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Interaction of L-Glutamate and Magnesium with Phencyclidine Recognition Sites in Rat Brain: Evidence for Multiple Affinity States of the Phencyclidine/*N*-Methyl-D-Aspartate Receptor Complex

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SUMMARY

Biochemical and electrophysiological studies have provided evidence that a complex comprising the *N*-methyl-D-aspartate (NMDA)-type excitatory amino acid (EAA) receptor and the phencyclidine (PCP) recognition site exists in mammalian brain. This complex, which has been compared to that established for the inhibitory amino acid, γ-aminobutyric acid, and the benzodiazepine anxiolytic, diazepam, is sensitive to the effects of the divalent cation Mg^{2+}, which has suggested the presence of a third, ion channel component. Using a radioreceptor assay for the PCP receptor, L-glutamate (L-Glu) produced a concentration-dependent increase in the binding of [^{3}H]thienyl cyclohexylpiperazine ([^{3}H]TCP) in well washed membranes from rat forebrain. The EAA produced a maximal increase in specific binding of 400%, with an EC_{50} value of 340 nM. The ability of L-Glu to enhance [^{3}H]TCP binding was 10-fold more potent in the presence of 30 μM Mg^{2+}, which inhibits NMDA-evoked responses in intact tissue preparations and produces a 50% increase in [^{3}H]TCP binding on its own. Analysis of saturation curves indicated that the effect of both L-Glu and Mg^{2+} could be attributed to an increase in receptor affinity as well as increases in the proportion of a high affinity state of the PCP-binding site. Assessment of the effect of a number of EAAs on basal [^{3}H]TCP binding (well washed membranes in the absence of either L-Glu or Mg^{2+}) showed that the EAA recognition site involved in the effects of L-Glu was the NMDA subtype. Further studies examined a series of compounds thought to interact with either the NMDA or PCP components of the receptor complex under four binding conditions: basal, +Mg^{2+}; +L-Glu; and +Mg^{2+}/L-Glu. These results showed that dissociative anesthetics, such as dexoxadrol and PCP, as well as the novel anticonvulsant MK-801, selectively interact with the high affinity state of the PCP receptor. NMDA antagonists, such as CPP, were also found to inhibit binding to the high affinity state of the PCP receptor, although not as potently as the dissociative anesthetics. Interestingly, the NMDA antagonists did not inhibit any of the binding to the low affinity state of the receptor. The σ ligands (±)-SKF 10,047 and haloperidol recognized two components of [^{3}H]TCP binding only in the presence of L-Glu. The results of the present study are consistent with the finding that agonists of the NMDA receptor induce a high affinity state of the PCP receptor.

PCP is a dissociative anesthetic which, in addition to its anesthetic properties, elicits psychotomimetic effects in the mammalian central nervous system. Considerable controversy exists concerning the mechanism by which this compound elicits its action at the membrane level (1). Several different neurotransmitter receptors, including the σ-type opiate receptor, have been implicated in this role. Currently, PCP is thought to bind to distinct recognition sites in brain tissue, which can be labeled by [^{3}H]PCP (2) or with higher affinity by the PCP analog [^{3}H]TCP (3).

Recent evidence (4–7) has indicated that the possible existence of a PCP receptor complex, consisting of the PCP recognition site, the NMDA EAA receptor subtype, and an ion channel. PCP inhibits NMDA-elicited action potentials in mammalian neurons (7), and similar effects have been seen in regard to the blockade of NMDA-evoked acetylcholine release in brain slices (5). Furthermore, Glu profoundly increases the binding of [^{3}H]TCP in washed rat forebrain membranes (8). Thus, it would appear that there may be a close association of NMDA-type Glu and PCP receptors in certain areas of rat brain. In this regard, it is interesting that Maragos *et al.* (9) have reported that there is a similar distribution of [^{3}H]TCP-

ABBREVIATIONS: PCP, phencyclidine; AP4, 2-amino-4-phosphonobutanoate; AP5, 2-amino-5-phosphonopentanoate; AP6, 2-amino-6-phosphonohexanoate; AP7, 2-amino-5-phosphonoheptanoate; BW 234U, cis-9-[3-(3,5-dimethyl-1-piperazinyl)propyl]-9H-carbazole dihydrochloride; CPP, 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid; EAA, excitatory amino acid; EDTA, ethylenediaminetetraacetate; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine; NMDA, *N*-methyl-D-aspartate; 3-PPP, 13-[3-hydroxyphenyl]-*N*-(1-propyl(piperidine; (±)-SKF 10,047, (±)-*N*-allylnormetazocine; TCP, 1-[1-(2-thienyl)cyclohexyl]piperidine.

and NMDA-sensitive Glu sites labeled by [^{3}H]Glu in rat brain. Recently, Jarvis *et al.* (10) have demonstrated similar findings using the radioligand [^{3}H]CPP to label the NMDA receptor.

The putative interaction between PCP and NMDA-binding sites may also involve magnesium. Several investigators have previously shown that excitatory amino acid-induced responses are Mg^{2+} sensitive (11–13). In addition, Honey *et al.* (14) have proposed, based on the ability of ketamine and PCP to block responses of L-aspartic acid, that an NMDA/PCP interaction may exist and that magnesium may be involved in this interaction. Additionally, it might be expected that if both PCP and Mg^{2+} antagonize NMDA effects, then there may be significant interactions between PCP and magnesium.

Using membranes prepared from rat forebrain and depleted of divalent cations and L-Glu by extensive washing, the effects of L-Glu, Mg^{2+}, and a number of pharmacological probes were examined for their effects on binding in order to better understand the relationship between the two recognition sites and the pharmacological significance of this interaction.

Materials and Methods

Membrane preparation. Forebrains from male Sprague-Dawley rats [150–250 g; Marland Farms, Mbf:(SD)] were homogenized in 50 volumes original tissue weight of 5 mM Tris-HCl buffer (pH 7.7) using a Brinkmann Polytron, setting 6, for 25 sec. Homogenates were centrifuged for 10 min at 50,000 × *g*. Pellets were resuspended in 50 volumes of 5 mM Tris-HCl containing 10 mM disodium EDTA, incubated at 37° for 10 min, and recentrifuged. Pellets were washed a second time by resuspension and centrifugation. The pellet from the second wash was resuspended in 50 volumes of 5 mM Tris-HCl and stored in suspension at −20° for at least 5 days. On the day of the experiment, the suspension was thawed, centrifuged, and washed an additional two times by resuspension and centrifugation. The final pellet was resuspended in 250 volumes original tissue weight of 5 mM Tris-HCl buffer, pH 7.7 (0.2 mg of protein/ml). This wash procedure has been shown to virtually eliminate endogenous Glu (8).

Receptor binding. Binding assays were initiated by the addition of 1.0 ml of membrane suspension to test tubes containing 5 nM [^{3}H] TCP (100 μl) and, when applicable, either 30 μM $MgCl_2$ (100 μl), 0.5 μM L-glutamic acid (100 μl) or Mg^{2+} and L-Glu, or 200 μl of drug or buffer. The remaining volume to bring the final assay volume to 2 ml consisted of 5 mM Tris-HCl buffer (pH 7.7). All assays were performed in triplicate.

The tubes were incubated for 2 hr in a 25° water bath. The reaction was terminated by filtration under reduced pressure through Whatman GF/B filters presoaked in 0.05% polyethylenimine. The filters were then rapidly washed with three aliquots of 6.5 ml of 5 mM Tris-HCl buffer. Radioactivity bound to the filter was measured by liquid scintillation spectrometry in 2.5 ml of Aquassure. Specific binding of [^{3}H] TCP was defined as total binding minus the binding of the presence of 100 μM dexoxadrol. This concentration of dexoxadrol was found to inhibit total binding to the same level of cpm as each compound that inhibited 100% of specific binding under all binding conditions.

In the experiments examining the effect of EAAs on [^{3}H]TCP binding, 9–12 different concentrations of the EAA were incubated with a radioligand concentration of 5 nM. In the saturation experiments, 20–26 different concentrations of radioligand ranging from 0.1 to 130 nM were examined. In the competition experiments, 8–12 different concentrations of competitor were incubated with a radioligand concentration of 5 nM.

Protein concentrations were determined using the Bio-Rad assay (15).

Drugs. Drugs generously donated by the following companies or agencies were: MK-801 (Merck, Sharp and Dohme Research Laboratories, West Point, PA), SKF 10,047 (Sterling-Winthrop Research Institute, Rensselaer, NY), haloperidol (McNeil Pharmaceuticals, Spring House, PA), BW 234U (Burroughs Wellcome Pharmaceuticals, Research Triangle Park, NC), tiletamine (Warner-Lambert, Morris Plains, NJ), and ketamine (Parke-Davis, Detroit, MI). Dexoxadrol, levoxadrol, PCP, CPP, (−)-3-PPP, and (+)-3- PPP were synthesized in the Drug Discovery Division, CIBA-GEIGY (Summit, NJ), and D,L-AP-7 was synthesized in Central Research, CIBA-GEIGY (Basel, Switzerland). D,L-AP4 and D,L-AP6 were purchased from Tocris Chemicals (Essex, England). D-AP5 was purchased from Cambridge Research Biochemicals, Ltd. (New York, NY). All other drugs were purchased from Sigma Chemical Co. (St. Louis, MO). Haloperidol and (±)-SKF 10,047 were dissolved in 10% ethanol/5 mM Tris buffer. All other compounds were dissolved in buffer.

Computer analysis of binding data. The results from replicate binding experiments were analyzed by nonlinear regression analysis using RS1 (Bolt, Beranek and Newman, Boston, MA). Binding equations describing the interaction of drug with two non-interconvertible classes of receptor were utilized. As described recently by Abramson *et al.* (16), these equations produce relatively accurate assessments of the concentration of the binding sites as well as the affinity of these components for competitive ligands, regardless of whether the two components are interconvertible or represent two distinct binding sites. Whether a two-component binding model fit the data significantly better than a one-component model was determined using the partial *F* test ($p < 0.01$). A three-component binding model was not found in any experiment to fit the data significantly better than a two-component model. The results shown are represented as the mean ± standard error generated by this analysis.

Results

The excitatory amino acid L-Glu has been shown to produce a substantial increase in the binding of [^{3}H]TCP to well washed rat forebrain homogenates (8). In the present study, L-Glu produced a concentration-dependent increase in specific [^{3}H] TCP binding (Fig. 1), with a maximal effect, occurring at a concentration range of 10–100 μM, of approximately 400% over basal binding (in the absence of either cation or L-Glu). The EC_{50} value was 340 ± 31 nM ($N = 3$; mean ± SE). Nonspecific binding was unaltered by the EAA.

Since Mg^{2+} appears to inhibit NMDA-induced excitation (11–13, 17), the effect of this cation on [^{3}H]TCP binding was also examined. Magnesium alone increased specific binding to a maximum of 50% at 300 μM (Fig. 2). When this experiment was repeated in the presence of 100 μM L-Glu (a concentration which produces a maximal increase in binding), binding of [^{3}H] TCP was found to be increased up to 400% over basal binding at a Mg^{2+} concentration of 30 μM. Interestingly, at concentrations greater than 30 μM, Mg^{2+} was found to inhibit the binding of [^{3}H]TCP in the absence or presence of L-Glu. Nonspecific binding was unaltered by the divalent cation.

To determine whether Mg^{2+} would enhance the effect of L-Glu, different concentrations of the EAA were again incubated in the [^{3}H]TCP binding assay, but now in the presence of 30 μM Mg^{2+}. As shown in Fig. 1, Mg^{2+} not only enhanced the maximal effect of L-Glu (to 500% over basal conditions), but also lowered the EC_{50} value for the amino acid by almost 10-fold (See Table 1).

Subsequently, the effects of other EAAs on [^{3}H]TCP binding were investigated. In the absence of Mg^{2+}, L-Glu and quisqualate produced the greatest maximal stimulation in [^{3}H]TCP binding, approximately 400% over that found under basal conditions (Table 1), whereas NMDA and D-Glu produced the smallest maximal increase in [^{3}H]TCP binding. Interestingly, in the presence of 30 μM Mg^{2+}, all of the EAAs produced an

increase over basal binding of approximately 500%. Comparison of the EC_{50} values for these compounds showed that L-Glu was at least 10-fold more active than any other EAA in the absence of Mg^{2+}, whereas D-Glu was approximately 30-fold less active than its L-isomer. Although quisqualate elicited a maximal stimulation of binding similar to that of L-Glu, it was the least potent EAA, being almost 100-fold less potent than L-Glu. In the presence of Mg^{2+}, a similar order of activity was noted, with the EC_{50} value being decreased by a factor of 8 (*l*-aspartate)- to 16-fold (quisqualate).

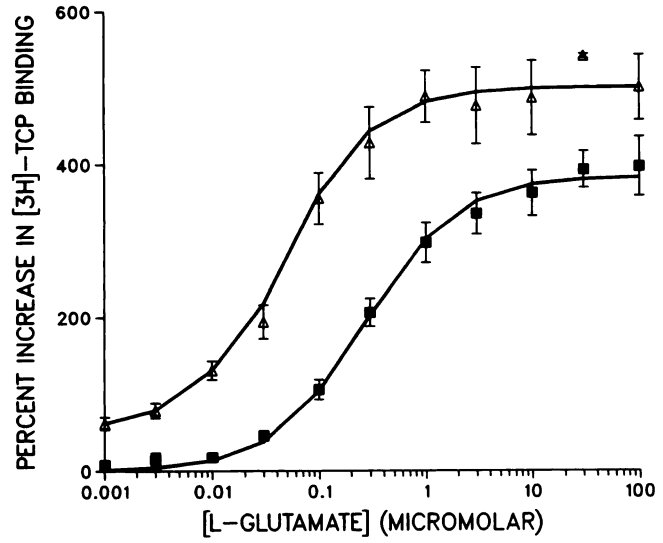

Fig. 1. Effect of L-Glu on specific binding of [^{3}H]TCP in the absence (■) and presence (Δ) of 30 μM Mg^{2+}. Values shown are the average ± standard error of three to four replicate experiments performed in triplicate.

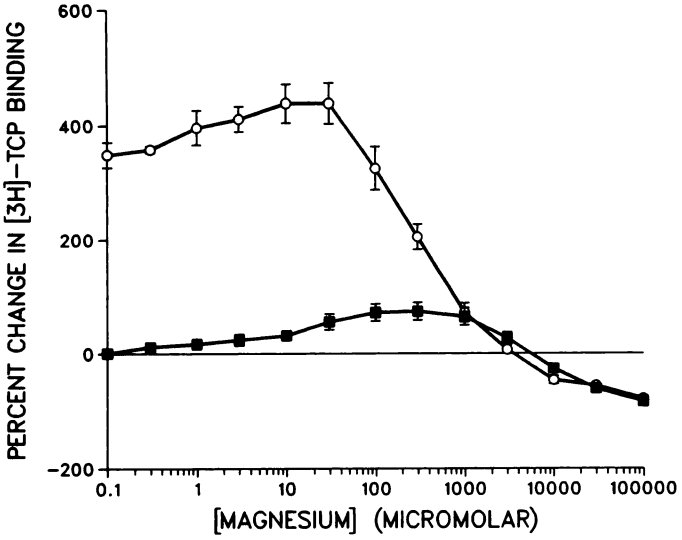

Fig. 2. Effect of Mg^{2+} on specific binding of [^{3}H]TCP in the absence (■) and presence (○) of 100 μM L-Glu. Values shown are the average ± standard error of three to four replicate experiments performed in triplicate.

To further examine the effects of L-Glu and Mg^{2+} on [^{3}H]TCP binding, a series of saturation experiments was conducted using 20–26 different concentrations of [^{3}H]TCP over the range of 0.1–130 nM. A single component was revealed by computer analysis under basal conditions (Fig. 3, Table 2), with a K_d value of 110 nM and an apparent B_{max} of 2700 fmol/mg of protein. In the presence of 30 μM Mg^{2+} or 100 μM L-Glu, a single binding component was again observed. Although a similar apparent B_{max} was obtained as determined under the basal conditions, the K_d value was decreased by 2-fold in the presence of Mg^{2+} to 53 nM, and by 6-fold in the presence of L-Glu to 19 nM (Table 2, Fig. 3). However, inclusion of L-Glu in the presence of 30 μM Mg^{2+} resulted in the appearance of two binding components: a high affinity component with a K_d value of 4 nM, and a lower affinity component with a K_d value of 92 nM, which is in good agreement with the value obtained under basal conditions (110 nM). The respective apparent B_{max} values were 1100 and 1700 fmol/mg of protein. The sum of these (2800 fmol/mg of protein) was in good agreement with the apparent number of binding sites seen under the other assay conditions. A lower concentration of L-Glu (500 nM) was also found to increase the affinity of [^{3}H]TCP (21 different ligand concentrations ranging from 0.1 to 55 nM) in the absence of Mg^{2+} to 28 ± 1 nM (Fig. 3C). In the presence of 30 μM Mg^{2+}, 500 nM L-Glu produced an effect on binding similar to that produced by 100 μM L-Glu under the same assay conditions (Fig. 3, E and F). Computer analysis of the binding data revealed B_{max} values of 570 ± 270 and 2000 ± 170 fmol/mg of protein, and K_d value of 3 ± 1 nM and 24 ± 8 nM for the high and low affinity components, respectively.

In order to obtain a better understanding of the pharmacology of the receptor labeled by [^{3}H]TCP under the different binding conditions, a series of compounds interacting with either NMDA- or PCP-related systems, or with the putative σ receptor were evaluated for their effects on [^{3}H]TCP binding. In this series of experiments, L-Glu was included at a final concentration of 500 nM, the concentration at which a 50% maximal response was seen in the absence of Mg^{2+} and a full response in the presence of the divalent cation.

The dissociative anesthetics dexoxadrol, ketamine, tiletamine, and PCP were examined for their ability to inhibit [^{3}H]TCP binding under the different binding conditions. The results of this experiment are summarized in Table 3 and the inhibition curves produced by dexoxadrol are shown in Fig. 4.

TABLE 1
Effect of excitatory amino acids on the binding of [^{3}H]TCP

Compound	No magnesium		Plus magnesium	
	% Increase	EC_{50}	% Increase	EC_{50}
		nM		nM
L-Glutamate	390 ± 6[a]	340 ± 31	500 ± 8	37 ± 4
D-Glutamate	280 ± 9	11,000 ± 1400	510 ± 9	1000 ± 95
NMDA	250 ± 5	4,000 ± 380	450 ± 12	350 ± 56
L-Aspartate	330 ± 5	2,000 ± 95	520 ± 10	240 ± 27
D-Aspartate	320 ± 6	2,000 ± 140	510 ± 8	160 ± 16
Quisqualate	400 ± 18	31,000 ± 4800	490 ± 12	2000 ± 240

[a] Values represent the mean ± standard error of three separate experiments performed in triplicate.

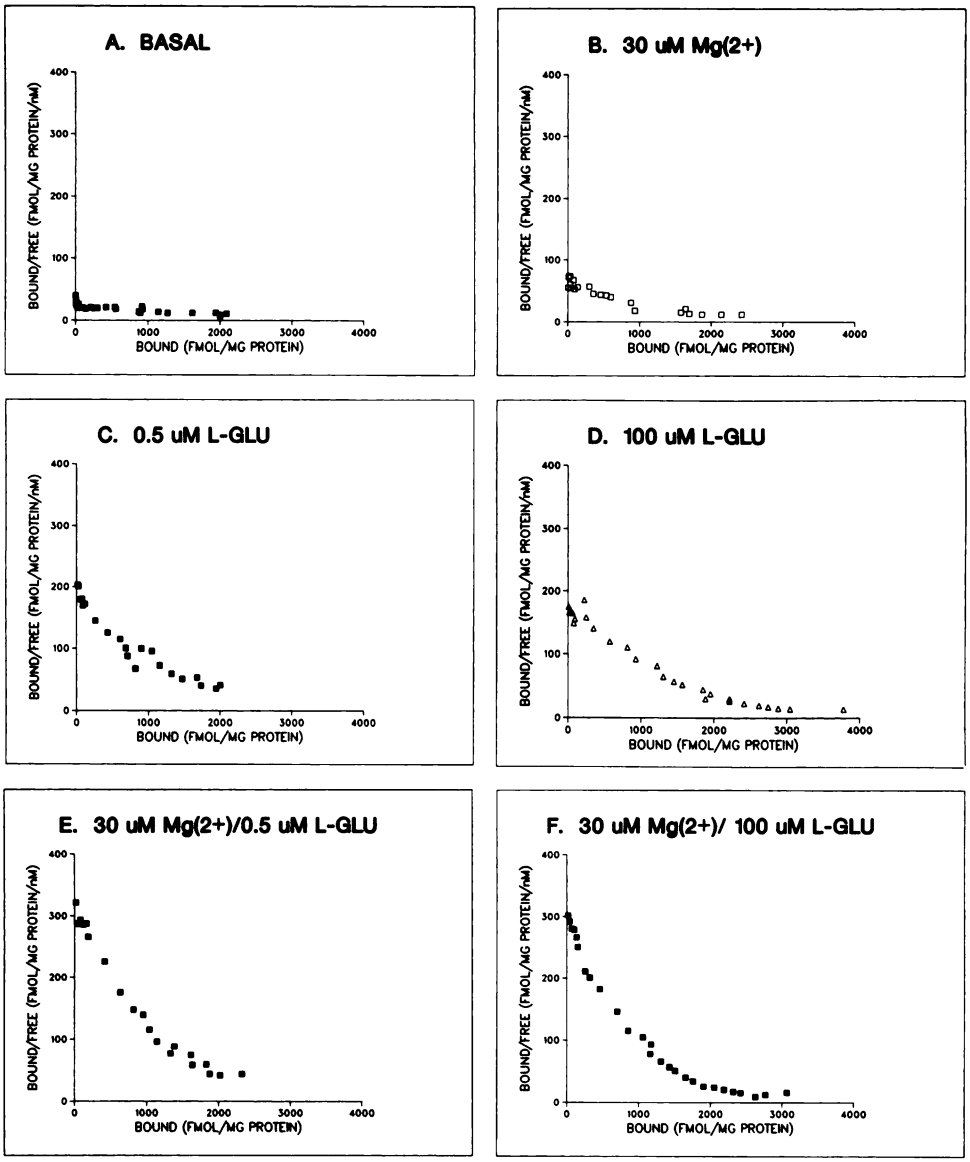

Fig. 3. Scatchard plots of [^{3}H]TCP binding: A, under basal conditions. B, in the presence of 30 μM Mg^{2+}; C, in the presence of 0.5 μM L-Glu; D, in the presence of 100 μM L-Glu; E, in the presence of 30 μM Mg^{2+} and 0.5 μM L-Glu; and, F, in the presence of 30 μM Mg^{2+} and 100 μM L-Glu. Each *curve* is representative of three separate experiments performed in triplicate, where 20–26 different concentrations of radioligand were examined for A, B, D, and F and 21 different concentrations were examined for C and E.

TABLE 2

Binding parameters derived from computer analysis of saturation experiments of [^{3}H]TCP binding

Binding conditions	B_{max} value	K_d value
	fmol/mg protein	*nM*
Basal	2700 ± 150[a]	110 ± 10
Magnesium (30 μM)	2200 ± 72	53 ± 4
Glutamate (100 μM)	2500 ± 47	19 ± 1
Magnesium (30 μM)/glutamate (100 μM)		
High	1100 ± 180	4 ± 1
Low	1700 ± 170	92 ± 45

[a] Values represent the mean ± standard error, as determined by computer analysis, of three separate experiments performed in triplicate.

All four of these compounds produced shallow competition curves ($n_H < 1.0$) under basal binding conditions (Table 3). Analysis of the inhibition curves indicated that a two- rather than a one-component model best described the data. The percentage of binding to the high affinity component was approximately 40% for dexoxadrol and PCP, and 60% for tiletamine and ketamine. The IC_{50} values for this high affinity component ranged from 14 nM for PCP to 3000 nM for ketamine. For the low affinity component, PCP demonstrated the highest affinity (990 nM), whereas ketamine was again the least potent inhibitor (124,000 nM).

In the presence of Mg^{2+}, shallow inhibition curves ($n_H < 1.0$) were again produced. Dexoxadrol, tiletamine, and ketamine were found to be more potent in competing for the binding of [^{3}H]TCP to the high affinity component, whereas the affinity for PCP was slightly reduced (Table 3). The ability of dexoxadrol, tiletamine, and ketamine to inhibit the lower affinity component was increased, whereas the capacity for PCP to inhibit binding to this component was unchanged. The percentage of total binding to the high affinity component ranged from 35% for dexoxadrol and tiletamine to 53% for ketamine.

In the presence of 500 nM L-Glu, dexoxadrol, tiletamine, and PCP still produced shallow inhibition curves. Binding to the high affinity component accounted for 38–84% of total specific binding. Interestingly, the affinity of this component for dexoxadrol and tiletamine decreased in the presence of L-Glu, whereas the affinity for PCP remained the same. Under these

TABLE 3

Binding parameters for dissociative anesthetics and MK-801 from competition curves of [^{3}H]TCP binding

	$\%B_H$	IC_{50H}	$\%B_L$	IC_{50L}	n_H
		nM		nM	
Dexoxadrol					
Basal	38 ± 4[a]	110 ± 38	60 ± 4	8,000 ± 1400	0.50 ± 0.02
Mg^{2+}	35 ± 6	13 ± 4	65 ± 3	2,000 ± 270	0.48 ± 0.02
L-Glu	38 ± 6	32 ± 10	62 ± 6	980 ± 190	0.64 ± 0.02
Mg^{2+}/L-Glu	98 ± 2	61 ± 5	–[b]	–	0.86 ± 0.04
Tiletamine					
Basal	61 ± 4	360 ± 68	39 ± 4	36,000 ± 13,000	0.51 ± 0.03
Mg^{2+}	35 ± 4	16 ± 6	65 ± 4	1,000 ± 250	0.55 ± 0.02
L-Glu	88 ± 1	91 ± 4	9 ± 1	21,000 ± 6200	0.77 ± 0.01
Mg^{2+}/L-Glu	92 ± 3	120 ± 8	7 ± 1	14,000 ± 9500	0.85 ± 0.02
PCP					
Basal	38 ± 3	14 ± 4	62 ± 3	990 ± 130	0.56 ± 0.02
Mg^{2+}	45 ± 7	32 ± 11	57 ± 8	710 ± 160	0.68 ± 0.03
L-Glu	84 ± 7	46 ± 2	13 ± 1	5,900 ± 1300	0.66 ± 0.02
Mg^{2+}/L-Glu	94 ± 1	60 ± 2	–	–	0.84 ± 0.02
Ketamine					
Basal	58 ± 5	3,000 ± 950	47 ± 5	120,000 ± 38,000	0.67 ± 0.03
Mg^{2+}	53 ± 6	1,000 ± 280	45 ± 6	27,000 ± 6,700	0.65 ± 0.01
L-Glu	100 ± 3	1,000 ± 140	–	–	0.65 ± 0.04
Mg^{2+}/L-Glu	106 ± 1	1,000 ± 95	–	–	0.45 ± 0.04
MK-801					
Basal	61 ± 2	190 ± 21	39 ± 2	56,000 ± 10,000	0.45 ± 0.04
Mg^{2+}	75 ± 2	56 ± 4	25 ± 1	35,000 ± 6,700	0.43 ± 0.01
L-Glu	75 ± 3	18 ± 2	25 ± 3	9,000 ± 4,800	0.48 ± 0.03
Mg^{2+}/L-Glu	89 ± 1	6 ± 0.5	11 ± 1	13,000 ± 7,100	0.64 ± 0.02
Levoxadrol					
Basal	93 ± 3	6,000 ± 950	–	–	0.75 ± 0.06
Mg^{2+}	96 ± 2	6,000 ± 480	–	–	0.87 ± 0.06
L-Glu	97 ± 1	3,000 ± 140	–	–	0.96 ± 0.02
Mg^{2+}/L-Glu	98 ± 1	9,000 ± 430	–	–	1.03 ± 0.03

[a] Values represent the mean ± standard error of three separate experiments.
[b] –, a one-component binding model adequately described the binding data.

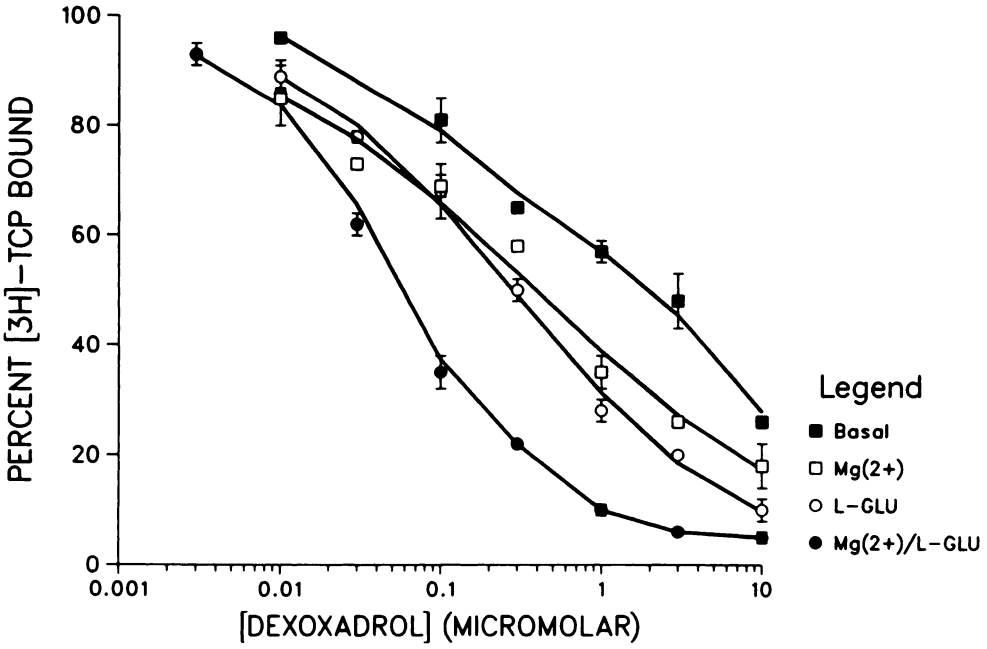

Fig. 4. Dexoxadrol inhibition of per cent of [^{3}H]TCP binding. Values shown are the average ± standard error of three separate experiments performed in triplicate. In these experiments, a minimum of eight concentrations of inhibitor were used; only those concentrations which were common to all three experiments are shown.

binding conditions, the IC_{50} value for PCP to inhibit binding to the low affinity component was increased 8-fold. In contrast, computer analysis indicated that a two-component binding model no longer described the data for ketamine inhibition significantly better than a one-component model. The IC_{50} value for ketamine is 1000 nM.

When L-Glu and Mg^{2+} were both included in the binding assay, steep inhibition curves were obtained for all compounds except for tiletamine, although the percentage of the low affinity component was only 7%. The IC_{50} values for these compounds ranged from 60 nM for PCP and dexoxadrol to 1000 nM for ketamine.

The binding profile of levoxadrol, the stereoisomer of dexoxadrol, is also shown in Table 3. This compound was far less potent in competing for the binding of [^{3}H]TCP and was found to inhibit this radioligand with approximately the same degree of potency under all four binding conditions.

Included in this group of compounds was the novel anticonvulsant MK-801 (18). This compound, like the true dissociative anesthetics, had a shallow inhibition curve in basal conditions. However, when Mg^{2+} and L-Glu were successively added to the binding assay, not only was the percentage of binding to the high affinity component increased, but the affinity of this component for MK-801 was successively increased as well (Table 3). Thus, although this compound produced a binding profile qualitatively similar to that of the dissociative anesthetics, significant quantitative differences exist between the competition curves for this class of compounds as compared to MK-801.

The NMDA antagonists D-AP5, CPP, and D,L-AP7 were examined for their ability to inhibit the binding of [^{3}H]TCP under the four conditions described above. The competition curves for CPP inhibition of [^{3}H]TCP binding are shown in Fig. 5. Under basal conditions, D-AP5 and CPP not only produced shallow inhibition curves, but were only able to inhibit 30–35% of specific binding (Table 4). In the presence of Mg^{2+}, shallow inhibition curves were again generated. However, a greater proportion (65–75%) of the binding was now inhibited and the competition curves were shifted to the left, with a corresponding lowering of the IC_{50} values (Table 4, Fig. 5A). In

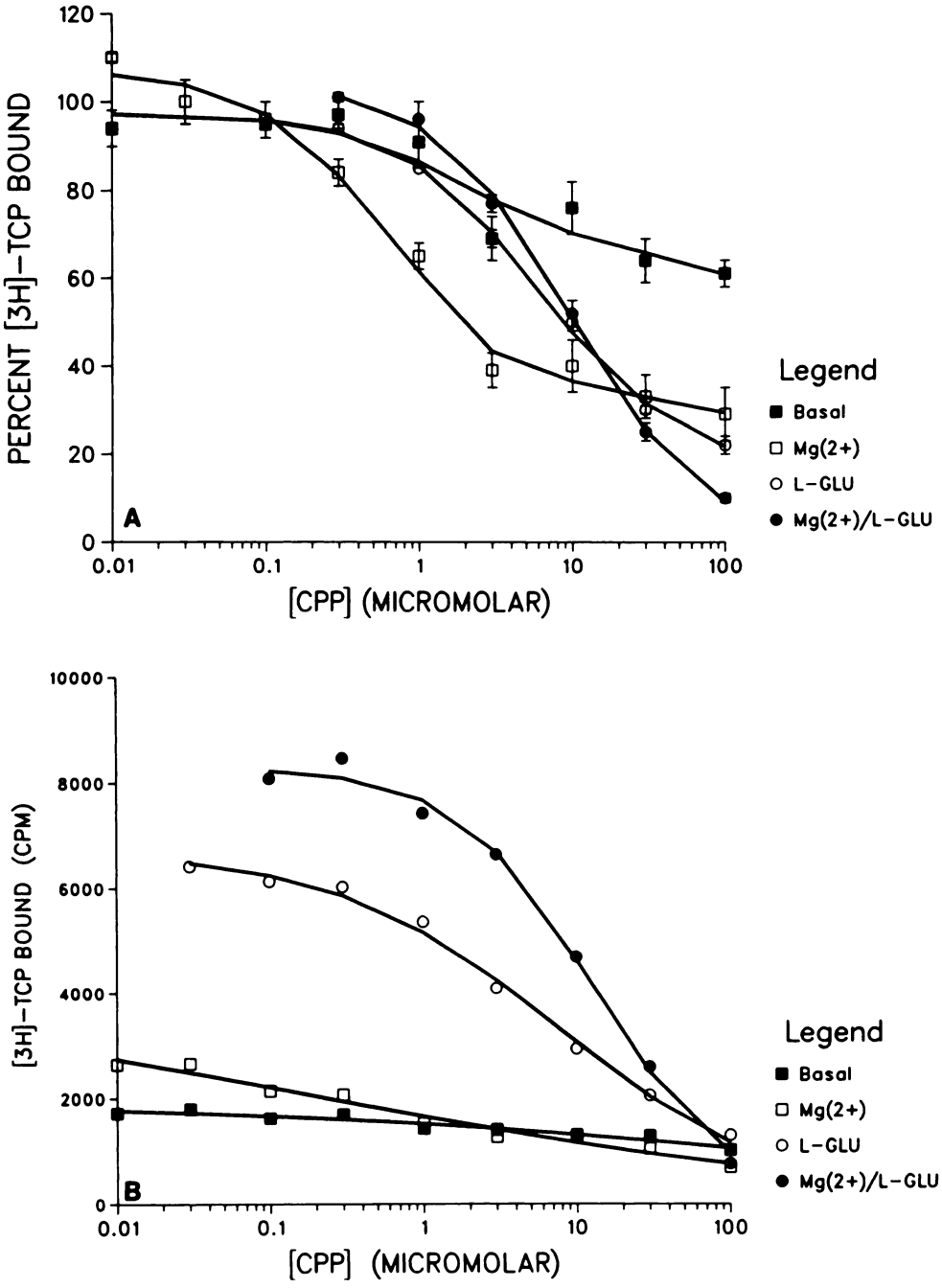

Fig. 5. CPP inhibition of per cent of [^{3}H] TCP binding (A) and amount of specific binding in cpm (B). Values shown are the average ± standard error of three separate experiments performed in triplicate (A) or are representative of the results of three separate experiments (B). For A, a minimum of eight concentrations of inhibitor were used; only those concentrations which were common to all three experiments are shown.

the presence of L-Glu, or Mg^{2+} and L-Glu, the competition curves were shifted to the right with a corresponding decrease in the affinity of the binding component for these compounds. This finding is in contrast to the results obtained for the dissociative anesthetics and MK-801, where Mg^{2+} and L-Glu produced a shift in the competition curves to the left. Additionally, D-AP5 and CPP were now able to inhibit almost all of the specific binding, and a one-component binding model was found to adequately describe the competition curve in the presence of L-Glu and Mg^{2+}.

Plotted as percentage of specific binding, the apparent ability of CPP and D-AP5 to inhibit different percentages of binding appears confounding. However, when the data are plotted as inhibition of amount of specific binding rather than as a percentage, it can be seen, as shown in Fig. 5B, that, actually, a certain *amount* of binding is not inhibited under any condition by CPP. Thus, these NMDA antagonist compounds displayed a completely different binding profile than the dissociative anesthetic compounds in inhibiting the binding of [^{3}H]TCP in the absence or presence of Mg^{2+} or L-Glu.

Unlike D-AP5 and CPP, D,L-AP7 was found to inhibit all of the specific binding of [^{3}H]TCP under all four conditions. In addition, D,L-AP7 showed a much lower affinity than the other antagonist compounds. However, the IC_{50} value was lowest in the presence of Mg^{2+}, as was found for the other NMDA antagonists (Table 4). The compounds D,L-AP4 and D,L-AP6 showed minimal activity under any binding condition; the IC_{50} values were >100,000 nM (n = 2). Thus, it would appear that the NMDA antagonists are able to interact preferentially with the [^{3}H]TCP-binding site in the presence of Mg^{2+}.

Recent studies have suggested that antipsychotic compounds that display activity at σ-type binding sites may interact with PCP-binding sites. To examine whether this class of compounds may selectively inhibit the binding of [^{3}H]TCP in the presence of Mg^{2+} and/or L-Glu, competition curves for the binding of [^{3}H]TCP were generated for (±)-SKF 10,047, (−)-3-PPP, haloperidol, and BW 234U. The results of this experiment are summarized in Table 5 and the competition curves for (±)-SKF 10,047 are shown in Fig. 6. Under basal binding conditions, only (±)-SKF 10,047 generated a shallow inhibition curve where a two-component binding model accurately described the binding data. When Mg^{2+} was included in the assay, with or without L-Glu, steep inhibition curves were generated for all four compounds. Interestingly, shallow inhibition curves were generated by haloperidol and (±)-SKF, 10,047 in the presence of L-Glu, whereas (−)-3-PPP and BW 234U produced steep inhibition curves under these binding conditions. The IC_{50} values for (±)-SKF 10,047 and haloperidol to inhibit the high affinity component were 160 nM and 670 nM, respectively. The compound (+)-3-PPP was inactive under all four binding conditions.

Examination of a series of centrally active compounds to evaluate the specificity of the effects produced at the binding site labeled by [^{3}H]TCP showed that D-ethylketocyclazocine, phenobarbital, metrazole, diazepam, verapamil, naloxone, γ-aminobutyric acid, and methysergide were inactive in their ability to inhibit the binding of [^{3}H]TCP at a concentration of 10,000 nM.

Discussion

The present study demonstrates that multiple components of the binding site for [^{3}H]TCP exist in rat forebrain. The initial finding that L-Glu could significantly increase the binding of [^{3}H]TCP (8) indicated that certain types of Glu receptors were modulating PCP receptors. Comparison of the potency of the different EAAs to increase [^{3}H]TCP binding revealed an order of potency of: L-Glu > L-Asp = D-Asp = NMDA > D-Glu > quisqualate. This is similar to the order of potency found for the inhibition of [^{3}H]-D-AP5 binding (19) and [^{3}H]-L-Glu binding to NMDA receptors (20), indicating that the Glu receptor subtype involved is most likely the NMDA receptor. Consistent with this idea are the findings that the NMDA receptor antagonists CPP and D-AP5 were able to inhibit a portion of the binding of [^{3}H]TCP with high affinity, and that Mg^{2+} is capable of modulating the PCP-binding site. In addition, Fagg and Baud (21) have reported that NMDA antagonists are capable of blocking Glu-induced increases in [^{3}H]TCP binding.

Interestingly, although quisqualate produced the highest degree of stimulation of [^{3}H]TCP binding in the absence of Mg^{2+} (along with L-Glu), it was the least potent of the EAAs examined. One interpretation of this finding is that, although quis-

TABLE 4
Binding parameters for NMDA antagonists from competition curves of [^{3}H]TCP binding

	$\%B_H$	IC_{50H}	$\%B_L$	IC_{50L}	n_H
		nM		nM	
CPP					
Basal	32 ± 5[a]	2,000 ± 950	65 ± 5	>100,000	0.29 ± 0.08
Mg^{2+}	75 ± 2	640 ± 85	25 ± 2	>100,000	0.52 ± 0.05
L-Glu	77 ± 3	5,700 ± 520	20 ± 3	>100,000	0.64 ± 0.03
Mg^{2+}/L-Glu	104 ± 1	9,500 ± 240	–[b]	>100,000	0.96 ± 0.02
D-AP5					
Basal	35 ± 4	790 ± 350	68 ± 3	>100,000	0.30 ± 0.04
Mg^{2+}	65 ± 3	460 ± 105	36 ± 3	>100,000	0.42 ± 0.03
L-Glu	100 ± 2	5,000 ± 480	–	–	0.60 ± 0.04
Mg^{2+}/L-Glu	96 ± 1	7,000 ± 380	–	–	0.79 ± 0.02
D,L-AP7					
Basal	98 ± 2	>100,000	–	–	0.52 ± 0.08
Mg^{2+}	94 ± 2	21,000 ± 1,400	–	–	0.70 ± 0.04
L-Glu	100 ± 2	94,000 ± 9,000	–	–	0.88 ± 0.08
Mg^{2+}/L-Glu	105 ± 2	120,000 ± 12,000	–	–	0.88 ± 0.10

[a] Values represent the mean ± standard error of three separate experiments.
[b] –, a one-component binding model adequately described the binding data.

TABLE 5

Binding parameters for σ-type compounds from competition curves of [^{3}H]TCP binding

	$\%B_H$	IC_{50H}	$\%B_L$	IC_{50L}	n_H
		nM		nM	
(±)-SKF 10,047					
Basal	66 ± 7[a]	820 ± 240	32 ± 8	31,000 ± 18,000	0.65 ± 0.03
Mg^{2+}	100 ± 2	700 ± 30	–[b]	–	0.84 ± 0.04
L-Glu	67 ± 8	160 ± 40	34 ± 9	2,000 ± 950	0.78 ± 0.02
Mg^{2+}/L-Glu	100 ± 1	1,000 ± 28	–	–	0.97 ± 0.02
(−)-3-PPP					
Basal	96 ± 2	28,000 ± 2400	–	–	0.87 ± 0.07
Mg^{2+}	96 ± 2	40,000 ± 480	–	–	0.86 ± 0.08
L-Glu	97 ± 1	28,000 ± 950	–	–	0.88 ± 0.02
Mg^{2+}/L-Glu	99 ± 1	74,000 ± 4300	–	–	0.93 ± 0.07
Haloperidol					
Basal	94 ± 2	7,000 ± 480	–	–	0.78 ± 0.04
Mg^{2+}	93 ± 3	10,000 ± 1400	–	–	0.75 ± 0.06
L-Glu	49 ± 18	670 ± 240	51 ± 9	21,000 ± 7,000	0.64 ± 0.04
Mg^{2+}/L-Glu	99 ± 2	20,000 ± 2400	–	–	0.96 ± 0.08
BW-234U					
Basal	96 ± 1	24,000 ± 1900	–	–	0.94 ± 0.06
Mg^{2+}	99 ± 1	25,000 ± 1900	–	–	1.24 ± 0.15
L-Glu	104 ± 1	63,000 ± 2900	–	–	1.34 ± 0.05
Mg^{2+}/L-Glu	102 ± 1	62,000 ± 2900	–	–	1.37 ± 0.04

[a] Values represent the mean ± standard error of three separate experiments.
[b] –, a one-component binding model adequately described the binding data.

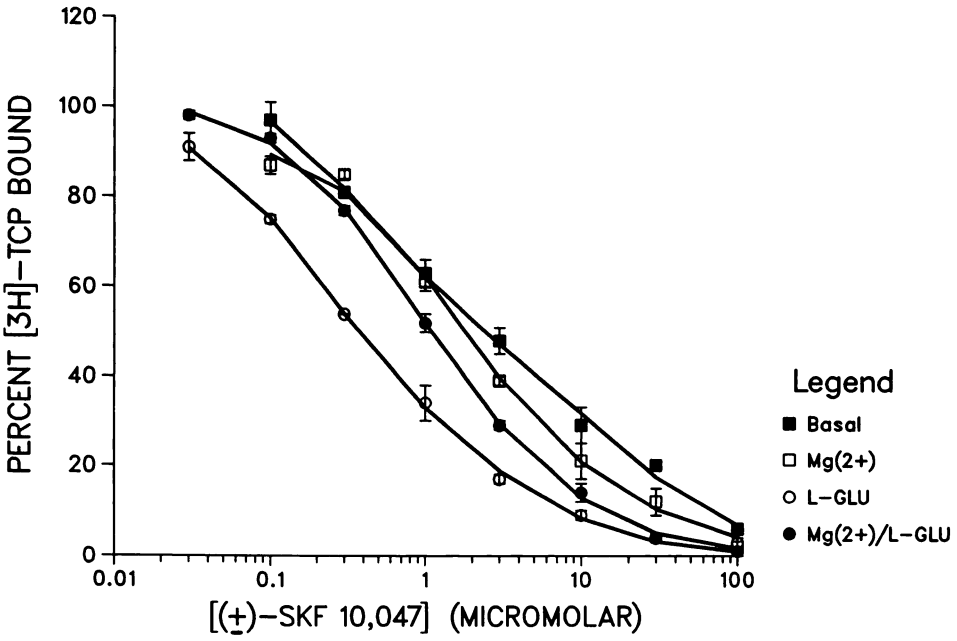

Fig. 6. (±)-SKF 10,047 inhibition of specific [^{3}H]TCP binding. Values shown are the average ± standard error of three separate experiments performed in triplicate. In these experiments, a minimum of eight concentrations of inhibitor were used; only those concentrations which were common to all three experiments are shown.

qualate is not as potent as L-Glu, it may possess a similar degree of intrinsic activity. Similarly, since NMDA produced only a 250% increase in binding as compared to 390% produced by L-Glu, this latter EAA may possess a higher intrinsic activity than NMDA.

Magnesium was also found to significantly affect [^{3}H]TCP binding in the present study. Although its ability to enhance binding at the PCP-binding site was significantly less than that of L-Glu, Mg^{2+} was able to shift the dose response curve for L-Glu to the left by almost 10-fold. These results imply that Mg^{2+} and L-Glu are altering [^{3}H]TCP binding in a dependent manner and suggest that they are producing effects at different sites associated with the PCP receptor. In previous studies, Mg^{2+} has been found to antagonize NMDA-elicited effects in a voltage-dependent manner (13). This is particularly interesting since PCP-like compounds have also been found to antagonize NMDA-evoked responses in a voltage-dependent manner.

Results from saturation experiments revealed that [^{3}H]TCP binds to one component under basal binding conditions or in the presence of either Mg^{2+} or L-Glu. However, in the presence of Mg^{2+} and L-Glu, the radioligand appeared to bind to two sites. This finding is puzzling since, in the experiments examining inhibition of 5 nM [^{3}H]TCP binding by the dissociative anesthetics, a two-component binding model best described the data under basal conditions, whereas a one component model adequately described the binding data for most of these compounds in the presence of Mg^{2+} and L-Glu. One explanation for this finding is that a high affinity component of binding is present, but the population may be too small to be detected by saturation analysis over a radioligand concentration range of

0.1–130 nM. To address whether a low capacity, high affinity component of binding might be present under all incubation conditions, Scatchard analysis of the saturation data was performed for the radioligand concentration range of 0.1–1.0 nM (Fig. 7). The B_{max} and K_d values obtained from this analysis are shown in Table 6. A high affinity binding component was observed under all six binding conditions. The K_d value, as determined by computer analysis, was essentially the same under all incubation conditions. The amount of this high affinity binding component was highest in the presence of Mg^{2+} and L-Glu and lowest under basal binding conditions. Again, the effect of L-Glu on [^{3}H]TCP binding was found to be dose dependent. In addition, the amount of the high affinity component was similar for Mg^{2+} in the presence of either 0.5 or 100 μM L-Glu. This finding is consistent with the data presented in Fig. 1, where a similar enhancement of [^{3}H]TCP binding was observed at these concentrations of the EAA. Although it would appear that two binding components might be present when only L-Glu is included in the binding assay, based on the slight curvilinearity of the Scatchard plots (Fig. 3, C and D), computer analysis revealed that a two-component model did not describe the binding data significantly better than a one-component model.

Using the values obtained from the saturation analyses, the percentage of the radioligand bound to the high and low affinity components at a radioligand concentration of 5 nM can be approximated (see Table 7, Footnote *a*). As seen under basal binding conditions, a large percentage of the high affinity component and only a small percentage of the low affinity component is labeled. Since the high affinity component is only a small proportion of the total binding compared to the low affinity component, the amount bound to each component is relatively the same, or approximately 50%. In contrast, since a large proportion of total binding is to the high affinity component in the presence of Mg^{2+} and L-Glu, most of the binding of 5 nM [^{3}H]TCP is to this high affinity component. Thus, at 5 nM [^{3}H]TCP, two binding components would be expected to be observed under basal binding conditions, whereas one component might be expected in the presence of Mg^{2+} and L-Glu. For most of the dissociative anesthetics and the NMDA antagonists, CPP and D-AP5, these were the results that were obtained.

The results obtained with Mg^{2+} and L-Glu thus far suggest that these compounds are elevating [^{3}H]TCP binding by either increasing the B_{max} value or increasing the affinity of the PCP receptor. Based on the results from the saturation experiments, where the high affinity component of binding is increased but the total B_{max} is essentially unchanged, it would appear that the effect is more complex. In other receptor systems, such as the α-adrenergic and serotoninergic systems, agonist interactions with the receptor have been shown to result in the formation of a high affinity state of the receptor (22, 23). Based on these findings, it would appear that the present results can best be explained by formation of a high affinity state of the receptor by Mg^{2+} and L-Glu. According to this model, a small proportion of binding (less than 10%) occurs to the high affinity state under basal conditions. In the presence of either 30 μM Mg^{2+}, 0.5 μM L-Glu, or 100 μM L-Glu, more of the high affinity state of the receptor is stabilized. Under these conditions, two different populations of sites were not detected. This may be attributable in part to the relatively low percentage of the high affinity component as compared to the total number of sites, or to the fact that the difference in the K_d values for the high and low affinity state (5 nM versus 110 nM) is at most 20-fold. Rather, what is detected by the one-site analysis is a lowering of the K_d value. In the presence of both Mg^{2+} and L-Glu, enough of the high affinity state of the receptor was formed to enable its detection in saturation experiments.

Other studies have, in general, reported a lower K_d value than 110 nM for [^{3}H]TCP binding (24, 25). This is most likely due to either the presence of endogenous Glu, which would tend to promote formation of the high affinity component, or the lack of inclusion of ligand concentrations which would detect binding to the low affinity component.

Alternative explanations for two components of binding include negative cooperativity and multiple receptor subtypes. However, the finding that L-Glu dose-dependently increases the high affinity component of binding indicates that these are

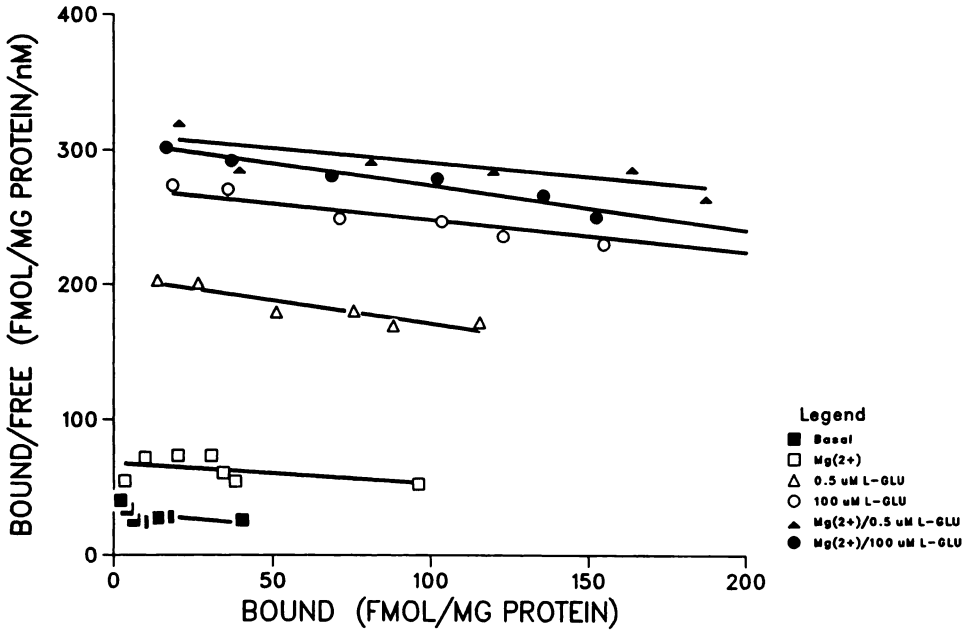

Fig. 7. Scatchard plots of [^{3}H]TCP binding over the range of 0.1–1.0 nM. Values shown are representative of the results from three separate experiments performed in triplicate.

TABLE 6

Binding parameters obtained from analysis of saturation data from 0.1 to 1.0 nM [^{3}H]TCP

Binding condition	B_{max} value	K_d value
	fmol/mg protein	nM
Basal	220 ± 85[a]	5.9 ± 2.8
Mg^{2+}	210 ± 91	2.7 ± 1.6
L-Glu (0.5 μM)	980 ± 220	6.8 ± 1.8
L-Glu (100 μM)	1500 ± 640	7.2 ± 3.5
Mg^{2+}/L-Glu (0.5 μM)	1500 ± 140	5.4 ± 0.6
Mg^{2+}/L-Glu (100 μM)	1400 ± 227	4.6 ± 0.9

[a] Values represent the mean ± standard error, as determined by computer analysis, of three separate experiments performed in triplicate.

not adequate explanations for the present binding data. In addition, the same proportion of high and low affinity binding components as well as similar IC_{50} values for compounds evaluated in competition experiments would be expected under all conditions if multiple subtypes were to account for the present data. Rather, as shown in the competition experiments, what is observed is that Mg^{2+} and L-Glu change the proportion of binding components as well as the ability of the compounds to inhibit [^{3}H]TCP binding.

It is important to note, however, that the present data do not attempt to address the question of whether or not multiple subtypes of the PCP-binding site exist, as recently reported (24, 26). Rather, these data suggest that a "sensitized" and "desensitized" state of the PCP receptor exist, as has been suggested in PCP/nicotinic receptor interactions (27).

In the competition experiments, using 5 nM [^{3}H]TCP, the ability of several classes of compounds to bind to PCP receptors was examined. Compounds in the dissociative anesthetic group, such as PCP, ketamine, and tiletamine, produced a qualitatively similar pattern of inhibition curves under the four conditions examined. Under basal conditions, a shallow inhibition curve was generated, indicating that these compounds were able to discriminate between the high and low affinity states of the receptor. Addition of Mg^{2+} and/or L-Glu revealed that these compounds show a high degree of selectivity for the high affinity state of the receptor.

Comparison of the IC_{50} values for these compounds reveal several interesting points. In general, these compounds appear to show the lowest affinity for the PCP receptor under basal conditions, except for PCP. It displayed approximately the same affinity for the high affinity state under all four conditions. Among the other compounds, dexoxadrol and tiletamine showed the lowest IC_{50} value in the presence of Mg^{2+}, whereas ketamine and MK-801 displayed the lowest IC_{50} value in the presence of the divalent cation and L-Glu.

In relation to these findings, the order of potency of these compounds was found to be different in the absence versus the presence of Mg^{2+} and L-Glu. Under basal binding conditions, the order of potency of the IC_{50} values for both the high and low affinity state were similar: PCP > MK-801 = dexoxadrol = tiletamine > ketamine. In contrast, the order of potency of the IC_{50} values for the high affinity state in the presence of Mg^{2+} and L-Glu was: MK-801 > PCP = dexoxadrol > tiletamine > ketamine. The significance of these findings is unclear at the present time. It would appear, however, that compounds in this group are able to interact potently with the high affinity state of the receptor. In this regard, it is important to note that MK-801 may noncompetitively block NMDA receptors through interaction with the PCP receptor, similar to the other dissociative anesthetics (18).

When the NMDA antagonists CPP and D-AP5 were examined, a different binding profile was found. Upon examination of the percentage of binding inhibited (Fig. 5A), it is unclear what components or states these compounds are inhibiting. However, when the actual amount of binding is examined (Fig. 5B), it becomes apparent that these compounds are unable to inhibit a certain amount of [^{3}H]TCP binding. Interestingly, this binding represents the amount of binding to the low affinity state of the receptor under each condition, as can be seen from Table 7. Whether [^{3}H]TCP binding is inhibited competitively or noncompetitively (e.g., inhibition by action at the NMDA receptor) by these compounds is presently unclear. However, the report by Fagg and Baud (21), that NMDA antagonists are capable of blocking the Glu-induced increase in [^{3}H]TCP binding, would be consistent with a noncompetitive type of inhibition.

As shown in Table 4, each NMDA antagonist examined inhibited [^{3}H]TCP binding most potently in the presence of Mg^{2+}. This finding, together with the ability of the divalent cation to shift the dose response curve for L-Glu approximately 10-fold to the left, indicates that Mg^{2+} is able to allow a better "coupling" between the NMDA and PCP receptor and suggests that the effects of the NMDA antagonists are produced noncompetitively; i.e., that these compounds inhibit [^{3}H]TCP binding through interaction at the NMDA receptor. The decrease in affinity seen when L-Glu was included in the binding assay may be due to a competitive interaction between L-Glu and the NMDA antagonists and would be consistent with a noncompetitive action of these compounds at the PCP-binding site.

For the σ-type compounds, (−)-3-PPP and BW 234U showed similar monophasic competition curves under all four binding conditions. Both compounds did not distinguish between the high and low affinity states under each condition. In contrast, (±)-SKF 10,047 and haloperidol produced complex results. Under basal binding conditions, or in the presence of Mg^{2+}, or Mg^{2+} and L-Glu, haloperidol produced relatively steep inhibition curves. However, in the presence of L-Glu, haloperidol was not able to differentiate between the two binding components. (±)-SKF 10,047 also produced steep inhibition curves in the presence of Mg^{2+} or Mg^{2+} plus L-Glu. However, shallow inhibition curves were generated in the presence of L-Glu or under basal conditions, indicating that this compound could discriminate between the high and low affinity states under these conditions. These findings indicate that haloperidol and (±)-SKF 10,047 are able to interact with or recognize the high affinity state of the receptor in the presence of L-Glu, but that Mg^{2+} produces a change in the state of the receptor so that these compounds can no longer differentiate this state from the

TABLE 7

Predicted amount of binding to the high and low affinity binding components at 5 nM [^{3}H]TCP

Binding conditions	$Bound_H$[a]	% B_H	$Bound_L$	% B_L
Basal	100[b]	44	116[b]	56
Mg^{2+}/L-Glu (100 μM)	608	87	88	13

[a] Values are derived from the equation:

$$B = \frac{B_{max}(H)}{1 + K_d(H)/L} + \frac{B_{max}(L)}{1 + K_d(L)/L}$$

where B_{max} and K_d values for the high and low affinity components were obtained from the saturation analysis experiments (see Tables 2 and 6).

[b] Values are expressed as fmol/mg of protein.

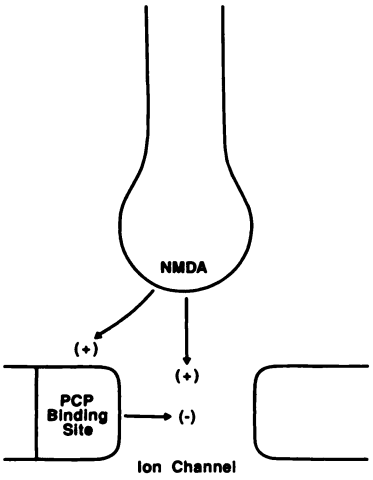

Fig. 8. Conceivable model of PCP/NMDA receptor interactions.

low affinity state. Since (±)-SKF 10,047 and haloperidol were, in general, less potent than the dissociative anesthetics, and D-ethylketocyclazocine was inactive under all four binding conditions, it would appear that the PCP-binding site measured in the present study is distinct from the σ-binding site (see Refs. 28 and 32).

In conclusion, the data presented in this study are consistent with the idea that NMDA agonists induce a high affinity state of the PCP receptor. The interaction between Mg^{2+}, L-Glu, and TCP is reminiscent of that found in the benzodiazepine complex where chloride ions and benzodiazepines have been found to interact with γ-aminobutyric acid (29). Thus, a complex consisting of an ion channel, an endogenous amino acid, and an exogenous drug that alters the binding states of both the ion channel and the amino acid may also occur in the present system. The PCP/NMDA interaction may also be analogous to the interaction of the dissociative anesthetic with the nicotinic cholinergic receptor (27). This latter possibility is reinforced by the use-dependent blockade phenomena seen in both the NMDA and nicotinic cholinergic systems. Since PCP and other dissociative anesthetics have not been found to inhibit the binding of the NMDA antagonist ligand [^{3}H]CPP (30), the present results, taken together with the current literature, would suggest the following model in rat forebrain. As shown in Fig. 8, NMDA-evoked responses are inhibited by PCP-like compounds in a noncompetitive manner. NMDA-type compounds are able to enhance the binding of [^{3}H]TCP, which would serve as a modulatory pathway to keep the NMDA pathway in check. It is important to note, however, that not all PCP-binding sites may be coupled to NMDA sites, since recent findings indicate that [^{3}H]TCP binding is minimally altered in the cerebellum by L-Glu (31).

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