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Allosteric modulation of [^{3}H]-CGP39653 binding through the glycine site of the NMDA receptor: further studies in rat and human brain

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1 Binding of D,L-(E)-2-amino-4-[^{3}H]-propyl-5-phosphono-3-pentenoic acid ([^{3}H]-CGP39653), a selective antagonist at the glutamate site of the NMDA receptor, is modulated by glycine in rat brain tissue. We have further investigated this phenomenon in rodent and human brain by means of receptor binding and quantitative autoradiography techniques.

2 In rat cerebral cortical membranes the glycine antagonist 3-[2-(Phenylaminocarbonyl)ethenyl]-4,6-dichloro-indole-2-carboxylic acid sodium salt (GV150526A) did not change basal [^{3}H]-CGP39653 binding, but competitively reversed the high affinity component of [^{3}H]-CGP39653 binding inhibition by glycine, with a pK_B value of 8.38, in line with its affinity for the glycine site ($pK_i = 8.49$ vs [^{3}H]-glycine). Glycine (10 μM) significantly decreased [^{3}H]-CGP39653 affinity for the NMDA receptor (with no change in the B_{max}), whereas enhanced L-glutamate affinity ($P < 0.05$, paired-samples Student's t-test).

3 In rat brain sections the addition of GV150526A (30 μM) to the incubation medium increased [^{3}H]-CGP39653 binding to 208% of control (average between areas), indicating the presence of endogenous glycine. The enhancement presented significant regional differences ($P < 0.05$, two-way ANOVA), with striatum higher than cerebral cortex (282 and 187% of control, respectively; $P < 0.05$, Fisher's LSD). On the contrary, there was not any significant variation in affinity values of [^{3}H]-CGP39653, L-glutamate, glycine and GV150526A in striatal and cortical membranes. These results confirmed the existence of regionally distinct NMDA receptors subtypes with different glycine/glutamate allosteric modulation.

4 Whole brain autoradiography revealed an uneven distribution of [^{3}H]-CGP39653 binding sites in human brain. High levels of binding were determined in hippocampus and in cingulate, frontoparietal and insular cortex. Intermediate to low levels of binding were found in diencephalic nuclei and basal ganglia. [^{3}H]-CGP39653 binding was increased to 216% of control (mean between areas) by 30 μM GV150526A. The enhancement, however, did not present significant regional differences.

5 These results introduce GV150526A as a useful tool to identify NMDA receptor subtypes by means of receptor autoradiography; moreover, they demonstrate that the allosteric inhibition of [^{3}H]-CGP39653 binding by glycine parallels an increase in receptor affinity to the endogenous ligand L-glutamate. Finally, this study provides the first detailed anatomical description of the regional distribution of [^{3}H]-CGP39653 binding sites in human brain.

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Abbreviations: 7-CKA, 7-chlorokynurenic acid; ANOVA, analysis of variance; BCA, bicinchoninic acid; CA1-4, fields CA1-4 of Ammon's horn; CaB, caudate nucleus, body; CGP39653, D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid; CM, centromedian nucleus; CPP, 3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; CPu, caudate putamen; D-AP5, D-2-amino-5-phosphonopentanoic acid; DG, dentate gyrus; DGmol, molecular layer of dentate gyrus; DGgr, granular layer of dentate gyrus; DLG, dorsal lateral geniculate nucleus; DM, dorsomedial nucleus; GV150526A, 3-[2-(Phenylaminocarbonyl)ethenyl]-4,6-dichloro-indole-2-carboxylic acid sodium salt; HF, hippocampal formation; LD, lateral dorsal nucleus; LG, lateral geniculate body; LSD, least significant difference; MCAo, middle cerebral artery occlusion; MG, medial geniculate body; MGD, medial geniculate nucleus, dorsal; MK801, (+)-5-methyl-10,11-dihydroxy-5H-dibenzo(a,d)cyclohepten-5,10-imine; NSB, non specific binding; PSL, phosphostimulated luminescence; Put, putamen; ROD, relative optical density; SB, specific binding; TCP, 1-(1-(2-thienyl)-cyclohexyl)-piperidine; VL, ventral lateral nucleus; VPL, ventral posterolateral nucleus

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Introduction

The *N*-methyl-D-aspartate (NMDA) receptor is a glutamate-gated ion channel with high calcium ions (Ca^{2+}) permeability,

voltage dependent regulation by magnesium ions (Mg^{2+}) and modulation by a variety of agents such as steroids, polyamines, reducing and oxidizing substances, protons (H^+) and zinc ions (Zn^+; Mayer & Westbrook, 1987; Collingridge & Lester, 1989; Monaghan *et al*., 1989; Scatton, 1993; McBain & Mayer, 1994). NMDA receptors are widely distributed in the mammalian brain (Nakanishi, 1992; Monyer *et al*., 1992; 1994; Mori & Mishina, 1995; Petralia *et al*., 1994a,b; Wenzel *et al*., 1995; Meoni *et al*., 1998) and they are thought to play a crucial role in important physiological mechanisms, such as regulation of excitatory transmission, neuronal plasticity, neuronal migration and long term potentiation (Collingridge & Singer, 1990; Komuro & Rakic, 1993; Bliss & Collingridge, 1993), and pathological processes, such as epilepsy, chronic pain and many neurodegenerative diseases (Meldrum & Garthwaite, 1990; Dickenson *et al*., 1990; Kemp & Leeson, 1993; Collingridge & Watkins, 1994).

One of the most interesting features of the NMDA receptor is the absolute requirement of glycine for channel activation (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). The discovery that the classical inhibitory neurotransmitter, glycine, markedly potentiates the effect of NMDA site agonists through an action at an independent, strychnine-insensitive site, has generated an enormous amount of interest, especially after the finding that antagonists at this site possess anticonvulsant and neuroprotective properties, but lack most side effects typical of other types of antagonists of the NMDA receptor (Kemp & Leeson, 1993; Danysz & Parsons, 1998).

In the last 10 years the application of the molecular cloning technology to the study of the glutamate receptor system has led to an explosion of knowledge about the structure, expression and function of NMDA receptors (for reviews, see Hollmann & Heinemann, 1994; Nakanishi & Masu, 1994; Mori & Mishina, 1995; Zukin & Bennett, 1995). Like other ligand-gated ion channels, the NMDA receptor is thought to be made up of four or five polypeptidic subunits, which assemble to generate a canal in the cell membrane (Betz *et al*., 1990). So far, two families of NMDA receptor subunits have been identified by molecular cloning: the NR1, which is composed of eight isoforms generated by alternative splicing of a single gene, and the NR2 family, which contains four subunits, belonging to four different genes. Functional NMDA receptors are generated by co-expression of NR1 with NR2 subunits, to give a hetero-oligomeric protein complex with distinct electrophysiological and pharmacological properties depending on the subunit composition (for reviews, see McBain & Mayer, 1994; Hollmann & Heinemann, 1994; Zukin & Bennett, 1995; Sucher *et al*., 1996; Kendrick *et al*., 1996; Buller & Monaghan, 1997; Kew *et al*., 1998; Vicini *et al*., 1998).

Site-directed mutagenesis and receptor binding to cloned glutamate receptors have shown that the glycine binding site resides in the NR1 subunits (Kuryatov *et al*., 1994; Wafford *et al*., 1995; Hirai *et al*., 1996; Williams *et al*., 1996; Wood *et al*., 1997; Uchino *et al*., 1997), whereas the major determinants of glutamate binding are in the extracellular domain of the NR2 subunits (Kendrick *et al*., 1996; Laube *et al*., 1997; Anson *et al*., 1998). In contrast to glutamate, however, binding of NMDA site antagonists requires the presence of both NR1 and NR2 subunits (Kendrick *et al*., 1996).

Despite the fact that their binding sites are located on different polypeptidic chains, glycine and glutamate not only interact to open the channel, but also reciprocally increase their binding at their respective recognition sites (Fadda *et al*., 1988; Monaghan *et al*., 1988a,b; Monahan *et al*., 1989; Kessler *et al*., 1989; Berger *et al*., 1995). The phenomenon of glycine/glutamate allosteric interactions becomes even more complex considering that glycine, while enhancing [^{3}H]-glutamate binding, negatively modulates binding of most NMDA site antagonists (Monaghan *et al*., 1988a,b; Kaplita & Ferkany, 1990; Sills *et al*., 1991; Mugnaini *et al*., 1993; for a review, see Thomson, 1990). In most of these studies, the allosteric modulation was reported only as an 'increase' or 'decrease' of binding, without investigating the overall effect on the affinity of the radioligand (in terms of dissociation constant, K_D) or on the maximum number of its binding sites (B_{max}). Similarly, in many electrophysiological studies the affinity of compounds (e.g. glutamate or NMDA) was determined at a single concentration of allosteric modulator (e.g. 300 nM glycine in Priestley *et al*., 1996) and only during the peak response, that is in non-equilibrium conditions (Priestley & Kemp, 1994; Priestley *et al*., 1996). Some authors found that glycine affinity decreased soon after NMDA receptor activation and suggested negative cooperativity between glutamate and glycine (Mayer *et al*., 1989; Vyklicky *et al*., 1990; Benveniste *et al*., 1990; Lerma *et al*., 1990; Parsons *et al*., 1993; see also reviews: McBain & Mayer, 1994 and Dingledine *et al*., 1999). In these experiments, however, the affinity of glycine, both during the peak response and in steady state conditions (at equilibrium), was measured at a single, constant concentration of NMDA or glutamate; no experiments were performed to determine the affinity of glycine at different NMDA or glutamate concentrations (neither at equilibrium nor during the peak response).

At present, only a few authors have described glycine/glutamate allosteric interactions in rodent brain tissue in terms of affinity values: Fadda *et al*. (1988) reported that the glycine-induced increase in [^{3}H]-glutamate binding corresponds to an increase in the affinity of glutamate for the NMDA binding site (higher pK_D) and Ransom & Deschenes (1990) and Berger *et al*. (1995) found an increase in the binding affinity of [^{3}H]-glycine by glutamate. Very little is known about the allosteric modulation in systems expressing recombinant NMDA receptors (Lynch *et al*., 1994), or in native human NMDA receptors (Ulas *et al*., 1992).

Nevertheless, experimental results from different laboratories indicate that the allosteric interactions between the glycine and the glutamate binding site can lead to the identification of native NMDA receptor subtypes in rat brain: Monaghan *et al*. (1988a,b) noticed a regional variation in the enhancement of [^{3}H]-glutamate binding by glycine in autoradiographic preparations; in line with these results, we found that inhibition of the binding of the NMDA site antagonist [^{3}H]-CGP39653 (D,L-(E)-2-amino-4-[^{3}H]-propyl-5-phosphono-3-pentenoic acid) by glycine was heterogeneous (Mugnaini *et al*., 1996).

In the light of the importance of NMDA receptors in so many physiological and pathological mechanisms, we have further characterized glycine/glutamate allosteric interaction in rat brain: (1) glycine negative modulation of [^{3}H]-CGP39653 binding was proved to be allosteric by counteracting glycine inhibition with the high affinity glycine site

antagonist 3-[2-(Phenylaminocarbonyl)ethenyl]-4,6-dichloro-indole-2-carboxylic acid sodium salt (GV150526A, Di Fabio *et al*., 1997); (2) the effect of glycine on the affinity value of [^{3}H]-CGP39653, glutamate and some other representative NMDA site ligands was determined; (3) the regional different allosteric interactions found previously with glycine (Mugnaini *et al*., 1996) were demonstrated also with GV150526A. Finally, we have looked for the possible existence of regionally distinct NMDA receptors with different glycine/glutamate interactions in the human brain, by means of large section autoradiography of [^{3}H]-CGP39653 binding.

A preliminary report of this work has been presented to the Federation of European Neuroscience Societies (Mugnaini *et al*., 1998).

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. Rats were killed by decapitation and the brains were removed and dissected. The research complied with national legislation and with the company policy on the Care of Use of Animals and with related codes of practice.

Human brains

Four different human brains were obtained (already frozen and cut in approximately 2 cm-thick coronal sections) from the Runwell Hospital Brain Bank (Wickford, Essex, SS11 7QE). The brains derived from four subjects (two males and two females) aged 72–82 years (average age: 78 ± 2 years; mean $\pm$ s.e.mean, $n=4$) who died immediately following myocardial infarction (three cases), with the exception of a man who was suffering from prostate carcinoma and died from bronchopneumonia (one case). The interval between the death and the autopsy ranged from 26–70.5 h (average delay: 40 ± 10 h).

Drugs and chemicals

[^{3}H]-CGP39653 (NET 1050, specific radioactivity 1098, 1091.5 and 1258 GBq/mmol) and [^{3}H]-glycine (NET 004, 1757.5 GBq/mmol) were from NEN Life Science Products, Cinisello Balsamo, Italy. NMDA, 7-chlorokynurenic acid (7-CKA), 3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), and D-2-amino-5-phosphonopentanoic acid (D-AP5) were obtained from Tocris Neuramin, Essex, U.K. L-glutamic acid·HCl and glycine·HCl were from Sigma, Sigma-Aldrich, Milan, Italy. GV150526A was synthesized at the Medicinal Chemistry Department of the Glaxo Wellcome Medicines Research Centre, Verona, Italy. Citric acid trisodium salt was from Merck, Germany. Tris(hydroxymethyl)amino-methane (Tris) and ethylenediamine-tetraacetic acid (EDTA) from Carlo Erba Reagenti, Milan, Italy. The BCA protein assay was purchased from Pierce, Illinois, U.S.A. Hybond-N membranes were from Amersham Italia, Milan, Italy, Filter Count from Canberra Packard, Milan, Italy and Solvable from NEN Life Science Products. Other salts and reagents were of highest analytical grade available.

GV 150526A and 7-CKA were dissolved in 10 mM KOH solution and subsequently diluted in the incubation medium. In the final tube, at the highest concentration of displacing compound (30 μM for GV150526A and 100 μM 7-CKA) the solution contained 200 μM KOH, which neither changed the pH of the incubation buffers nor significantly affected total [^{3}H]-CGP39653 or [^{3}H]-glycine binding. All other compounds were dissolved directly in the buffer solution.

Membrane preparation

Crude synaptic membranes were prepared according to Mugnaini *et al*. (1993). All steps were performed at 4°C, unless otherwise indicated. Briefly, rats were killed by decapitation, the cerebral cortex or striatum immediately dissected and homogenized in 15 volumes of ice-cold 0.32 M sucrose (pH 7) with a Potter homogenizer (12 strokes at 700 r.p.m). The homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was centrifuged at $17{,}000 \times g$ for 20 min and the resultant pellet was resuspended with 40 volumes of deionized water (Milli-Q system, Millipore) and lysed with a Polytron. Following incubation at 35°C for 30 min, the membranes were centrifuged at $48{,}000 \times g$ for 15 min and washed twice (by resuspension in 40 volumes of water and centrifugation at $48{,}000 \times g$ for 15 min). The final pellet was resuspended in three volumes of water and divided into aliquots that were quick frozen in liquid N_2.

[^{3}H]-CGP39653 binding

On the day of the experiment, membranes were thawed and washed twice by resuspension in 40 volumes of water and centrifugation at $48{,}000 \times g$ for 10 min. The final pellet was resuspended in 100 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 1–60 nM. Specific binding was defined as the portion of [^{3}H]-CGP39653 binding that was displaceable by 100 μM L-glutamic acid. Displacement binding curves were obtained at 2 nM [^{3}H]-CGP39653. 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 with bovine serum albumin as the standard.

To determine the effect of 10 μM glycine on (i) the affinity of [^{3}H]-CGP39653, (ii) the maximum number of [^{3}H]-CGP39653 binding sites and (iii) the potency of competitive inhibitors, saturation or displacement curves were performed in the absence and presence of glycine, within the same experiment. This was to minimize the interexperiment variability due to different membrane and radioligand concentrations. Preliminary experiments showed that both in normal conditions and in the presence of 10 μM glycine (which non-competitively inhibits [^{3}H]-CGP39653 binding) the association rate at 2 nM [^{3}H]-CGP39653 and 25°C was relatively fast and equilibrium was obtained within a few

minutes. Therefore, the standard incubation time of 25 min was used also for the experiments with 10 μM glycine.

[^{3}H]-Glycine binding

Displacement binding curves were obtained with 20 nM [^{3}H]-glycine (specific activity 175.75 GBq mmol^{-1}, obtained by a 10 fold isotopic dilution with unlabelled glycine). The incubations were carried out directly in scintillation Biovials (Beckman), in a final volume of 1 ml. The reaction was started by the addition of 750 μl of the membrane suspension and lasted 20 min at 4°C. Samples were centrifuged at $30{,}100 \times g$ for 20 min, after which the supernatants were aspirated and discarded, whereas the pellets were washed twice with 2 ml of buffer and then digested in 30 min with 150 μl of Solvable. After solubilization, the radioactivity was estimated by liquid scintillation counting. Non specific binding was determined by adding 100 μM unlabelled glycine.

Receptor autoradiography with rat brain

[^{3}H]-CGP39653 receptor autoradiography was performed as described by Mugnaini *et al.* (1996). Animals were killed by intracardiac perfusion, under urethane anaesthesia (1.5 mg kg^{-1}, i.p.), 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 coronal and horizontal sections (corresponding approximately to Tables 104–105 of Paxinos & Watson, 1986) were cut on a cryostat, mounted on poly-L-lysinated or gelatine coated glass slides and stored at −20°C for up to 2 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). After this, incubation was performed by applying over each section 200 μl of buffer solution containing 20 nM [^{3}H]-CGP39653. An incubation period of 25 min at 25°C of temperature was chosen as the standard incubation conditions since previous studies on cerebral cortical membranes (see above) had shown that the association rate of 2 nM [^{3}H]-CGP39653 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 (see also Mugnaini *et al.*, 1993; Zuo *et al.*, 1993).

Dried sections were apposed to tritium sensitive films (Hyperfilm-[^{3}H], Amersham Italia, Milan, Italy) with [^{3}H]-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.

Receptor autoradiography with human brains

When used for autoradiography, the 2 cm-thick human brain slices were embedded in carboxymethylcellulose matrix, placed in a cryomicrotome at −20°C and cut into 20 μm-thick coronal sections, corresponding approximately to Figures 24, 25, 26 and 27 of De Armond *et al.* (1989) and 40, 41 and 42 of Duvernoy (1988). Each section, which could be composed of only one or both the hemispheres, was picked up from the knife by using nylon membranes (Hybond-N, Amersham). Preliminary experiments had shown that, among all supports tested, nylon membranes presented the lowest level of [^{3}H]-CGP39653 binding (0.40%), which was similar to that found with glass (0.06%). Mounted sections were air dried and conserved at −80°C for up to 2 weeks.

For large section autoradiography experiments with human brain slices [^{3}H]-CGP39653 binding conditions were similar to those reported for the rat. Sections were allowed to reach the room temperature, air dried and subjected to a prewashing step (30 min, 37°C) in 50 mM Tris HCl buffer solution containing 2.5 mM $CaCl_2$ (final pH 7.6). After this, sections were again air dried and the incubation (25 min, 25°C) was performed by applying over each hemisphere 3 ml of buffer solution containing 20 nM [^{3}H]-CGP39653. During incubation, a squared glass slab was put above the sections (held up by four thin coverslips), to obtain a homogeneous distribu-

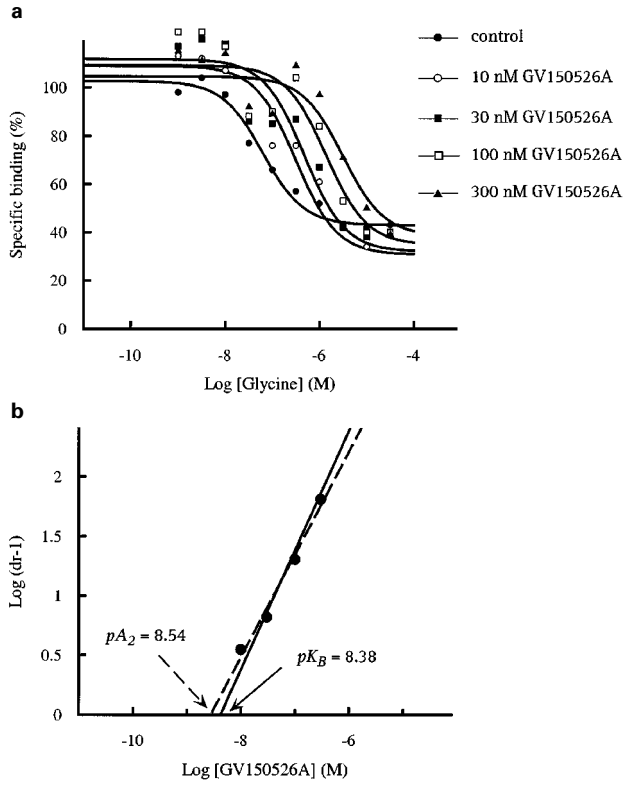

Figure 1 Competitive antagonism by GV150526A of the high affinity glycine-induced inhibition of [^{3}H]-CGP39653 binding. (a) The figure shows the rightward shift of the high affinity glycine-induced inhibition of [^{3}H]-CGP39653 binding by increasing concentrations of GV150526A (indicated to the right of the graph). Each data point is the average of three determinations. (b) Schild plot derived from the above data. Schild slope was either fitted by the computer (dashed line) or constrained to 1 (straight line). In the first case, the intercept on the x axis represents the pA_2 value of GV150526A (dashed arrow). In the second case, the intercept is the pK_B value (straight arrow). dr, dose ratio.

tion of the incubation liquid volume. Three hundred μM L-glutamic acid was used to determine non-specific binding.

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 and dried under a stream of air. Dried sections were apposed to tritium sensitive films with [^{3}H]-standards for 8 weeks. Alternatively, slides were exposed to Fuji Imaging Plates BAS-TR2025 for 3–4 days and analysed with the Bio-image Analyzer BAS5000 (Fuji Photo Film Co., Kanagawa, Japan).

Data analysis

Data of saturation and displacement experiments, to determine the dissociation constant (K_D) and the binding site density (B_{max}) of the radioligand and the inhibition constants of the displacer ligands (K_i), were analysed using the non linear curve fitting program LIGAND (Munson & Rodbard, 1980). The concentration of compounds inhibiting 50% of binding (IC_{50}) was obtained using ALLFIT (De Lean *et al*., 1978). The potency of GV150526A in antagonizing the allosteric modulation of [^{3}H]-CGP39653 binding by glycine was expressed in terms of A_2 (the concentration of antagonist which would produce a 2 fold shift in the concentration-response curve for an agonist) and K_B (the dissociation constant for a competitive antagonist) and calculated according to the method of Arunlakshana & Schild (1959).

Quantitative analysis of autoradiograms was performed by computer assisted image analysis (AIS, Imaging Research, St. Catharines, Ontario, Canada). In general, each area of interest was selected and the relative optical density (ROD) or the photostimulated luminescence (PSL) per mm^2 (PSL/mm^2) measured integrally. Regional values were converted to the corresponding radioligand concentration by reference to tritium standards on the same film or plate. Specific binding (SB) was determined after subtraction of the non-specific binding (NSB, defined as the binding measured in sections treated with an excess of a cold competitive ligand).

In the case of layers of laminated structures in the rat brain (oriens layer, pyramidal cell layer and stratum radiatum of hippocampus; molecular and granular layer of dentate gyrus; molecular and granular layer of cerebellum) values were the average of 5–10 determinations per section. When the human hippocampus was analysed, a unique, wide band of dense labelling was found in Ammon's horn, without a clear laminar distinction. This band was the only one selected and measured in the CA1, CA2 and CA3 regions. Conversely, the molecular and the granular layer of the dentate gyrus were easily distinguishable. Concerning the human cerebral cortex, five representative regions were selected and measured integrally: the cingulate sulcus, the superior frontal sulcus, the parietal operculum, the insular gyri and the middle temporal gyrus (see Figure 25 of De armond *et al*., 1989).

Comparisons were determined by independent-samples or paired-samples Student's *t*-test or, in the case of multiple comparisons, by one-way or two-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference (LSD) on least squares means method. Statistical analyses were performed with SPSS (SPSS Inc., Chicago, U.S.A.). All results are expressed as mean ± s.e.mean.

Results

Reversibility of the high affinity glycine-induced inhibition of [^{3}H]-CGP39653 binding by GV150526A

As previously reported (Mugnaini *et al*., 1993) glycine inhibited [^{3}H]-CGP39653 binding to rat cerebral cortical membranes with a biphasic displacement curve, characterized by a high affinity component of approximately 50% of specific binding ($pK_{iH} = 6.98 \pm 0.02$, $n = 7$) and a low affinity component with millimolar affinity. Seven-CKA and GV150526A did not change specific [^{3}H]-CGP39653 binding, but reversed the high affinity component of glycine inhibition, without affecting the low affinity displacement (Mugnaini *et al*., 1993). To determine the potency of GV150526A in counteracting glycine, high affinity glycine inhibition curves of [^{3}H]-CGP39653 binding were produced in control conditions and in the presence of increasing concentrations of GV150526A (see Figure 1a). Parallel rightward shifts of the glycine inhibition curve were obtained, with no significant variation of the maximal response and slope factor, as

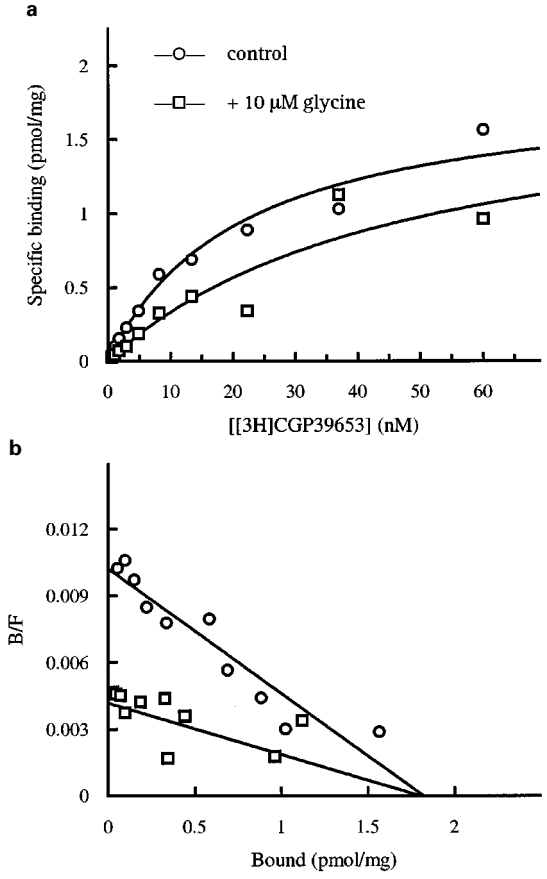

Figure 2 Glycine decreases the affinity of [^{3}H]-CGP39653 binding, without changing the number of receptor binding sites. (a) Cerebral cortical membranes were incubated at 25°C for 25 min in the presence of increasing concentrations of [^{3}H]-CGP39653 (from 1 to 60 nM), either in control conditions (○) or in the presence of 10 μM glycine (□), within the same experiment. Non-specific binding (NSB) was determined by the addition of 100 μM L-glutamic acid. (b) Scatchard representation of the same data. Note the lower slope in the presence of 10 μM glycine, which corresponds to a smaller pK_D value, without change in the abscissal intercept (B_{max} value).

Table 1 [^{3}H]-CGP39653 binding characteristics to rat cerebral cortex membranes, in the presence and absence of 10 μM glycine

Compound		*Standard condition*	*In the presence of 10 μM glycine*
[^{3}H]-CGP39653	pK_D	7.86±0.03 (3)	7.61±0.07 (3)*
	B_{max}	2.0±0.2 (3) pmol mg^{-1}	1.7±0.2 (3) pmol mg^{-1}
D-AP5	pK_i	6.61±0.07 (3)	6.40±0.04 (3)*
L-glutamate HCI	pK_i	6.49±0.01 (3)	6.70±0.01 (3)*
NMDA	pK_i	5.23±0.05 (3)	5.30±0.06 (3)*
CPP	pK_i	6.77±0.01 (3)	7.03±0.04 (3)*

K_D = dissociation constant of the radioligand; B_{max} = maximum number of binding sites; K_i = inhibition constant of the displacer ligand. *Significantly different from the value of the same parameter determined in the absence of glycine ($P<0.05$, paired-samples Student's *t*-test). Results are the mean±s.e.mean of (*n*) experiments. The same parameter was measured in the presence and absence of 10 μM glycine within the same experiment (see Methods).

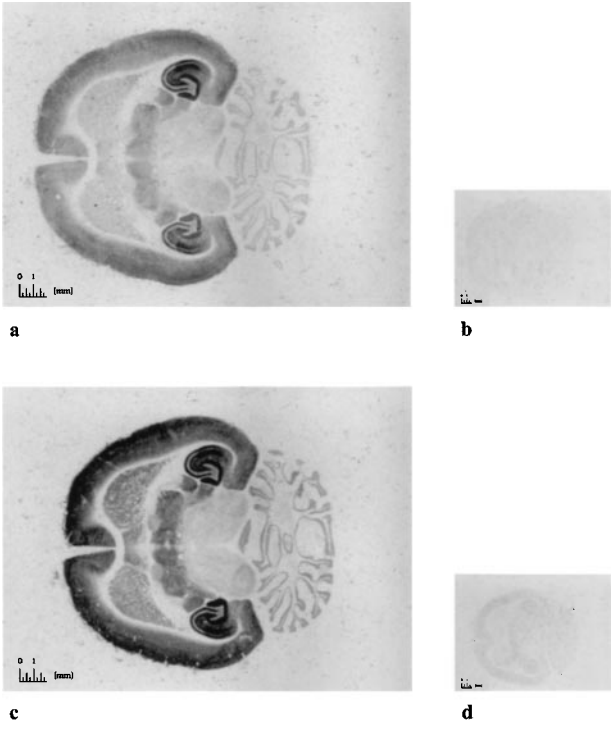

Figure 3 Enhancement of [^{3}H]CGP39653 binding by GV150526A in rat brain. Horizontal rat brain sections (corresponding approximately to Table 105 of Paxinos & Watson, 1986) were incubated in 20 nM [^{3}H]-CGP39653 for 25 min at 25°C, either in the absence (a,b) or in the presence (c,d) of 30 μM GV1505626A. (a,c) Total binding; (b,d) non specific binding, in the presence of 100 μM L-glutamate. Calibration bar corresponds to 2 mm.

demonstrated with ALLFIT analysis (De Lean *et al.*, 1978). The Arunlakshana & Schild (1959) analysis revealed an intercept of 8.54±0.12 (pA_2) and a slope factor of 0.86±0.08, which was not significantly different from one, confirming the competitive nature of GV150526A antagonism. A pK_B value of 8.38±0.06 was obtained for GV150526A when the slope was constrained to one (see Figure 1b).

[^{3}H]-CGP39653 saturation binding studies in the presence of 10 μM glycine

The effect of glycine on the affinity value and the maximum number of [^{3}H]-CGP39653 binding sites was investigated in three experiments, performed as explained in Methods. In control conditions (i.e. in the absence of added glycine), [^{3}H]-CGP39653 bound to cerebral cortex membranes in a saturable manner, with a pK_D value of 7.86±0.03 ($n=3$) and a receptor density of 2.0±0.2 pmol mg^{-1} protein (see Figure 2). In the presence of 10 μM glycine, [^{3}H]-CGP39653 bound to the membranes with a pK_D value of 7.61±0.07 ($n=3$), which was significantly lower than that found in the absence of glycine ($P<0.05$, paired-samples Student's *t*-test). In contrast, no significant differences were observed between the B_{max} values determined in the presence and absence of 10 μM glycine (see Figure 2 and Table 1).

[^{3}H]-CGP39653 inhibition binding studies in the presence of glycine

Inhibition binding studies in the absence and presence of 10 μM glycine were performed with some representative

Table 2 Regional GV150526A-induced enhancement of basal [^{3}H]-CGP39653 binding in rat brain sections

Brain region	*Basal* (fmol mg^{-1})	*Specific [^{3}H]-CGP39653 binding + 30 μM GV150526A* (fmol mg^{-1})	(% basal)[a]
Cortex	104±4	195±6	187±7^{S,M}
CPu	31±2	87±8	282±14^{C,T,M,D,H}
Thalamus	64±6	124±6	195±10^{S,M,H}
MGD	53±4	120±6	229±10^{C,S,T,D,H}
DLG	62±10	106±18	172±10^{S,M}
Hippocampal formation	159±5	257±10	162±4^{S,T,M}

Results are expressed as fmol mg^{-1} of brain tissue or as a percentage of specific [^{3}H]-CGP39653 binding determined in the same area in the absence of added compounds. Data represent the mean±s.e.mean of three different animals (two sections per animal). Differences were considered significant for $P<0.05$. CPu, caudate putamen, MGD, medial geniculate nucleus, dorsal; DLG, dorsal lateral geniculate nucleus. [a]Significant differences between areas, $P<0.0001$ (two-way ANOVA). [C]Significantly different from Cortex (Fisher's LSD). [S]Significantly different from CPu (Fisher's LSD). [T]Significantly different from Thalamus (Fisher's LSD). [M]Significantly different from MGD (Fisher's LSD). [D]Significantly different from DLG (Fisher's LSD). [H]Significantly different from Hippocampus (Fisher's LSD).

NMDA site ligands, namely L-glutamic acid, NMDA, D-AP5 and CPP. As indicated in Table 1, in the presence of glycine the endogenous agonist L-glutamic acid had a significantly higher affinity with respect to normal conditions. Similarly, the agonist NMDA displayed a slightly but significantly higher pK_i value in the presence of glycine, whereas the affinity of the antagonist D-AP5, similarly to that of the radioligand, was lower in the presence of glycine. Interestingly, the antagonist CPP, like the agonists L-glutamic acid and NMDA, showed a higher affinity for the receptor in the presence of glycine with respect to standard conditions.

Enhancement of basal [^{3}H]-CGP39653 binding to rat brain sections by GV150526A

Differently from rat cerebral cortical membranes, where 30 μM GV150526A had no significant effect on basal [^{3}H]-CGP39653 binding, the same concentration of GV150526A was able to greatly increase [^{3}H]-CGP39653 binding in rat brain slices (see Figure 3). This fact revealed the presence of endogenous glycine in the sections, counteracted by GV150526A. On average, in the presence of GV150526A the binding level was twice the control ($205 \pm 18\%$ of control, mean between areas). Nevertheless, the enhancement presented marked regional differences ($P < 0.0001$; two-way ANOVA; see Table 2). Caudate putamen (CPu) was the region most sensitive to the increase in [^{3}H]-CGP39653 binding by GV150526A. In this area binding in the presence of the glycine antagonist was $282 \pm 14\%$ ($n = 3$) of the basal level and was significantly different ($P < 0.05$, Fisher's protected LSD) from all the other regions tested. The lowest enhancement was found in the hippocampus, where GV150526A increased the binding up to $162 \pm 4\%$ of control. The cerebral cortex was midway between these two regions: the increase tended to be higher than that of hippocampus and much lower than that of CPu. The difference between hippocampus and cerebral cortex, however, failed to be significant.

[^{3}H]-Glycine binding studies

Saturation binding experiments showed that [^{3}H]-glycine bound in a specific and saturable manner to rat cerebral cortex membranes, with a curve according to a single-site binding model (see Figure 4). The presence of a single site was confirmed by the linear Scatchard plot. A receptor density of 2.9 ± 0.2 pmol mg^{-1} protein and an affinity value (K_D) of 97.7 nM ($pK_D = 7.01 \pm 0.04$, $n = 3$) were determined for [^{3}H]-glycine in these experiments. This affinity value was in line with that calculated by means of homologous displacement experiments ($pK_i = 6.74 \pm 0.06$, $n = 5$).

Binding of [^{3}H]-glycine in striatum presented many similarities with respect to the cortex. By means of saturation experiments, it was found that [^{3}H]-glycine bound to a single binding site in rat striatal membranes, with a K_D value of 83.2 nM ($pK_D = 7.08 \pm 0.08$, $n = 3$) and a B_{max} of 1.8 ± 0.2 pmol mg^{-1} protein (see Figure 4). This affinity value, confirmed by homologous displacement experiments ($pK_i = 7.00 \pm 0.09$, $n = 3$), was not significantly different from that found in the cerebral cortex. Displacement experiments performed with 7-CKA and GV150526A revealed that there were no differences in the affinity of these compounds between the striatum and the cerebral cortex (see Table 3).

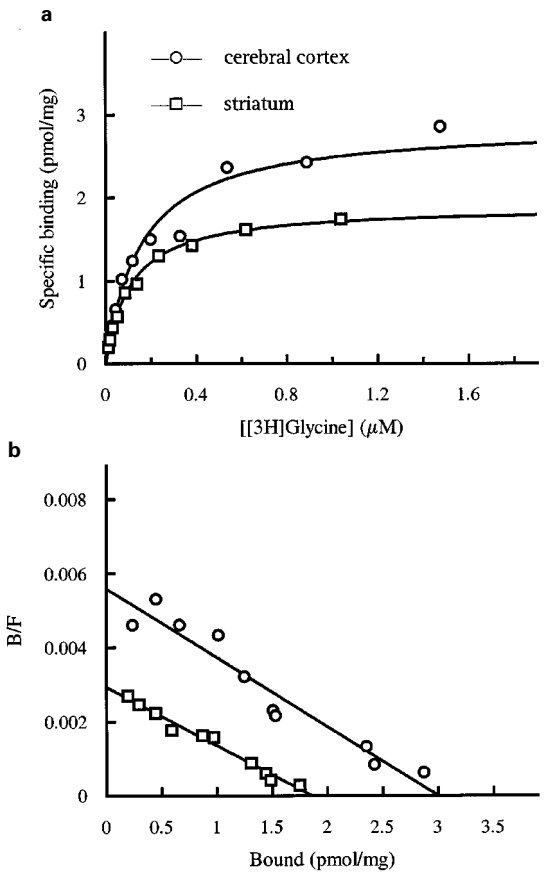

Figure 4 Representative saturation isotherms of [^{3}H]-glycine binding to rat cerebral cortical and striatal membranes. (a) Cerebral cortical (○) and striatal (□) membranes were incubated, in indipendent experiments, at 4°C for 20 min in the presence of increasing concentrations of [^{3}H]-glycine (from 20 nM to 1.5 μM or 1.1 μM, respectively). Non-specific binding (NSB) was determined by the addition of 1 mM glycine. (b) Scatchard representation of the same data. Note the similar slope (which corresponds to comparable pK_D values) and the different abscissal intercept (which corresponds to different B_{max} values) for the striatal and cortical Scatchard plots.

Distribution of [^{3}H]-CGP39653 binding sites in human brain

[^{3}H]-CGP39653 binding sites were distributed unevenly in human brain sections. An example of the distribution is shown in Figure 5. High levels of binding density were present in the hippocampal formation and in the cerebral cortex, low to intermediate levels in the thalamus and low to very low levels in the basal ganglia. Non specific binding was weak and homogeneous in all regions (see Figure 5, part d). In the cerebral cortex, non specific binding was 25% of total binding.

Densitometric analysis (see Table 4) of autoradiograms indicated that the highest concentration of specifically bound [^{3}H]-CGP39653 occurred in the CA1 region of the hippocampus (11.5 fmol mg^{-1} tissue) and in the molecular layer of the dentate gyrus (11.8 fmol mg^{-1}). The lowest levels were detected in the globus pallidus (1.6 fmol mg^{-1}) and in the centromedian nucleus of the thalamus (1.7 fmol mg^{-1}).

Within the hippocampal formation, a unique, wide band of dense labelling was present in Ammon's horn, without a clear laminar distinction (see Figure 6). Among the different

regions of the cornu, the binding density agreed with the following rank order: CA1 > CA3 > CA2 ≅ CA4. Conversely, in the dentate gyrus the molecular layer was clearly distinguishable from the granular layer, where the level of binding was relatively low. Cerebral cortex regions exhibited moderate (temporal cortex) to high (cingulate, frontal, insular, parietal) levels of binding, with higher binding density in the intermedial layers than superficial or internal layers. An uneven distribution was found among the diencephalic nuclei. The highest level of signal was found in the lateral dorsal nucleus and in the lateral geniculate body. The medial geniculate body and the dorsomedial and ventral lateral nuclei of the thalamus contained low levels of [^{3}H]-CGP39653 binding. In the centromedian nucleus the signal was extremely weak.

In general, the basal ganglia contained low levels of binding sites. Also these regions revealed a heterogeneous pattern of distribution: the signal was slightly lower in the caudate nucleus with respect to the putamen and the globus pallidus and the claustrum contained very low binding levels. No significant differences were found between the tail and the body of the caudate nucleus.

Much lower [^{3}H]-CGP39653 binding levels were found in the human brain compared with the rat brain (average between areas: 6.2 and 165.1 fmol mg^{-1} of protein, respectively). Moreover, a very high intersubject variability was found between the human brains, as revealed by the standard error of the mean, which was 13% of the mean in the CA1 region of the hippocampus and 30% or more in regions containing low binding such as the basal ganglia (see Table 4). In the rat (see Table 3), the standard error was only 6% of the mean in the stratum radiatum of the CA1 region of hippocampus and 15% in the caudate putamen.

Table 3 Comparison between the characteristics of [^{3}H]-glycine binding to rat cerebral cortical and striatal membranes

Compound	*Cerebral cortex*	*Striatum*
[^{3}H]-glycine	pK_D 7.01 ± 0.04 (3)	7.08 ± 0.08 (3)
	B_{max} 2.9 ± 0.2 (pmol mg^{-1})	1.8 ± 0.2 (pmol mg^{-1})*
Glycine	pK_i 6.75 ± 0.02 (5)	7.00 ± 0.09 (3)
7-CKA	pK_i 6.71 ± 0.07 (5)	6.89 ± 0.09 (3)
GV150526A	pK_i 8.49 ± 0.02 (3)	8.47 ± 0.10 (3)

K_D = dissociation constant of the radioligand; B_{max} = maximum number of binding sites; K_i = inhibition constant of the displacer ligand. *Significantly different from the value of the same parameter measured in the cerebral cortex ($P < 0.05$, independent-samples Student's t-test). Results are expressed as the mean ± s.e.mean of (n) experiments.

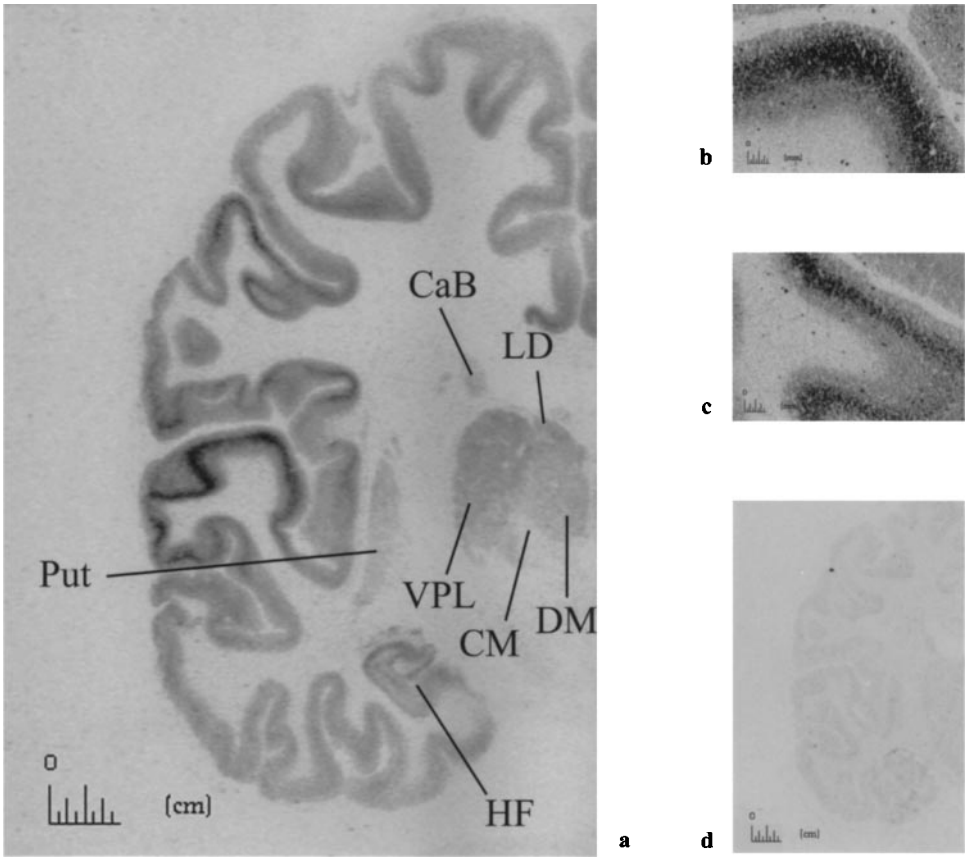

Figure 5 Distribution of [^{3}H]-CGP39653 binding sites in human brain. Coronal human brain sections (corresponding approximately to Figures 25 and 26 of De Armond *et al*., 1989 and Figures 40 and 41 of Duvernoy, 1988) were incubated in 20 nM [^{3}H]-CGP39653 for 25 min at 25°C. (a) Total binding; (b) temporal cortex, at the level of the operculum; (c) frontal cortex, at the level of the precentral sulcus; (d) non specific binding, in the presence of 300 μM L-glutamate. CaB, caudate nucleus, body; Put, putamen; HF, hippocampal formation; LD, lateral dorsal nucleus; DM, dorsomedial nucleus; VPL, ventral posterolateral nucleus; CM, centromedian nucleus. Calibration bar corresponds to 1 cm (a,d) or 1 mm (b,c).

Table 4 Distribution of [^{3}H]-CGP39653 binding sites in human brain

Area	*Specific [^{3}H]-CGP39653 binding* (fmol mg$^{\pm 1}$ *brain tissue*)	*Binding relative to DG (molecular layer)* (%)
Cerebral cortex		
Cingulate	9.5±0.8	81
Frontal	10.8±0.9	92
Parietal	9.7±2.0	82
Insular	9.8±1.6	83
Temporal	6.8±0.7	58
Basal ganglia		
Caudate nucleus, body	3.1±1.1	26
Caudate nucleus, tail	2.8±1.2	24
Putamen	3.6±1.2	31
Globus pallidus	1.6±1.3	14
Claustrum	2.2±0.6	19
Thalamus		
LD	6.0±1.2	51
DM	3.2±1.0	27
VL	4.9±0.9	42
CM	1.7±0.4	14
Other diencephalic nuclei		
MG	3.1±0.9	26
LG	6.9±2.8	59
Hippocampal formation		
Subiculum	5.9±1.6	50
CA1	11.5±1.5	97
CA2	6.5±1.3	55
CA3	9.5±1.9	81
CA4	7.8±1.4	66
DG, molecular layer	11.8±2.3	100
DG, granular layer	4.4±0.4	37

LD, lateral dorsal nucleus; DM, dorsomedial nucleus; VL, ventral lateral nucleus; CM, centromedian median nucleus; MG medial geniculate body; LG, lateral geniculate body; CA1–4, fields CA1–4 of Ammon's horn; DG, dentate gyrus. Values represent the mean±s.e.mean of four different brains. For each brain, binding levels are the result of one or two experiments, performed in triplicate (three consecutive sections per experiment).

Both in human and rat brain, the highest levels of [^{3}H]-CGP39653 binding were found in the CA1 region of hippocampus and in the molecular layer of dentate gyrus. Nevertheless, most regions, such as all the cerebral cortical areas measured, the putamen and the caudate nucleus and certain diencephalic nuclei (namely the lateral dorsal and the ventral lateral nucleus and the lateral geniculate body) contained relative to the molecular layer of the dentate gyrus, a much higher level of [^{3}H]-CGP39653 binding in the human than in the rat brain (compare Table 4 and Table 2). In contrast, other regions such as the dorsomedial nucleus of thalamus and medial geniculate body had comparable levels of binding in the two species.

Different laminar distributions between the rat and human brain were found both in the cerebral cortex and the Ammon's horn. In rat brain, [^{3}H]-CGP39653 binding density was higher in the superficial than the internal layers of the cerebral cortex, whereas in the human cerebral cortex the highest levels were found in the intermediate bands (see Figure 5, part b and c). Within the hippocampal formation, a unique, wide band of dense labelling was present in Ammon's horn, without a clear laminar distinction between strata oriens, pyramidal and radiatum as found in the rat (see Figure 6).

Enhancement of basal [^{3}H]-CGP39653 binding by GV150526A

In human brain slices 30 μM GV150526A was able to greatly increase [^{3}H]-CGP39653 binding as in rat tissue (see Figures 6 and 7). In general, an increase of almost twice the basal binding (216±9% of control, average between areas) was found in the presence of the glycine antagonist. In the cingulate sulcus, for instance, the binding increased from 10.0 to 19.4 fmol mg^{-1} protein (191±23% of control, $n=4$). The enhancement did not present regional differences. Values of [^{3}H]-CGP39653 binding in the presence of 30 μM GV150526A and the percentage of increase with respect to control are found in Table 5. In general, a marked variability was found between subjects in this increase, as can be noted from the high s.e.mean of the samplings (see Table 5). In putamen, for example, the standard error (±33%) was around 16% of the average enhancement (213%, see Table 5). On the contrary, in rat caudate putamen the s.e. (±14%) was only 5% of the value (282%, see Table 2).

Discussion

We have previously shown that binding of the antagonist [^{3}H]-CPG39653 to the glutamate site of the NMDA receptor can be allosterically modulated by glycine in rat brain (Mugnaini *et al*., 1993). The extent of this effect differed between regions and revealed the existence of NMDA receptor subtypes which may have a different sensitivity to endogenous ligands as well as to potential therapeutic agents (Mugnaini *et al*., 1996). The aim of the present study was to confirm these findings in rat brain and look for the possible existence of similar NMDA receptor subtypes in human brain.

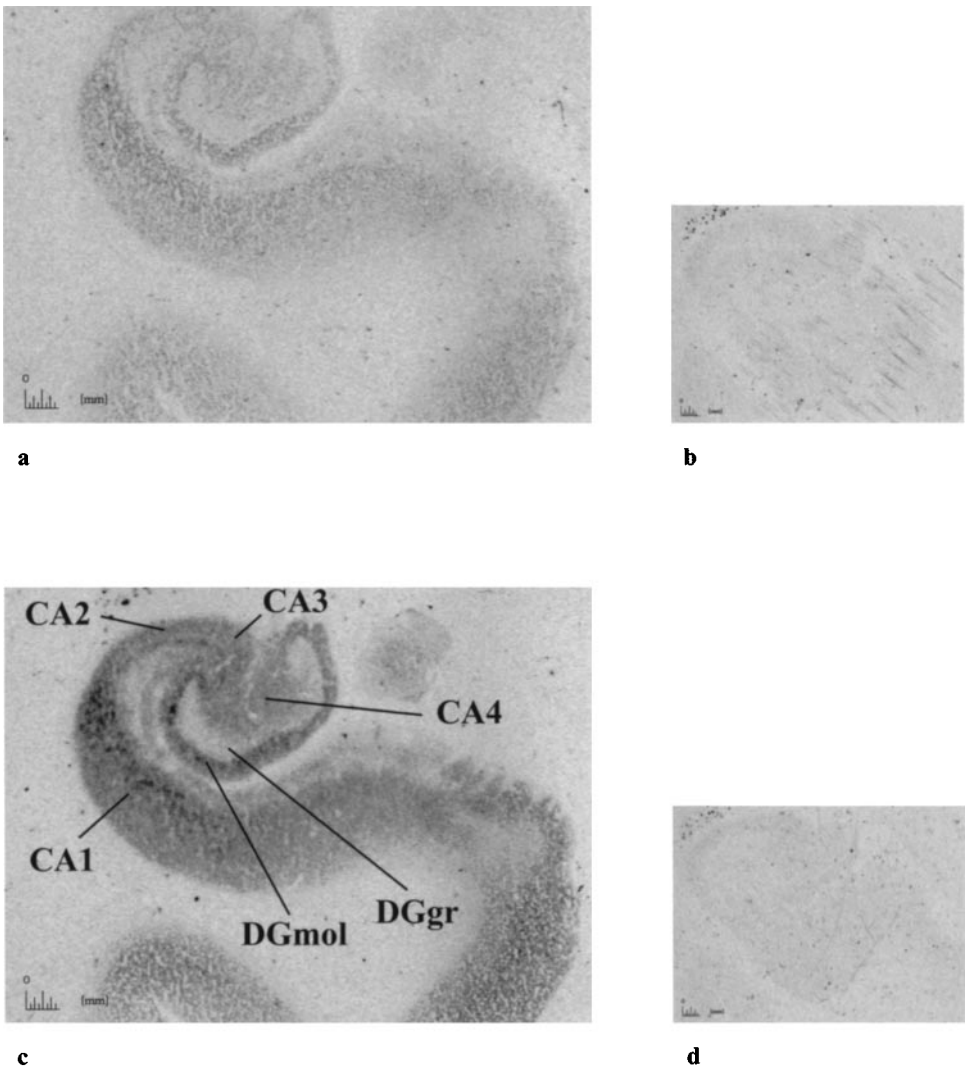

Figure 6 Enhancement of [^{3}H]-CGP39653 binding by GV150526A in human hippocampus. Coronal human brain sections (corresponding approximately to Figure 25 of De Armond *et al*., 1989) were incubated in 20 nM radioligand for 25 min at 25°C, either in the absence (a,b) and in the presence (c,d) of 30 μM GV1505626A. (a,c) Total binding; (b,d) non specific binding, in the presence of 300 μM L-glutamate. The image shows only the hippocampal formation and corresponds to Figure 58.B of Duvernoy (1988). CA1–4, fields CA1–4 of Ammon's horn; DGmol, molecular layer of dentate gyrus; DGgr, granular layer of dentate gyrus. Calibration bar corresponds to 1 mm.

The non-competitive, allosteric nature of the high affinity component of [^{3}H]-CGP39653 binding inhibition by glycine was confirmed with the use of GV150526A, a glycine site antagonist with nanomolar affinity (Di Fabio *et al*., 1997; Mugnaini *et al*., 2000; Bordi *et al*., 2000). GV150526A did not affect [^{3}H]-CGP39653 binding directly, but competitively reversed the high affinity component of glycine inhibition, with a potency in line with its affinity for the glycine site ($pK_B = 8.38$ and $pK_i = 8.49$, respectively). The low affinity component of the inhibitory action of glycine (Mugnaini *et al*., 1993) was not affected by GV150526A or by 7-CKA, suggesting that at high concentrations (>10 μM) glycine interacted with the NMDA binding site in a different manner.

Glycine decreased the affinity of [^{3}H]-CGP39653 for the NMDA binding site, without any changes in receptor binding density. Moreover, in the presence of glycine there was an increase in the affinity of the agonists L-glutamic acid and NMDA and a parallel decrease in the affinity of the antagonist D-AP5. Interestingly, the affinity of CPP, in contrast to the other NMDA site antagonists, CGP39653 and D-AP5, was increased by glycine.

Fadda *et al*. (1988) have already reported that activation of the glycine site by glycine caused an increase in [^{3}H]-glutamate affinity for the NMDA recognition site, and Robichon *et al*. (1997) have found a decrease in [^{3}H]-CGP39653 affinity in the presence of 10 μM glycine. However, the present results are the first demonstrating that activation of the glycine site causes a change in the NMDA receptor, shifting the receptor from an 'antagonist-' to an 'agonist-preferring' state or conformation.

The finding that glycine increases the affinity of the antagonist CPP, however, does not appear to support this model. Possibly CPP, although it is a pure antagonist, binds to the 'agonist-preferring' conformation of the receptor. Contrasting results, though, were reported on the effect of glycine site ligands on [^{3}H]-CPP binding: some authors

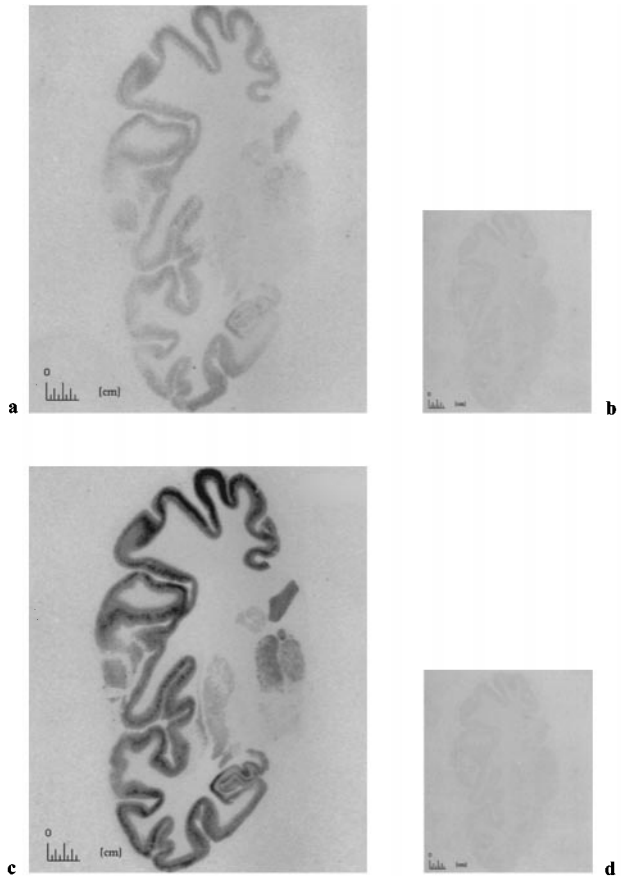

Figure 7 Enhancement of [^{3}H]-CGP39653 binding by GV150526A in human brain. Coronal human brain sections (corresponding approximately to Figure 24 of De Armond *et al*., 1989 and Figure 40 of Duvernoy, 1988) were incubated in 20 nM [^{3}H]-CGP39653 for 25 min at 25°C, in the absence (a,b) and presence (c,d) of 30 μM GV150526A. (a,b) Total binding; (c,d) non specific binding, in the presence of 300 μM L-glutamate. Calibration bars correspond to 1 cm.

observed an inhibition of binding by D-serine (Monaghan *et al*., 1988a,b), whereas others found that [^{3}H]-CPP binding was not significantly altered by glycine (Van Amsterdam *et al*., 1992; Kaplita & Ferkany, 1990). Moreover, CPP recognizes two binding sites (Porter *et al*., 1992; Van Amsterdam *et al*., 1992), which might be differently modulated by glycine. As a consequence, the glycine-induced increase in CPP affinity found in the present work may be the result of a combination of multiple effects.

GV150526A potently increased basal [^{3}H]-CGP39653 binding throughout the rodent and human brain, counteracting the inhibitory effect of the endogenous glycine. The enhancement was uneven in rat brain: in CPu, for example, the increase was higher than in the cerebral cortex. This was in line with the previous finding that 1 mM glycine was able to inhibit basal [^{3}H]-CGP39653 binding to a smaller extent in CPu than in the cerebral cortex (Mugnaini *et al*., 1996).

These results are unlikely to be justified by the presence of different levels of endogenous glycine in the brain regions examined. It has been shown by *in vivo* microdialysis that striatum contains a lower level of endogenous free glycine than the cerebral cortex (Globus *et al*., 1991; Hashimoto *et al*., 1995), which is the converse of what would be predicted from the present data. Instead they might be explained by considering the regionally different potencies of glycine in the allosteric inhibition of [^{3}H]-CGP39653 binding, or different potencies of GV150526A to reverse glycine inhibition. In line with this hypothesis, we found a greater potency of glycine in allosterically inhibiting [^{3}H]-CGP39653 binding in striatal than in cortical membranes ($pK_{iH}=7.48$ and 6.98, respectively; Mugnaini *et al*., 1996). On the contrary, there was not any significant variation in affinity values of glycine, GV150526A, [^{3}H]-CGP39653 and L-glutamate.

In summary, these data indicate that NMDA receptors exist, in rat brain, which conserve their characteristics in terms of affinity values for NMDA or glycine site ligands, whilst differing in the potency of the allosteric modulation between the glycine and the glutamate binding site, i.e. in the capability of interconverting from the 'antagonist-' to the 'agonist-preferring' state or conformation. As a consequence of that, these receptor subtypes may present a different sensitivity to the helpful effects of potential neuroprotective agents acting through these sites, such as CGP39653 or GV150526A. The higher affinity of glutamate for striatal than cortical NMDA receptors (in the presence of the same concentration of endogenous glycine) may be one of the possible reasons for which, in the rat middle cerebral artery occlusion (MCAo) model of cerebral ischaemia, NMDA antagonists reduce the damage to a lower extent in striatum than in the cerebral cortex (Maier *et al*., 1995; Park *et al*., 1988).

In autoradiographic preparations of rat brain, Monaghan *et al*. (1988a,b) described the existence of NMDA receptors with relatively higher affinity for the antagonists and receptors with relatively higher affinity for the agonists, and called them 'antagonist-' and 'agonist-preferring' NMDA receptor subtypes; noteworthy, there was a good regional correlation between the relative proportion of 'antagonist-' to 'agonist-preferring' receptors and the size of the enhancement of [^{3}H]-glutamate binding by glycine, which made the authors hypothesise the existence, in alternative or in addition to these receptor subtypes, of 'antagonist-' and 'agonist-preferring' conformations interconverted by glycine. The present results support the existence of both the receptor subtypes and of the interconverting forms in rodent brain. The 'agonist-preferring' receptors are NMDA receptors which can shift from the 'antagonist-' to the 'agonist-' receptor state more easily (i.e. with a lower concentration of glycine) than the 'antagonist-preferring' receptors, and vice versa.

The existence of similar NMDA receptor subtypes in the human brain was explored by means of large-section autoradiography experiments. The hippocampus contained the highest density of binding sites for [^{3}H]-CGP39653, together with the cingulate, frontoparietal and insular cortex. High levels were also found in the temporal cortex, the lateral geniculate body and the lateral dorsal nucleus of thalamus. Intermediate to low levels of binding sites were found in the other diencephalic nuclei and in the basal ganglia (caudate, putamen, globus pallidus and claustrum).

The distribution of [^{3}H]-CGP39653 binding sites in the hippocampus was quite similar to that of most other radioligands for the NMDA receptor (namely [^{3}H]-(+)-5-methyl-10,11-dihydroxy-5H-dibenzo(a,d)cyclohepten-5,10-imine ([^{3}H]-MK801), [^{3}H]-1-(1-(2-thienyl)-cyclohexyl)-piperi-

Table 5 Increase of [3H]-CGP39653 binding by GV150526A in human brain

Area	Basal (fmol mg^{-1})	Specific [3H]-CGP39653 binding +30 μM GV150526A (fmol mg^{-1})	(% basal)[a]
Cerebral cortex			
Cingulate	10.0±1.1	19.4±3.5	191±23
Frontal	10.9±1.4	18.2±2.4	168±12
Parietal	10.9±2.0	18.5±4.7	163±16
Insular	10.7±1.7	23.6±3.4	222±14
Temporal	7.2±1.4	11.3±3.5	151±22
Basal ganglia			
Caudate nucleus, body	3.1±1.1	4.5±1.0	160±28
Caudate nucleus, tail	3.1±1.3	5.0±1.3	229±44
Putamen	3.6±1.2	7.0±1.8	213±33
Globus pallidus	1.6±1.3	2.1±1.3	215±99
Claustrum	2.2±0.6	5.0±0.9	272±71
Thalamus			
LD	6.6±1.4	11.7±2.8	182±35
DM	3.8±1.0	6.7±1.7	176±13
VL	5.3±1.2	10.4±1.6	206±15
CM	1.8±0.3	2.6±0.4	141±11
Other diencephalic nuclei			
MG	3.1±0.9	7.4±1.0	263±47
LG	6.9±2.8	16.1±4.3	286±86
Hippocampal formation			
Subiculum	6.0±1.3	12.0±3.2	218±53
CA1	11.4±1.4	26.9±5.6	235±39
CA2	7.0±1.3	16.1±4.5	238±44
CA3	9.2±1.5	21.9±4.2	245±44
CA4	7.7±1.1	20.1±3.4	275±51
DG, molecular layer	11.3±1.9	26.8±4.3	255±48
DG, granular layer	4.4±0.3	10.5±1.5	249±46

Results are expressed as fmol mg^{-1} of brain tissue or as percentage of specific [3H]-CGP39653 binding determined in the same area in the absence of added compounds. Values represent the mean±s.e.mean of five experiments. Each experiment was performed with adiacent sections, in triplicate. Abbreviations are as reported in Table 4.

dine ([3H]-TCP), [3H]-CPP, [3H]-glutamate and [3H]-glycine): indeed the highest density was found in the CA1 region and the molecular layer of dentate gyrus, with lower binding in the CA3 region and the granular layer of dentate gyrus (Jansen *et al*., 1989a; Ulas *et al*., 1992; Meoni *et al*., 1998). Similarly, within the basal ganglia, there was a good correlation between the present study and that of Ball *et al*. (1994), who found, using [3H]-MK801 and [3H]-glycine, a moderate binding level in caudate and putamen and low binding levels in globus pallidus and claustrum. Nevertheless, in contrast to the other ligands, [3H]-CGP39653 binding density was significantly greater in certain thalamic nuclei, such as the ventral lateral nucleus, and in the insular cortex. Other differences were also found in the laminar distribution within the cerebral cortex. [3H]-CGP39653 was concentrated in the intermediate layers of the cerebral cortex, whereas that of other radioligands such as [3H]-glutamic acid, [3H]-glycine and [3H]-TCP showed the highest binding density in laminae I-III (Jansen *et al*., 1989b). At present, however, it is not clear whether these differences are due to the radioligand (e.g. [3H]-CGP39653 *vs* [3H]-glutamic acid or [3H]-glycine) or to the different techniques employed (i.e. the use of whole-brain sections instead of dissected parts of a brain).

Much lower [3H]-CGP39653 binding levels and higher intersubject variability were found in the human than in the rat brain. The lower binding may be related to the long post mortem delay of the four brains used in the present study, with the consequent receptor degradation and glutamate release. Indeed, Jaarsma *et al*. (1993) demonstrated that addition of glutamate dehydrogenase considerably increased [3H]-CGP39653 binding in a human brain subsection. Conversely, the very high intersubject variability may originate from the different post mortem delays, as well as from the different ages, sex, and, as suggested by Ulas *et al*. (1992), from the different genetic background or 'accumulation of life experiences'.

Basal [3H]-CGP39653 binding was stimulated to around 200% of control by GV150526A in the human brain. This result indicated that human NMDA receptors also demonstrate allosteric modulation and that human brain slices probably contain comparable levels of endogenous glycine. The existence of putative allosteric interactions in the human hippocampus has been reported by Ulas *et al*. (1992), who found a slight (20–30%) stimulation of [3H]-glutamate binding by glycine and of [3H]-CPP binding by HA966. With the use of [3H]-CGP39653 and GV150526A the present study confirms this finding and extends it to many other regions of the human brain. Regional differences in the allosteric modulation of [3H]-CGP39653 binding through the glycine site have not been found in the human brain, in contrast to the rat brain. At present, however, it is not possible to understand whether the high intersubject variability has hindered the detection of any regional variation, or if in the human brain there exists a more homogeneous NMDA receptor population. In contrast with the latter hypothesis, in a recent receptor binding study with the channel blocker [3H]-MK801, Mortensen *et al*. (1999) found subtle but significant differences, across a range of four human brain cortical regions, in the potency of spermine and glutamate to activate

the NMDA receptor. The affinity of glutamate was not determined in this study, neither in the absence nor in the presence of glycine, and therefore it is not possible to know if the differences in glutamate potency (which was determined at constant glycine concentration) were due to regional different glycine/glutamate allosteric interactions, or to other factors.

In conclusion, this study has provided the first detailed anatomical description of the regional distribution of [^{3}H]-CGP39653 binding sites in the human brain. Moreover, it has proved the existence of glycine/glutamate allosteric modulation for NMDA receptors of most regions of the human brain.

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