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A partial agonist model used in the allosteric modulation of the NMDA receptor

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Abstract

We used a partial agonist model to understand further the allosteric modulation of D,L-(*E*)-2-amino-4-propyl-5-phosphono-3-pentenoic acid ([^{3}H]CGP-39653) binding by glycine, 1-hydroxy-3-amino-2-pyrrolidone (HA-966) and 5,7-dichlorokynurenic acid at the NMDA receptor. Binding of [^{3}H]CGP-39653 was investigated in homogenates of cortex, hippocampus and cerebellum of adult rat. Glycine, HA-966 and 5,7-dichlorokynurenic acid maximally decreased the binding of 10 nM of [^{3}H]CGP-39653 by approximately 50, 40 and 22%, respectively. Glycine, HA-966 and 5,7-dichlorokynurenic acid reduced [^{3}H]CGP-39653 binding with IC_{50} values of 0.31, 11 and 0.044 μM, respectively. The decrease in [^{3}H]CGP-39653 binding was due to a reduced affinity (K_d) and number of binding sites (B_{max}) by all three drugs at concentrations where approximately maximum inhibition was observed. Glycine, HA-966 and 5,7-dichlorokynurenic acid lowered the B_{max} by approximately 29, 16 and 10%, respectively, whereas the K_d values were increased by approximately 84, 44 and 32%, respectively, in cortex and hippocampus. There was no change in the binding of [^{3}H]CGP-39653 in the cerebellum. The model used revealed that neither 5,7-dichlorokynurenic acid nor HA-966 had partial agonist characteristics in respect with the allosteric modulation of [^{3}H]CGP-39653 binding. Furthermore, the results showed that brain regions have different pharmacological profiles which may depend on the NMDA receptor subunit composition. © 1997 Elsevier Science B.V.

Keywords: [^{3}H]CGP-39653; Partial agonist; Binding; Allosteric modulation

1. Introduction

The NMDA receptor is a ligand-gated ion channel receptor activated by the excitatory amino acid glutamate. A unique aspect of this receptor is the presence of a large number of modulatory sites. Glycine, MK-801 or phencyclidine, Mg^{2+}, Zn^{2+}, and polyamines all influence channel function by interacting at specific binding sites (for review see Scatton, 1993). Although glutamate is considered to be the endogenous agonist, glycine is an absolute requirement for channel activation and can be considered a co-agonist (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988).

Early work suggested a simple reciprocal relationship between agonists and antagonists at each site. Glycine site agonists increased the affinity of glutamate site agonists while decreasing the affinity of glutamate site antagonists (Kaplita and Ferkani, 1990; Fadda et al., 1988). This model is clearly consistent with the idea of an antagonist-preferring state of the glutamate site which could be converted to an agonist-preferring conformation by the action of glycine (Monaghan et al., 1988).

More recent evidence suggests that the affinity of different glutamate site antagonists may respond differently to activation or blockade of the glycine site, or to other modulators (Danysz et al., 1989; Compton et al., 1990; Porter et al., 1992). Glycine modulates the glutamate site by decreasing the binding of [^{3}H]CGS-19755 (D,L-*cis*-4-(phosphonomethyl)piperidine-2-carboxylic acid) and [^{3}H]CGP-39653 (D,L-(*E*)-2-amino-4-propyl-5-phosphono-3-pentenoic acid) by approximately 60% whereas it does not affect the binding of [^{3}H]CPP (3[(±)2-carboxypiperazine-4-yl]propyl-1-phosphonic acid) (Kaplita and Ferkani, 1990; Sills et al., 1991; Mugnaini et al., 1993; Grimwood et al., 1993) or slightly increases it by 20% (Porter et al., 1992). The binding of glutamate is enhanced in presence of glycine or D-serine. The glycine site antagonists kynurenic acid and its derivatives have complex modulatory effects on the binding of drugs targeting the glutamate

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site. Kynurenic acid, 7-chlorokynurenic acid and 5,7-dichlorokynurenic acid (Baron et al., 1990, 1991) reduce the binding of [^{3}H]CPP, whereas [^{3}H]CGP-39653 binding is enhanced in presence of 7-chlorokynurenic acid but reduced with 5,7-dichlorokynurenic acid (Danysz et al., 1989; Porter et al., 1992; Reynolds, 1994). Another group of drugs acting at the glycine site are the partial agonists HA-966 (1-hydroxy-3-amino-2-pyrrolidone) and L-687,414 ((3*R*)-(+)-*cis*-4-methyl-HA-966). The binding of [^{3}H]CPP is increased in presence of HA-966 and L-687,414, whereas [^{3}H]CGP-39653 binding is reduced by HA-966 (Danysz et al., 1989; Porter et al., 1992). Thus, the modulation of the glutamate site by the glycine co-agonist site is rather complex and may depend on the chemical structures of the drugs used. This characterization may have some useful therapeutic application (Coderre and Van Empel, 1994).

At least part of the differential effects of modulators on the affinity of different ligands may be the result of receptor heterogeneity. So far, five NMDA receptor subunits named NR1, NR2A, NR2B, NR2C and NR2D have been isolated from the rat brain (Moriyoshi et al., 1991; Monyer et al., 1992; Ishii et al., 1993). The distribution of these subunits (Moriyoshi et al., 1991; Monyer et al., 1992; Akasawa et al., 1994) as well as their pharmacological characteristics (Lynch et al., 1994; Laurie and Seeburg, 1994) vary throughout the brain. The cortex and the hippocampus are known to be rich in NR2A subunits which in heteromeric complex with the NR1 subunit have a high affinity for 5,7-dichlorokynurenic acid and [^{3}H]CGP-39653. The NR1/NR2B heteromeric complex also present in these regions binds glutamate with a greater affinity than antagonists (Lynch et al., 1994; Laurie and Seeburg, 1994). The NR1/NR2C heteromeric complex, almost exclusively found in the cerebellum, shows a high affinity for glycine and 5,7-dichlorokynurenic acid.

In the present paper we investigated the interaction of glycine and glutamate sites in cortex, hippocampus and cerebellum of the adult male rat brain with a new approach. Specifically, we determined the effects of glycine, a glycine site antagonist (5,7-dichlorokynurenic acid) and a glycine site partial agonist (HA-966) on the binding of the high affinity and highly selective NMDA receptor antagonist [^{3}H]CGP-39653, with the use of a partial agonist model.

2. Materials and methods

2.1. Tissue preparation

Adult male Sprague-Dawley rats (220–250 g) were decapitated and their brains quickly removed. Following brain dissection, the cerebral cortex, hippocampus and cerebellum were isolated and immediately immersed in 5 mM Tris-HCl buffer (pH 7.7 at room temperature). The tissue was prepared according to Sills et al. (1991) with some modifications. The brain regions were homogenized in 35 ml of Tris-HCl buffer using a SDT Tissumizer (Tekmar Company, Cincinnati, OH, USA) setting 35 for 10 s. The homogenate was centrifuged at $48\,400 \times g$ for 10 min. Following the first wash, the pellets were homogenized in 35 ml Tris-HCl/EDTA (5 mM/10 mM) and placed in shakers for 10 min at 37°C. Pellets were washed and rinsed twice again. The final suspension was kept in the freezer for at least 4 days at −20°C.

On the day of the experiment, the homogenates of cortex, hippocampus and cerebellum were thawed at room temperature. The homogenates of cortex and hippocampus were then centrifuged and rinsed once, and the cerebellum twice, in Tris-HCl buffer as previously described. The final suspensions of cortex, hippocampus and cerebellum were resuspended in Tris-HCl buffer, such that the final concentration of protein was approximately 100–180 μg/400 μl. Protein concentration was determined by the Lowry method (Lowry et al., 1951).

2.2. [^{3}H]CGP-39653 binding

For allosteric modulation curves, total binding was assessed with 100 μl [^{3}H]CGP-39653 (10 nM), 400 μl tissue and 100 μl of buffer containing glycine, 5,7-dichlorokynurenic acid or HA-966 at various concentrations. Non-specific binding was determined by addition of 10 μM glutamate. Total volume for both total and non-specific binding was 1 ml. The concentrations for glycine and 5,7-dichlorokynurenic acid ranged from 10^{-8} to 10^{-4} M at equal logarithmic intervals. The concentration of HA-966 ranged from 3×10^{-8} to 3×10^{-4} M also at equal logarithmic intervals.

The effect of 5,7-dichlorokynurenic acid on glycine-induced displacement of 10 nM [^{3}H]CGP-39653 was assessed with 0, 30 nM and 3 μM of 5,7-dichlorokynurenic acid. For displacement of 10 nM [^{3}H]CGP-39653 by glycine in presence of HA-966, we used the same procedure but with 0, 3 and 100 μM of HA-966.

For saturation experiments, total binding was measured with 11 concentrations of [^{3}H]CGP-39653 ranging from 0.4 to 60 nM, 400 μl of tissue and either 10 μM glycine, 3 μM 5,7-dichlorokynurenic acid or 100 μM HA-966. We used the concentration of glycine, 5,7-dichlorokynurenic acid and HA-966 at which maximum displacement of [^{3}H]CGP-39653 was observed. Non-specific binding was determined with 10 μM glutamate. Total volume was 1 ml. All assays were run in duplicate and incubated on ice for 2 h.

Binding of [^{3}H]CGP-39653 to membranes was determined by vacuum filtration through Whatman glass fiber filters (FPB-148 GF/B Fired) using a Brandel Cell Harvester. The filters were washed 3 times with ice-cold Tris-HCl buffer (5 mM) and then placed in scintillation vials containing 5 ml of Beckman Ready Protein cocktail. The vials were then left in the dark overnight before counting.

Values of the parameters K_d and B_{max} were determined with the program LIGAND (Munson and Rodbard, 1980). The slopes, IC_{50} and maximum inhibition values were determined with SPSS, using non-linear regression. Non-specific binding represented less than 30% of total binding.

2.3. Statistical analysis

Effects of glycine, HA-966 and 5,7-dichlorokynurenic acid on [^{3}H]CGP-39653 binding were evaluated with the analysis of variance (ANOVA) using a split-plot design in which drug concentration was a within- and brain region was a between-plot variable. Data were analyzed using SPSS version 4.0. Individual comparisons were tested using Dunnett's test.

2.4. Partial agonist model

Some aspects of the 5,7-dichlorokynurenic acid effect on [^{3}H]CGP-39653 binding were reminiscent of those of a partial agonist and it has been suggested that HA-966 is a partial agonist because of its limited ability to completely block glutamate effects in electrophysiological preparations (Foster and Kemp, 1989). We examined this possibility by analyzing the dose-response curves for glycine inhibition of [^{3}H]CGP-39653 binding in the presence of different concentrations of 5,7-dichlorokynurenic acid or HA-966. The model employed was that of Pöch and Zimmermann (1988) in which the dose-response curve to the full agonist (glycine) in the presence of the partial agonist is expressed as a right-shifted version of the dose-additive effect. Since the dose-additive curve is shifted by $1 + [B]/K_b$ (where K_b is the dissociation constant of the putative partial agonist and [B] its concentration) fits of this model can be used to determine the K_b of a partial agonist.

This model was fit directly using the nonlinear regression (NLR) routine of the SPSS statistical package. In order to test whether the partial agonist model would explain the data, we performed two fits, one in which the curves at the different 'partial agonist' concentrations were constrained to have the same K_b and one in which this parameter was permitted to vary between the curves (unconstrained fit). A residual *F*-test was computed to determine whether sharing this parameter (as would be the case if the drug were a partial agonist) significantly degraded the fit. In addition, the fits of the partial agonist model for the individual 'partial agonist' concentrations were tested against fits in which unconstrained logistic functions were used to describe the dose-response curves.

The partial agonist model of Pöch and Zimmermann (1988) was fit in SPSS. This model assumes that the full agonist dose-response curve obtained in the presence of a partial agonist can be represented by a parallel shifted version (shifted by $1 + [B]/K_b$) where [B] is the concentration and K_b the dissociation constant of the partial agonist. Control curves were modeled with a 4-parameter logistic equation:

$$y = \frac{(\min - \max)}{1 + e^{(\text{slope}(\ln(x) - A))}} + \max$$

where y is the response, x, the dose of the drug, min, the response when $x = 0$, max, the response at infinite concentration of x and A is the natural log of the IC_{50}.

To model the curve in the presence of the partial agonist, an equivalent dose, D_e, of full agonist producing the same response as the partial agonist alone was first determined using the inverse logistic function where min, max, A and slope are the parameters of the control curve defined above, and resp is the response to the partial agonist alone (fit as a parameter).

$$D_e = e^{\frac{\ln(\frac{\min - \text{resp}}{\text{resp} - \max}) + \text{slope} * A}{\text{slope}}}$$

This dose, D_e, was added to that of the full agonist, the result equaling the dose of full agonist representing dose-additivity, DA. Finally, the curve was shifted $1 + \frac{[B]}{K_b}$ to the right by subtracting the $\ln\left(1 + \frac{[B]}{K_b}\right)$ from $\ln(DA)$ in the logistic equation representing the control curve. Thus, the response in the presence of the partial agonist was represented as:

$$y = \frac{(\min - \max)}{1 + e^{(\text{slope}(\ln(DA) - A - \ln(1 + \frac{[B]}{K_b})))}} + \max.$$

Further details are available from the authors by request.

2.5. Materials

Adult male Sprague-Dawley rats aged 2 months were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) and housed at the Animal Resources Center of The University of Texas. [^{3}H]CGP-39653 was purchased from NEN-Dupont (Boston, MA, USA) with a specific activity of 44.1 Ci/mmol. TRIZMA hydrochloride (Tris-HCl), EDTA, glycine and L-glutamic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5,7-Dichlorokynurenic acid was obtained from RBI (Natick, MA, USA) and HA-966 from Tocris Cookson (Bristol, UK). The scintillation cocktail Ready Protein was obtained from Beckman Instruments (Fullerton, CA, USA). Glass fiber filters were purchased from Brandel (Gaithersburg, MD, USA).

3. Results

3.1. Allosteric modulation of [^{3}H]CGP-39653 by glycine, 5,7-dichlorokynurenic acid and HA-966

The allosteric inhibition of [^{3}H]CGP-39653 binding by glycine, 5,7-dichlorokynurenic acid and HA-966 in cortex

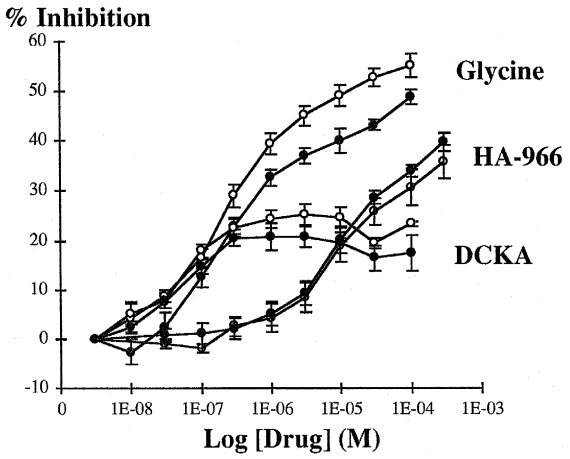

Fig. 1. Reduction of 10 nM [^{3}H]CGP-39653 binding by glycine, HA-966 and 5,7-dichlorokynurenic acid in cortex (open circle) and hippocampus (filled circle). The curves represent the means ± S.E.M. of 5, 4 and 4 experiments, respectively, expressed as percent inhibition.

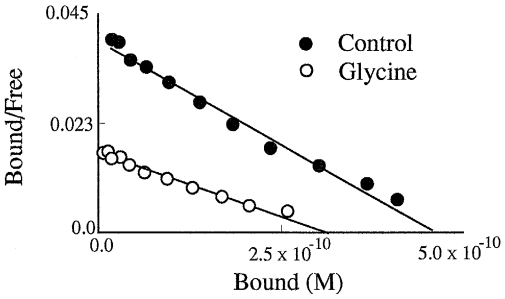

Fig. 2. Scatchard plot representative of a set of experiments run with 10 μM glycine, 100 μM HA-966 or 3 μM 5,7-dichlorokynurenic acid in cortex or hippocampus. $P < 0.001$ for both K_d and B_{max} in all treatment conditions. Scatchard plot analysis with the program LIGAND revealed a single binding site. r^2 approximately equal to 0.95.

and hippocampus is represented in Fig. 1. The inhibition of [^{3}H]CGP-39653 binding was concentration-dependent with all three drug treatments, in both cortex and hippocampus ($P < 0.001$). The allosteric modulation of [^{3}H]CGP-39653 binding by HA-966 and 5,7-dichlorokynurenic acid was similar in cortex and hippocampus ($P > 0.05$). There was a statistical difference ($P < 0.01$) between the effect of glycine on [^{3}H]CGP-39653 binding in cortex and in hippocampus. The curve parameters corresponding to Fig. 1 are shown in Table 1. The three drugs decreased the binding of [^{3}H]CGP-39653 in cortex and hippocampus with different potency: 5,7-dichlorokynurenic acid > glycine > HA-966, as revealed by the different values of IC_{50}. This order is the same as the order of the affinity of the drugs for the glycine site of the receptor as revealed by their K_d values. The drug 5,7-dichlorokynurenic acid has the highest affinity followed by glycine and HA-966 (Yoneda et al., 1993; O'Shea et al., 1991). However, the maximum reduction of [^{3}H]CGP-39653 binding was greater with glycine followed by HA-966 and 5,7-dichlorokynurenic acid.

We further measured the effect of all three drugs on K_d and B_{max} parameters, by conducting saturation experiments. Scatchard plots were found to fit a single binding site model with an r^2 of approximately 0.95 for cortex and hippocampus (Fig. 2). The results of the assays run with 10 μM glycine, 3 μM 5,7-dichlorokynurenic acid or 100 μM HA-966 are represented in Table 2. These values represent the concentrations at which the inhibition of [^{3}H]CGP-39653 binding was maximum or near to maximum, in cortex and hippocampus. There was a statistically significant increase in K_d values and diminution in B_{max} values with all three drug treatments ($P < 0.001$), but 5,7-dichlorokynurenic acid had the weakest effect compared to glycine and HA-966. Decrease in B_{max} values was greatest with glycine followed by HA-966 and 5,7-dichlorokynurenic acid ($P < 0.05$). Whereas the decrease in [^{3}H]CGP-39653 binding by glycine and HA-966 was due to an alteration of both K_d and B_{max} values, the 5,7-dichlorokynurenic acid effect was more a consequence of a decrease in the affinity of [^{3}H]CGP-39653 for the receptor than a diminution of the number of binding sites (Table 2).

Whereas glycine and 5,7-dichlorokynurenic acid had an inhibitory effect on the binding of [^{3}H]CGP-39653 in cortex and hippocampus, they did not, overall, alter the binding of the ligand in cerebellum, as shown in Fig. 3. The only statistically significant decrease in [^{3}H]CGP-

Table 1
Allosteric modulation of [^{3}H]CGP-39653 binding by glycine, HA-966 and 5,7-dichlorokynurenic acid

Drug treatment	Brain regions					
	Cortex			Hippocampus		
	IC_{50}	Slope	Max	IC_{50}	Slope	Max
Glycine	0.31 ± 0.06	0.75 ± 0.08	55%	0.33 ± 0.05	0.91 ± 0.09	46%
HA-966	11 ± 2	1.00 ± 0.23	37%	17 ± 5	0.9 ± 0.2	44%
DCKA	0.044 ± 0.006	1.38 ± 0.17	24%	0.046 ± 0.010	1.82 ± 0.35	20%

Values of IC_{50} are expressed in μM. The slope and the IC_{50} values represent the means ± S.E.M. of at least 4 experiments run in duplicate in both cortex and hippocampus. Max means the maximum percent inhibition compared to control values and represents the mean of at least 4 experiments run in duplicate in both cortex and hippocampus. DCKA, 5,7-dichlorokynurenic acid.

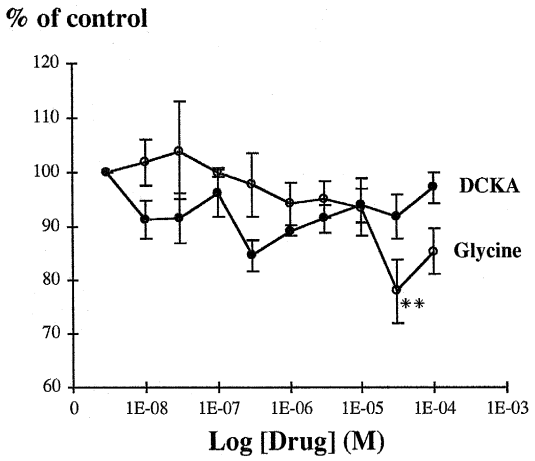

Fig. 3. Displacement of [^{3}H]CGP-39653 (10 nM) by glycine and 5,7-dichlorokynurenic acid in cerebellum. The curves represent the means ± S.E.M. of 6 and 4 experiments run with glycine and 5,7-dichlorokynurenic acid, respectively. Glycine had a slight effect on [^{3}H]CGP-39653 binding, but only at a single concentration (30 μM) indicated by ** ($P < 0.05$).

39653 binding ($P < 0.05$) was noticed at a very high concentration of glycine (30 μM) and may not be biologically relevant. Because of the lack of allosteric modulation of glycine and 5,7-dichlorokynurenic acid on [^{3}H]CGP-39653 binding in the cerebellum, we did not run any concentration–response curve experiments in presence of HA-966.

3.2. Characterization of the nature of 5,7-dichlorokynurenic acid and HA-966 with respect to the allosteric modulation of [^{3}H]CGP-39653 binding

The maximum response for HA-966 observed in Fig. 1 was lower than that of glycine in cortex but not in hippocampus. Sharing the maximum response parameters for the two drugs in cortex significantly increased the residual of variances ($P < 0.001$) of the fit. This was not the case in hippocampus ($P > 0.05$). It has been shown that HA-966

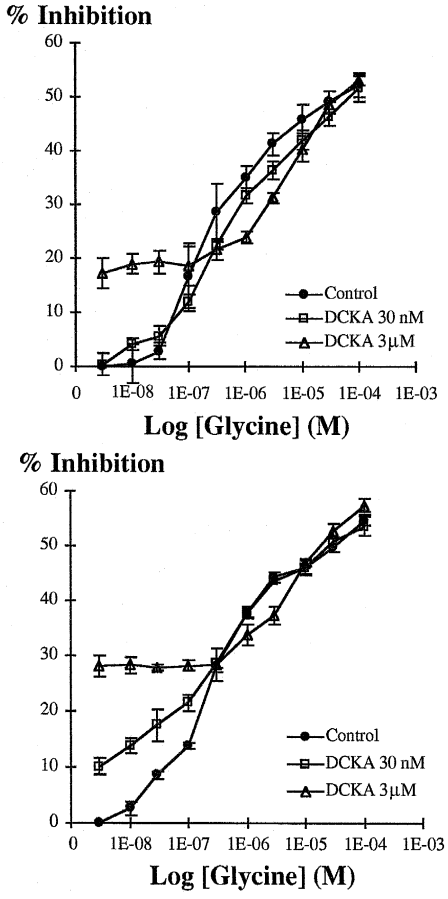

Fig. 4. Characterization of 5,7-dichlorokynurenic acid as potential partial agonist. The curves are the inhibition of [^{3}H]CGP-39653 by glycine in the presence of two concentrations of 5,7-dichlorokynurenic acid (3×10^{-8} M and 3×10^{-6} M) in cortex (top) and hippocampus (bottom). The curves represent the mean ± S.E.M. of 4 and 3 experiments run in duplicate in cortex and hippocampus, respectively. Control binding was specific binding of 10 nM [^{3}H]CGP-39653 without glycine or 5,7-dichlorokynurenic acid.

behaved as a partial agonist in some cases (Foster and Kemp, 1989). As shown in Fig. 1, 5,7-dichlorokynurenic acid produced a reduction of [^{3}H]CGP-39653 binding con-

Table 2
Saturation experiments of [^{3}H]CGP-39653 in presence of glycine, 5,7-dichlorokynurenic acid or HA-966

Drug treatment	Brain regions					
	Cortex			Hippocampus		
	K_d	B_{max}	n_H	K_d	B_{max}	n_H
Control	11.24 ± 0.13	2574 ± 131	0.97 ± 0.03	10.31 ± 0.28	3435 ± 131	0.89 ± 0.07
Glycine	20.37 ± 0.72	1808 ± 105	0.99 ± 0.03	19.14 ± 0.51	2472 ± 108	1.02 ± 0.06
Control	12.42 ± 0.43	2162 ± 49	0.91 ± 0.03	12.10 ± 0.59	2763 ± 171	0.88 ± 0.03
DCKA	15.75 ± 0.62	1911 ± 67	0.88 ± 0.03	16.47 ± 0.78	2509 ± 135	0.84 ± 0.03
Control	12.07 ± 0.60	2617 ± 45	0.87 ± 0.06	11.50 ± 0.38	3647 ± 112	0.95 ± 0.03
HA-966	17.03 ± 0.56	2197 ± 63	0.99 ± 0.04	16.80 ± 0.33	3048 ± 135	0.97 ± 0.02

Values of K_d and B_{max} are expressed in nM and fmol/mg protein respectively. Concentration of glycine, 5,7-dichlorokynurenic (DCKA) acid and HA-966 was 10, 3 and 100 μM, respectively. Values represent the means ± S.E.M. of 6, 7 and 4 experiments run in duplicate with glycine, 5,7-dichlorokynurenic acid or HA-966, respectively. n_H represents the Hill coefficient. Values of K_d and B_{max} significantly affected by glycine, 5,7-dichlorokynurenic acid or HA-966 ($P < 0.001$).

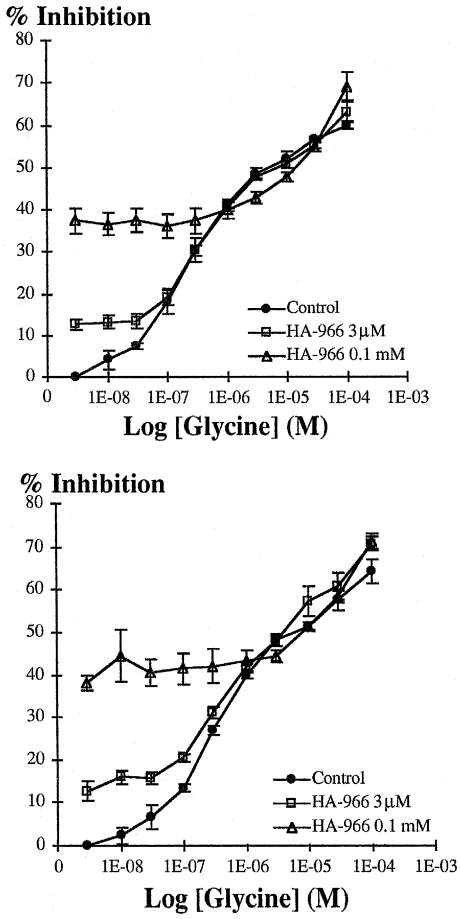

Fig. 5. Characterization of HA-966 as potential partial agonist. The curves are the inhibition of [^{3}H]CGP-39653 by glycine in the presence of two concentrations of HA-966 (3×10^{-6} M and 10^{-4} M) in cortex (top) and hippocampus (bottom). The curves represent the mean ± S.E.M. of 4 experiments run in duplicate. Control binding was specific binding of 10 nM [^{3}H]CGP-39653 without glycine or HA-966.

sistent with that of a partial agonist since the maximum percent of inhibition was statistically lower than HA-966 or glycine. This suggests that HA-966 and 5,7-dichlorokynurenic acid might behave as partial agonists with respect to the allosteric modulation of [^{3}H]CGP-39653 binding.

To address the question of additivity, we conducted assays of concentration–response curves for glycine in presence of two concentrations of 5,7-dichlorokynurenic acid or HA-966 as shown in Figs. 4 and 5, respectively. The IC_{50} values of the curves are represented in Table 3. There was no 5,7-dichlorokynurenic acid or HA-966 effect on the slopes ($P > 0.05$). Furthermore, as it can be seen in Figs. 4 and 5, none of the curves were shifted to the left compared to the control curve. This indicates that there was no additivity effect between glycine and 5,7-dichlorokynurenic acid or between glycine and HA-966.

3.3. Partial-agonist model

Figs. 4 and 5 show a small shift to the right compared to the control curve in both cortex and hippocampus, mostly at high concentration of either 5,7-dichlorokynurenic acid or HA-966. The data were then fit with a partial agonist model (Pöch and Zimmermann, 1988) to determine whether both 5,7-dichlorokynurenic acid and HA-966 might be partial agonists with respect to the allosteric reduction in [^{3}H]CGP-39653 binding. In both cases, independent logistic fits of the dose-response curves had significantly smaller variances than did fits of the partial agonist model. Further, the effects of the different concentrations of 5,7-dichlorokynurenic acid and HA-966 were inconsistent with a single K_d value, i.e., forcing the K_b for the two curves to be equal, significantly increased the residual variance of the fit ($P < 0.001$ in all cases tested). Finally, the small shift to the right observed at high concentration of 5,7-dichlorokynurenic acid and HA-966 is not reminiscent of a typical partial-agonist shift ($1 + ([\text{partial agonist}]/K_b)$). Thus, these results suggest that neither 5,7-dichlorokynurenic acid nor HA-966 had partial-agonist characteristics with respect to the allosteric modulation of [^{3}H]CGP-39653 binding at the NMDA receptor.

4. Discussion

This project was designed to study the allosteric modulation of the competitive antagonist ([^{3}H]CGP-39653) binding by a glycine site agonist (glycine), partial agonist (HA-966) and a full antagonist (5,7-dichlorokynurenic acid) in cortex, hippocampus and cerebellum of adult rat brain.

Glycine and glycine-site antagonists reduced the binding of [^{3}H]CGP-39653 in cortex and hippocampus, but not in cerebellum. 5,7-Dichlorokynurenic acid was weak in decreasing the binding of the competitive antagonist compared to glycine and HA-966. Thus, it appears that drugs with agonist properties were of higher efficacy in decreasing [^{3}H]CGP-39653 binding than an antagonist, 5,7-di-

Table 3
Values of IC_{50} resulting from the concentration-response curves shown in Figs. 4 and 5

Brain regions	5,7-Dichlorokynurenic acid (M)			HA-966 (M)		
	0	3×10^{-8}	3×10^{-6}	0	3×10^{-6}	10^{-4}
Cortex	3.6 ± 0.8	6.5 ± 1.4	74.8 ± 13.0	2.8 ± 0.6	6.3 ± 0.8	217.4 ± 76.8
Hippocampus	2.9 ± 0.1	4.9 ± 0.5	81.7 ± 22.8	5.8 ± 1.3	25.5 ± 18.6	329.2 ± 154.9

IC_{50} values are expressed in 10^{-7} M. The values represent the means ± S.E.M. of at least 3 experiments run in duplicate in cortex and hippocampus.

chlorokynurenic acid. It is unclear why both the agonist glycine and glycine site antagonists reduced the binding of [^{3}H]CGP-39653. One possibility is that both 5,7-dichlorokynurenic acid and HA-966 might behave as weak partial agonists in the allosteric modulation of [^{3}H]CGP-39653 binding. Previous work showed that HA-966 has partial agonist characteristics (Foster and Kemp, 1989; Pullan et al., 1991) but also that it may bind to a separate domain of the NMDA receptor (Kloog et al., 1990). In our study, as shown in Fig. 5 and as explained in Section 3, HA-966 did not exhibit partial-agonist-like properties with regards to the allosteric modulation of [^{3}H]CGP-39653 binding. At high concentration of glycine no right shift of the curves equivalent to $(1 + ([\text{partial agonist}]/K_b))$ was observed. Further, the effect of increasing concentrations of a partial agonist should be systematically larger deviations from dose-additivity, i.e. the shift from the dose-additive model should equal $1 + [B]/K_b$ where [B] is the concentration of partial agonist and K_b, the dissociation constant of the partial agonist. This was not the case as is indicated by an increase in the residual around the fit when the K_b parameter is shared between curves. One possible explanation of this discrepancy might be that the glycine dose-response curve is contaminated by the reciprocal allosteric action of the binding of [^{3}H]CGP-39653 on binding at the glycine site. Direct simulations of binding equilibrium under these conditions (data not shown) suggest that the rightward shift in the glycine dose-response curve should be at least as great as that predicted by the partial agonist model itself.

It has been suggested that there are two binding sites for glycine and two binding sites for glutamate at the NMDA receptor (Benveniste et al., 1990; Benveniste and Mayer, 1991). Full activation of the receptor is achieved following the binding of two molecules of glycine and two molecules of NMDA to their respective sites (Benveniste and Mayer, 1991). Our results showed no shift (or very small) of the glycine concentration–response curves in presence of two different concentrations of HA-966 or 5,7-dichlorokynurenic acid compared to control curves. Second, although all three drugs had an inhibitory effect on [^{3}H]CGP-39653 binding, when taken together as shown in Figs. 4 and 5 they did not show signs of additivity. Third, at concentrations of glycine higher than 3×10^{-7} M (in presence of 5,7-dichlorokynurenic acid) and 10^{-6} M (in presence of HA-966), the curves were barely different from the control curve. These results may indicate that at low concentrations of glycine, one molecule of glycine is binding to its site along with 5,7-dichlorokynurenic acid or HA-966, but has no effect on the binding of [^{3}H]CGP-39653. As the concentration of glycine increases, glycine may fill both sites, after HA-966 or 5,7-dichlorokynurenic acid has been displaced, and then decreases the binding of [^{3}H]CGP-39653. Thus, at concentrations higher than 3×10^{-7} M (in presence of 5,7-dichlorokynurenic acid) and 10^{-6} M (in presence of HA-966) only glycine is present at the glycine site. This would confirm that both glycine sites have to be occupied by glycine to be fully active.

Saturation experiments in presence of glycine, HA-966 or 5,7-dichlorokynurenic acid showed that the reduction of [^{3}H]CGP-39653 binding was due to a decrease in both K_d and B_{max}. Thus, the three drugs may induce a change in conformation of the NMDA receptor. This change of conformation may be such that the number of accessible sites for [^{3}H]CGP-39653 is reduced. However, 5,7-dichlorokynurenic acid had a different behavior on [^{3}H]CGP-39653 binding compared to glycine and HA-966. With 5,7-dichlorokynurenic acid, the maximum reduction of [^{3}H]CGP-39653 was very small, the slope of the modulation curve had a value higher than 1 and the B_{max} was the least decreased as compared to glycine and HA-966. The effect of 5,7-dichlorokynurenic acid on the slope may indicate a complex interaction between [^{3}H]CGP-39653 and its binding site when 5,7-dichlorokynurenic acid is present.

Glycine decreases the binding of CGS-19755 by approximately 68% in frontal cortical membranes (Kaplita and Ferkani, 1990) and increases the binding of glutamate in brain hemispheres (Fadda et al., 1988). Glycine is without effect (Kaplita and Ferkani, 1990) or slightly increases CPP binding in brain devoid of cerebellum (Porter et al., 1992). Also, HA-966 increases the binding of CPP (Danysz et al., 1989; Compton et al., 1990; Porter et al., 1992), but decreases the binding of glutamate (Danysz et al., 1989). Taken together these results combined with our work, confirm a modulatory effect of the glycine site on the glutamate site. They do not, however, explain why both glycine-site agonist and antagonists reduce the binding of [^{3}H]CGP-39653.

It is generally agreed that native NMDA receptors are comprised of heteromeric complex, NR1 and one or more of the NR2 subunits. The NR1 subunit is widespread throughout the brain (Moriyoshi et al., 1991), while the NR2A subunit is more concentrated in the forebrain and the cerebellum. The NR2B subunit is concentrated in the forebrain and the NR2C homomer shows the highest concentration in the cerebellum (Monyer et al., 1992). The NR2D subunit is moderately expressed in the brainstem regions and cortex of the olfactory bulb (Akasawa et al., 1994). Thus, the cerebellum contrasts with the cortex and the hippocampus by a higher concentration of NR2C subunits and a lower concentration of NR2A subunits (Monyer et al., 1992; Akasawa et al., 1994). Furthermore, previous studies showed that the cerebellum behaves differently from the forebrain in terms of the allosteric modulation of [^{3}H]CGS-19755 binding by glycine, 7-chlorokynurenic acid and L-689,560 (Widdowson et al., 1995). These authors showed that [^{3}H]CGS-19755 binding was increased by glycine site antagonists and decreased by glycine site agonists. The modulatory effect of the drugs on [^{3}H]CGS-19755 binding was stronger in the cerebellum compared to the forebrain (Widdowson et al., 1995). Conversely, in our

study, glycine and 5,7-dichlorokynurenic acid did not alter [^{3}H]CGP-39653 binding in cerebellum. Thus, glycine and glycine site antagonists may modulate the binding of [^{3}H]CGS-19755 and [^{3}H]CGP-39653 in a different manner in the cerebellum. Furthermore, different populations of NMDA receptors present in the cerebellum may contribute these differences in findings.

In conclusion, our study showed a decrease in [^{3}H]CGP-39653 binding in cortex, hippocampus and cerebellum in presence of glycine and glycine site antagonists, due to a decrease in [^{3}H]CGP-39653 affinity and number of binding sites. This modulation varies with the drugs used and is independent of any partial agonism properties of the drugs, but depends on the brain regions investigated. Thus, this work shows that the role of chemical structures of drugs and heteromeric associations of the receptor subunits throughout the brain have important consequences on the modulation of the NMDA receptor.

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