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Receptor binding characteristics of the novel NMDA receptor glycine site antagonist [^{3}H]GV150526A in rat cerebral cortical membranes

Manolo Mugnaini *, Giovanna Dal Forno, Mauro Corsi, Bernd Bunnemann

Glaxo Wellcome, Medicines Research Centre, Via Fleming 4, 37135 Verona, Italy

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Abstract

Binding of the glycine site antagonist 3-[2-(Phenylamino-carbonyl)ethenyl]-4,6-dichloro-indole-2-carboxylic acid sodium salt ([^{3}H]GV150526A) was characterised in rat cerebral cortical membranes. Saturation experiments indicated the existence of a high affinity binding site, with a pK_d value of 9.08 ($K_d = 0.8$ nM) and a B_{max} of 3.4 pmol/mg of protein. A strong linear correlation was observed between the displacement potencies for [^{3}H]GV150526A and [^{3}H]glycine of 13 glycine site ligands ($r = 0.991$). The association kinetics of [^{3}H]GV150526A binding was monophasic, with a k_{on} value of 0.047 $(nM)^{-1}$ min^{-1}. Dissociation was induced by the addition of an excess of glycine, GV150526A, or 5,7-dichlorokynurenic acid (DCKA), another glycine antagonist. With GV150526A and DCKA, the dissociation curves presented similar k_{off} values (0.068 and 0.069 min^{-1}, respectively), as expected from ligands binding to the same site. Conversely, a significantly lower k_{off} value (0.027 min^{-1}) was found with glycine. Although these data may suggest that glycine agonists and antagonists bind to discrete sites with an allosteric linkage (rather than interacting competitively), the reason for this difference remains to be elucidated. It is concluded that [^{3}H]GV150526A can be considered a new valuable tool to further investigate the properties of the glycine site of the NMDA receptor.

Keywords: NMDA receptor; Glycine site; Receptor binding; GV150526A

1. Introduction

The NMDA receptor is a ligand-gated ion channel belonging to the family of glutamate receptors (Betz, 1990; Meldrum, 1991; Seeburg, 1993; Collingridge and Watkins, 1994; Nakanishi and Masu, 1994). High calcium (Ca^{2+}) ions permeability, voltage-dependent regulation by magnesium (Mg^{2+}) ions, non-competitive antagonism by steric blockage of the channel, and modulation by steroids, polyamines, reducing agents, protons (H^+) and zinc (Zn^{2+}) ions are some of the particular features which differentiate the NMDA receptor from other glutamate-gated ion channels (Mayer and Westbrook, 1987; Collingridge and Lester, 1989; Monaghan et al., 1989; Scatton, 1993; Mc Bain and Mayer, 1994).

The absolute requirement of glycine for channel activation, however, remains one of the most striking characteristics of the NMDA receptor. Since its discovery (Jonhson and Ascher, 1987), the strychnine-insensitive glycine site of the NMDA receptor-channel complex has generated an enormous amount of interest, especially after the finding that antagonists at this site possess anticonvulsant and neuroprotective properties, but lack some of the side-effects of other types of antagonists of the NMDA receptor (for references, see Kemp and Leeson, 1993; Danysz and Parsons, 1998). As a consequence, a large number of glycine site ligands have been synthesised (see reviews Thomson, 1990; Palfreyman and Baron, 1991; Leeson and Iversen, 1994; Lodge et al., 1994; Kulagowski, 1996; for a more recent update, see Danysz and Parsons, 1998). To date, glycine itself is the most potent agonist, whereas the most potent glycine site antagonists include (±)-4-(*trans*)-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetra-hydroquinoline (L689560; Leeson et al., 1991), 5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione (ACEA1021; Keana et al., 1995), and 3-[2-(Phenylamino-carbonyl)ethenyl]-4,6-dichloro-indole-2-carboxylic acid sodium salt (GV150526A); Di Fabio et al., 1997).

Like other ligand-gated ion channels, the NMDA receptor is thought to consist of four or five polypeptidic

* Corresponding author. Tel.: +39-045-9218-955; fax: +39-045-9218-047.

E-mail address: mm25927@glaxowellcome.co.uk (M. Mugnaini).

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subunits, which assemble in the cell membrane to generate a receptor-channel complex (Betz, 1990). Indeed, recent molecular cloning studies have revealed the existence of two families of NMDA receptor subunits: the NR1, present in eight isoforms generated by alternative splicing of a single gene, and the NR2 family, which contains four subunits transcribed from four different genes (for reviews, see Hollmann and Heinemann, 1994; Nakanishi and Masu, 1994; Mori and Mishina, 1995; Zukin and Bennett, 1995). Both homomeric NR1 and NR2 receptors are inactive in mammalian expression systems (Chazot et al., 1992; Monyer et al., 1992). Differently from the NR2, the NR1 subunits assemble into functional ion channels when expressed as homomers in *Xenopus* oocytes. Recently, however, Soloviev and Barnard (1997) have demonstrated that oocytes endogenously express a new glutamate receptor subunit, XenU1 (Ishimaru et al., 1996), which might assemble with the rat NR1 subunit to give functional receptors, as well as it does with the *Xenopus* NR1 subunit (XenNR1; Soloviev et al., 1996). Conversely, co-expression of NR1 with NR2 subunits greatly enhances the response of homomeric NR1 receptors in oocytes and leads to formation of functional channels in mammalian cells (Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993), supporting the theory that native NMDA receptors result from heteromeric NR1/NR2 combination.

Each of the recombinantly expressed, homomeric NR1 or heteromeric NR1/NR2 subunit receptor combination has distinctive, subunit-dependent biophysical and pharmacological signatures, which include: the strength of Mg^{2+} block; sensitivity to modulation by glycine, reducing agents, polyamines and phosphorylation; desensitisation and offset decay kinetics; affinity for specific agonists and antagonists (for reviews, see Hollmann and Heinemann, 1994; Mc Bain and Mayer, 1994; Zukin and Bennett, 1995; Sucher et al., 1996). Even more complications arise if recombinant receptors containing more than one type of NR2 subunits and/or NR1 splice variants are considered (Kendrick et al., 1996; Buller and Monaghan, 1997; Kew et al., 1998; Vicini et al., 1998). The existence of four distinct genes encoding different NR2 subunits (namely the NR2A, NR2B, NR2C and NR2D subunits), together with the alternative RNA splicing of the NR1 gene, might be responsible for a theoretically enormous molecular diversity in neuronal NMDA receptors. Therefore, in the years to come, one of the most important aims of pharmacology will be to design subtype specific NMDA receptor antagonists.

Valuable tools to study the characteristics of both native and recombinant NMDA receptors are radioligands specific for the different binding sites of the receptor-channel complex. Considerable effort has been put in introducing radioactive ligands to facilitate the study of the glycine binding site because of its potential therapeutic role. [^{3}H]glycine was the first compound used for this purpose; a strychnine-insensitive [^{3}H]glycine binding site had been described by Kishimoto et al. (1981) and Bristow et al. (1986) even before the discovery of the co-agonist action of glycine on the NMDA receptor (Jonhson and Ascher, 1987). In addition to [^{3}H]glycine (Snell et al., 1988; Marvizon et al., 1989; Mc Donald et al., 1990), other more selective ligands have been introduced for labeling the glycine site of the NMDA receptor, such as the agonist [^{3}H]D-serine (Danysz et al., 1990), the partial agonist 1-aminocyclopropyl-1-carboxylate ([^{3}H]ACC; Monahan et al., 1990; Popik et al., 1995), the antagonist 5,7-dichlorokynurenic acid ([^{3}H]DCKA, Baron et al., 1991; Canton et al., 1992; Mugnaini et al., 1998), and the high-affinity antagonists [^{3}H]L689560 (Grimwood et al., 1992) and (*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid ([^{3}H]MDL105519; Baron et al., 1991). More recently, another compound emerged as a glycine-site selective radioligand: the antagonist (±)-*trans*-4-[2-(4-azidophenyl)acetylamino]-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-carboxylic acid ([^{3}H]CGP61594; Honer et al., 1998; Benke et al., 1999). Interestingly, [^{3}H]CGP61594 is also a photo affinity label and can be used as a tool for the identification of structural elements of the glycine binding sites in situ; moreover, it is the first glycine site radioligand which has a preferential affinity for the NR1/NR2B receptors (Honer et al., 1998).

GV150526A is a new glycine site antagonist which has been shown to reduce the infarct area and to protect somatosensory evoked potentials (SEP) in the middle cerebral artery occlusion (MCAo) model of focal ischemia in the rat (Bordi et al., 1997). The compound, which inhibits [^{3}H]glycine binding with a K_i value of 3 nM ($pK_i = 8.49$; Di Fabio et al., 1997), has subsequently been radio labeled. We report here on [^{3}H]GV150526A, as a new, high affinity radioligand for the glycine site of the NMDA receptor.

A preliminary report of the study has been presented to the British Pharmacological Society (Mugnaini et al., 1997).

2. Materials and methods

2.1. Materials

[^{3}H]GV150526A (TRQ7148, specific activity 2.59 TBq/mmol) was custom synthesised by Amersham International, Buckinghamshire, UK. [^{3}H]glycine (NET 004, 1757.5 GBq/mmol) was purchased from Dupont New England Nuclear, Frankfurt, Germany.

NMDA, 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP), D-2-amino-5-phosphonopentanoic acid (D-AP5), *S*-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (S-AMPA), 7-Chlorokynurenic acid (7-CKA), DCKA, 6,7-dinitroquinoxaline-2,3-dione (DNQX), 5,7-dinitroquinoxaline-2,3-dione (MNQX), 1-amino-1-carboxycyclobutane (ACBC), and L-aspartic acid were obtained from Tocris Neuramin, Essex, UK. L-glutamic acid · HCl,

kainic acid, strychnine, glycine · HCl, D-serine, and D-alanine were from Sigma, USA. ACC was purchased from Aldrich, USA. HA966, (+)-5-methyl-10,11-dihydroxy-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine (MK801) and *cis*-4-(phosphonomethyl)-piperidine-2-carboxylic acid (CGS19755) were from RBI, USA. GV150526A and ACEA1021 were synthesised at the Medicinal Chemistry Department of the Glaxo Wellcome Medicines Research Centre, Verona, Italy.

DNQX and MNQX were dissolved in DMSO and subsequently diluted in the incubation medium (50 mM Tris citrate buffer, pH 7.1). In the final tube, at the highest concentration of displacing compound (300 μM), the solution contained 2% DMSO, which caused a slight enhancement of [^{3}H]glycine binding (+10% of total binding) and inhibition of [^{3}H]GV150526A binding (−10% of total binding). GV150526A, ACEA1021, D-serine, 7-CKA, and DCKA 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 ACEA1021, 100 μM for 7-CKA and DCKA, 300 μM for L-aspartic acid and 1 mM for D-serine), the solution contained 200 μM KOH, which neither changed the pH of the incubation buffer nor affected significantly total [^{3}H]glycine or [^{3}H]GV150526A binding. Alternatively, in some experiments GV150526A, ACEA1021, 7-CKA, and DCKA were dissolved in DMSO (highest concentration tested, with 2% DMSO: 100 μM). All other compounds were dissolved directly in the buffer solution.

Citric acid trisodium salt was from Merck, Germany. Tris(hydroxymethyl)amino-methane (Tris) and ethylenediaminetetraacetic acid (EDTA) from Carlo Erba Reagenti, Milan, Italy. The BCA protein assay was purchased from Pierce, IL, USA.

2.2. Animals

Male Sprague–Dawley rats (200–250 g) were used. Animals were supplied by Charles River (Italy) and were kept under standard laboratory conditions. The research complied with national legislation and with the company policy on the Care of Use of Animals and with related codes of practice.

2.3. Membrane preparation

Crude synaptic membranes were prepared as described by Mugnaini et al. (1998), with only slight modifications. All steps were performed at 4°C, unless otherwise indicated. Briefly, rats were killed by decapitation and the cerebral cortex immediately dissected. The tissue was homogenised in 10 volumes of ice-cold 0.32 M sucrose (pH 7) with a Potter homogeniser (10 strokes at 500 rpm). The homogenate was then centrifuged at $1000 \times g$ for 10 min. The pellet was discarded, whereas the supernatant was centrifuged at $17{,}000 \times g$ for 20 min. The resultant pellet was resuspended with 20 volumes of deionised water (Milli-Q system, Millipore) and lysed with a Polytron. Following incubation at 37°C for 30 min, the membranes were centrifuged at $48{,}000 \times g$ for 15 min and washed twice (by resuspension in 20 volumes of water and centrifugation at $48{,}000 \times g$ for 15 min). The final pellet was resuspended in 3 volumes of water and divided into aliquots that were quick frozen in liquid N_2, stored at −80°C and used within 3 months.

Preliminary experiments showed that the modifications introduced in this protocol, with respect to that reported previously (Mugnaini et al., 1998) did not change significantly the characteristics of [^{3}H]glycine binding to rat cerebral cortex membranes (in terms of specific to non-specific binding ratio, affinity value and maximum binding density; data not shown).

2.4. [^{3}H]GV150526A binding assay

On the day of the experiment, pellets were thawed and resuspended in 20 volumes of 50 mM Tris citrate buffer (pH 7.1) and then incubated at 37°C for 20 min. The suspension was centrifuged at $48{,}000 \times g$ for 10 min. The pellets were washed twice by resuspension in 20 volumes of buffer and centrifugation at $48{,}000 \times g$ for 10 min. The final pellets were resuspended in 250 volumes of buffer and immediately used for binding assay.

The incubations were performed in glass tubes, in a final volume of 1 ml. Saturation binding experiments were carried out using a range of concentrations from 0.3 to 30 nM [^{3}H]GV150526A. Displacement binding curves were obtained at 1 nM radioligand. Non-specific binding was determined in the presence of 300 nM unlabeled GV150526A. The reaction was started by the addition of 750 μl of the membrane suspension and lasted 90 min at 4°C. Incubation was stopped 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 BCA method (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as standard.

Association of the radioligand to the receptor was studied by incubating the membranes with 1 nM [^{3}H]GV150526A at increasing times (from 1 to 240 min). For dissociation experiments, membranes were incubated with 1 nM radioligand for 90 min; separation of the [^{3}H]GV150526A from the receptor was then obtained by blocking the re-association of the radioligand through the addition of 300 nM unlabeled GV150526A, 10 μM DCKA or 1 mM glycine. The amount of radioligand bound to the membranes was measured at increasing times from the beginning of dissociation (from 1 to 150 min).

A centrifugation protocol could not be utilized in these experiments, because of the very high level of non-specific [^{3}H]GV150526A binding to the plastic centrifugation tubes

(scintillation Biovials, Beckman). BSA prevented adhesion of the radioligand to plastic. Nevertheless, since [^{3}H]GV150526A extensively binds to serum albumin (Rowley et al., 1997), it was decided not to add BSA to the incubation medium, which might have given an inaccurate estimate of [^{3}H]GV150526A binding affinity.

2.5. [^{3}H]glycine binding assay

In the case of [^{3}H]glycine, displacement binding curves were obtained with 20 nM [^{3}H]glycine (specific activity 175.75 GBq/mmol, obtained by a tenfold isotopic dilution with unlabeled 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 (Dupont). After solubilization, the radioactivity was estimated by liquid scintillation counting. Non specific binding was determined by adding 100 μM unlabeled glycine.

2.6. Data analysis

To determine the dissociation constant (K_d) and the maximum binding density (B_{max}) of the radioligand and the inhibition constants of displacer ligands (K_i), data of saturation and displacement experiments were analysed using the non-linear curve fitting program LIGAND (Munson and Rodbard, 1980). The observed kinetic association rate constant (k_{obs}) and the kinetic dissociation rate constant (k_{off}) were determined by non-linear regression analysis using ENZFITTER (R.J. Leatherbarrow, BioSoft, UK). The kinetic association rate constant (k_{on}) and the kinetically-derived dissociation constant were calculated as described by Weiland and Molinoff (1981).

Statistical analyses were performed with SPSS (SPSS, Chicago, USA). Comparisons were determined by independent-samples Student's *t*-test. The assumption of two binding sites in saturation, displacement and association and dissociation kinetic curves was determined on the basis of the *F*-ratio test and was accepted only if the test indicated an improvement of the fit with $P < 0.05$. Correlation analysis was performed with ORIGIN (Microcal Software, Northampton, USA). All results are expressed as mean ± S.E.M.

3. Results

3.1. Optimizing [^{3}H]GV150526A binding

Preliminary displacement experiments were performed with GV150526A, DCKA and glycine, to define the level of non-specific binding. In these experiments, membranes were incubated for 90 min at 4°C with 1 nM radioligand.

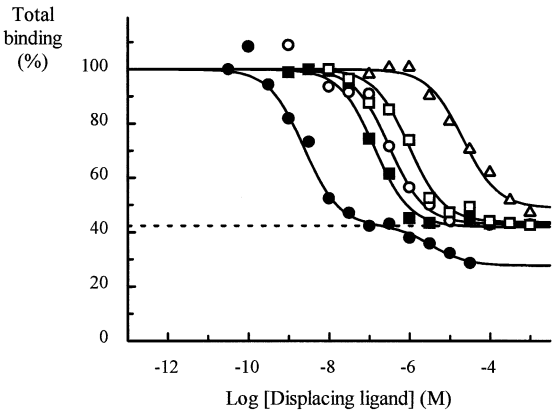

Fig. 1. Representative displacement curves of total [^{3}H]GV150526A binding by some glycine site ligands: GV 150526A (●), DCKA (■), glycine (○), D-serine (□), and HA966 (△). GV150526A inhibition curve was better fitted by a two-site binding model ($P < 0.001$). Dotted line represents the amount of binding displaced by 300 nM GV150526A.

Both DCKA and glycine gave the same maximal inhibition of total [^{3}H]GV150526A binding, with displacement curves according to a single-site binding model (see Fig. 1). Indeed, 1 mM glycine was able to displace 58 ± 4% ($n = 4$) of total [^{3}H]GV150526A binding, which was not significantly different from the percentage of inhibition produced by 100 μM DCKA (59 ± 3%, $n = 3$). Conversely, homologous displacement curves of GV150526A binding were described significantly better by a two-site than a one-site fit ($P < 0.001$). In these experiments, 65 ± 4% ($n = 5$) and 22 ± 2% ($n = 5$) of total [^{3}H]GV150526A binding were associated to the high and the low affinity components, respectively. The percentage of total binding displaced by GV150526A in the high affinity component (65 ± 4%, $n = 5$) was not significantly different from that produced by 1 mM glycine or 100 μM DCKA ($P > 0.05$, Student's *t*-test).

Following these results, non-specific binding was defined as the binding in the presence of 300 nM unlabeled GV150526A, and the low affinity, glycine-insensitive component of [^{3}H]GV150526A binding was no further investigated.

3.2. Kinetic studies

Kinetic experiments revealed that the association of 1 nM [^{3}H]GV150526A binding to rat cerebral cortex membranes was quite slow at 4°C (Fig. 2, upper panel). Equilibrium was reached by 1 h (see Fig. 2, upper panel) and the percentage of specific binding (determined as the difference between total binding and binding in the presence of 300 nM GV150526A) remained steady up to 4 h (data not shown). An observed association rate constant (k_{obs}) of 0.115 ± 0.024 $(nM)^{-1}$ min^{-1} ($n = 4$) was ob-

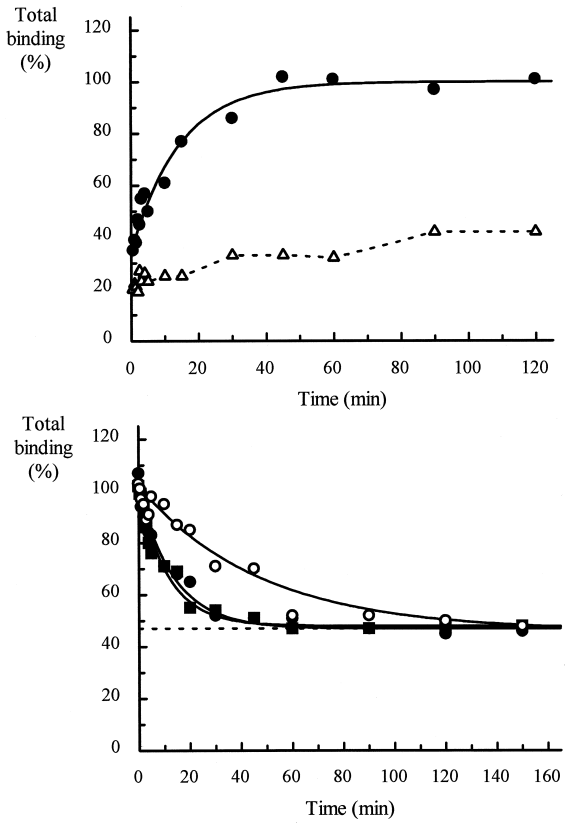

Fig. 2. Upper panel. Representative association curve of 1 nM [^{3}H]GV150526A binding to rat cerebral cortical membranes, performed at 4°C. Straight line represents total [^{3}H]GV150526A binding (●). Dotted line shows non-specific binding, determined in the presence of 300 nM GV150526A (△). Lower panel. Representative dissociation curves of total [^{3}H]GV150526A binding. Membranes were first incubated with 1 nM radioligand for 90 min at 4°C; dissociation was then induced by the addition of 300 nM GV150526A (●), 10 μM DCKA (■), or 1 mM glycine (○). Non-specific binding was determined in the presence of 300 nM GV150526A (dotted line). Note that GV150526A and DCKA produced similar [^{3}H]GV150526A dissociation curves, with almost equivalent k_{off} values (0.068 and 0.069 min^{-1}, respectively), whereas dissociation in the presence of glycine was slower (k_{off} = 0.027 min^{-1}; see text).

tained, with a corresponding half time ($t_{1/2}$) of 6 min. These values indicated that after 45 min, 99% of ligand-receptor complex formation had occurred. As a consequence of these findings, the initial binding conditions, consisting in an incubation time of 90 min and a temperature of 4°C, were chosen as standard incubation conditions for the rest of the experiments.

After such an incubation (90 min, 4°C), dissociation of 1 nM [^{3}H]GV150526A from the membranes was initiated by the addition of an excess (300 nM) of unlabeled GV150526A. As shown in Fig. 2 (lower panel), similarly to the association, also the dissociation of 1 nM radioligand from the receptor was quite slow at 4°C. A dissociation rate constant (k_{off}) value of 0.068 ± 0.001 min^{-1} (n = 4) was determined, with a $t_{1/2}$ of 10 min. An association rate constant (k_{on}) value of 0.047 (nM)$^{-1}$ min^{-1} and a kinetically-derived dissociation constant value of 1.5 nM (pK_d = 8.84) were calculated for [^{3}H]GV150526A according to Weiland and Molinoff (1981).

When dissociation was started by the addition of 10 μM DCKA, a k_{off} value of 0.069 ± 0.009 min^{-1} (n = 3) was found (corresponding $t_{1/2}$ = 10 min), which was not significantly different from that found with GV150526A. Conversely, when dissociation of the radioligand from the receptor was started by the addition of an excess (1 mM) of glycine, the kinetics was quite slower (see Fig. 2, lower panel). Indeed, a k_{off} value of 0.027 ± 0.004 min^{-1} (n = 3) was found ($t_{1/2}$ = 25 min), which was significantly lower than that determined by means of GV150526A or DCKA addition ($P < 0.05$; indipendent-samples Student's t-test).

All kinetic curves accorded to a single site binding model.

3.3. Saturation analysis

Computer analysis of saturation experiments of [^{3}H]GV150526A binding, performed at radioligand concentrations ranging from 0.3 to 30 nM, indicated the presence of a single high affinity binding site, with a dissociation constant of 0.8 nM (pK_d = 9.08 ± 0.08, n = 4) and a receptor density (B_{max}) of 3.4 ± 0.2 pmol/mg protein (see Fig. 3, upper panel). Scatchard analysis of the

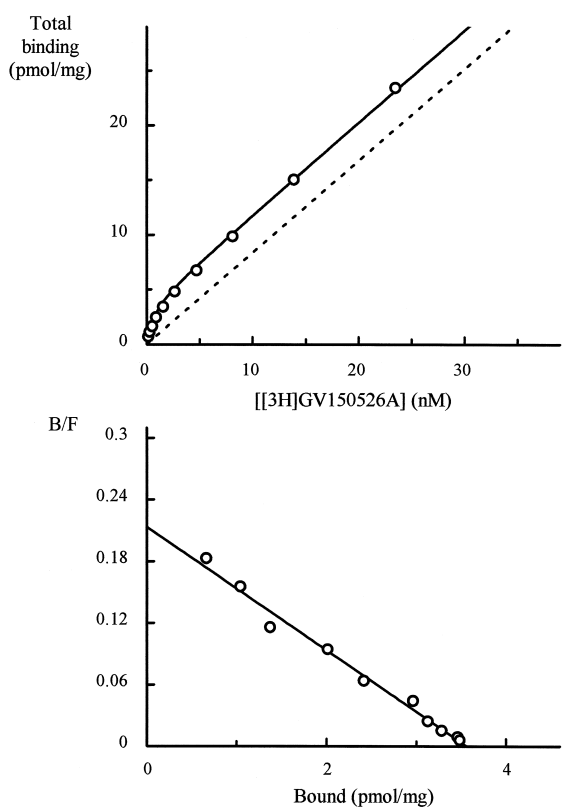

Fig. 3. Upper panel. Representative saturation isotherm of total [^{3}H]GV150526A binding, performed at radioligand concentrations ranging from 0.3 to 24 nM. Dotted line represents non-specific binding, determined in the presence of 300 nM GV150526A. Lower panel. Scatchard representation, showing the one-site fit of the data presented.

isotherms confirmed the one-site fit of the curves (see Fig. 3, lower panel).

3.4. Competition binding studies

Displacement of [^{3}H]GV150526A binding was performed by a number of different ligands known to interact with the NMDA receptor channel complex (see Table 1). Ligands specific for the glycine site of the NMDA receptor completely displaced specific [^{3}H]GV150526A binding, with a curve according to a single-site binding model (see Fig. 1). The only exception to this model was GV150526A, which, as explained above, produced biphasic inhibition curves, with an inhibition constant value of 1.7 nM ($pK_{iH} = 8.76 \pm 0.02$, $n = 5$) for the high affinity, glycine-sensitive component and 4.6 μM ($pK_{iL} = 5.34 \pm 0.16$, $n = 5$) for the low affinity, glycine-insensitive component of binding. The inhibition constant value for the high affinity component ($pK_{iH} = 8.76$) was in line with the dissociation constant values from the saturation and kinetic experiments ($pK_d = 9.08$ and 8.84, respectively; see above).

Table 1
Effect of NMDA and non-NMDA ligands on [^{3}H]GV150526A and [^{3}H]glycine binding to rat cerebral cortex membranes

K_i, inhibition constant of the displacer ligand. ↑, the compound caused an increase of binding. NE, the compound had no effect on binding. Inhibition is expressed as percentage of inhibition of specific binding (defined as the difference between total binding and binding in the presence of 300 nM GV150526A) at the highest concentration of displacer tested (ACBC, ACC, D-alanine and HA966: 1 mM; D-cycloserine: 300 μM; ACEA1021 and GV150526A: 30 μM; all other compounds: 100 μM); negative values correspond to an enhancement of binding. Two pK_i and 'percent inhibition' values are reported for GV150526A, as it inhibited [^{3}H]GV150526A binding with a biphasic displacement curve. Data represent the mean ± S.E.M. of at least three experiments. Part of [^{3}H]glycine data were obtained previously (Mugnaini et al., 1998).

	[^{3}H]GV150526A		[^{3}H]glycine	
	pK_i	Inhibition (%)	pK_i	Inhibition (%)
Glycine site antagonists				
GV 150526A	8.76 ± 0.02	100*	8.49 ± 0.02	97 ± 5
	5.34 ± 0.16	134 ± 3		
DCKA	7.16 ± 0.02	91 ± 5	7.20 ± 0.04	100 ± 5
7-CKA	6.51 ± 0.01	86 ± 8	6.71 ± 0.07	98 ± 2
MNQX	6.90 ± 0.14	102 ± 2	7.02 ± 0.08	98 ± 3
DNQX	6.18 ± 0.10	100 ± 3	6.13 ± 0.18	100 ± 1
ACEA1021	8.44 ± 0.12	91 ± 2	8.21 ± 0.11	99 ± 3
Glycine site agonists				
Glycine	6.78 ± 0.07	89 ± 6	6.75 ± 0.02	100
D-Serine	6.29 ± 0.06	85 ± 6	6.49 ± 0.04	94 ± 2
D-Alanine	5.98 ± 0.08	86 ± 1	6.30 ± 0.12	99 ± 2
Glycine site partial agonists				
ACC	6.67 ± 0.04	99 ± 2	6.78 ± 0.11	98 ± 5
ACBC	4.66 ± 0.09	77 ± 4	5.05 ± 0.15	100 ± 1
D-Cycloserine	4.02 ± 0.07	54 ± 2	3.95 ± 0.08	73 ± 4
HA-966	5.00 ± 0.05	85 ± 4	5.15 ± 0.06	108 ± 3
NMDA site antagonists				
CGS 19755	6.11 ± 0.05	30 ± 4	5.83 ± 0.08	54 ± 2
D-AP5	< 3	NE	5.26 ± 0.18	49 ± 2
CPP	< 3	NE	↑	−30 ± 8
NMDA site agonists				
NMDA	< 3	NE	↑	−8 ± 3
L-Glutamic acid	< 3	NE	↑	−29 ± 11
L-Aspartic acid	< 3	NE	↑	−14 ± 3
Other ligands				
MK801	< 3	NE	< 3	NE
AMPA	< 3	NE	< 3	NE
Kainic acid	< 3	NE	< 3	NE
Strychnine	< 3	NE	< 3	NE

* Inhibition by 300 nM GV150526A.

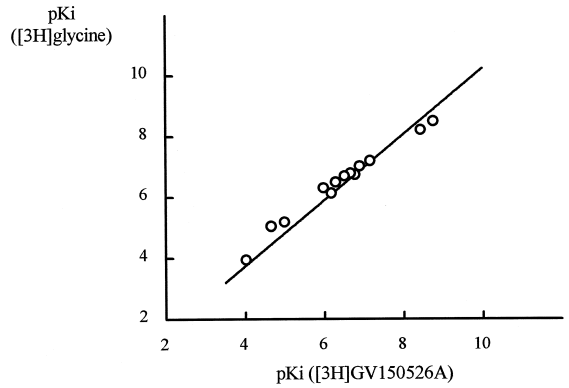

Fig. 4. Comparison between the affinity values estimated from [^{3}H]GV150526A and [^{3}H]glycine binding of a series of glycine site ligands. K_i, inhibition constant of the displacer ligand. Data are from Table 1.

A strong linear correlation ($r = 0.991$, $P < 0.0001$), with a slope not significantly ($P > 0.05$) different from 1 (slope = 1.08) and an intercept not significantly ($P > 0.05$) different from 0 (intercept = −0.58) was observed between the displacement potencies for [^{3}H]glycine and [^{3}H]GV150526A (see Fig. 4).

Ligands for the glutamate or NMDA site of the receptor complex (namely NMDA, L-aspartic acid, L-glutamic acid, D-AP5, and CPP) had no effect on [^{3}H]GV150526A binding, with the exception of the NMDA site antagonist CGS19755 which produced a slight but significant inhibition of specific binding.

Non-NMDA ligands (strychnine, S-AMPA, kainic acid) and the NMDA receptor-channel blocker MK801 showed no displacement of specific binding up to 100 μM.

4. Discussion

In the present study we have characterised [^{3}H]-GV15026A as a new radioligand for the glycine recognition site of the NMDA receptor–channel complex. Computerized non-linear curve fitting of saturation isotherms revealed the presence of a single high affinity binding site in rat cerebral cortex membranes, with a dissociation constant (K_d) value of 0.8 nM and a receptor density (B_{max}) of 3.4 pmol/mg protein. With this K_d value, approxi-

mately four to five times lower than that of [^{3}H]L689560 (K_d = 3.0 nM; Grimwood et al., 1992) and [^{3}H]MDL105519 (K_d = 3.8 nM; Baron et al., 1996), [^{3}H]GV15026A is the radioligand with the highest affinity for the glycine site of the NMDA receptor.

Inhibition studies of [^{3}H]GV150526A and [^{3}H]glycine binding with a series of ligands confirmed the identity of the high affinity [^{3}H]GV150526A binding site with the strychnine-insensitive glycine recognition site; molecules known to interact with this site displayed a potency that was in good agreement with literature data (Palfreyman and Baron, 1991; Kemp and Leeson, 1993), whereas compounds selective for receptors other than NMDA had no effect on [^{3}H]GV150526A binding. In addition to the high affinity site, homologous displacement experiments revealed the presence of a low affinity binding site for [^{3}H]GV150526A, not recognised by glycine. The presence of an agonist-insensitive component of binding of micromolar affinity had already been reported for GV150526A and other glycine site antagonists (Grimwood et al., 1992; Mugnaini et al., 1998). These studies, however, did not reveal whether this second binding site was related to the NMDA receptor or to a distinct target, such as an aminoacids uptake system. In the present work, we used a filtration system to terminate the experiments, which was not appropriate to detect a binding component in the micromolar range. On the other hand, the centrifugation method could not be used because of the high level of aspecific binding to the plastic centrifugation tubes. Consequently, the second site of [^{3}H]GV150526A binding was not further investigated.

No modulation of [^{3}H]GV150526A binding was observed by ligands which bind to the glutamate binding site of the NMDA receptor channel–complex, with the only exception of the NMDA site antagonist CGS19755, which produced a slight (30%) inhibition of specific binding. These findings apparently contrast with the results of many authors, which reported that most NMDA site agonists and antagonists respectively enhance and inhibit the binding of some other glycine site radioligands, namely [^{3}H]glycine, [^{3}H]DCKA and [^{3}H]L689560 (Kessler et al., 1989; Hood et al., 1990; Kaplita and Ferkany, 1990; Baron et al., 1991; Grimwood et al., 1993). Conversely, they are more in line with what was found for [^{3}H]MDL105519, the binding of which was not modulated by NMDA site ligands (Baron et al., 1996). At present, it is difficult to understand if these differences depend on the radioligand used or are due to other factors, such as the level of endogenous glutamate in the membrane preparation, or the time of incubation. Interestingly, it has been shown that allosteric effects greatly change if measured at different times of the association (Grimwood et al., 1992, 1993).

The main kinetic features of [^{3}H]GV150526A binding were represented by monoexponential association and dissociation curves, with a kinetically-derived K_d value (1.5 nM) in line with the equilibrium-derived estimate obtained from saturation and competition binding studies (K_d = 0.8 nM and K_{iH} = 1.7 nM, respectively). The kinetics of the low affinity, glycine-insensitive component of [^{3}H]GV-150526A binding was not detected in these experiments, since a concentration of unlabeled GV150526A able to displace only the high affinity component of the radioligand binding (300 nM; see Fig. 1) was used both to start the dissociation and to determine the level of non-specific binding.

Noteworthy, dissociation was considerably slower if initiated by the addition of an excess of glycine (instead of GV150526A), as revealed by the significantly lower dissociation rate constant value (k_{off} = 0.027 min^{-1}, in comparison with 0.068 min^{-1} obtained with GV150526A). Conversely, the kinetic features of [^{3}H]GV150526A binding were unchanged if dissociation was started with the addition of an excess of the competitive glycine site antagonist DCKA.

At present, the kinetic features of [^{3}H]GV150526A cannot be easily compared with those of other radio labeled glycine site antagonists, such as [^{3}H]DCKA, [^{3}H]MDL-105519, and [^{3}H]L689560. Only glycine was used to initiate dissociation of these radioligands from the receptor (Baron et al., 1991, 1996; Canton et al., 1992; Grimwood et al., 1992); consequently, the effect of glycine on the dissociation kinetics of [^{3}H]DCKA, [^{3}H]MDL105519, and [^{3}H]L689560 cannot be compared with the effect of other glycine site ligands such as DCKA. In addition, differently from [^{3}H]GV150526A, [^{3}H]L-689560 showed biexponential association and dissociation curves (Grimwood et al., 1992), which makes it difficult to compare the kinetic parameters for the two radioligands because of the different models.

The present results seem to suggest that [^{3}H]GV150-526A and DCKA compete for the same binding site, whereas glycine inhibits [^{3}H]GV150526A binding via a different mechanism. According to this hypothesis, it may be argued that glycine agonists and antagonists bind to distinct sites on the NMDA receptor, which are allosterically coupled in a negative manner.

A similar model has been recently proposed also to describe the interaction between polyamine site agonists and antagonists (Kew and Kemp, 1998). Although ifenprodil (or its analogues) and spermine displace each other in an apparently competitive manner (Schoemaker et al., 1990; Mercer et al., 1993; Mutel et al., 1998), many experimental results suggest that these compounds act at distinct sites in a non-competitive allosteric way (Reynolds and Miller, 1989; Legendre and Westbrook, 1991; Williams, 1993; Kew and Kemp, 1998).

Recently, many site directed mutagenesis studies, followed by functional analysis, have supported the evidence that glycine site agonists and antagonists bind to overlapping, but different sites on the NMDA receptor (Kuryatov et al., 1994; Wafford et al., 1995). Moreover, polyamine ligands were shown to produce a divergent modulation of

[^{3}H]glycine and [^{3}H]DCKA binding (Yoneda et al., 1994), whereas opposite changes in potency of agonists and antagonists were found when [^{3}H]DCKA was used compared to [^{3}H]glycine (Canton et al., 1992).

From these data and the results of our work, however, it is not possible to clarify the nature of the different dissociation kinetics of [^{3}H]GV150526A binding in the presence of glycine. Further experiments have to be performed, using more glycine site ligands, to understand if the differences between GV150526A (or DCKA) and glycine are due to the agonist/antagonist nature of the ligand used to induce [^{3}H]GV150526A dissociation or to other unidentified factors.

In summary, this study has supported the identity of the high affinity site labeled by [^{3}H]GV150526A with that of the strychnine-insensitive glycine site of the NMDA receptor-channel complex. Moreover, it has shown that different glycine site ligands have a distinct effect on the radioligand dissociation kinetics. These characteristics, together with its high affinity and selectivity, make [^{3}H]GV150526A a valuable tool to further investigate the properties of the glycine site of the NMDA receptor.

References

Baron, B.M., Siegel, B.W., Slone, A.L., Harrison, B.L., Palfreyman, M.G., Hurt, S.D., 1991. [^{3}H]5,7-Dichlorokynurenic acid, a novel radioligand labels NMDA receptor-associated glycine binding sites. Eur. J. Pharmacol., Mol. Pharmacol. Sect. 206, 149–154.

Baron, B.M., Siegel, B.W., Harrison, B.J.L., Gross, R.S., Hawes, C., Towers, P., 1996. [^{3}H]MDL105519, a high-affinity radioligand for the *N*-methyl-D-aspartate receptor-associated glycine recognition site. J. Pharmacol. Exp. Ther. 279, 62–68.

Benke, D., Honer, M., Heckendorn, R., Pozza, M.F., Allgeier, H., Angst, C., Mohler, H., 1999. [^{3}H]CGP61594, the first photoaffinity ligand for the glycine site of NMDA receptors. Neuropharmacology 38, 233–242.

Betz, H., 1990. Ligand-gated ion channels in the brain: the amino acid receptor superfamily. Neuron 5, 383–392.

Bordi, F., Pietra, C., Ziviani, L., Reggiani, A., 1997. The glycine antagonist GV150526 protects somatosensory evoked potentials and reduces the infarct area in the MCAo model of focal ischemia in the rat. Exp. Neurol. 145, 425–433.

Bristow, D., Bowery, N.G., Woodruff, G.N., 1986. Light microscopic autoradiographic localisation of [^{3}H]glycine and [^{3}H]strychnine binding sites in rat brain. Eur. J. Pharmacol. 126, 303–307.

Buller, A.L., Monaghan, D.T., 1997. Pharmacological heterogeneity of NMDA receptors: characterization of NR1a/NR2D heteromers expressed in *Xenopus* oocytes. Eur. J. Pharmacol. 320, 87–94.

Canton, T., Doble, A., Miquet, J.M., Jimonet, P., Blanchard, J.-C., 1992. A rapid filtration assay for the glycine binding site on the NMDA receptor in rat cortical membranes using [^{3}H]Dichlorokynurenic acid. J. Pharm. Pharmacol. 44, 812–816.

Chazot, P.L., Cik, M., Stephenson, F.A., 1992. Immunological detection of the NMDAR1 glutamate receptor subunit expressed in human embryonic kidney 293 cells and in rat brain. J. Neurochem. 59 (3), 1176–1180.

Collingridge, G.L., Lester, R.A.J., 1989. Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol. Rev. 41, 143–210.

Collingridge, G.L., Watkins, J.C., 1994. The NMDA receptor. 2nd edn. Oxford Univ. Press, Oxford.

Danysz, W., Fadda, E., Wroblewski, J.T., Costa, E., 1990. [^{3}H]D-serine labels strychnine-insensitive glycine recognition sites of rat central nervous system. Life Sci. 46 (3), 155–164.

Danysz, W., Parsons, C.G., 1998. Glycine and *N*-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacol. Rev. 50 (4), 597–664.

Di Fabio, R., Capelli, A.M., Conti, N., Cugola, A., Donati, D., Feriani, A., Gastaldi, P., Gaviraghi, G., Hewkin, C.T., Micheli, F., Missio, A., Mugnaini, M., Pecunioso, A., Quaglia, A.M., Ratti, A., Rossi, L., Tedesco, G., Reggiani, A., 1997. Substituted indole-2-carboxylates as in vivo potent antagonists acting at the strychnine-insensitive glycine binding site. J. Med. Chem. 40 (6), 841–850.

Grimwood, S., Moseley, A.M., Carling, R.W., Leeson, P.D., Foster, A.C., 1992. Characterization of the binding of [^{3}H]L-689560, an antagonist for the glycine site on the *N*-methyl-D-aspartate receptor, to rat brain membranes. Mol. Pharmacol. 41, 923–930.

Grimwood, S., Wilde, G.J.C., Foster, A., 1993. Interactions between the glutamate and glycine recognition sites of the *N*-methyl-D-aspartate receptor from rat brain, as revealed from radioligand binding studies. J. Neurochem. 60, 1729–1738.

Hollmann, M., Heinemann, S., 1994. Cloned glutamate receptors. Annu. Rev. Neurosci. 17, 31–108.

Honer, M., Benke, D., Laube, B., Kuhse, J., Heckendorn, R., Allgeier, H., Angst, C., Monyer, H., Seeburg, P.H., Betz, H., Mohler, H., 1998. Differentiation of glycine antagonist sites of *N*-methyl-D-aspartate receptor subtypes. J. Biol. Chem. 273 (18), 11158–11163.

Hood, W.F., Compton, R.P., Monahan, J.B., 1990. *N*-methyl-D-aspartate recognition site ligands modulate activity at the coupled glycine recognition site. J. Neurochem. 54, 1040–1046.

Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M., Nakanishi, S., 1993. Molecular characterization of the family of the *N*-methyl-D-aspartate receptor subunits. J. Biol. Chem. 268 (4), 2836–2843.

Ishimaru, H., Kamboj, R., Ambrosini, A., Henley, J.M., Soloviev, M.M., Sudan, H., Rossier, J., Abutidze, K., Rampersad, V., Usherwood, P.N.R., Bateson, A.N., Barnard, E.A., 1996. A unitary non-NMDA receptor short subunit from *Xenopus*: DNA cloning and expression. Recept. Channels 4, 31–49.

Jonhson, J.W., Ascher, P., 1987. Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325, 529–531.

Kaplita, P.V., Ferkany, J.W., 1990. Evidence for direct interactions between the NMDA and glycine recognition sites in brain. Eur. J. Pharmacol., Mol. Pharmacol. Sect. 188, 175–179.

Keana, J.F.W., Kher, S.M., Cai, S.X., Dinsmore, C.M., Glenn, A.G., Guastella, J., Huang, J.C., Lu, Y., Ilyn, V., Lu, Y., Mouser, P.L., Woodward, R.M., Weber, E., 1995. Synthesis and structure-activity relationships of substituted 1,4-dihydroquinoxaline-2,3-diones (QXs): antagonists of the glycine site on the NMDA receptor and of non-NMDA glutamate receptors. J. Med. Chem. 38, 4367–4379.

Kemp, J.A., Leeson, P.D., 1993. The glycine site of the NMDA receptor — five years on. Trends Pharmacol. Sci. 14, 20–25.

Kendrick, S.J., Lynch, D.R., Pritchett, D.B., 1996. Characterization of glutamate binding sites in receptors assembled from transfected NMDA receptor subunits. J. Neurochem. 67 (2), 608–616.

Kessler, M., Terramani, T., Lynch, G., Baudry, M., 1989. A glycine site associated with *N*-methyl-D-aspartic acid receptors: characterization and identification of a new class of antagonists. J. Neurochem. 52, 1319–1328.

Kew, J.N.C., Kemp, J.A., 1998. An allosteric interaction between the NMDA receptor polyamine and ifenprodil sites in rat cultured cortical neurones. J. Physiol. 512.1, 17–28.

Kew, J.N.C., Richards, G., Mutel, V., Kemp, J.A., 1998. Developmental changes in NMDA receptor affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. J. Neurosci. 18 (6), 1935–1943.

Kishimoto, H., Simon, J.R., Aprison, M.H., 1981. Determination of the equilibrium dissociation constants and number of glycine binding sites in several areas of the rat central nervous system, using a sodium-indipendent system. J. Neurochem. 37, 1015–1024.

Kulagowski, J.J., 1996. Glycine-site NMDA receptor antagonists: an update. Exp. Opin. Ther. Pat. 6 (10), 1069–1079.

Kuryatov, A., Laube, B., Betz, H., Kuhse, J., 1994. Mutational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins. Neuron 12, 1291–1300.

Leeson, P.D., Carling, R.W., Smith, J.D., Baker, R., Foster, A.C., Kemp, J.A., 1991. *Trans* 2-carboxy-4-substituted tetrahydroquinolines. Potent glygine-site NMDA receptor antagonists. Med. Chem. Res. 1, 64–73.

Leeson, P.D., Iversen, L.L., 1994. The glycine site on the NMDA receptor: structure–activity relationships and therapeutic potential. J. Med. Chem. 37, 4053–4067.

Legendre, P., Westbrook, G.L., 1991. Ifenprodil blocks *N*-methyl-D-aspartate receptors by a two-component mechanism. Mol. Pharmacol. 40, 289–298.

Lodge, D., Jones, M., Fletcher, E., 1994. Non-competitive antagonists of *N*-methyl-D-aspartate. In: Collingridge, G.L., Watkins, J.C. (Eds.), The NMDA Receptor. 2nd edn. Oxford Univ. Press, Oxford, pp. 105–131.

Marvizon, J.-C.G., Lewin, A.H., Skolnick, P., 1989. 1-Aminocyclopropane carboxylic acid: a potent and selective ligand for the glycine modulatory site of the *N*-methyl-D-aspartate receptor complex. J. Neurochem. 52, 992–994.

Mayer, M.L., Westbrook, G.L., 1987. The physiology of excitatory amino acids in the vertebrate central nervous system. Prog. Neurobiol. 28, 197–276.

Mc Bain, C.J., Mayer, M.L., 1994. *N*-methyl-D-aspartatic acid receptor structure and function. Phys. Rev. 74 (3), 723–760.

Mc Donald, J.W., Penney, J.B., Johnston, M.V., Young, A.B., 1990. Characterization and regional distribution of strychnine-insensitive [^{3}H]glycine binding sites in rat brain by quantitative receptor autoradiography. Neuroscience 35 (3), 653–668.

Meguro, H., Mori, H., Araki, K., Kushiya, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K., Mishina, M., 1992. Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. Nature 357, 70–74.

Meldrum, B.S., 1991. Excitatory Amino Acid Antagonists. Blackwell, Oxford.

Mercer, L.D., Jarrot, B., Beart, P.M., 1993. ^{125}I-ifenprodil: synthesis and characterization of binding by a polyamine-sensitive site in cerebral cortical membranes. J. Neurochem. 61, 120–126.

Monaghan, D.T., Bridges, R.J., Cotman, W.C., 1989. The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. Annu. Rev. Pharmacol. Toxicol. 29, 365–402.

Monahan, J.B., Biesterfeldt, J.P., Hood, W.F., Compton, R.P., Dappen, M.S., Cordi, A.A., 1990. [^{3}H]1-Aminocyclopropyl-1-carboxilate: a selective, high affinity ligand for labeling the *N*-Methyl-D-Aspartate receptor-linked glycine recognition site. Eur. J. Pharmacol. 183, 944–945.

Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B., Seeburg, P.H., 1992. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256, 1217–1221.

Mori, H., Mishina, M., 1995. Structure and function of the NMDA receptor channel. Neuropharmacology 34, 1219–1237.

Mugnaini, M., Antolini, M., Corsi, M., van Amsterdam, F.T.M., 1998. [^{3}H]5,7-Dichlorokynurenic acid recognizes two binding sites in rat cerebral cortex membranes. J. Recept. Signal Transduction Res. 18 (2 and 3), 91–112.

Mugnaini, M., Dal Forno, G., Corsi, M., Trist, D.G., 1997. Receptor binding characteristics of the novel NMDA receptor glycine site antagonist [^{3}H]GV150526A in rat cerebral cortical membranes. Br. J. Pharmacol. 122 (Proceedings Supplement), 63P.

Munson, P.J., Rodbard, D., 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding data. Anal. Biochem. 107, 220–239.

Mutel, V., Buchy, D., Klingelschmidt, A., Messer, J., Bleuel, Z., Kemp, J.A., Grayson Richards, J., 1998. In vitro binding properties in rat brain of [^{3}H]Ro25-6981, a potent and selective antagonist of NMDA receptors containing NR2B subunits. J. Neurochem. 70 (5), 2147–2155.

Nakanishi, S., Masu, M., 1994. Molecular diversity and functions of glutamate receptors. Annu. Rev. Biophys. Biomol. Struct. 23, 319–348.

Palfreyman, M.G., Baron, B.M., 1991. Non-competitive NMDA antagonists, acting on the glycine site. In: Meldrum, B.S. (Ed.), Excitatory Amino Acid Antagonists. Blackwell, Oxford, pp. 101–129.

Popik, P., Lewin, A., Berrang, B., Nowak, G., Layer, R., Skolnick, P., 1995. [^{3}H]-Aminocyclopropanecarboxylic acid, a novel probe for strychnine insensitive glycine receptors. Eur. J. Pharmacol., Mol. Pharmacol. Sect. 291, 221–227.

Reynolds, I.J., Miller, R.J., 1989. Ifenprodil is a novel type of *N*-methyl-D-aspartate receptor antagonist: interaction with polyamines. Mol. Pharmacol. 36, 758–765.

Rowley, M., Kulagowski, J.J., Watt, A.P., Rathbone, D., Stevenson, G.I., Carling, R.W., Baker, R., Marshall, G.R., Kemp, J.A., Foster, A.C., Grimwood, S., Hargreaves, R., Hurley, C., Saywell, K.L., Tricklebank, M.D., Leeson, P.D., 1997. Effect of plasma protein binding on in vivo activity and brain penetration of glycine/NMDA receptor antagonists. J. Med. Chem. 40, 4053–4068.

Scatton, B., 1993. The NMDA receptor complex. Fundam. Clin. Pharmacol. 7, 389–400.

Schoemaker, H., Allen, J., Langer, S.Z., 1990. Binding of [^{3}H]ifenprodil, a NMDA antagonist, to a polyamine-sensitive site in the rat cerebral cortex. Eur. J. Pharmacol. 176, 249–250.

Snell, L.D., Morter, R.S., Johnson, K.M., 1988. Structural requirements for activation of the glycine receptor that modulates the *N*-methyl-D-aspartate operated ion channel. Eur. J. Pharmacol. 156, 105–110.

Soloviev, M.M., Barnard, E.A., 1997. *Xenopus* oocytes express a unitary glutamate receptor endogenously. J. Mol. Biol. 273, 14–18.

Soloviev, M.M., Brierley, M.J., Shao, Z.Y., Mellor, I.R., Volkova, T.M., Kamboj, R., Ishimaru, H., Sudan, H., Harris, J., Foldes, R.L., Grishin, E.V., Usherwood, P.N.R., Barnard, E.A., 1996. Functional expression of a recombinant unitary glutamate receptor from *Xenopus*, which contains *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor subunits. J. Biol. Chem. 271 (51), 32572–32579.

Sucher, N.J., Awobuluyi, M., Choi, Y.-B., Lipton, S.A., 1996. NMDA receptors: from genes to channels. Trends Pharmacol. Sci. 17, 348–355.

Thomson, A.M., 1990. Glycine is a coagonist at the NMDA receptor/channel complex. Prog. Neurobiol. 35, 53–74.

Vicini, S., Wang, J.F., Li, J.H., Zhu, W.J., Wang, Y.H., Luo, J.H., Wolfe, B.B., Grayson, D.R., 1998. Functional and pharmacological differences between recombinant *N*-methyl-D-aspartate receptors. J. Neurophysiol. 79, 555–566.

Weiland, G.A., Molinoff, P.B., 1981. Quantitative analysis of drug-receptor interactions: I. Determination of kinetic and equilibrium properties. Life Sci. 29, 313–330.

Williams, K., 1993. Ifenprodil discriminates subtypes of the *N*-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. Mol. Pharmacol. 44, 851–859.

Yoneda, Y., Suzuki, T., Ogita, K., 1994. Different profiles of binding of a radiolabeled agonist and antagonist at a glycine recognition domain on the *N*-methyl-D-aspartate receptor ionophore complex in rat brain. J. Neurochem. 62, 102–112.

Zukin, R.S., Bennett, M.V.L., 1995. Alternatively spliced isoforms of the NMDAR1 receptor subunit. Trends Neurosci. 18 (7), 306–313.