0	83.4	102	40.3	33.8	49.6	6.5	13.3
	10.13	16.9	49.5	61.7	20.6	15.9	33.7	4.8	9.5
	10.13	19.4	70.9	88.2	39.8	34.9	36.0	4.9	8.8
CIAA-et:	9.79	6.23	71.9	18.9	47.9	40.7	31.2	7.2	2.4
	9.82	4.15	54.1	21.0	24.7	21.1	32.9	3.5	2.1
	9.78	4.75	76.3	22.4	26.5	21.6	54.7	4.9	3.0
	10.03	5.51	63.7	28.6	32.2	28.3	35.4	3.9	2.7
NQX:	9.80	1.22	67.3	2.56	21.8	10.7	56.6	11.1	0.90
	9.97	3.39	50.4	8.96	18.4	11.5	38.9	6.9	2.18
	10.13	1.24	48.0	3.25	18.3	11.5	36.5	6.8	0.78
DCKA:	9.94	0.070	56.8	0.320	36.7	33.1	23.7	3.6	0.025
	10.33	0.044	56.0	0.171	37.4	32.1	23.9	4.3	0.016
	9.94	0.059	98.1	0.287	77.7	73.6	24.5	4.1	0.013
	9.72	0.047	68.5	0.204	47.8	43.5	25.0	4.3	0.015
	9.81	0.073	50.2	0.346	23.7	20.2	30.0	3.5	0.036

IC_{50}, K_{obs}, K_D, inhibitor concentration providing halfmaximal inhibition, apparent dissociation constant of [^{3}H]glycine binding, and true dissociation constant, in the absence of spermine; $\tilde{IC}_{50}$, $\tilde{K}_{obs}$, $\tilde{K}_D$, same meaning, but in the presence of 100-μmol spermine per liter; L, [^{3}H]glycine concentration; L_E, environmental (background) glycine concentration; K_i, spermine-independent inhibition constant; L_E was calculated by using Equation 6, K_D-values were obtained by subtracting L_E from the respective K_{obs}-values (Equation 3), and K_i-values were calculated from Equation 4 (see data analysis).

CIAA, 2-carboxy-1H-indole-3-acetic acid; CIAA-et, 2-ethylester of CIAA; NQX, 2,3-dihydroxy-6-nitroquinoxaline; DCKA, 5,7-dichlorokynurenic acid.

ments by use of Equation 6 are listed in Table 3 and ranged between 11 and 74 nmol/L. For three of the four antagonists used (CIAA, CIAA-et, NQX), the same "true" affinity constant of [^{3}H]glycine was obtained: without spermine K_D = 40.1 ± 8.4 nmol/L, with 100-μmol spermine per liter K_D = 7.2 ± 3.7 nmol/L (n = 13). The use of DCKA, however, resulted in significantly lower values (Table 3, $p < .05$, analysis of variance).

Glycine Stimulated [^{3}H]MK-801 Binding

The main results of nine experiments are summarized in Table 4. In the nominal absence of glycine, binding of [^{3}H]MK-801 (B_o in Table 4) varied between 27% and 79% of the saturation level. Fitting of the results obtained with increasing glycine concentrations to Equation 7 yielded similar EC_{50} values for all experiments (between 22 and 35 nmol/L, mean 28.6 nmol/L), whereas extra glycine levels varied between 10 and 111 nmol/L (mean 36.5 nmol/L). Figure 3 illustrates the results for two groups of experiments, one with low (*open symbols*) and the other with medium extra glycine levels (*filled symbols*). After calculation of background glycine levels by computerized curve fitting, specific binding of [^{3}H]MK-801 was plotted against the logarithm of the true glycine concentration (sum of added and background glycine), resulting in a typical sigmoidal stimulation curve. The Hill coefficient of this stimulation was 0.98 and thus close to unity, a natural consequence of the function (Equation 7) used for fitting the data.

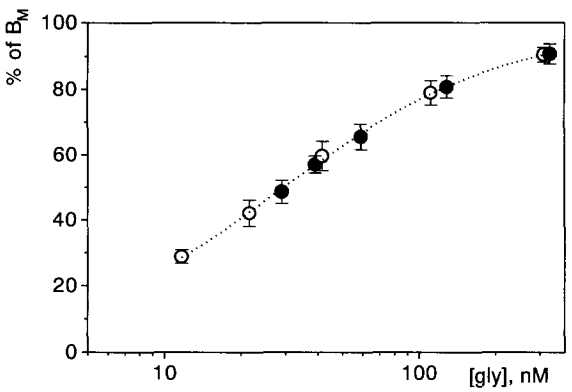

Figure 3. Stimulation of [^{3}H]MK-801 binding (5 nmol/L) by glycine. Summary of three experiments with low (mean 11 nmol/L, open circles) and three experiments with medium environmental glycine (mean 29 nmol/L, filled circles; bars indicate SD). The percentage of maximal binding is plotted against the real glycine concentration calculated from the added concentration and background glycine (see Table 4).

Inhibition by Established Glycine Site Ligands

Relying on a specific "mixed" pipetting protocol, IC_{50} values for several established ligands of the NMDA receptor associated glycine site were determined concomitantly with the apparent equilibrium dissociation constant (K_{obs}) of [^{3}H]glycine. Variability in K_{obs} was interpreted to reflect variability in the background concentration of unlabeled glycine. Based on the evidence given above (and discussed below), the true equilibrium constant (K_D) was assumed to have been close to 40 nmol/L in all these experiments. Consequently, for the calculation of K_i values, a modification of the Cheng–Prusoff correction was used:

$$K_i = IC_{50}/[1 + (+ K_{obs} - K_D)/K_D] \quad (8)$$

where $K_{obs} - K_D$ is the background glycine concentration (Equation 3), and $L + K_{obs} - K_D$ the total ligand

Table 4. Stimulation of [^{3}H]MK-801 Binding by Glycine, in Presence of 10-μmol Glutamic Acid per Liter

Experiment	B_M*	EC_{50}†	$[gly]_E$†	B_o*	$B_o \cdot EC_{50}/(B_M - B_o)$†
1.	37.8	23.9	9.7	11.7	12.8
2.	40.1	29.5	11.8	10.7	10.3
3.	40.3	32.0	13.5	11.7	11.7
mean ± SD (3)		28.5 ± 4.1	11.7 ± 1.9		
4.	35.1	22.1	25.7	18.4	31.5
5.	41.0	32.5	30.4	18.6	31.5
6.	37.5	34.9	31.3	18.0	26.4
mean ± SD (3)		29.8 ± 6.8	29.1 ± 3.0		
7.	43.5	21.6	47.2	30.2	64.9
8.	35.9	32.3	47.7	21.1	40.6
9.	40.5	29.1	111	32.1	108
mean ± SD (9)		28.6 ± 4.9	36.5 ± 31		

*fmol/mg tissue; †nmol/L.

B_M, EC_{50}, $[gly]_E$, obtained by computerized curve fitting to the function $B = B_M \cdot ([gly]_{nom} + [gly]_E)/(EC_{50} + [gly]_{nom} + [gly]_E)$, where $[gly]_{nom}$ and $[gly]_E$ are the nominal (added) and the extra (background) glycine concentrations; B_o, specific binding of [^{3}H]MK-801 in nominal absence of glycine; $B_o \cdot EC_{50}/(B_M - B_o)$ gives an estimate for $[gly]_E$ relying on B_o and the mean of all obtained EC_{50} values (28.6 nmol/L).

JPM Vol. 34, No. 2
October 1995:79–88

concentration competing with the inhibitor. The equation can be simplified to

$$K_i = IC_{50} \cdot K_D/(L + K_{obs}) \quad (8a)$$

Setting K_D equal to 40 nmol/L, the mean K_i values summarized in Table 5 were obtained. The following sequence of potencies was observed: AC3CA > DCKA > D-ser = 7-Cl-PHQ >>NQX = CNQX > BICA = KYNA = CIAA > PHQ = QX = 3-OH-QCA > FICA > ICA > MQX. The presently available literature describes K_i values at least 2 times weaker for these substances. In two of three studies, ICA was found inactive (Baron et al., 1991; Gray et al., 1991; Hood et al., 1992). No K_i value has been published for the N-methylated form of QX.

Table 5. K_i Values for Several Inhibitors of [^{3}H]glycine Binding to Hippocampal Membranes

Compound	K_i (μmol/L) ± SD	n_H ± SD
AC3CA	0.0133 ± 0.0035 (4)	0.86 ± 0.08 (3)
DCKA	0.044 ± 0.017 (9)	0.89 ± 0.04 (5)
D-serine	0.088 ± 0.022 (6)	0.96 ± 0.08 (3)
7-Cl-PHQ	0.14 ± 0.04 (4)	0.88 ± 0.06 (4)
NQX	1.43 ± 0.53 (8)	0.89 ± 0.10 (6)
CNQX	2.19 ± 0.53 (6)	0.92 ± 0.06 (3)
BICA	5.30 ± 1.75 (5)	0.84 ± 0.06 (3)
KYNA	7.33 ± 3.31 (6)	0.85 ± 0.06 (3)
CIAA	8.44 ± 1.46 (13)	0.92 ± 0.17 (6)
PHQ	12.2 ± 5.2 (4)	ND
QX	12.6 ± 4.1 (4)	ND
3-OH-QCA	16.1 ± 4.9 (3)	ND
FICA	27.9 ± 5.3 (4)	ND
ICA	43.4 ± 5.7 (4)	ND
MQX	81.3 ± 5.7 (3)	ND

Calculated by using the equation $K_i = IC_{50} \cdot 40/(L + K_{obs})$ (L, concentration of [^{3}H]glycine, K_{obs}, observed affinity constant of [^{3}H]-glycine, see Equation 8a), based on the assumption that the true K_D = 40 nmol/L. Mean K_i-values separated by free lines are significantly different from each other ($p < .05$, analysis of variance; number of determinations in parentheses).

AC3CA, aminocyclopropane carboxylic acid; DCKA, 5,7-dichlorokynurenic acid; 7-Cl-PHQ, 7-Cl-derivative of PHQ; NQX, 6-nitro-QX; CNQX, 7-cyano-NQX; BICA, 1H-benzimidazole-2-carboxylic acid; KYNA, kynurenic acid; CIAA, 2-carboxy-1H-indole-3-acetic acid; PHQ, 3-phenyl-4-hydroxyquinolin-2(1H)-one (McQuaid et al., 1992); QX, 2,3-dihydroxyquinoxaline; 3-OH-QCA, 3-hydroxyquinoxaline-2-carboxylic acid; FICA, 5-fluoro-ICA; ICA, 1H-indole-2 carboxylic acid; MQX, N-methyl-QX; n_H, Hill coefficient; ND, not determined (for computer analysis, n_H was set equal to unity).

Discussion

During the last few years since the discovery of the NMDA receptor associated glycine site (Johnson and Ascher, 1987), the affinity of [^{3}H]glycine to this binding site has been determined in more than two dozen published studies. The main reason for this apparent redundancy seems to be a technical one: the high variability of the obtained data. The published values may not be scattered around the true value, but may rather be distorted always to the same direction, to a variable extent, by influences that are difficult to control. As a working hypothesis, the observed variability in the apparent K_D-values can be interpreted as the consequence of varying amounts of background glycine arising from environmental contamination. Because most values obtained in the present study were scattered between 40 and 80 nmol/L (see Figure 2), this hypothesis predicted a true affinity constant around the lower boundary and contaminating glycine concentrations between the boundaries.

Contamination by glycine may originate from several sources. Residual glycine may be liberated from the membranes during incubation; it may be present on the surface of assay tubes and pipette tips; or, finally, it may be contained in the water or the chemicals used for the preparation of the assay buffer. The results of the binding assays presented above suggested background glycine concentrations between 10 and 30 nmol/L, in exceptional cases up to 100 nmol/L and more. Using highly sensitive HPLC analysis of amino acids, practically no glycine (≤6 nmol/L) was found in most water samples used for the preparation of the assay buffer. This was surprising, because no special efforts were applied to the purification of the water (conventional ion exchange resin). In a footnote, Kleckner and Dingledine (1988) indicated 20–50-nmol glycine per liter in water distilled in normal, unbaked glassware. Interestingly, in the present study no glycine could be detected even in tap water, a possible indication for the high quality of tap water in Vienna (*Hochquellwasser*). Any additional purification procedures most likely would have increased instead of reduced the risk of glycine contamination. Thus, contamination of water by glycine is unlikely to explain for persistent contamination by glycine observed in the present study. No effort was undertaken to quantify a possible glycine contamination in Tris or acetic acid, but it should be kept in mind that 1 ppm glycine in 50-mmol Tris-acetate per liter would result in 50-nmol glycine per liter. The manufacturer (Merck) does not explicitly exclude such a contamination. In a preliminary experiment, residual glycine in the membrane preparation was determined by incubating membranes (1-mg original tissue weight/mL) for 2 hr at room temperature in deionized water. The supernatant and

the water sample used were analyzed in parallel by HPLC; around 40-nmol glycine per liter was found in the supernatant (not illustrated), and only 2 nmol/L in the water (experiment G, Table 1). This result, however, should be interpreted with caution; no calibration with glycine-supplemented membrane suspensions was performed, and the membranes had been incubated in water, not in buffer. Unfortunately Tris, as a primary amine, consumes the derivatization reagents and yields an enormous peak in the chromatogram, precluding any high-sensitivity detection of glycine in Tris buffer by that technique.

A possibility to determine an unknown quantity of glycine directly in the suspension of the [^{3}H]glycine binding assay is offered by the observation that the polyamine spermine increased the affinity of glycine to its NMDA receptor-associated binding site without influencing the inhibition constant (K_i) of glycine antagonists (Sacaan and Johnson, 1989; Ransom and Deschenes, 1990). Data obtained on the influence of spermine on the IC_{50}s of glycine antagonists were compatible with a glycine background concentration between 11 and 74 nmol/L, and with a true K_D-value for [^{3}H]glycine between 24 and 55 nmol/L. Thus, the variability in the observed affinity constant was indeed, to a considerable extent, attributed to variable concentrations of contaminating glycine. The data, however, also pointed to a possible influence of spermine on the K_i of one of the glycine antagonists used. K_D-values calculated from experiments with DCKA were significantly lower than from experiments with the other antagonists (Table 3). To reconcile the results obtained with different glycine antagonists with each other, it must be assumed that either spermine increased the K_i of DCKA (i.e., weakened its inhibitory potency), or decreased the K_is of the other antagonists. The first explanation is in agreement with recent results obtained with [^{3}H]DCKA (Yoneda et al., 1994) and the structurally related [^{3}H]L-689.560 (Grimwood et al., 1994); however, more detailed studies on a broader selection of glycine antagonists are necessary to clarify this issue.

Also the stimulation of [^{3}H]MK-801 binding to neuronal membranes by glycine has been studied in considerable detail. Here, a number of authors reported EC_{50}-values below 100 nmol/L (Wong et al., 1987; Ransom and Stec, 1988; Foster et al., 1992), most likely a consequence of the use of 50-μmol glutamic acid per liter or 100-μmol NMDA per liter, respectively, in these studies. Data shown in Table 4 of the present study point to a "true" EC_{50}-value for stimulation of [^{3}H]MK-801 binding by glycine below the lowest value published, namely around 30 nmol/L. Background glycine concentrations suggested by these data ranged between 10 and 111 nmol/L, in agreement with the range of scattering of apparent K_D-values for [^{3}H]glycine binding, and with the evidence from the experiments with spermine. The at-least equal potency of glycine in stimulating binding of [^{3}H]MK-801 at room temperature, as compared to the affinity of [^{3}H]glycine to the NMDA receptor complex on ice, stands in contradiction to the known negative temperature dependence of [^{3}H]glycine binding (Schneider and Urwyler, 1992). However, the "true" EC_{50} for stimulation of [^{3}H]MK-801 binding by glycine must not be expected to match the "true" K_D of [^{3}H]glycine binding; although both assays were conducted in the presence of 10-μmol glutamic acid per liter, also other assay parameters in addition to the temperature have been different (incubation time, details in the membrane preparation procedure).

Weighing all the evidence discussed above, the "true" affinity constant of glycine for the NMDA receptor complex most likely amounts to 30–40 nmol/L in the presence of glutamate, and in its nominal absence still to 55–65 nmol/L; the physiological polyamine spermine even pushes this value below 10 nmol/L. This is a surprisingly strong affinity for a small molecule with only two functional groups available for interaction with the receptor protein. In that respect, [^{3}H]glycine seems to be no weaker as a ligand than the recently introduced [^{3}H]DCKA (Baron et al., 1991). In light of this high affinity, a permanent stimulatory ("constitutive") influence of glycine on the NMDA receptor complex should be expected. However, several studies on rats and mice indicate that the neuronal NMDA receptor is not saturated in vivo by glycine (Singh et al., 1990; Peterson, 1991; Budai et al., 1992). Thus, highly efficient uptake processes seem to keep the levels of glycine low in the neighborhood of the receptor, or glycine has to compete in vivo with endogenous competitive antagonists as kynurenic acid (Swartz et al., 1990; Schwarcz et al., 1992) or other still unknown compounds.

I thank C.R. Noe for providing BICA, CIAA, CIAAet, ICA, PHQ, 7-Cl-PHQ, QX, and NQX; and I thank H. Reither for high sensitivity adaptation of the HPLC apparatus.

References

Baron BM, Siegel BW, Slone AL, Harrison BL, Palfreyman MG, Hurt SD (1991) [^{3}H]5,7-Dichlorokynurenic acid, a novel radioligand labels NMDA receptor-associated glycine binding sites. *Eur J Pharmacol* 206:149–154.

Berger ML, Charton G, Ben-Ari Y (1986) Effect of seizures induced by intra-amygdaloid kainic acid on kainic acid binding sites in rat hippocampus and amygdala. *J Neurochem* 47:720–727.

Bristow DR, Bowery NG, Woodruff GN (1986) Light microscopic autoradiographic localisation of [^{3}H]glycine and [^{3}H]strychnine binding sites in rat brain. *Eur J Pharmacol* 126:303–307.

Budai D, Wilcox GL, Larson AA (1992) Enhancement of NMDA-evoked neuronal activity by glycine in the rat spinal cord in vivo. *Neurosci Lett* 135:265–268.

Cheng Y-C, Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* 22:3099–3108.

Donzanti BA, Yamamoto BK (1988) An improved and rapid HPLC-EC method for the isocratic separation of amino acid neurotransmitters from brain tissue and microdialysis perfusates. *Life Sci* 43:913–922.

Foster AC, Kemp JA, Leeson PD, Grimwood S, Donald AE, Marshall GR, Priestley T, Smith JD, Carling RW (1992) Kynurenic acid analogues with improved affinity and selectivity for the glycine site on the NMDA receptor from rat brain. *Mol Pharmacol* 41:914–922.

Gray NM, Dappen MS, Cheng BK, Cordi AA, Biesterfeldt JP, Hood WF, Monahan JB (1991) Novel indole-2-carboxylates as ligands for the strychnine-insensitive NMDA-linked glycine receptor. *J Med Chem* 34:1283–1292.

Grimwood S, Moseley AM, Carling RW, Leeson PD, Foster AC (1992) Characterization of the binding of [^{3}H]L-689.560, an antagonist for the glycine site on the N-methyl-D-aspartate receptor, to rat brain membranes. *Mol Pharmacol* 41:923–930.

Grimwood S, Struthers L, Foster AC (1994) Polyamines modulate [^{3}H]L-689.560 binding to the glycine site of the NMDA receptor from rat brain. *Eur J Pharmacol* 266:43–50.

Hood WH, Compton RP, Monahan JB (1990) N-methyl-D-aspartate recognition site ligands modulate activity at the coupled glycine recognition site. *J Neurochem* 54:1040–1046.

Hood WF, Gray NM, Dappen MS, Watson GB, Compton RP, Cordi AA, Lanthorn TH, Monahan JB (1992) Characterization of indole-2-carboxylate derivatives as antagonists of NMDA receptor activity at the associated glycine recognition site. *J Pharmacol Exp Ther* 262:654–660.

Johnson JW, Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 325:529–531.

Johnson JW, Ascher P (1992) Equilibrium and kinetic study of glycine action on the N-methyl-D-aspartate receptor in cultured mouse brain neurons. *J Physiol* 455:339–365.

Jones MS, Snell DL, Johnson KM (1989) Characterization of the binding of radioligands to the N-methyl-D-aspartate, phencyclidine, and glycine receptors in buffy coat membranes. *J Pharmacol Methods* 21:161–168.

Kemp JA, Leeson PD (1993) The glycine site of the NMDA receptor—five years on. *Trends Pharmacol Sci* 14:20–25.

Kessler M, Terramani T, Lynch G, Baudry M (1989) A glycine site associated with N-methyl-D-aspartic acid receptors: Characterization and identification of a new class of antagonists. *J Neurochem* 52:1319–1328.

Kishimoto H, Simon JR, Aprison MH (1981) Determination of the equilibrium dissociation constants and number of glycine binding sites in several areas of the rat central nervous system, using a sodium-independent system. *J Neurochem* 37:1015–1024.

Kleckner NW, Dingledine R (1988) Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. *Science* 241:835–837.

Marvizón J-CG, Lewin AH, Skolnick P (1989) 1-Aminocyclopropane carboxylic acid: A potent and selective ligand for the glycine modulatory site of the N-methyl-D-aspartate receptor complex. *J Neurochem* 52:992–994.

McNamara D, Smith ECR, Calligaro DO, O'Malley PJ, McQuaid LA, Dingledine R (1990) 5,7-Dichlorokynurenic acid, a potent and selective competitive antagonist of the glycine site on NMDA receptors. *Neurosci Lett* 120:17–20.

McQuaid LA, Smith ECR, Lodge D, Pralong E, Wikel JH, Calligaro DO, O'Malley PJ (1992) 3-Phenyl-4-hydroxyquinolin-2(1H)-ones: Potent and selective antagonists at the strychnine-insensitive glycine site on the N-methyl-D-aspartate receptor complex. *J Med Chem* 35:3423–3425.

Monahan JB, Corpus VM, Hood WH, Thomas JW, Compton RP (1989) Characterization of a [^{3}H]glycine recognition site as a modulatory site of the N-methyl-D-aspartate receptor complex. *J Neurochem* 53:370–375.

Ogita K, Suzuki T, Yoneda Y (1989) Strychnine-insensitive binding of [^{3}H]glycine to synaptic membranes in rat brain, treated with Triton X-100. *Neuropharmacology* 28:1263–1270.

Peterson SL (1991) Anticonvulsant drug potentiation by glycine in maximal electroshock seizures is mimicked by D-serine and antagonized by 7-chlorokynurenic acid. *Eur J Pharmacol* 199:341–348.

Ransom RW, Deschenes NL (1990) Polyamines regulate glycine interaction with the N-methyl-D-aspartate receptor. *Synapse* 5:294–298.

Ransom RW, Stec NL (1988) Cooperative modulation of [^{3}H]MK-801 binding to the NMDA receptor-ion channel complex by L-glutamate, glycine, and polyamines. *J Neurochem* 51:830–836.

Sacaan AI, Johnson KM (1989) Spermine enhances binding to the glycine site associated with the N-methyl-D-aspartate receptor complex. *Mol Pharmacol* 36:836–839.

Schneider CI, Urwyler S (1992) 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. *Biochem Pharmacol* 43:1693–1699.

Schwarcz R, Du F, Schmidt W, Turski WA, Gramsbergen JBP, Okuno E, Roberts RC (1992) Kynurenic acid: A potential pathogen in brain disorders. *Ann NY Acad Sci* 648:140–153.

Singh L, Oles RJ, Tricklebank MD (1990) Modulation of seizure susceptibility in the mouse by the strychnine-insensitive glycine recognition site of the NMDA receptor/ion channel complex. *Br J Pharmacol* 99:285–288.

Swartz KJ, During MJ, Freese A, Beal MF (1990) Cerebral synthesis and release of kynurenic acid: An endogenous antagonist of excitatory amino acid receptors. *J Neurosci* 10:2965–2973.

Thedinga KH, Benedict MS, Fagg GE (1989) The N-methyl-D-aspartate (NMDA) receptor complex: A stoichiometric analysis of radioligand binding domains. *Neurosci Lett* 104:217–222.

Williams K, Romano C, Dichter MA, Molinoff PB (1991) Modulation of the NMDA receptor by polyamines. *Life Sciences* 48:469–498.

Wong EHF, Knight AR, Ransom R (1987) Glycine modulates [^{3}H]MK-801 binding to the NMDA receptor in rat brain. *Eur J Pharmacol* 142:487–488.

Yoneda Y, Suzuki T, Ogita K (1994) Differential profiles of binding of a radiolabeled agonist and antagonist at a glycine recognition domain on the NMDA receptor ionophore complex in rat brain. *J Neurochem* 62:102–112.