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CHARACTERIZATION AND REGIONAL DISTRIBUTION OF STRYCHNINE-INSENSITIVE [^{3}H]GLYCINE BINDING SITES IN RAT BRAIN BY QUANTITATIVE RECEPTOR AUTORADIOGRAPHY

J. W. McDONALD,* J. B. PENNEY,† M. V. JOHNSTON‡ and A. B. YOUNG†§

*Neuroscience and Medical Scientist Training Program, †Department of Neurology, University of Michigan, Ann Arbor, MI 48104, U.S.A.
‡Departments of Pediatrics and Neurology, Johns Hopkins University, Baltimore, MD, U.S.A.

Abstract—Recent evidence suggests that a strychnine-insensitive glycine modulatory site is associated with the *N*-methyl-D-aspartate receptor–channel complex. A quantitative autoradiographic method was used to characterize the pharmacological specificity and anatomical distribution of strychnine-insensitive [^{3}H]glycine binding sites in rat brain. [^{3}H]Glycine binding was specific, saturable, reversible, pH and temperature-sensitive and of high affinity. [^{3}H]Glycine interacted with a single population of sites having a K_D of approximately 200 nM and a maximum density of 6.2 pmol/mg protein (stratum radiatum, CA1). Binding exhibited a pharmacological profile similar to the physiologically defined strychnine-insensitive glycine modulatory site. Binding was stereoselective; the rank order of potency of simple amino acids as displacers of binding was: glycine > D-serine > D-alanine > L-serine > L-alanine > L-valine > D-valine. Binding was not altered by the inhibitory glycine receptor ligand, strychnine, by the glutamate agonists, quisqualate and kainate, or by GABA receptor selective ligands. Most competitive agonists or antagonists of the *N*-methyl-D-aspartate recognition site were ineffective displacers of glycine binding. The exceptions were the aminophosphono series of antagonists, D-alpha-aminoadipate, gamma-D-glutamylglycine and beta-D-aspartylaminomethylphosphonic acid. However, the inhibition of [^{3}H]glycine binding produced by the aminophosphono compounds could be accounted for by the level of glycine contamination present in these compounds. The non-competitive NMDA receptor–channel blockers, phencyclidine, its thienyl derivative, and MK-801 did not alter glycine binding. Kynurenate, glycine methylester, L-serine-*O*-sulfate, L-homocysteic acid, and several glycine-containing dipeptides were effective displacers of glycine binding. Structure–activity relations of agonists and antagonists of the strychnine-insensitive glycine binding site are discussed. The distribution of strychnine-insensitive [^{3}H]glycine binding was heterogeneous with the following rank order of binding densities: hippocampus > cerebral cortex > caudate–putamen ≥ thalamus > cerebellum > brain stem. This distribution of binding was correlated with *N*-methyl-D-aspartate-sensitive [^{3}H]glutamate binding ($r^2 = 0.77$; $P < 0.001$; Pearson product-moment) and [^{3}H]thienylcyclohexylpiperidine binding ($r^2 = 0.72$; $P < 0.001$).

These observations are consistent with the hypothesis that the strychnine-insensitive glycine binding site is closely associated with the *N*-methyl-D-aspartate receptor–channel complex. These data also suggest that the stoichiometry between these binding sites is approximately 1:3:4 (thienylcyclohexylpiperidine: glycine:glutamate) in stratum radiatum of area CA1. However, in other brain regions, there were more glycine than glutamate binding sites suggesting that there may be heterogeneity in the *N*-methyl-D-aspartate receptor–channel complex.

The *N*-methyl-D-aspartate (NMDA)-subtype of glutamate receptors is part of a receptor–ion channel complex with multiple regulatory sites including distinct sites for magnesium and zinc, and a channel binding site (phencyclidine, PCP receptor) for dissociative anesthetics and {(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate} (MK-801).[21,22,34] The complex also has a strychnine-insensitive glycine binding site.[23,27,58] The NMDA operated channel is gated by magnesium in a voltage-dependent manner.[32,36,45] Magnesium also alters the binding characteristics of [^{3}H]trichloroacetic acid ([^{3}H]TCP) and [^{3}H]MK-801 to the NMDA associated channel.[8,52,64] Zinc selectively antagonizes NMDA-mediated responses at a site distinct from that of magnesium.[49,61] Zinc also inhibits [^{3}H]glutamate binding to NMDA recognition sites and reduces [^{3}H]MK-801 binding to the channel.[44,52]

PCP receptor ligands block NMDA responses in a non-competitive, activity-dependent manner probably by binding within the ion channel.[1,62]

§To whom correspondence should be addressed.

Abbreviations: AP5, DL-2-amino-5-phosphonopentanoic acid; AP7, DL-2-amino-7-phosphonoheptanoic acid; APB, DL-2-amino-4-phosphonobutyric acid; ASP–AMP, beta-D-aspartyl-aminomethylphosphonic acid; DGG, gamma-D-glutamylglycine; GAMS, gamma-D-glutamylamino-methylsulfonate; GDEE, L-glutamyl-diethylester; HA-966, 3-amino-1-hydroxypyrollid-2-one; HPLC, high pressure liquid chromatography; LGG, gamma-L-glutamylglycine; L-SOS, L-serine-*O*-sulfate; MK-801, {(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate}; NAAG, *N*-acetyl-L-aspartyl-L-glutamic acid; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; TCP, *N*-(1-[2-thienyl]cyclohexyl)3,4-piperidine; THA, tetrahydroamino acridine; TLC, thin layer chromatography.

Modulation of NMDA receptor activation regulates [^{3}H]TCP and [^{3}H]MK-801 binding.[22,31] However, reciprocal interactions have not been demonstrated.[44,44a]

Johnson and Ascher[23] demonstrated, in outside-out patches, that glycine potentiates NMDA-induced increases in cation conductance in a strychnine-insensitive manner. Following this observation, several reports have indicated that glycine and structurally-related analogs also potentiate NMDA-mediated events in other systems: enhancement of NMDA-stimulated electrophysiological responses,[4,15,29] [^{3}H]TCP and [^{3}H]MK-801 binding,[5,51,55,63] and [^{3}H]norepinephrine release.[50]

Furthermore, reciprocal interactions between the NMDA recognition site and the glycine modulatory site have been described.[9,11,27,43] Glycine can stimulate agonist binding to the NMDA recognition site in membrane homogenates. Furthermore, NMDA receptor agonists stimulate [^{3}H]glycine binding whereas antagonists inhibit [^{3}H]glycine binding. However, additional studies have demonstrated no interaction between glycine and NMDA recognition sites.[5a,44a]

The anatomical localization of strychnine-insensitive [^{3}H]glycine sites and the regional relationship between this site and NMDA recognition and PCP sites have not been described in detail. It is not clear whether these binding sites are always associated or if they can exist independently of one another.

Although there have been previous reports of strychnine-insensitive glycine binding in rat brain,[6,9,27,55,56] we now provide the first detailed characterization of the regional distribution and pharmacology of glycine binding sites in rat brain sections using quantitative autoradiography. The topography of strychnine-insensitive glycine binding sites is compared with the distribution of NMDA-sensitive [^{3}H]glutamate, and [^{3}H]TCP binding sites. Some of this work has been presented in preliminary form.[39]

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]Glycine was obtained from Amersham (Arlington Heights, IL, U.S.A., specific activity = 13.3–19 Ci/mmol). DL-2-Amino-4-phosphonobutyric acid (APB), DL-2-amino-5-phosphonopentanoic acid (AP5), DL-2-amino-7-phosphonoheptanoic acid (AP7), gamma-D-glutamylamino-methylsulfonate (GAMS), gamma-D-glutamylglycine (DGG), gamma-L-glutamylglycine (LGG), L-glutamyl-diethylester (GDEE), beta-D-aspartyl-aminomethylphosphonic acid (ASP-AMP), D-homocysteine sulfinic acid, 7-chlorokynurenate, and 3-amino-1-hydroxypyrollid-2-one (HA-966) were obtained from Tocris Neuramin (Essex, U.K.). *N*-Acetyl-L-aspartyl-L-glutamic acid (NAAG) and glycyl-L-glutamic acid were purchased from Bachem (Torrance, CA, U.S.A.). MK-801 was a gift from Dr P. Anderson (Merck, Sharp & Dohme, West Point, PA, U.S.A.). TCP and PCP were gifts from Dr E. Domino, University of Michigan (Ann Arbor, MI, U.S.A.). All other compounds were purchased from Sigma.

Detection of glycine contamination

DL-AP5, DL-AP7, kynurenate, glycine methyl ester, D-alpha-aminoadipic acid, L-homocysteic acid, ASP-AMP, and five glycine-containing dipeptides were tested for possible glycine contamination by thin layer chromatography (TLC) with acidic (*n*-butanol:acetic acid:water, 12:3:5) and basic (ethanol:ammonia:water, 20:1:4) solvent systems. Ninhydrin was used to visualize glycine. Additionally, DL-AP5, DL-AP7, kynurenate, NMDA and glutamate were tested for glycine contamination using high pressure liquid chromatography (HPLC). Electrochemical detection of glycine was carried out using a modification of previously described *o*-phthaldialdehyde derivitization.[24a,30a] Compounds were examined by HPLC at concentrations (100 nM–1 mM) that allowed detection of at least 0.001% glycine contamination. Multiple sources of compounds were tested when available.

Tissue preparation

Adult male Sprague-Dawley albino rats (three months old) were killed by decapitation. The brains were quickly removed, frozen on dry ice, and mounted on cryostat chucks. Mounted brains were allowed to equilibrate to the temperature of the cryostat (−12 to −20°C) and 20-μM horizontal sections were cut and thaw-mounted onto gelatin-coated slides. Sections were stored at −20°C for less than 24 h. Preliminary experiments determined that [^{3}H]glycine binding was not altered by frozen storage for 24 h.

Strychnine-insensitive [^{3}H]glycine autoradiography

Tissue sections were preincubated in 50 mM Tris citrate (pH 7.4) at 4°C for 30 min to remove endogenous compounds and then dried under a stream of cool air. In preliminary experiments this length of preincubation was determined to maximize [^{3}H]glycine binding while maintaining tissue integrity. Longer preincubation periods of up to 2 h at 4°C did not further enhance binding. In regional distribution studies, sections were then incubated in the same buffer (pH 7.4) containing 100 nM [^{3}H]glycine for 35 min at 4°C (final volume = 8 ml); under these conditions, less than 5% of free ligand was bound and "zone A" conditions were maintained. In preliminary experiments, [^{3}H]glycine binding in rat forebrain was found to be insensitive to the addition of 100 μM strychnine. In saturation studies, the concentration of [^{3}H]glycine was 40 nM and varying amounts of unlabeled glycine were included in the incubation buffer to give final concentrations of glycine ranging from 41 nM to 2.5 μM. Non-specific binding was determined in the presence of 1 mM unlabeled glycine and represented less than 10% of total binding at a concentration of 100 nM [^{3}H]glycine.[39]

Following the incubation, sections were rinsed three times with 2 ml ice-cold buffer followed by a final rinse with 2 ml ice-cold glutaraldehyde (49%):acetone mixture (1:19 v/v) and quickly dried under a warm stream of air to minimize uneven ligand dissociation. Preliminary experiments determined that this rinse procedure optimized the ratio of specific to non-specific binding. Maximum rinse time was less than 5 s.

Tissue sections were apposed to tritium-sensitive film (LKB Ultrofilm ^{3}H) for two to six weeks. A set of radioactive standards (American Radiochemicals, ARC C14) calibrated against whole brain pastes with known amounts of protein–tritium was exposed with each film. Protein values of whole brain paste sections were determined by the Lowry method. Quantitative analysis of the resulting autoradiograms was performed densitometrically using a microcomputer based video densitometer system (Imaging Research, St Catherines, Ontario). Optical density values

were converted to pmol/mg protein using a computer generated polynomial regression analysis which compared film densities produced by the tissue sections to those of radioactive standards.[47] Ten readings per area, bilaterally, were averaged in triplicate sections from at least three animals. Values are expressed relative to protein and represent the mean ± S.E.M. unless otherwise stated.

N-Methyl-D-aspartate-sensitive [^{3}H]glutamate binding

NMDA receptors were labeled with [^{3}H]glutamate under conditions which select for binding to NMDA receptors.[18] Briefly, following a 30-min pre-rinse, sections were incubated with 200 nM [^{3}H]glutamate plus 2.5 μM quisqualate in 50 mM Tris–acetate, pH 7.4, at 4°C. Sections were rinsed three times with 2 ml of buffer and once with a glutaraldehyde–acetone solution.

[^{3}H]Thienylcyclohexylpiperidine binding

PCP receptors were labeled with 20 nM [^{3}H]TCP in 50 mM Tris–acetate in the presence of 1 mM Mg^{2+} at 4°C for 45 min, following a 30-min pre-rinse.[33] One millimolar Mg^{2+} maximizes [^{3}H]TCP binding in frozen rat brain sections.[8] Using this method, binding equilibrium is achieved by approximately 45 min in the presence of 1 mM Mg^{2+}. Sections were rinsed 3 × 1 min with ice-cold buffer.

Data analysis

IC_{50} values for competitors were calculated by logit–log analysis. K_I values were determined by the method of Cheng and Prusoff[7] using the formula $K_I = IC_{50}/(1 + ([L]/K_D))$. Glycine, NMDA and PCP receptor binding distributions were compared with the Pearson product-moment coefficient and linear regression analysis.

RESULTS

Effects of temperature, pH and incubation buffer on strychnine-insensitive [^{3}H]glycine binding

Binding to the strychnine-insensitive glycine site in hippocampal area CA1 was reduced by raising the temperature from 4 to 37°C. Relative to 4°C, binding at 23°C was reduced by 52 ± 6%. Incubation at 37°C reduced binding by 68 ± 8%.

Strychnine-insensitive glycine binding was very sensitive to changes in pH (Fig. 1). Specific binding of [^{3}H]glycine was studied at 10 pH values ranging from 4.0 to 10.0. Binding in the stratum radiatum of CA1 was optimal in the narrow pH range 7.0–7.5.

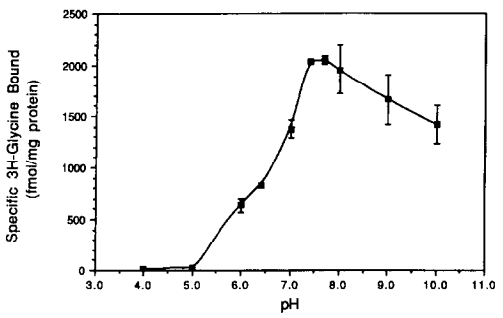

Fig. 1. Effect of pH on strychnine-insensitive [^{3}H]glycine binding in stratum radiatum of the CA1 hippocampal subfield. Rat brain sections were incubated with 100 nM [^{3}H]glycine (4°C) in 50 mM Tris–citrate at the pH indicated. Data represent specific binding (mean ± S.E.M., $n = 3$).

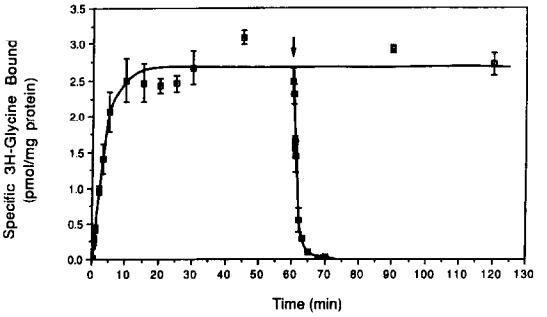

Fig. 2. Time dependence and reversibility of strychnine-insensitive [^{3}H]glycine binding in stratum radiatum of the CA1 area in rat brain sections. Sections were incubated at 4°C with 100 nM [^{3}H]glycine for various times. At 60 min, dissociation was initiated by "infinite dilution" (arrow). Data represent specific binding (binding displaceable by 1 mM unlabeled glycine) and are expressed as mean ± S.E.M. ($n = 3$) in terms of pmol/mg protein.

Specific binding decreased faster at acidic rather than alkaline pH values; there was no specific binding at a pH value of 5. In contrast, at a pH value of 10 binding was reduced by 30%.

The effects of three buffers on strychnine-insensitive glycine binding were evaluated: Tris–citrate, Tris–acetate and Tris–HCl (pH 7.4). Tris–base (50 mM) was used in each of the three buffer systems. Specific equilibrium binding in stratum radiatum of CA1 was greatest in Tris–citrate buffer and was, in comparison, reduced by 11 ± 5% in Tris–acetate buffer and by 19 ± 4% in HCl buffer. The qualitative distribution of glycine binding under each of the buffer conditions was similar.

Kinetics of strychnine-insensitive [^{3}H]glycine binding

At a concentration of 100 nM [^{3}H]glycine, binding in the stratum radiatum of the CA1 area in rat brain reached equilibrium rapidly at 4°C and remained at equilibrium for at least 2 h (Fig. 2). Dissociation of glycine binding (by infinite dilution) was very rapid with a rate constant of 0.76/min. The half-time for receptor–ligand dissociation was approximately 0.92 min. Since the dissociation of glycine was very rapid, the association rate constant was determined to be 2.05 × 10^6/M per min assuming second order kinetics. These rate constant values indicate a dissociation constant (K_D) value of 369 ± 41 nM in the stratum radiatum of hippocampal area CA1.

Saturation of strychnine-insensitive glycine binding

Saturation experiments were performed over the range of 40 nM–2.5 μM glycine (Table 1; Fig. 3). Scatchard transformation of the saturation isotherm demonstrated maximal binding (B_{max}) of 6.2 ± 0.04 pmol/mg protein (stratum radiatum of CA1) to a single population of sites with a K_D of 196 ± 14 nM (Fig. 3 inset). The Hill coefficient from this data was 0.96 ± 0.02 indicating no cooperativity between sites. The discrepancy between K_D determination by kinetic experiments and the equilibrium K_D value (Fig. 3)

Table 1. Regional comparison of parameters of strychnine-insensitive [^{3}H]glycine binding

Region	K_D (nM)	B_{max} (pmol/mg protein)	Hill
SR CA1	196 ± 10	6.2 ± 0.04	0.96 ± 0.02
SR CA3	198 ± 23	4.6 ± 0.4	0.97 ± 0.05
SMDG	169 ± 17	4.3 ± 0.2	1.01 ± 0.03
Med-CPu	200 ± 10	2.9 ± 0.04	1.04 ± 0.07
CICX	250 ± 16	4.9 ± 0.4	1.03 ± 0.03
AV THAL	213 ± 28	3.6 ± 0.3	0.96 ± 0.01

Parameters of strychnine-insensitive [^{3}H]glycine equilibrium binding in six brain regions were determined by Scatchard analysis of saturation isotherms. Rat brain sections were incubated with 40 nM [^{3}H]glycine in the presence of 1 nM to 2.5 μM unlabeled glycine at 4 C for 35 min as described in Experimental Procedures. Values represent specific binding and are presented as mean ± S.E.M. ($n = 3$). There were no significant differences between regional K_D values. SR, stratum radiatum; SMDG, stratum moleculare dentate gyrus; Med-CPu, medial caudate putamen; CICX, layers I, II cingulate cortex; AV THAL, anterior ventral thalamus.

probably reflects the difficulty in accurately determining the association rate constant due to fast dissociation of glycine. Similar K_D values were obtained in other brain regions (Table 1).

Contamination of compounds with glycine

The level of glycine contamination in commercial compounds as assessed by either thin layer chromatography or high pressure liquid chromatography varied considerably between compounds. Less than 0.1% glycine contamination was present in the majority of compounds tested. However, DL-AP5 (from two sources) contained approximately 1% glycine. NMDA and glutamate were also contaminated with low levels of glycine (0.01–0.1%).

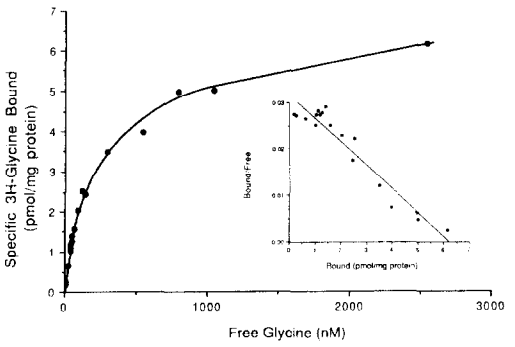

Fig. 3. Representative saturation isotherm and Scatchard analysis (inset) of strychnine-insensitive glycine equilibrium binding in stratum radiatum of the CA1 hippocampal subfield. Rat brain sections were incubated with 40 nM [^{3}H]glycine in the presence of 1 nM to 2.5 μM unlabeled glycine at 4 C for 35 min as described in Experimental Procedures. Each point represents specific binding (total binding minus binding present in adjacent sections incubated in the presence of 1 mM unlabeled glycine). The experiment was repeated six times in six animals with similar results. Averaged data represent mean ± S.E.M.

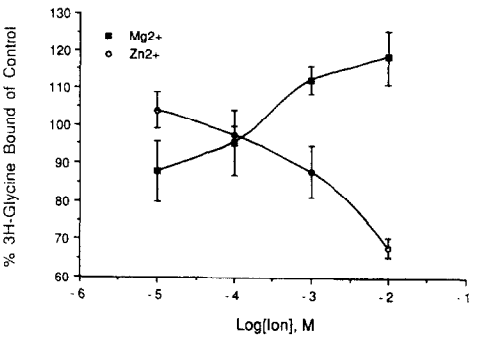

Fig. 4. Effects of magnesium and zinc ions on strychnine-insensitive [^{3}H]glycine binding in stratum radiatum of the CA1 hippocampal area. Data are mean ± S.E.M. ($n = 3$).

Pharmacological characterization of strychnine-insensitive glycine binding sites

All compounds were tested at a single concentration of 100 μM (unless stated otherwise) for their ability to alter strychnine-insensitive [^{3}H]glycine binding (100 nM [^{3}H]glycine). Values represent the mean ± S.E.M. of three animals and were taken from stratum radiatum of hippocampal area CA1 (Fig. 4 and Tables 2 and 3).

Effect of magnesium and zinc

Distinct binding sites on the NMDA receptor–channel complex have been described for the cations, magnesium and zinc. The differential effects of these two ions on specific strychnine-insensitive glycine binding is illustrated in Fig. 4. Magnesium enhanced [^{3}H]glycine binding over the concentration range, 0.1–10 mM ($P < 0.05$, ANOVA). Binding was maximally increased to 120 ± 7% of control at 10 mM magnesium. Binding was slightly reduced at 10 μM magnesium. In contrast, zinc inhibited glycine binding over the concentration range of 10 μM–10 mM ($P < 0.005$, ANOVA). Ten millimolar zinc maximally reduced binding to 68 ± 3% of the control value.

Table 2. Competition for strychnine-insensitive [^{3}H]glycine binding sites in stratum radiatum of CA1 by glycine and structurally related amino acids

Displacer	K_I (μM)	S.E.M.
Glycine	0.13	0.02
D-Serine	0.15	0.02
D-Alanine	0.53	0.14
L-Serine	16.32	3.21
L-Alanine	46.12	8.43
L-Valine	4.24 mM	1.42
D-Valine	> 10 mM	—

Values represent mean ± S.E.M. ($n = 3$). Assays were performed using 100 nM [^{3}H]glycine as described in Experimental Procedures. K_I values were determined assuming a K_D of 198 nM for glycine.

Table 3. Pharmacological profile of strychnine-insensitive [^{3}H]glycine binding

Compounds tested (100 μM)	Percentage inhibition or stimulation of strychnine-insensitive [^{3}H]glycine binding		
	Mean	S.E.M.	*P-value
Inhibitory glycine receptor ligands			
beta-alanine	−44.43	6.15	0.001
Non-active compounds			
Strychnine			
NMDA receptor antagonists			
DL-APV	−61.96	4.67	0.001
DL-APH	−35.04	2.22	0.002
THA	43.44	8.49	0.038
Non-active compounds			
CPP			
PCP			
TCP			
MK-801			
Dextromethorphan			
Glutamate receptor antagonists			
Kynurenate	−83.07	5.1	0.001
DL-2-Amino-4-phosphonobutyric acid (APB)	−26.48	6.2	0.014
Non-active compounds			
gamma-D-Glutamylaminomethylsulfonate (GAMS)			
L-Glutamyl-diethyl ester (GDEE)			
Glutamate receptor agonists			
Non-active compounds			
L-Glutamic acid			
D-Aspartic acid			
L-Aspartic acid			
NMDA			
Ibotenic acid			
Quinolinic acid			
Quisqualic acid			
Kainic acid			
Amino acid-containing compounds			
D-alpha-Aminoadipic acid	−57.33	4.47	0.007
Glycine methyl ester	−100	1.29	0.001
L-Homocysteic acid	−45.34	11.96	0.031
L-Serine-*O*-sulfate (L-SOS)	−63.07	8.12	0.003
O-Phospho-DL-tyrosine	−45.58	24.44	0.03
beta-D-Aspartyl-aminomethylphosphonic acid (ASP-AMP)	−60.37	8.49	0.005
gamma-D-Glutamylglycine	−80.21	6.41	0.001
gamma-L-Glutamylglycine	−99.07	3.59	0.001
gamma-L-Aspartylglycine	−93.97	3.5	0.001
Glycylglycine	−62.07	4.6	0.001
Non-active compounds			
D-Asparagine			
L-Asparagine			
D-Aspartic acid			
L-Aspartic acid			
L-Arginine			
L-Cysteic acid			
L-Cysteine			
L-Cysteine sulfonic acid			
L-Glutamic acid			
Homocarnosine sulfate			
D-Homocysteine sulfinic acid			
D-Homocysteic acid			
D-Phenylalanine			
L-Phenylalanine			
L-Proline			
Taurine			
N-Acetyl-L-aspartic acid			
N-Acetyl-L-glutamic acid			
N-Acetyl-L-aspartyl-L-glutamic acid (NAAG)			
alpha-L-Glutamyl-L-glutamic acid			
Glycyl-L-glutamic acid			

Continued

Table 3.—*Continued*

Compounds tested (100 μM)	Percentage inhibition or stimulation of strychnine-insensitive [^{3}H]glycine binding		
	Mean	S.E.M.	*P-value
GABA receptor ligands			
Non-active compounds			
GABA			
delta-Amino-*N*-valeric acid			
N-Benzoyl-*d*-amino valeric acid			
DL-2,4-Diaminobutyric acid			

Data represent mean ± S.E.M. ($n = 3$) and are expressed as percentage stimulation or inhibition (−) of binding in the presence of displacer relative to the amount of binding in the absence of displacer. All readings were taken from stratum radiatum of the CA1 hippocampal area. Binding assays were performed as described in Experimental Procedures using 100 nM [^{3}H]glycine. All displacers were present at single 100 μM concentrations. Statistical comparisons were made by one-way ANOVA, binding in presence of displacer vs total binding (non-active compounds produced non-significant alterations in [^{3}H]glycine binding, i.e. $P > 0.05$). See Refs 24, 37, 46, 60 for review of the pharmacology of some of these compounds.

Effect of simple amino acids

The potencies of glycine and both the D- and L-isomers of serine, alanine and valine are presented in Table 2. Of these amino acids, glycine was the most potent inhibitor of strychnine-insensitive glycine binding with a K_i value of 0.13 ± 0.02 μM. In general the D-amino acid isomers were more potent than the corresponding L-forms. The rank order of potency in displacing glycine binding was: glycine > D-serine > D-alanine > L-serine > L-alanine > L-valine > D-valine. All these amino acids produced complete inhibition of glycine binding with the exceptions of D- and L-valine which were only evaluated up to concentrations of 10 mM. All Hill coefficients were near unity. The potencies of these amino acids as displacers of [^{3}H]glycine binding in our assay were highly correlated with their ability to inhibit strychnine-insensitive [^{3}H]glycine binding in homogenate studies[28,56,63] ($r^2 > 0.82$; $P < 0.01$; Pearson product-moment), enhance NMDA-stimulated [^{3}H]TCP[56] ($r^2 = 0.98$; $P < 0.01$) and [^{3}H]MK-801[63] ($r^2 = 0.92$; $P < 0.01$) binding in membranes, and stimulate NMDA-mediated [^{3}H]norepinephrine release in whole brain sections[50] ($r^2 = 0.83$; $P < 0.01$). However, much higher concentrations (eight to 40 times greater) were necessary to enhance NMDA-mediated [^{3}H]norepinephrine release.[50]

Effect of strychnine-insensitive glycine receptor antagonists

HA-966 and 7-chlorokynurenate, two competitive antagonists of the strychnine-insensitive glycine site,[14,26] inhibited [^{3}H]glycine binding in a dose-dependent manner (Fig. 5). In stratum radiatum of CA1, 7-chlorokynurenate was 41 times more potent than HA-966 in inhibiting [^{3}H]glycine binding; K_i values were 0.27 ± 0.03 μM (7-chlorokynurenate) and 11.11 ± 0.89 μM (HA-966). Hill coefficients were near unity indicating that these ligands are acting at a single population of glycine sites under the present conditions.

Effect of inhibitory glycine receptor ligands

Strychnine, an antagonist at the inhibitory glycine receptor, did not alter glycine binding at 100 μM. In contrast, beta-alanine significantly reduced strychnine-insensitive glycine binding by 44 ± 6% at a concentration of 100 μM ($P < 0.001$ vs total binding, ANOVA).

Effect of glutamate receptor antagonists

Of the ligands that bind to the NMDA receptor-operated ion channel, only tetrahydroaminoacridine (THA) significantly affected strychnine-insensitive glycine binding; 100 μM THA enhanced glycine binding by 43%. In contrast, similar concentrations of the dissociative anesthetics PCP and its thienyl derivative, TCP, did not alter glycine binding. An effect of MK-801 was also absent. The selective, high affinity, competitive NMDA antagonist, CPP, did not alter binding. In contrast, the aminophosphono series of compounds significantly reduced glycine binding. The rank order of effectiveness of these compounds in inhibiting glycine binding, at a single

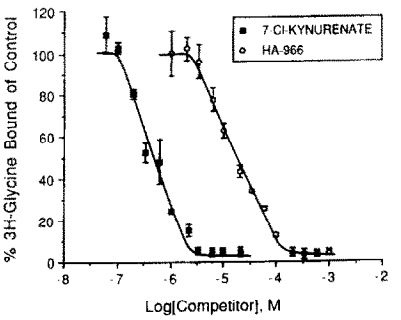

Fig. 5. Displacement of strychnine-insensitive [^{3}H]glycine binding by two competitive antagonists, 7-chlorokynurenate and HA-966. Sections were incubated with 100 nM [^{3}H]glycine at 4 °C in the presence of various concentrations of competitors. Measurements were made in stratum radiatum of hippocampal area CA1. Data are mean ± S.E.M. ($n = 4$).

100 μM concentration, was DL-AP5 > DL-AP7 > DL-AP4 ($-62 \pm 5\%$, $-35 \pm 2\%$, $-26 \pm 6\%$; vs total binding, $P < 0.05$, ANOVA). Additionally, the amino acid-containing NMDA antagonists, D-alpha aminoadipate, ASP–AMP, and gamma-D-glutamylglycine all produced greater than 50% inhibition of glycine binding. Of these compounds, gamma-D-glutamylglycine was the most effective displacer of binding (binding was reduced by 80%). The L-isomer of glutamylglycine completely displaced glycine binding. The non-selective glutamate receptor antagonist, kynurenate, significantly antagonized glycine binding, displacing 83% of binding at a 100 μM concentration ($P < 0.001$, ANOVA). Two additional antagonists with selectivity favoring non-NMDA receptors, GAMS and GDEE, did not alter binding.

Effect of glutamate receptor agonists

Neither L-glutamate nor D- and L-aspartate affected strychnine-insensitive [^{3}H]glycine binding significantly although trends suggested that the D-form of aspartate, in comparison to the L-isomer, produced a greater reduction in binding. None of the prototypic selective glutamate receptor-subtype agonists altered glycine binding. However, the NMDA agonists, L-homocysteic acid and L-serine-*O*-sulfate, both significantly reduced binding by 45 and 63%, respectively ($P < 0.03$, ANOVA).

Effect of amino acid-containing compounds

With the exception of the simple amino acids described above, none of the other amino acids tested altered glycine binding. Of note, taurine, which has been demonstrated to inhibit high affinity glycine uptake in the rat forebrain, does not displace strychnine-insensitive [^{3}H]glycine binding. The methyl ester derivative of glycine completely inhibited glycine binding.

In addition to the amino acid-containing compounds above, several dipeptides also significantly reduced binding. Glutamyl- and aspartyl-glycine both reduced binding by greater than 80% while *N*-glycylglycine only reduced binding by 62% ($P < 0.001$, ANOVA).

Effect of GABA receptor ligands

None of the GABA derivatives tested significantly altered strychnine-insensitive glycine binding at concentrations of 100 μM.

Regional distribution of strychnine-insensitive glycine binding sites

Strychnine-insensitive glycine binding to rat brain sections exhibited a marked regional heterogeneity, with the highest binding density present in the stratum radiatum of the CA1 hippocampal subfield (Table 4 and Fig. 6). Essentially all brain regions showed strychnine-insensitive glycine binding. Within laminated structures, dendritic zones generally had the greatest density of binding with less binding in cellular layers. In general, binding decreased at caudal levels of the neuroaxis. Within the telencephalon, the rank order of binding density was hippocampal formation > cerebral cortex > olfactory nuclei > basal ganglia $\geq$ diencephalic nuclei. Midbrain structures exhibited the least amount of binding and the cerebellum was intermediate.

Within the olfactory bulb, a distinct laminar distribution of glycine binding was present (Fig. 6b); the greatest density of binding was in the external plexiform layer (43% of maximum), intermediate levels in the internal granule layer, and lowest density in the glomerular layer. In addition to the high binding density in the olfactory bulb, the medial and lateral olfactory nuclei also exhibited a high degree of binding (75% of the maximum density of glycine sites).

Cerebral cortical regions demonstrated moderate to high levels of binding. The density of binding varied widely within cortical layers with greatest variation between different cortical regions (up to two-fold). Generally, binding density decreased from superficial to deep cortical layers with the homogeneous distribution pattern present in the entorhinal cortex being the exception. Of the cortical regions, the cingulate had the highest binding density and the frontal and frontoparietal regions exhibited lower levels of glycine binding.

The basal ganglia also revealed a heterogeneous pattern of binding. Binding was greatest in the caudate–putamen, moderate in nucleus accumbens, and low in globus pallidus. Very little binding was present in the entopeduncular nucleus. Marked variation in binding was also observed within these structures. In the corpus striatum, 69% of maximal CA1 binding was present in the ventral posterior region, 55% ventroanteriorally, 44% dorsomedially, and 38% dorsolaterally. In addition, binding densities in the posterior globus pallidus were nearly three times that present more anteriorally. The lateral septum exhibited 43% of maximal CA1 binding while less binding was present in the other basal forebrain structures.

In general, the diencephalic nuclei had moderate levels of [^{3}H]glycine binding. Various nuclei were readily distinguished by the amount of glycine bound. The medial dorsal nucleus had the greatest density of binding, and the habenula contained the fewest binding sites. The anterior ventral, ventral lateral and ventral posterior medial nuclei possessed equivalent levels of binding (46% maximum). The medial and lateral geniculate were readily distinguishable with slightly higher binding in the former. Binding in the nucleus reunions was only 35% of maximum.

The hippocampal formation exhibited a very distinctive laminar distribution of glycine binding (Fig. 6a) that was similar to the topography of NMDA-sensitive glutamate binding (Fig. 7).[18,33,42] The maximal density of binding sites observed anywhere in the brain was found in the stratum radiatum of area CA1. Within the hippocampus, levels were

Table 4. Regional distribution of strychnine-insensitive [^{3}H]glycine binding sites in rat brain

Brain region	Abbreviations	[^{3}H]Glycine bound (fmol/mg protein) Mean ($n = 4$)	S.E.M.	Relative to stratum radiatum of CA1 (%)
Olfactory region				
Glomerular layer	GL	371	16	15
External plexiform layer	EPL	1096	43	43
Internal granule layer	IGL	603	28	24
Medial anterior olfactory nucleus	Med AON	1888	79	74
Lateral anterior olfactory nucleus	Lat AON	1902	86	75
Primary olfactory cortex	POC	1910	87	75
Cortex				
Entorhinal, layers I and II	1,2 ENT	951	142	37
Entorhinal, layer IV	4 ENT	1049	131	41
Entorhinal, layers V and VI	5,6 ENT	1020	95	40
Frontoparietal, layers I and II	1,2 FrPa	1550	100	61
Frontoparietal, layer IV	4 FrPa	1296	95	51
Frontoparietal, layers V and VI	5,6 FrPa	1088	84	43
Frontal, layers I and II	1,2 FRCX	1506	167	59
Frontal, layer IV	4 FRCX	1351	96	53
Frontal, layers V and VI	5,6 FRCX	955	56	38
Cingulate, layers I and II	1,2 CICX	1919	128	76
Cingulate, layers V and VI	5,6 CICX	1566	130	62
Basal ganglia				
Caudate–putamen, medial	Med-CPu	1120	75	44
Caudate–putamen, lateral	Lat-CPu	955	97	38
Caudate–putamen, anterior	Ant-CPu	1396	52	55
Caudate–putamen, posterior	Post-CPu	1748	30	69
Globus pallidus, anterior	Ant-GP	285	7	11
Globus pallidus, posterior	Post-GP	686	13	27
Accumbens nucleus	Acb	1085	66	43
Entopeduncular nucleus	EPN	126	15	5
Basal forebrain				
Ventral limb diagonal band	VLDB	417	25	16
Bed nucleus stria terminalis	BNST	617	47	24
Lateral septum	Lat-SEP	1163	125	46
Diencephalic nuclei				
Medial dorsal	MD-THAL	1396	91	55
Anterior ventral	AV-THAL	1183	81	47
Ventral lateral	VL-THAL	1155	74	46
Ventral posterior medial	VPM-THAL	1172	62	46
Habenula	HB	99	55	4
Lateral geniculate	LG	1164	71	46
Medial geniculate	MG	1312	91	52
Reunions	RE	887	76	35
Hippocampal formation				
CA1, stratum lacunosum moleculare	SLM-CA1	1791	133	71
CA1, stratum radiatum	SR-CA1	2536	144	100
CA1, stratum pyramidale	SP-CA1	1635	142	64
CA1, stratum oriens	SO-CA1	2062	119	81
CA3, stratum radiatum	SR-CA3	1707	100	67
CA3, stratum pyramidale	SP-CA3	741	75	29
CA3, stratum oriens	SO-CA3	1566	100	62
CA4	CA4	796	87	31
Dentate gyrus, stratum moleculare	SMDG	2260	142	89
Brainstem				
Central gray	CG	410	60	16
Inferior colliculus	IC	152	39	6
Substantia nigra	SNR	275	37	11
Cerebellum				
Molecular layer	Mol-Cb	106	54	4
Granule layer	Gr-Cb	753	89	30

Values are presented as the mean ± S.E.M. ($n = 4$) and as a percentage relative to stratum radiatum of the CA1 hippocampal subfield. [^{3}H]Glycine concentration was 100 nM. Autoradiography and quantitation of autoradiograms were performed as described in Experimental Procedures.

greater in CA1 than in CA3 and lowest in the hilus. The rank order of binding densities within CA1 and CA3 subfields was stratum radiatum > stratum oriens > stratum pyramidale. Eighty-nine per cent of maximal binding was present in the stratum moleculare of the dentate gyrus.

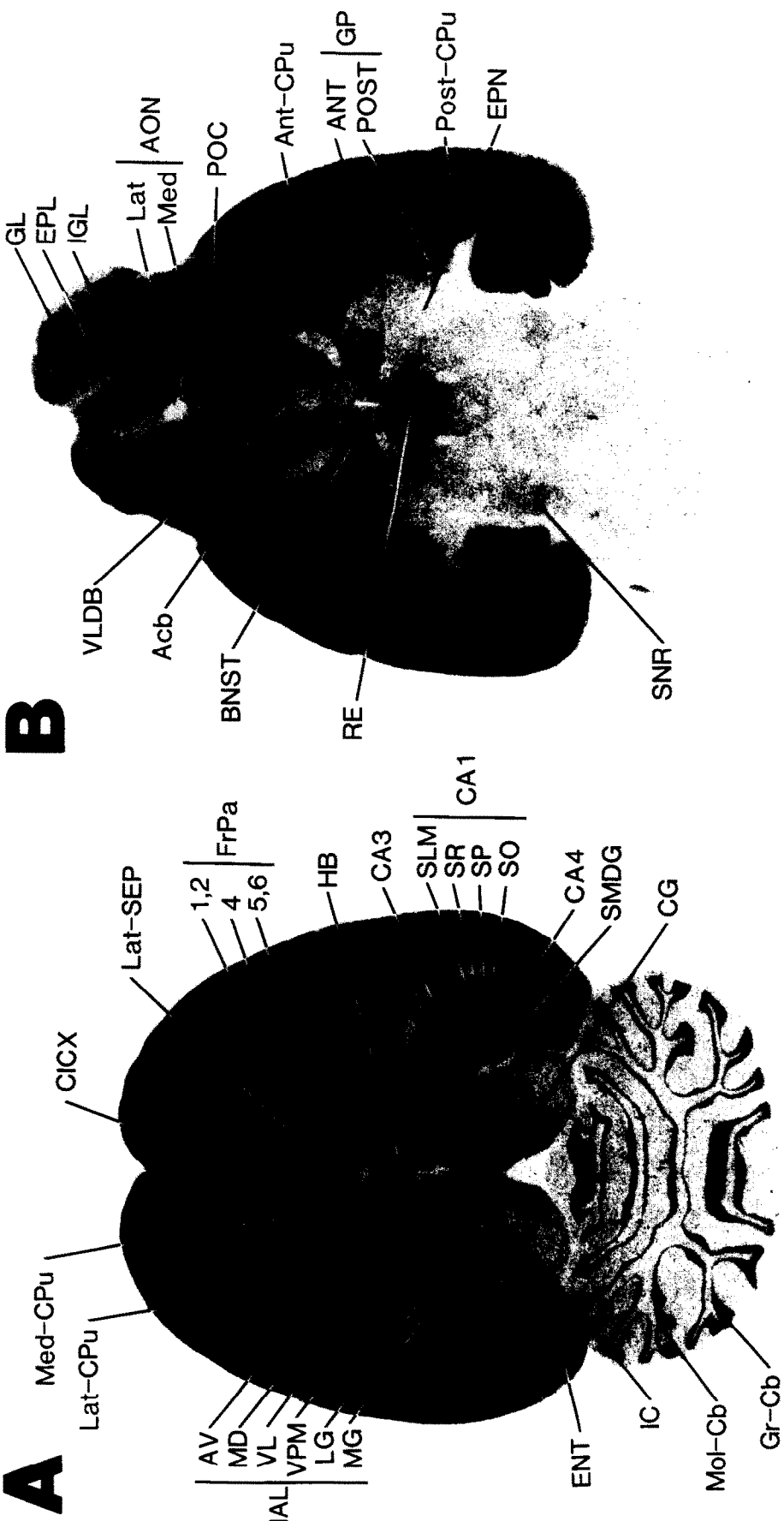

Fig. 6. Representative autoradiograms of strychnine-insensitive [^{3}H]glycine binding to horizontal sections of rat brain (A, dorsal section; B, ventral section). Sections were incubated in 100 nM [^{3}H]glycine and autoradiograms were generated as described in Experimental Procedures. See Table 4 for abbreviations.

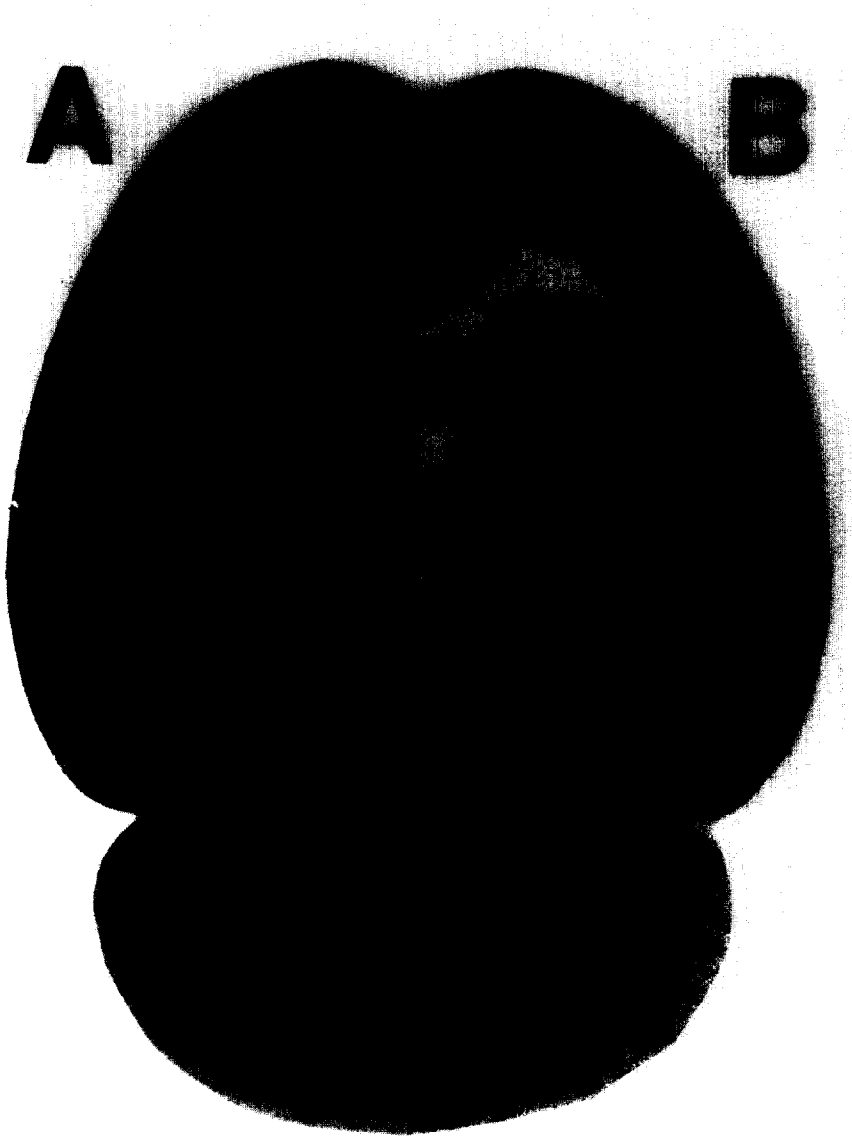

Fig. 7. Comparison of representative autoradiograms of strychnine-insensitive [^{3}H]glycine binding (A) and NMDA-sensitive glutamate binding (B). Glycine binding was carried out using 100 nM [^{3}H]glycine as described in Experimental Procedures. NMDA-sensitive [^{3}H]glutamate binding was performed with 200 nM [^{3}H]glutamate in the presence of 2.5 μM quisqualate as described in detail previously.[33] Qualitatively, the distribution of the two binding sites was nearly identical.

Brainstem structures exhibited low levels of binding with the exception of moderate levels present in the central gray, the inferior colliculus and in the substantia nigra. In the cerebellum, distinct laminar binding was observed. Density levels were low in the molecular layer which is the Purkinje cell dendritic zone. Binding in the granule cell layer of the cerebellum was 30% of maximal binding density (seven times greater than the molecular layer).

Comparison of the distribution of binding sites comprising the N-*methyl*-D-*aspartate receptor–channel complex*

A representative comparison of the distribution of strychnine-insensitive [^{3}H]glycine binding sites and NMDA-sensitive [^{3}H]glutamate binding sites in rat brain is illustrated in Fig. 7. Overall, the regional distribution of the two sites was qualitatively very similar. Linear regression analysis comparing the

distribution of strychnine-insensitive [^{3}H]glycine binding, NMDA-sensitive [^{3}H]glutamate binding, and [^{3}H]TCP binding in 30 brain regions revealed a marked degree of concordance using single concentration equilibrium binding values ($r^2 = 0.77$, $P < 0.001$, Pearson product-moment, [^{3}H]glycine vs [^{3}H]glutamate; $r^2 = 0.73$, $P < 0.001$, [^{3}H]glycine vs [^{3}H]TCP).

As we have previously reported,[33] the distribution of NMDA-sensitive [^{3}H]glutamate binding and [^{3}H]TCP binding sites in rat brain is nearly identical ($r^2 = 0.91$, $P < 0.001$) with the exception of the cerebellar granule layer. However, greater variation is observed between the distribution of these two binding sites and the distribution of strychnine-insensitive glycine binding sites. In stratum radiatum of the CA1 hippocampal subfield, the ratio of maximum binding densities obtained from saturation experiments is approximately 1:3.4:4, TCP:glycine:glutamate.

Comparison of single point equilibrium binding density ratios reveals a general pattern among brain regions. Glycine and glutamate binding ratios are presented relative to a TCP binding value of one. In contrast to the lower ratio of glycine to glutamate binding present in the hippocampal formation and cerebral cortical regions, the ratio of glycine to glutamate binding in most other brain regions is reversed: olfactory structures, 8.5:7; basal ganglia, 12:6.6; basal forebrain structures, 10:8.6; thalamic nuclei, 12.6:8.6; brainstem structures, 16:9. The cerebellar granule layer exhibits the largest ratio of glycine and glutamate to TCP binding (60:50:1). The glycine:glutamate binding ratio also varies within structures. The ratio in stratum pyramidale of CA1 is 17:8, compared with a ratio of 7.6:8.2 in the same layer of area CA3.

DISCUSSION

Correspondence to physiologically defined strychnine-insensitive glycine site

In rat brain sections, [^{3}H]glycine interacts with a single population of sites in a saturable and reversible manner. The linear Scatchard plots of saturation data with corresponding Hill coefficients near unity and the displacement studies support binding to a single receptor population. The inability of strychnine and the relative inability of beta-alanine and taurine to displace [^{3}H]glycine binding in our assay distinguishes these sites from the classical inhibitory glycine receptor which is present in high concentrations in spinal cord. Furthermore, the kinetics, pharmacological selectivity, narrow pH dependency, and regional brain distribution of [^{3}H]glycine binding suggest that the sites labeled in this study correspond to the physiologically defined strychnine-insensitive glycine sites.

Strychnine-insensitive glycine binding sites exhibit the appropriate kinetics expected for the physiologically defined site.[23,29] The equilibrium dissociation constant (K_D) derived in this study corresponds well with not only the IC_{50} value for glycine inhibition of [^{3}H]glycine binding in the present study, but also with EC_{50} values for glycine enhancement of NMDA-mediated [^{3}H]TCP and [^{3}H]MK-801 binding.[51,55] The K_D value of 198 nM calculated from rat brain sections in the present study is nearly identical to values reported in membrane homogenate studies.[6,28]

Strychnine-insensitive glycine binding sites also exhibit a ligand specificity indicative of the physiologically defined strychnine-insensitive glycine receptor. The rank order of potency of glycine and the D- and L-isomers of serine, alanine and valine as displacers of strychnine-insensitive [^{3}H]glycine binding in this study is highly correlated with the ability of these ligands to stimulate NMDA-mediated [^{3}H]norepinephrine release[50] and enhance [^{3}H]TCP and [^{3}H]MK-801 binding in biochemical studies.[56,63] A similar amino acid profile has been observed for enhancement of NMDA-induced conductances and NMDA-stimulated calcium influx.[23,51] Furthermore, the threshold concentration of glycine necessary to displace [^{3}H]glycine binding is very similar to the glycine levels sufficient to enhance NMDA-mediated currents in patch clamp studies.[23,29] Except for glycine, serine, alanine and valine, none of the other simple amino acids were effective displacers of [^{3}H]glycine binding in agreement with patch clamp studies and with previous biochemical reports.[5,23,50,51,63] The ability of 7-chlorokynurenate and HA-966 to inhibit [^{3}H]glycine binding in our assay is consistent with their ability to block NMDA-responses and limit NMDA-mediated brain injury.[14,26,41] The ability of several dipeptides to inhibit [^{3}H]glycine binding may be related to their ability to antagonize NMDA responses.[60] Kainate- and quisqualate-type glutamate receptor and GABA receptor selective ligands failed to displace specific strychnine-insensitive glycine binding in agreement with previous biochemical and electrophysiological studies.[3,23]

It is unlikely that [^{3}H]glycine binding represents an enzymatic or transport binding site[20] or simple sequestration since strychnine-insensitive glycine binding was maximal at 4°C and was greatly reduced at 23 or 37°C. In addition, the K_D determined by kinetic experiments was similar to equilibrium K_D suggesting that sequestration is unlikely to play a role in binding. Binding was highest in areas where glycine uptake is lowest. Furthermore, inhibitors of high affinity glycine uptake described in rat forebrain, such as taurine, are ineffective displacers of binding. Taken together with the evidence described above, the high degree of concordance between the distribution of strychnine-insensitive glycine binding sites and the distributions of [^{3}H]TCP and NMDA-sensitive glutamate binding sites strongly suggest that the site labelled by [^{3}H]glycine in this study represents the physiologically defined receptor.

Interactions with magnesium and zinc

Two distinct binding sites on the NMDA receptor–channel complex have been described for the prototypic cations magnesium and zinc. Magnesium gates the NMDA channel in a voltage-dependent manner[13,32,36,45] and has also been shown to both enhance and inhibit [^{3}H]TCP and [^{3}H]MK-801 binding depending on the concentration of magnesium and the state of channel activation.[8,52,64] Magnesium, however, does not alter NMDA-sensitive glutamate binding.[8,44] We found that concentrations of magnesium from 0.1 to 10 mM stimulate glycine binding. This concentration range is consistent with the concentrations needed to block NMDA associated ion channels and alter [^{3}H]TCP and [^{3}H]MK-801 binding.

Zinc also selectively antagonizes the NMDA receptor–channel in electrophysiological, biochemical, and neurotoxicity studies but at a site which is distinct from the magnesium binding site.[15,30,49,52,61] Monahan and Michel[44] have shown that 1 mM zinc antagonizes NMDA-sensitive glutamate binding in membrane homogenates. The concentration range of zinc that inhibits strychnine-insensitive glycine binding (10 μM–10 mM) is in the range of concentrations that attenuate NMDA neuroexcitation (5 μM–1 mM) and NMDA-mediated neuronal injury (30 μM–0.5 mM) in cortical and hippocampal cell cultures and reduce [^{3}H]MK-801 binding (1 μM–1 mM) to rat brain membranes.[19a,30,49,52,61] The antagonistic action of zinc on NMDA-mediated responses and neurotoxicity and [^{3}H]MK-801 binding may be due, in part, to a reduction in agonist activity at NMDA and glycine recognition sites. The distribution of zinc containing presynaptic boutons in rat forebrain (Timm staining method) closely parallels the distribution of glycine, NMDA and TCP recognition sites.[33,48] Staining is highest in the hippocampus, cerebral cortex, olfactory bulb, caudate–putamen and lateral septum, with little staining present in the brainstem and cerebellum. Since high micromolar concentrations of zinc may be achieved in the synaptic cleft upon stimulation, zinc may act as a negative modulator of the NMDA receptor–channel *in vivo*.[2,15]

Relationship of strychnine-insensitive glycine binding sites to the N-*methyl*-D-*aspartate receptor–channel complex*

Qualitatively, the distribution of glycine, NMDA and TCP recognition sites is very similar with the following order of binding densities: hippocampus > cerebral cortex > caudate–putamen ≥ thalamus > cerebellum > brainstem. These data strongly suggest, in addition to the physiological evidence of interactions between sites,[12,22,23,25,31] that these sites are closely associated. Saturation data indicate that the stoichiometry of these sites is 1:3.4:4 (TCP:glycine:glutamate) in the stratum radiatum of the CA1 hippocampal subfield. In the majority of brain regions the ratio of TCP and NMDA-sensitive glutamate binding densities is relatively constant.[33] In contrast, ratios of strychnine-insensitive glycine binding to NMDA-sensitive glutamate binding and TCP binding are much more variable between brain structures. Using single concentration equilibrium binding values, TCP:glycine:glutamate binding ratios varied from 1:7:9 in area CA1 to 1:13:9 in basal ganglia and thalamus, and 1:16:9 in the brainstem. The largest discrepancy occurred in the cerebellum (1:60:50). Since the K_Ds of these binding sites do not vary considerably between brain regions (see Results and Table 1), the single concentration equilibrium values reflect mainly alterations in the maximum number of binding sites and are unlikely to reflect different endogenous concentrations of glycine among various brain regions. The absolute stoichiometric numbers are only estimates of the actual receptor stoichiometry since the single ligand concentration:K_D ratios differed between receptor binding assays [concentration:K_D ratios were: TCP (1:1), glutamate (1:1), glycine (1:2)]. However, the different ligand concentration:K_D ratios do not alter the basic conclusion that the receptor stoichiometry differs between brain regions. These data suggest there may be heterogeneity to the NMDA receptor–channel complex as has been seen with the GABA receptor–channel complex[57,59] and/or that some glycine sites may not be associated with the NMDA receptor–channel complex. The strychnine-insensitive glycine site may have several genetic forms that are expressed differentially among brain regions. For example, a family of nicotinic acetylcholine receptor genes, presumably coding for receptor subtypes, has been described that are expressed in different regions of brain.[10,17]

In contrast to reports describing reciprocal interactions between the NMDA recognition site and the strychnine-insensitive glycine modulatory site,[9,11,27,43] neither selective competitive agonists nor antagonists of the NMDA recognition site were effective displacers of [^{3}H]glycine binding in the present study. Neither L-glutamate, NMDA, nor quinolinate at 100 μM concentrations enhanced [^{3}H]glycine binding. Another report has demonstrated similar results.[35] We found that the selective competitive NMDA antagonist, CPP, does not produce substantial inhibition of [^{3}H]glycine binding. Determination of the allosteric interactions between NMDA and glycine binding sites awaits the development of more selective glycine antagonists. Since dissociative anesthetics and MK-801 did not alter glycine binding, it appears that there is no direct reciprocal interaction between the channel binding site and the strychnine-insensitive glycine site. This observation is consistent with other reports which demonstrated that dissociative anesthetics do not directly affect NMDA-sensitive glutamate binding.[42,44]

In contrast to the effect of CPP, the aminophosphono family of compounds (AP4, AP5, AP7)

were all effective displacers of glycine binding as were several dipeptides (especially those containing glycine). However, the inhibition of [^{3}H]glycine binding by the amino phosphono compounds can be accounted for by the level of glycine contamination present in these compounds. HPLC analysis of glycine content indicated that AP5 (from two sources; Tocris Neuramin, Sigma) contained approximately 1% glycine. At 100 μM, DL-AP5 inhibited [^{3}H]glycine binding by 62%. At this concentration of AP5, 1 μM glycine contamination would be present. Since the K_D of glycine is 200 nM, this level of glycine contamination would be expected to produce approximately 80% inhibition of [^{3}H]glycine binding. The contamination could account for the level of [^{3}H]glycine displacement produced by 100 μM AP5. AP4 and AP7 contained less than 0.1% glycine and this level of glycine contamination would be expected to inhibit [^{3}H]glycine binding by less than 30% at 100 μM AP4 or AP7 (accounting for the degree of inhibition of binding produced by these two compounds). In contrast, the level of glycine present in the glycine containing dipeptides (less than 0.1%) cannot explain the inhibition of [^{3}H]glycine produced by these dipeptides since they all produced 60–100% inhibition of binding at 100 μM concentrations. Most other effective displacers of [^{3}H]glycine binding as well as inactive compounds (NMDA, glutamate) contained low levels of glycine contamination which could not account for their effects on [^{3}H]glycine binding.

The problem of glycine contamination of commercial compounds, water supplies, and glassware may complicate the interpretation of studies examining the function of excitatory amino acid receptor modulators. In autoradiographic experiments, endogenous glycine in the tissue sections is always an issue despite extensive washes. In some patch clamp or binding studies using extensively washed membranes where glycine contamination is thought to be minimal, the levels of glycine in commercial materials becomes problematic. The purity of compounds used in studies of this modulator site should be considered in the interpretation of the results.

Structural requirements of strychnine-insensitive glycine receptor ligands

The structure–activity relations of glycine receptor ligands that can be determined from the inhibition studies of [^{3}H]glycine binding in Tables 1 and 2 confirm and extend results from previous studies of structure–activity relations.[56] The D-forms of simple alpha-amino acids are in general more potent displacers than the L-forms (about 80 times) of strychnine-insensitive glycine binding. This stereospecificity is similar to the structural requirements of antagonists of the NMDA recognition site.[46] A hydrogen on the alpha carbon (glycine), a methylene-hydroxyl group (serine), or a methylene-*O*-sulfate group L-serine-*O*-sulfate (L-SOS) is more active than a single methyl group while all compounds with aspartate length side chains are not active. Of compounds with glutamate-length side chains, the order of activity with respect to the terminal acid group is $SO_3H > PO_3H_2 > COOH$.

The number of atoms separating the amino and carboxyl terminals is also an important factor in determining activity at the strychnine-insensitive glycine receptor. In comparison to glycine, interterminal chain lengths greater than one carbon reduce (beta-alanine, two carbons) or eliminate (GABA, three carbons) activity. This is also true for activity of ligands at the NMDA recognition site.[46,60]

Substitutions of the amino and carboxyl terminals are also important determinants of activity. Replacement of the carboxyl terminal of beta-alanine with a sulfonic group (taurine) eliminates activity. In contrast, addition of a methylester moiety to the carboxyl terminal of glycine (glycine methylester) maintains pronounced activity. Methylation of the primary amine of glycine (*N*-methyl-glycine) eliminates activity at the glycine binding site.[51,56] Of note, methylation of aspartate (*N*-methyl-aspartate) increases activity at the NMDA receptor, whereas larger alkylations decrease activity.[46]

Addition of heteroatoms in the carbon chain connecting the acidic groups appears to be well tolerated. Replacement of the methylene group of homocysteic acid with an oxygen atom (serine-*O*-sulfate) maintains activity. Of the dipeptides with glycine at the gamma-carboxy terminal the order of potency of the acidic terminal of glycine is $COOH > PO_3H_2 > SO_3H$. Furthermore, dipeptides with glycine at the N-terminal are less effective than those with glycine at the C-terminal and non-glycine containing dipeptides are not active at all.

Several common structural features of reported antagonists of the strychnine-insensitive glycine site imply that comformationally restricted and heterocyclic structures are important determinants of antagonists of the glycine receptor. These include the cyclic anhydric analog of GABA, HA-966,[14,60] cycloleucine,[55a] kynurenate, its analog 7-chlorokynurenate,[26] and two heterocyclic quinoxaline derivatives 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX).[4a] The common structural features of these glycine receptor antagonists include planar conformational restriction and functional groups up to at least four atoms from the alpha-amino acid carbon.

Several other putative antagonists of the NMDA recognition site also inhibit strychnine-insensitive glycine binding (gamma-D-glutamylglycine, ASP–AMP, D-alpha-aminoadipate, kynurenate).[24,46,60] In fact, gamma-D-glutamylglycine is more effective in inhibiting glycine binding than NMDA-sensitive glutamate binding (unpublished observation). The effects of these compounds on [^{3}H]glycine binding cannot be explained entirely by non-competitive,

allosteric interations with NMDA recognition sites because the selective NMDA antagonist, CPP, did not reduce [^{3}H]glycine binding. Furthermore, the NMDA agonists, L-SOS and L-homocysteic acid, also inhibited [^{3}H]glycine binding. Glycine contamination cannot account for the inhibition of [^{3}H]glycine binding produced by these compounds. The structural features of the dipeptide-type NMDA antagonists indicated above are consistent with the features of glycine receptor ligands, particularly the antagonists. It is therefore likely that the structural requirements for antagonists of both NMDA and glycine sites partially overlap.

CONCLUSION

The results presented in this paper provide the first detailed information about the regional brain distribution of strychnine-insensitive [^{3}H]glycine binding. The data extend earlier descriptions of the pharmacology of strychnine-insensitive [^{3}H]glycine binding and demonstrate that this labeled site corresponds to the physiologically defined site. The results also indicate that the stoichiometry between the modulatory sites comprising the NMDA receptor complex vary among brain regions. The preliminary studies of the structure–activity relations of strychnine-insensitive glycine receptor ligands could be of considerable value in the development of selective agonists and antagonists for the glycine modulatory site. Antagonists of the strychnine-insensitive glycine site may prove to be an effective treatment in inherited juvenile non-ketotic hyperglycinemia[16] as well as other neurologic disorders.[19,38,40,41,53,54]

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