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Regionally different N-methyl-D-aspartate receptors distinguished by ligand binding and quantitative autoradiography of [^{3}H]-CGP 39653 in rat brain

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1 Binding of D,L-(E)-2-amino-4-[^{3}H]-propyl-5-phosphono-3-pentenoic acid ([^{3}H]-CGP 39653), a high affinity, selective antagonist at the glutamate site of the N-methyl-D-aspartate (NMDA) receptor, was investigated in rat brain by means of receptor binding and quantitative autoradiography techniques.

2 [^{3}H]-CGP 39653 interacted with striatal and cerebellar membranes in a saturable manner and to a single binding site, with K_D values of 15.5 nM and 10.0 nM and receptor binding densities (B_{max} values) of 3.1 and 0.5 pmol mg^{-1} protein, respectively. These K_D values were not significantly different from that previously reported in the cerebral cortex (10.7 nM).

3 Displacement analyses of [^{3}H]-CGP 39653 in striatum and cerebellum, performed with L-glutamic acid, 3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) and glycine showed a pharmacological profile similar to that reported in the cerebral cortex. L-Glutamic acid and CPP produced complete displacement of specific binding with K_i values not significantly different from the cerebral cortex. Glycine inhibited [^{3}H]-CGP 39653 binding with shallow, biphasic curves, characterized by a high and a low affinity component. Furthermore, glycine discriminated between these regions ($P<0.005$, one-way ANOVA), since the apparent K_i of the high affinity component of the glycine inhibition curve (K_{iH}) was significantly lower (Fisher's protected LSD) in the striatum than the cortex (33 nM and 104 nM, respectively).

4 Regional binding of [^{3}H]-CGP 39653 to horizontal sections of rat brain revealed a heterogeneous distribution of binding sites, similar to that reported for other radiolabelled antagonists at the NMDA site (D-2-[^{3}H]-amino-5-phosphonopentanoic acid ([^{3}H]-D-AP5) and [^{3}H]-CPP). High values of binding were detected in the hippocampal formation, cerebral cortex and thalamus, with low levels in striatum and cerebellum.

5 [^{3}H]-CGP 39653 binding was inhibited by increasing concentrations of L-glutamic acid, CPP and glycine. L-Glutamic acid and CPP completely displaced specific binding in all regions tested, with similar IC_{50} values throughout. Similarly, glycine was able to inhibit the binding in all areas considered: 10 μM and 1 mM glycine reduced the binding to 80% and 65% of control (average between areas) respectively. The percentage of specific [^{3}H]-CGP 39653 binding inhibited by 1 mM glycine varied among regions ($P<0.05$, two-ways ANOVA). Multiple comparison, performed by Fisher's protected LSD method, showed that the inhibition was lower in striatum (72% of control), with respect to cortex (66% of control) and hippocampal formation (58% of control).

6 The inhibitory action of 10 μM glycine was reversed by 100 μM 7-chloro-kynurenic acid (7-CKA), a competitive antagonist of the glycine site of the NMDA receptor channel complex, in all areas tested. Moreover, reversal by 7-CKA was not the same in all regions ($P<0.05$, two-ways ANOVA). In fact, in the presence of 10 μM glycine and 100 μM 7-KCA, specific [^{3}H]-CGP 39653 binding in the striatum was 131% of control, which was significantly greater (Fisher's protected LSD) than binding in the hippocampus and the thalamus (104% and 112% of control, respectively).

7 These results demonstrate that [^{3}H]-CGP 39653 binding can be inhibited by glycine in rat brain regions containing NMDA receptors; moreover, they suggest the existence of regionally distinct NMDA receptor subtypes with a different allosteric mechanism of [^{3}H]-CGP 39653 binding modulation through the associated glycine site.

Keywords: CGP 39653; glycine; glutamic acid; NMDA receptor; allosteric modulation; ligand affinity; autoradiography

Introduction

The N-methyl-D-aspartate (NMDA) receptor plays an important role in regulating excitatory transmission, neuronal plasticity (Collingridge & Singer, 1990) and neuronal migration (Komuro & Rakic, 1993). Moreover, NMDA receptor overactivation is involved in many neurodegenerative diseases (Meldrum & Garthwaite, 1990).

The NMDA receptor complex consists of a ligand-gated ion channel with high Ca^{2+} permeability, voltage-dependent Mg^{2+} blockade, inhibition by Zn^{2+} and several selective channel blockers. Moreover, the receptor is subject to complex allosteric modulations through three different sites: the NMDA binding site, to which the endogenous ligand glutamate binds, the strychnine-insensitive glycine site and the polyamine site (Wong & Kemp, 1991). Like other ligand-gated ion channels, the NMDA receptor is thought to be made up of a number of subunits. So far, two families of NMDA receptor subunits have been identified by molecular cloning: the NR_1 (Moriyoshi *et al.*, 1991; Yamazaki *et al.*, 1992), which is composed of eight isoforms generated by alternative splicing of a single gene (Sugihara

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et al., 1992; Hollmann et al., 1993) and the NR_2 family which contains four subunits (Monyer et al., 1992; Meguro et al., 1992; Kutsuwada et al., 1992; Ikeda et al., 1992; Ishii et al., 1993), namely NR_{2A}, NR_{2B}, NR_{2C} and NR_{2D}. Functional studies suggest that native receptors are heterometric complexes composed of one or more members of the NR_2 group and at least one NR_1 subunit (Monyer et al., 1992; Meguro et al., 1992).

The distribution in rat and mouse brain of the mRNA encoding for these subunits has been determined by *in situ* hybridization (Nakanishi, 1992; Watanabe et al., 1993) whereas recent immunocytochemistry experiments (Petralia et al., 1994a,b; Aoki et al., 1994; Wenzel et al., 1995) have detected the translated proteins of some members of the NMDA subunit family using specific antibodies. Autoradiography binding studies, performed with radioligands selective for the different binding sites of the NMDA receptor channel complex, have revealed the localization of the heteromeric receptors, in their final, quaternary structure (Jarvis et al., 1987; Maragos et al., 1988; Bowery et al., 1988; McDonald et al., 1990). Several experiments with recombinant receptors have demonstrated that the pharmacological and binding characteristics of NMDA receptors vary significantly with changes in the subunit composition (Williams, 1993; Lynch et al., 1994; Laurie & Seeburg, 1994; Priestley et al., 1995). Therefore, specific binding properties in a certain brain region may help to provide information about the possible subunit composition of the complex in that region. Recently Laurie & Seeburg (1994) and Buller et al. (1994) have formed hypotheses on the subunit composition of four NMDA receptors subtypes, previously described mainly on the basis of regional heterogeneity of [3H]-glutamic acid binding and [3H]-3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid ([3H]-CPP) binding characteristics at the NMDA site of the complex (Monaghan et al., 1988; Monaghan & Beaton, 1991; Beaton et al., 1992) and differences in the pharmacology of (+)-[3H]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate ([3H]-MK-801) binding (Reynolds & Palmer, 1991; Yoneda & Ogita, 1991; Ebert et al., 1991; Sakurai et al., 1993). According to these authors, the 'antagonist preferring', the 'agonist preferring', the 'cerebellar' and 'medial thalamic' subtypes of native NMDA receptors defined in these studies have pharmacological and binding characteristics which might correspond with NR_1/NR_{2A}, NR_1/NR_{2B}, NR_1/NR_{2C} and NR_1/NR_{2D} recombinant receptors, respectively.

Recently, a new radioligand for the NMDA binding site of the NMDA receptor channel complex has been described: D,L-(E)-2-amino-4-[3H]-propyl-5-phosphono-3-pentenoic acid ([3H]-CGP 39653, Sills et al., 1991). This antagonist binds, in rat cerebral cortex membranes, to a single binding site which has the same characteristics of the high affinity binding site recognized by [3H]-CPP (Van Amsterdam et al., 1992; Porter et al., 1992). Nevertheless, in contrast to [3H]-CPP, its binding is modified by the activation of the associated strychnine-insensitive glycine site of the NMDA receptor-channel complex (Mugnaini et al., 1993; Zuo et al., 1993). Because of its high affinity and selectivity, [3H]-CGP 39653 is the radioligand of choice for the localization and quantitative determination of the NMDA binding sites by autoradiography. Moreover, its binding characteristics have never been studied in brain regions other than the cortex.

In this paper, the binding of [3H]-CGP 39653 is described in different regions of rat brain, by means of quantitative autoradiography and receptor binding using tissue homogenates. A preliminary report of the study has been presented to the British Pharmacological Society (Mugnaini et al., 1996).

Methods

Animals

Male Sprague-Dawley rats (200–250 g) were used. Animals were supplied by Charles River, Italy and were kept under standard laboratory conditions.

Receptor autoradiography

Animals were killed by intracardiac perfusion, under pentobarbitone sodium anaesthesia (60 mg kg^{-1}), with 100 ml ice cold saline. The brains were quickly removed from the skulls, immediately frozen in dry ice pre-cooled isopentane and stored at −80°C. Fourteen micron thick horizontal sections (corresponding to Table 104 and 105 of Paxinos & Watson, 1986) were cut on a cryostat, mounted on gelatine coated glass slides and stored at −20°C for up to two weeks. On the day of the experiment, sections were brought to room temperature and preincubated (30 min, 37°C) in 50 mM Tris HCl buffer solution containing 2.5 mM $CaCl_2$ (final pH 7.6). This step was necessary to decrease and equilibrate in all brain regions the levels of endogenous ligands (glutamate and glycine). After this, incubation was performed by applying over each section 200 µl of buffer solution containing 20 nM [3H]-CGP 39653. An incubation period of 25 min at 25°C of temperature was chosen as the standard incubation conditions for autoradiography as well as for binding experiments (see below). Previous studies on cortex membranes (Mugnaini et al., 1993) had shown that the association rate of [3H]-CGP 39653 was rather fast at 25°C and equilibrium was obtained within a few minutes.

The reaction was stopped by rapidly aspirating the radiolabelled mixture and washing (5 min, 4°C) the slides in buffer solution). The sections were then quickly rinsed in purified water (Milli-Q system, Millipore) to remove the excess of buffer salts and dried under a stream of air. Preliminary experiments determined that this rinse procedure, as well as the preincubation washing step, optimized the ratio of specific to non specific binding. In these experiments, the slices were swabbed up with a wet Whatman GF/C filter and the bound radioactivity measured by scintillation spectrometry.

Dried sections were apposed to tritum sensitive films (Hyperfilm-[3H], Amersham International plc, Buckinghamshire, England) with 3H-standards (Microscales, Amersham) for 28 days. Varying amounts of different unlabelled ligands were included in the incubation medium of consecutive sections for displacement binding studies, whereas 100 µM L-glutamic acid was used to determine non specific binding.

Binding assay

Briefly, rats were killed by decapitation, the striatum and cerebellum immediately dissected and crude synaptic membranes prepared as described previously (Mugnaini et al., 1993). The final pellet was resuspended in 3 volumes of water and divided into aliquots that were quick frozen in liquid N_2. On the day of the experiment, membranes were thawed and washed twice by resuspension in 40 volumes of water and centrifugation at 48,000 *g* for 10 min. The final pellet was resuspended in 75 (striatum) or 50 (cerebellum) volumes of 50 mM Tris HCl buffer solution containing 2.5 mM $CaCl_2$ (final pH 7.6).

Saturation binding experiments were performed at radioligand concentrations ranging from 1 to 60 nM. Specific binding was defined as the portion of [3H]-CGP 39653 binding that was displaceable by 30 µM L-glutamic acid. Displacement binding curves were obtained at 2 nM [3H]-CGP 39653. The incubation, performed (25 min, 25°C) in a final volume of 1 ml, was initiated by the addition of 500 (saturation) or 750 (displacement) µl membrane suspension and terminated by dilution with ice cold Tris buffer solution and filtration over Whatman GF/C filters using a Brandel M/48R cell harvester. Filters were washed twice and bound radioactivity estimated by liquid scintillation counting, using a Packard TRI-CARB 1900 CA. Protein content was determined by the method of BCA (Pierce, Rockford, Illinois, U.S.A.) with bovine serum albumin as the standard.

Chemicals

[^{3}H]-CGP 39653 (NET 1050, specific radioactivity 1098 and 1091.5 GBq mmol^{-1}) was purchased from Dupont New England Nuclear, U.S.A. CPP and 7-CKA were obtained form Tocris, England. L-Glutamic acid HCl and glycine HCl were from Sigma Chemical Co., U.S.A. Filter count was from Packard. Other salts and reagents were of highest analytical grade available.

Data analysis

Data of saturation and displacement experiments to determine, respectively, the dissociation constant of the radioligand (K_D) and the inhibition constants of displacer ligands (K_i), were analysed by the non linear curve fitting programme, LIGAND (Munson & Rodbard, 1980). Statistical comparison was determined by one-way analysis of variance (ANOVA), followed by Fisher's protected LSD (least significant difference) on least squares means method.

Densitometric analysis of autoradiograms was performed by computer assisted image analysis (MCID, Imaging Research, St. Catherines, Ontario, Canada). Each area of interest was selected and the optical density measured integrally. In the case of layers of laminated structures (oriens layer, pyramidal cell layer and stratum radiatum of hippocampus; molecular and granular layer of dentate gyrus; molecular and granular layer of cerebellum) density values were the average of 5 to 10 determinations per section. Regional film densities were converted to the corresponding ligand concentration by reference to tritium standards on the same film. To study the potency of displacer ligands in different regions, inhibition curves were obtained with 9 different concentrations of displacers. The concentration of compounds inhibiting 50% of binding (IC_{50}) was obtained using ALLFIT (De Lean *et al.*, 1978). Statistical comparison was determined by two-ways analysis of variance (ANOVA), followed by Fisher's protected LSD method. Results are all expressed as mean ± s.e.mean values.

Results

Distribution of [^{3}H]-CGP 39653 binding sites

[^{3}H]-CGP 39653 binding sites were distributed unevenly in rat brain sections. An example of the distribution is shown in Figure 1. A very high concentration of binding sites was present in the hippocampal formation, with intermediate to high

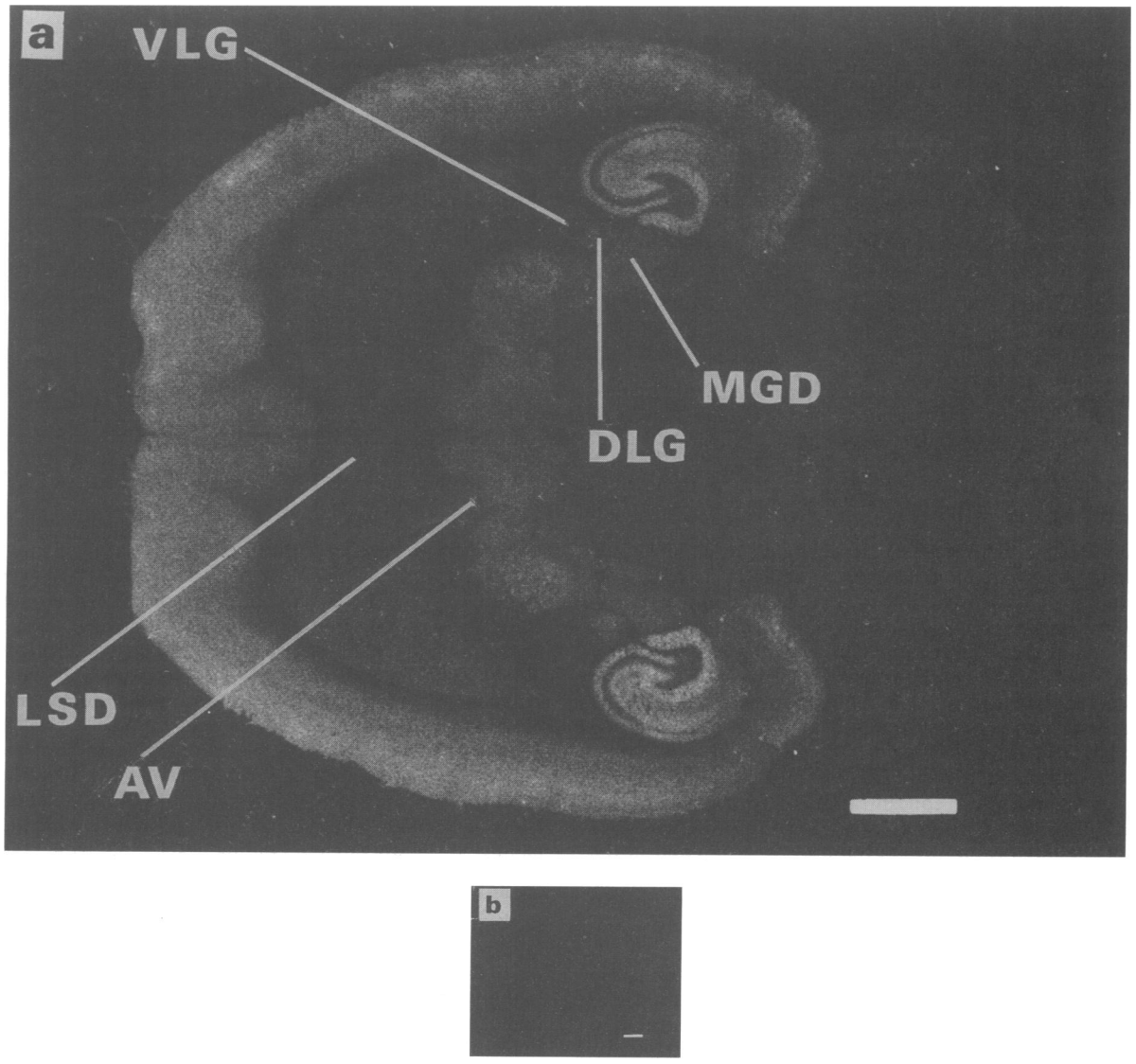

Figure 1 Distribution of [^{3}H]-CGP 39653 binding sites in rat brain sections: (a) total binding; (b) non specific binding, in the presence of 100 μM L-glutamic acid. LSD, lateral septal nucleus, dorsal; DLG, dorsal lateral geniculate nucleus; VLG, ventral lateral geniculate nucleus. Calibration bar corresponds to 2.0 mm.

Table 1 Distribution of [^{3}H]-CGP 39653 binding sites in rat brain

Area	*Specific [^{3}H]-CGP 39653 binding* (fmol mg^{-1} brain tissue)	*Binding relative to hippocampal formation (overall)* (%)
Cerebral cortex (overall)	169 ± 10	60
Basal ganglia (CPu)	65 ± 10	23
Septal area (LSD)	72 ± 18	25
Thalamus		
Overall	145 ± 17	51
AV	47 ± 6	17
Other diencephalic nuclei		
MGD	114 ± 9	40
DLG	152 ± 11	54
VLG	27 ± 7	10
Hippocampal formation		
Overall	283 ± 15	100
CA1, oriens layer	357 ± 24	126
CA1, pyramidal cell layer	176 ± 7	62
CA1, stratum radiatum	432 ± 25	153
CA3, oriens layer	322 ± 10	114
CA3, pyramidal cell layer	65 ± 1	23
CA3, stratum radiatum	374 ± 10	132
DG, molecular layer	429 ± 25	152
DG, granular layer	105 ± 16	37
Cerebellum		
Overall	19 ± 7	7
Molecular layer	9 ± 5	3
Granular layer	72 ± 8	25
Midbrain (overall)	33 ± 10	12

CPu, caudate putamen; LSD, lateral septal nucleus, dorsal; AV, anteroventral thalamic nuclei; MGD, medial geniculate nucleus, dorsal; DLG, dorsal lateral geniculate nucleus; VLG, ventral lateral geniculate nucleus; CA1-3, fields CA1-3 of Ammon's horn; DG, dentate gyrus. Values represent the mean ± s.e.mean of three animals (two sections per animal).

Table 2 [^{3}H]-CGP 39653 binding profile in striatum, cerebellum and cerebral cortex tissue homogenates

Region	*[^{3}H]-CGP 39653* (^{a}pK_D)	*L-Glutamic acid* (^{a}pK_i)	*CPP* (^{a}pK_i)	*Glycine* (^{b}pK_{iH})	(^{a}pK_{iL})
Striatum	7.81 ± 0.05 (3)	6.66 ± 0.06 (3)	6.97 ± 0.01 (3)	7.48 ± 0.05 (3)^C	3.89 ± 0.06 (3)
Cerebellum	8.00 ± 0.08 (3)	6.72 ± 0.26 (3)	7.17 ± 0.12 (3)	7.26 ± 0.13 (4)	3.65 ± 0.37 (4)
Cerebral cortex	7.97 ± 0.07 (4)	6.53 ± 0.09 (3)	6.97 ± 0.12 (3)	6.98 ± 0.02 (7)^O	3.49 ± 0.11 (7)

K_D dissociation constant of the radioligand; K_i inhibition constant of the displacer ligand; K_{iH} and K_{iL}, inhibition constant of the high and the low affinity component of the displacement curve, respectively. Results are expressed as the mean ± s.e.mean of (*n*) experiments. Each experiment was performed in duplicate. Data for the cerebral cortex are from Mugnaini *et al.*, 1993. ^{a}No significant differences between areas, $P > 0.05$ (one-way ANOVA). ^{b}Significant differences between areas, $P < 0.005$ (one-way ANOVA). ^{C}Significantly different from cerebral cortex (Fisher's protected LSD). ^{D}Significantly different from striatum (Fisher's protected LSD).

levels in the cortex and in many diencephalic nuclei, low levels in striatum, septal area and cerebellum and very low levels in the brainstem. Within laminated structures (hippocampus, cerebellum), dendritic zones had the greatest density of binding with less binding in the cellular layers. Non specific binding was very low and homogeneous in all regions (see Figure 1b). In the cerebral cortex, non specific binding was 14% of total binding, in accordance with the level obtained in membrane binding studies (Mugnaini *et al.*, 1993).

Densitometric analysis (see Table 1) of autoradiograms indicated that the highest concentration of specifically bound [^{3}H]-CGP 39653 occurred in the stratum radiatum of the CA1 region of the hippocampus (432 fmol mg^{-1} tissue) and in the molecular layer of the dentate gyrus (429 fmol mg^{-1}). By contrast, the lowest levels were detected in the brainstem (33 fmol mg^{-1}) and the molecular layer of the cerebellum (9 fmol mg^{-1}).

Within the hippocampal formation, the stratum radiatum and oriens layers had very high densities of binding sites (stratum radiatum > oriens layer), which were comparable to that detected in the molecular layer of dentate gyrus. By contrast, the pyramidal cell layer of the hippocampus and the granular layer of the dentate gyrus had relatively low levels of binding. Densitites were greater in CA1 than in CA3 region. Cerebral cortex regions exhibited moderate (temporal cortex) to high (frontoparietal, cingulate and enthorinal cortex) levels of binding, with the binding density decreasing from superficial to deep layers. Generally, the diencephalic nuclei contained high levels of binding. Many thalamic nuclei, such as the ventral lateral, ventral posteromedial and posterolater, posterior, centrolateral, mediodorsal, intermediodorsal, paraventricular, paratenial and parafascicular nuclei possessed nearly equivalent, high levels of binding. The overall binding density of these nuclei was 145 fmol mg^{-1}. In contrast, very low levels of binding (47 fmol mg^{-1}) were found in the anteroventral thalamic group. Dorsomedial geniculate and dorsolateral geniculate were clearly distinguishable, with the latter having a slightly higher binding level (152 with respect to 114 fmol mg^{-1}). Ventrolateral geniculate contained a very low density of binding sites (27 fmol mg^{-1}). The basal ganglia and the septal area contained low levels of binding sites. These regions also revealed a heterogeneous pattern of distribution: medial striatum had a slightly lower density with respect to the lateral striatum and the dorsal lateral septum nuclei had a higher density of binding sites than intermediate lateral septum nuclei. In cerebellum, distinct laminar binding was observed. Density levels were very low in the molecular layer (9 fmol mg^{-1}), which is the Purkinje cell dendritic zone. Binding in the granule cell layer of the cerebellum was much higher (72 fmol mg^{-1}).

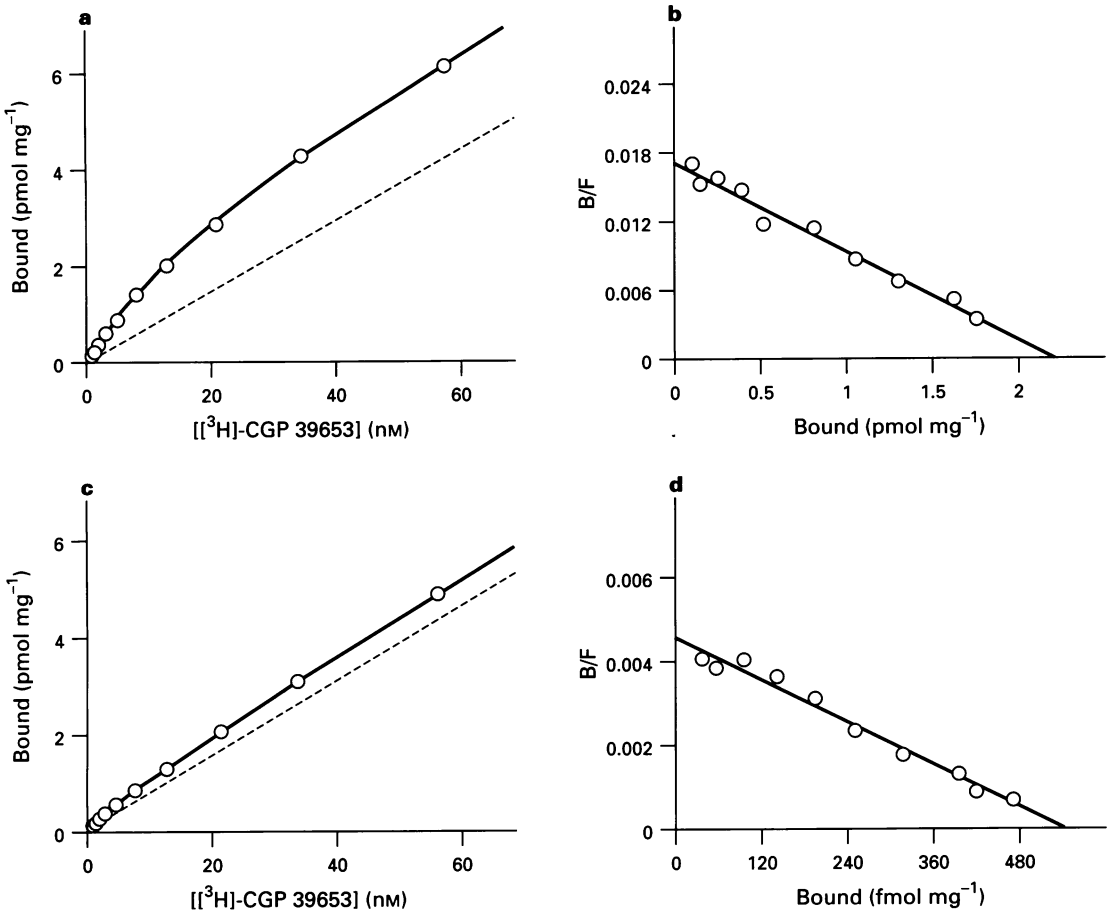

Figure 2 Saturation analysis of [^{3}H]-CGP 39653 binding to striatum (a, b) and cerebellum (c, d) membranes. (a, c) Saturation isotherm (dotted line represents non specific binding); (b, d) Scatchard analysis.

[^{3}H]-CGP 39653 binding characteristics in striatum and cerebellum membranes

Analysis of the saturation binding curves by LIGAND revealed, in both striatum and cerebellum membranes, a high affinity binding site for [^{3}H]-CGP 39653, with dissociation constants of 15.5 nM ($pK_D = 7.81 \pm 0.05$, $n = 3$) and 10.0 nM ($pK_D = 8.00 \pm 0.08$, $n = 3$) and B_{max} values of 3.1 ± 0.5 ($n = 3$) and 0.5 ± 0.1 ($n = 3$) pmol/mg^{-1} protein, respectively. Analysis of variance revealed that there were no significant differences between the pK_D of [^{3}H]-CGP 39653 in striatum, cerebellum and cerebral cortex (see Table 2). The presence of a single binding site was confirmed in both cases by a linear Scatchard representation (see Figure 2).

Displacement of [^{3}H]-CGP 39653 binding performed in striatum and cerebellum by CPP and L-glutamic acid showed that these compounds inhibited the binding in a competitive manner, according to a single site binding model (see Figure 3). The most active compound was CPP, with pK_i values of 6.97 ± 0.01 ($n = 3$) in striatum and 7.17 ± 0.12 ($n = 3$) in cerebellum. L-Glutamic acid had pK_i values of 6.66 ± 0.06 ($n = 3$) and 6.72 ± 0.26 ($n = 3$) in striatum and cerebellum respectively. The affinity values of CPP and L-glutamic acid in these two tissues were not significantly different from the values obtained in cerebral cortex membranes (see Table 2).

Glycine inhibited [^{3}H]-CGP 39653 binding in both striatum and cerebellum with a biphasic displacement curve (see Figure 3), characterized by a high affinity component of approximately 50% of specific [^{3}H]-CGP 39653 binding and a low affinity component with millimolar affinity. One-way analysis of variance by ANOVA revealed significant differences ($P < 0.005$) between the values of the apparent inhibition constant of the high affinity component (K_{iH}) of glycine displacement in striatum, cerebellum and cerebral cortex. Multiple comparison by Fisher's protected LSD method revealed, in fact, that the pK_{iH} value of glycine in striatum was significantly different from that observed in the cortex (7.48 ± 0.05, $n = 3$ with respect to 6.98 ± 0.02, $n = 7$).

Inhibition of [^{3}H]-CGP 39653 binding determined by means of autoradiography

L-Glutamic acid (1 nM – 100 μM) and CPP (1 nM – 100 μM) inhibited [^{3}H]-CGP 39653 binding in all the brain regions examined. CPP completely displaced the specifically bound [^{3}H]-CGP 39653. Certain representative regions were chosen to evaluate the displacing potency of these two ligands in different parts of the brain. At doses giving intermediate to high levels of inhibition of [^{3}H]-CGP 39653 binding, the granular cell layer of the cerebellum was hardly distinguishable from the neighbouring molecular layer; also if the cerebellum was selected and measured integrally, the level of binding was very low and difficult to quantify. As a consequence, reproducible inhibition curves were not obtained for cerebellum and this region was not further considered in this study.

In the cerebral cortex, L-glutamic acid was able to inhibit binding with a pIC_{50} of 6.00 ± 0.11 ($n = 3$). The affinity of L-glutamic acid in the cortex could be determined because the dissociation constant of [^{3}H]-CGP 39653 in this tissue had previously been obtained in binding experiments with membranes (Mugnaini *et al.*, 1993). The pK_i value obtained for L-glutamic acid, applying the Cheng & Prusoff (1973) equation, 6.47 ± 0.08 ($n = 3$), was not significantly different from the

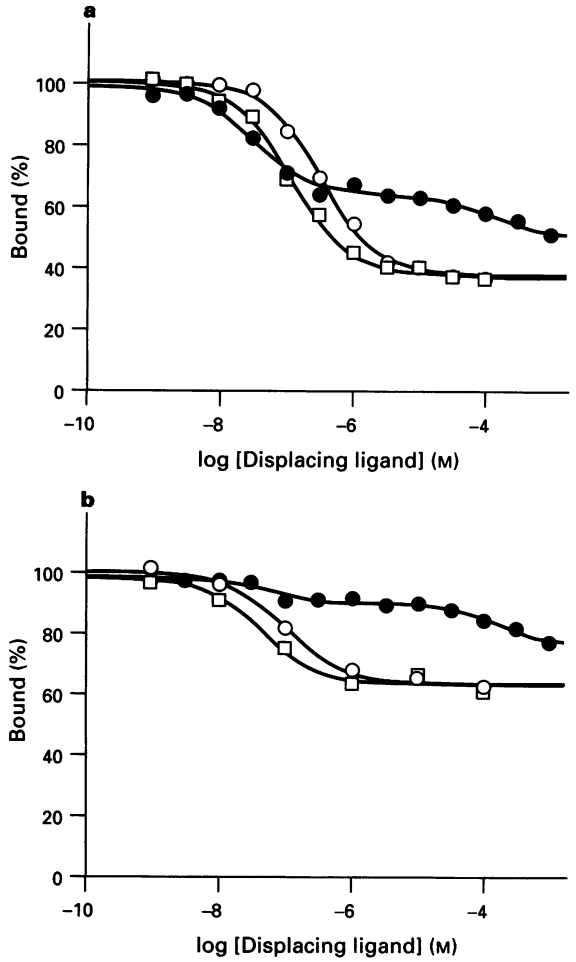

Figure 3 Representative inhibition curves of [^{3}H]-CGP 39653 binding by CPP (□), L-glutamic acid (○) and glycine (●) in striatum (a) and cerebellum (b) membranes.

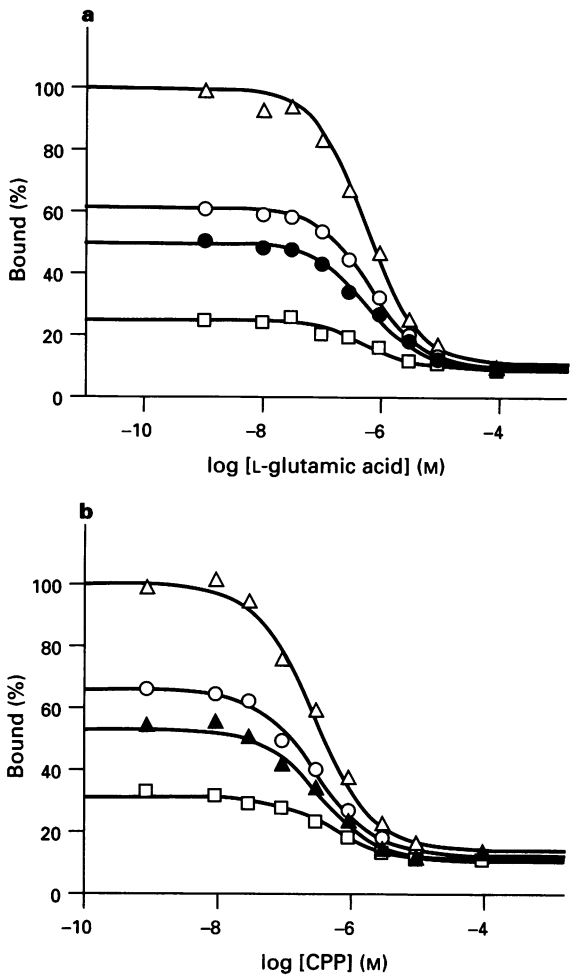

Figure 4 Inhibition curves of [^{3}H]-CGP 39653 binding to some representative brain regions by L-glutamic acid (a) and CPP (b), determined by means of autoradiographic analysis; (○) cortex; (●) DLG; (△) hippocampal formation; (▲) thalamus and (□) CPu. Data are expressed as percentage of the binding density determined in the hippocampal formation. For abbreviations, see Table 1.

Table 3 Regional inhibition of [^{3}H]-CGP 39653 binding by L-glutamic acid and CPP

Area	*L-Glutamic acid* (^{a}pIC$_{50}$)	*CPP* (^{a}pIC$_{50}$)
Cerebral cortex	6.00±0.11	6.46±0.02
CPu	5.98±0.16	6.19±0.08
Thalamus	6.03±0.11	6.41±0.08
MGD	5.99±0.08	6.32±0.05
DLG	5.97±0.12	6.42±0.05
Hippocampal formation	6.11±0.09	6.44±0.03

IC$_{50}$, concentration of compounds inhibiting 50% of binding. Data represent the mean±s.e.mean of three experiments, from three different animals. Each experiment was performed in duplicate (two sections per animal). For abbreviations, see Table 1. ^{a}No significant differences between areas, $P>0.05$ (two-ways ANOVA).

value obtained in cortical homogenates (pK_i=6.53±0.09, n=3). Analysis of the data by LIGAND gave displacement curves indicating a single site binding model and equivalent pK_i values. CPP also inhibited [^{3}H]-CGP 39653 binding in the cerebral cortex with a monophasic displacement curve and a pIC$_{50}$ value of 6.46±0.02 (n=3). The calculated pK_i (6.92±0.003, n=3) was not significantly different from the value found in cortical homogenates (6.97±0.12, n=3).

The potency of L-glutamic acid and CPP in displacing [^{3}H]-CGP 39653 binding was the same in all regions tested (see Table 3). In fact, there were no significant differences between the pIC$_{50}$ values obtained in the different regions. Displacement curves had the same characteristics of the curve obtained in the cortex, with complete displacement of specific binding. Representative inhibition of curves of L-glutamic acid and CPP are shown in Figure 4.

Glycine inhibited [^{3}H]-CGP 39653 binding in all regions tested, with shallow, biphasic displacement curves (not shown). The inhibitory effect of glycine was generally lower with respect to that found previously in rat cerebrocortical membrane homogenates at the same concentration, which may be due to higher levels of endogenous glycine remaining

Table 4 Regional glycine-induced inhibition of [3H]-CGP 39653 binding and reversal by 7-CKA

Area	*Specific [3H]-CGP 39653 binding* (%)	*+Glycine (10 μM)* ([a]%)	*+Glycine (1 mM)* ([b]%)	*+Glycine (10 μM) +7-CKA (100 μM)* ([b]%)
Cortex	100	80±5	$66\pm2^{S,H}$	120 ± 6^{H}
CPu	100	82±5	$72\pm3^{C,H}$	$131\pm6^{T,H}$
Thalamus	100	79±4	66 ± 2^{H}	112 ± 5^{S}
MGD	100	80±1	66±8	113±5
DLG	100	86±1	64±3	114±1
Hippocampal formation	100	70±5	$58\pm3^{S,C,T}$	$104\pm2^{S,C}$

Results are expressed as percentage of specific [3H]-CGP 39653 binding determined in the same area in the absence of added bis compounds. Data represent the mean±s.e.mean of four experiments, from four different animals. Each experiment was performed in duplicate (two sections per animal). For abbreviations, see Table 1. [a]No significant differences between areas, $P>0.05$ (two-ways ANOVA). [b]Significant differences between areas, $P<0.05$ (two-ways ANOVA). [C]Significantly different from cortex (Fisher's protected LSD). [S]Significantly different from CPu (Fisher's protected LSD). [T] Significantly different from Thalamus (Fisher's protected LSD). [H] Significantly different from hippocampal formation (Fisher's protected LSD).

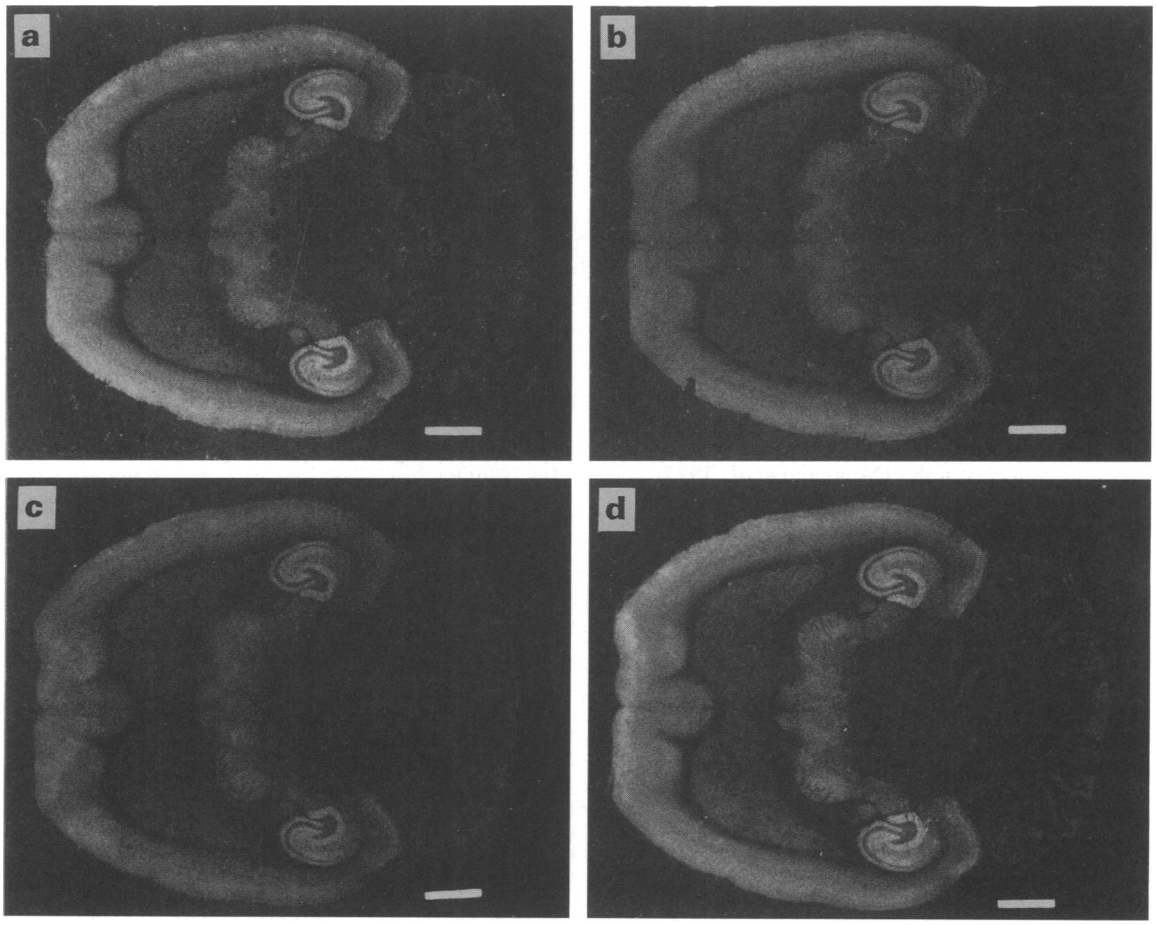

Figure 5 Reversal of glycine-induced inhibition of [3H]-CGP 39653 binding by 7-CKA: (a) total binding; (b) +10 μM glycine; (c) +1 mM glycine; (d) +10 μM glycine+100 μM 7-CKA. Calibration bar corresponds to 2.0 mm.

in the slices. There were no regional differences in the percentage of inhibition of [3H]-CGP 39653 specific binding by 10 μM glycine (see Table 4). Conversely, 1 mM glycine was also able to inhibit the binding to a different extent in the different areas considered ($P<0.05$, two-ways ANOVA). In the caudate putamen (CPu) for instance, 1 mM glycine reduced the binding to only 72±3% of control, whereas in the cortex and the hippocampus the inhibition was significantly (Fisher's protected LSD) greater (66±2% and 58±3% of control, respectively). Furthermore, the inhibition of [3H]-CGP 39653 specific binding by 10 μM glycine could be reversed differently ($P<0.05$, two-way ANOVA) in these regions by 100 μM 7-CKA (see Figure 5). In hippocampus, for example, the presence of 100 μM 7-CKA restored the specific binding to the basal value (104±2%) whereas in the other regions this substance caused an increase of the specific binding with respect to the initial level. The reversal by 7-CKA was significantly greater (Fisher's protected LSD) in CPu (131±6%) with respect to the thalamus (112±5%) and the hippocampal formation (104±2%).

Discussion

Receptor autoradiography studies with [3H]-CGP 39653 have been performed by other groups to determine the distribution of the NMDA binding sites in rodent brain (Cimino *et al.*, 1993; Laurie & Seeburg, 1994). The present data are largely in agreement with the previous studies: indeed high binding of [3H]-CGP 39653 was found in hippocampus, cerebral cortex and thalamus and low binding was detected in striatum, septum and cerebellum. Within the hippocampus, the region with the highest density of binding sites, the striatum oriens and striatum radiatum of both CA1 and CA3 domains of Ammon's horn and the molecular layer of dentate gyrus were the most intensely labelled. In the cerebral cortex, a lower density was evident in the deep layers with respect to the superficial layers. High levels of [3H]-CGP 39653 binding were also pre-sent in many diencephalic nuclei like the ventrolateral, medial and posterior thalamic nuclei, the lateral and medial geniculate nuclei. This pattern of distribution was consistent with that of other antagonists used to label the NMDA binding sites, such as D-2-[3H]-amino-5-phosphonopentanoic acid (D-[3H]-AP5, Monaghan *et al.*, 1984) and [3H]-CPP (Jarvis *et al.*, 1987), whereas differences could be detected with respect to the localization of the agonist [3H]-glutamic acid. In fact, [3H]-CGP 39653 binding, as well as that of [3H]-CPP (Monaghan *et al.*, 1988; Buller *et al.*, 1994) was relatively low, compared with [3H]-glutamic acid, in striatum, septum and cerebellum but higher in the thalamus and inner cerebral cortex.

[3H]-CGP 39653 had similar binding characteristics in the various rat brain regions examined. The affinity of the radioligand was the same in the cerebral cortex, striatum and cerebellum and the competitive ligands CPP and L-glutamic acid completely displaced the specific binding of [3H]-CGP 39653 in all regions, with comparable potencies throughout. In addition, glycine also inhibited the binding in all areas. The effect was antagonized by the glycine antagonist, 7-CKA, in all regions, indicating that the possibility of negative allosteric modulation of [3H]-CGP 39653 binding, through the activation of the associated strychnine-insensitive glycine site of the NMDA receptor channel complex (Mugnaini *et al.*, 1993), was not restricted to the cerebral cortex alone. Nevertheless, even if in striatum homogenates, glycine affected [3H]-CGP 39653 binding with the same modality found in the cerebral cortex, i.e. with biphasic inhibition curves and nearly the same amounts of specific binding displaced by glycine, the apparent pK_{iH} was higher in the striatum than in the cortex. In other words, in the striatum glycine was able to inhibit [3H]-CGP 39653 binding at lower concentrations.

These results indicate the presence of NMDA receptors in the striatum, which differ from those of the cortex in the diverse coupling of the glutamate and glycine binding sites, which may reflect a different sensitivity to endogenous ligands, as well as to potential therapeutic agents (such as CGP 39653) antagonizing the functioning of the NMDA receptor complex through action at one of these sites.

The hypothesis of the existence of regionally distinct NMDA receptors, with a different allosteric modulation of [3H]-CGP 39653 binding, was supported by the results of receptor autoradiography experiments. 7-CKA was able to reverse the inhibitory effect of a low dose (10 μM) of glycine on [3H]-GCP 39653 binding throughout the brain. Generally, 7-CKA was able to increase the binding to a level that was higher than the basal level, suggesting that the initial level of [3H]-CGP 39653 binding was already partially inhibited by the endogenous glycine. This enhancement was greater in some regions than others: in the CPu, for example, the increase was higher with respect to the thalamus and the hippocampus. If it is assumed that the same level of endogenous glycine was present in all regions after the prewashing step, these data indicate, again, different potencies of glycine in inhibiting [3H]-CGP 39653 binding, or of 7-CKA in counteracting the glycine effect. In addition, a high dose of glycine (1 mM) was able to decrease [3H]-CGP 39653 binding to a different extent in the brain regions considered. Interestingly, in the CPu the inhibition by glycine was significantly lower than in the cerebral cortex. This result confirmed what was found with tissue homogenates. In the CPu, in fact, the low concentration of endogenous glycine, because of its greater apparent pK_{iH}, caused an inhibition of basal [3H]-CGP 39653 binding that was higher with respect to the cortex. As a consequence, since the maximal inhibition by glycine was the same in these two regions, the inhibition of [3H]-CGP 39653 binding by a high dose of glycine was found to be lower in the CPu than in the cerebral cortex.

So far, it has been shown that specific, high affinity [3H]-CGP 39653 binding is possible only when the subunit NR_{2A} is present in the heteromeric receptor (Lynch *et al.*, 1993; Marti *et al.*, 1993; Laurie & Seeburg, 1994). These results were consistent with the fact that the distribution of [3H]-CGP 39653 binding, like that of [3H]-CPP (Buller *et al.*, 1994) strictly paralleled that of the mRNA encoding for the NR_{2A} subunit (Laurie & Seeburg, 1994). [3H]-CGP 39653 binding characteristics at the NR_1/NR_{2A} receptor were very similar to those found in the brain: nanomolar affinity was reported for the radioligand, similar pK_i values were found for competitive displacers and a biphasic inhibition curve was obtained with glycine (Lynch *et al.*, 1994). Nevertheless, the binding characteristics of [3H]-CGP 39653 at other kinds of recombinant receptors have never been studied. Laurie & Seeburg (1994), for instance, showed that CGP 39653 was able, although with low potency, to displace [3H]-glutamic acid binding from the NR_1/NR_{2B}, NR_1/NR_{2C} and NR_1/NR_{2D} heteromeric receptors, indicating that low affinity CGP 36953 binding is possible also with combinations other than NR_1/NR_{2A}. Moreover, recent results indicate that native receptors may be formed of the NR_1 subunit and more than one member of the NR_2 family (Wafford *et al.*, 1993; Chazot *et al.*, 1994). The presence of an additional NR_2 subunit different from the NR_{2A} in the NR_1/NR_{2A} combination (to give, for instance, a $NR_1/NR_{2A}/NR_{2C}$ or a $NR_1/NR_{2A}/NR_{2B}$ heteromeric receptor) or of a mixture of receptors (e.g. NR_1/NR_{2A} and NR_1/NR_{2B}), may account for the regional differences that we found in the negative allosteric modulation of [3H]-CGP 39653 binding by glycine.

Unlike other authors (Monaghan *et al.*, 1988; Reynolds *et al.*, 1991; Yoneda *et al.*, 1991; Sakurai *et al.*, 1993) we found that the agonist L-glutamic acid, as well as the antagonist CPP, had the same potency in all rat brain areas tested. These discrepancies may have arisen because, in our case, an NMDA site antagonist was used to label the NMDA receptor. Molecular target size analyses of the NMDA receptor complex revealed that glutamate and CPP bind to proteins with different molecular weights (Honore' *et al.*, 1989), suggesting that NMDA agonists and antagonists bind to overlapping but distinct sites. Recently, two binding sites were described in native receptors for the antagonists *cis*-4-phosphonomethyl-2-[3H]-piperidine carboxylic acid ([3H]-CGS 19755, Murphy *et al.*, 1988) and [3H]-CPP (Porter *et al.*, 1992; Van Amsterdam *et al.*, 1992): a high affinity site, which corresponds to the site recognized by [3H]-CGP 39653 and a low affinity site, probably related to the known physiological response (Van Amsterdam *et al.*, 1992). Interestingly, displacement binding studies of [3H]-glutamic acid revealed pK_i values for CPP and CGS 19755 which were more in accord with the low affinity site (Fagg *et al.*, 1990; Grimwood *et al.*, 1991). When the potency of glutamate and CPP was determined by means of [3H]-MK-801 binding, which requires the receptor in an active conformation, or [3H]-glutamic acid, the low affinity, agonist preferring site probably played a main role. Conversely, when [3H]-CGP 39653 was used with the same purpose, only the high affinity, antagonist-preferring site was labelled. In conclusion, the involvement of low affinity components, not apparent when using [3H]-CGP 39653 to label the receptor, may account for the regional differences in the potency of glutamic acid and CPP which other authors obtained with [3H]-glutamic acid or

[3H]-MK-801. According to our results, no significant regional differences were found in the potency of either CPP or glutamic acid towards the high affinity, antagonist-preferring site or conformation of the NMDA receptor.

In line with other NMDA site antagonists, the pattern of distribution of [3H]-CGP 39653 binding differed from that of the agonist [3H]-glutamic acid in being relatively lower in striatum, but higher in the thalamus and cerebral cortex (Monaghan *et al.*, 1988; Buller *et al.*, 1994). It may be noteworthy that in our data, because of the different allosteric modulation, basal [3H]-CGP 39653 binding was already partially inhibited by the endogenous glycine to a greater extent in striatum with respect to the cerebral cortex and the thalamus. Although hypothetically, it can be argued that the presence of endogenous glycine, which was reported to increase NMDA-sensitive [3H]-glutamic acid binding (Fadda *et al.*, 1988; Monaghan *et al.*, 1988), might in part explain the partially different distribution of these two radioligands, especially if regional differences were also present in the positive allosteric modulation of [3H]-glutamic acid binding. The same basal level of endogenous glycine present in the autoradiographic experiments, in fact, would oppose to different extents, in different regions, the binding of these two compounds. However, to prove the validity of this hypothesis further autoradiography experiments should be performed, with both radioligands, in the presence of a high concentration of a potent glycine site antagonist, counteracting completely the effect of the endogenous glycine.

In conclusion, the present results show that some of the main binding characteristics of [3H]-CGP 39653, represented by high affinity, displacement by L-glutamic acid and CPP and non competitive inhibition by glycine, are maintained in all the brain regions examined and are very similar to the binding characteristics of the radioligand reported for the NR_1/NR_{2A} recombinant receptor. Nevertheless, regional differences were found, in the potency of glycine, with regard to the allosteric modulation of the binding at the NMDA site. These results underline the importance of the glycine site in the functioning of the channel and suggest the possibility of counteracting, selectively, the activity of different NMDA receptor subtypes with glycine antagonists.

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