The pharmacological specificity of N-methyl-D-aspartate receptors in rat cerebral cortex: correspondence between radioligand binding and electrophysiological measurements

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1 The pharmacological specificity of N-methyl-D-aspartate (NMDA) receptors has been investigated in the rat cerebral cortex by use of radioligand binding and electrophysiological techniques.

2 A comparison was made between a functional assay (NMDA-induced depolarizations in a rat cortical slice preparation) and NMDA-sensitive L-[³H]-glutamate binding in the same brain region and species, to provide accurate affinity values for agonists and antagonists at the NMDA recognition site.

3 In a preparation of crude postsynaptic densities (PSD) from rat cortex, L-[³H]-glutamate bound with high affinity to an NMDA-sensitive population of sites with K_D (geometric mean (–s.e.mean, +s.e.mean) = 120 (114, 126) nM, B_{max} (mean ± s.e.mean) = 11.4 ± 0.8 pmol mg^{–1} protein and Hill coefficient (mean ± s.e.mean) = 1.2 ± 0.17 ($n = 3$).

4 There was a good agreement between the relative affinities in radioligand binding and electrophysiological assays for the receptor agonists NMDA, N-methyl-L-aspartate, quinolinate and *trans*-2,3-piperidine dicarboxylate, which are poor substrates of acidic amino acid transport systems. However, agonists which are good substrates for high affinity uptake systems (L- and D-glutamate, L- and D-aspartate, D-aspartate- β -hydroxamate and L-glutamate- γ -hydroxamate) were much weaker in the electrophysiological experiments.

5 Schild analysis of the antagonism of NMDA responses in the rat cortical slice by DL-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate, D- and DL-2-amino-5-phosphonovalerate, D- and DL-2-amino-7-phosphonoheptanoate, D- β -aspartylaminomethylphosphonate, D- γ -glutamylglycine and D- α -aminoadipate (D-AA) indicated a competitive interaction with respective pA_2 values of 6.17, 5.62, 5.24, 5.28, 5.20, 5.00, 4.43 and 3.97.

6 In the radioligand binding experiments the same antagonists inhibited only the NMDA-sensitive component of L-[³H]-glutamate binding. IC_{50} values showed a good correlation with the pA_2 values (correlation coefficient = 0.96), with the exception of D-AA which was more potent than anticipated in the binding experiments ($IC_{50} = 9.8 \mu M$).

7 These results confirm that NMDA-sensitive L-[³H]-glutamate binding sites represent the NMDA recognition site of the NMDA receptor and provide affinity values for both agonists and antagonists in the rat cerebral cortex, agreeing well with previous estimates in this and other tissues.

Keywords: NMDA; glutamate; aspartate; cortex; radioligand binding; cortical slice; NMDA receptor

Introduction

N-methyl-D-aspartate (NMDA) preferentially activates a subtype of receptors for the excitatory amino acid neurotransmitter L-glutamate (Watkins & Evans, 1981; Fagg *et al.*, 1986). NMDA receptors are a focus of considerable interest due to their specialized role in neuronal signalling, plasticity and proposed involvement in certain neuropathological conditions including epilepsy, spasticity and cerebral ischaemia (Watkins & Collingridge, 1989). The NMDA receptor possesses at least three functional domains: a recognition site for acidic amino acids such as L-glutamate or NMDA, a recognition site for neutral amino acids such as glycine and a cationic channel (Foster & Fagg, 1987b).

The structure-activity requirements of the recognition site for NMDA have been investigated for several years (see Watkins & Olverman, 1987). Accurate measurement of receptor affinities can best be made *in vitro* where the concentrations of drugs are easily controlled. In general, two approaches can be used to obtain such information, analysis of radioligand binding to membrane fragments or measurements of functional responses to receptor activation in tissue preparations. Both types of experiment have inherent advantages and disadvantages. In radioligand binding assays, a

direct measurement of affinity for the receptor can be made in conditions under which cellular influences such as uptake and metabolism are eliminated or minimized. However, the value of this technique rests upon the assumption that the site being labelled is the receptor of interest, a supposition which must be supported by a careful comparison of the pharmacology of the binding site and functionally-defined receptor. A functional assay has the advantages that the response measured is evoked by activation of a receptor in 'living tissue', and that the efficacy of pharmacological agents can be determined (e.g. agonist, partial agonist, antagonist). Nevertheless, estimation of agonist affinity in a functional assay is complicated by differing degrees of efficacy, receptor reserve, receptor desensitization, uptake and metabolism of compounds and in the case of excitatory amino acids, neurotoxic effects. Voltage- and patch-clamp studies give a more direct measure of agonist affinity and efficacy but suffer from the disadvantage that they are usually performed on isolated cultured cells taken from immature animals or expressed in the foreign environment of a *Xenopus* oocyte. Ideally, quantitative determination of receptor affinities should be obtained with radioligand binding and functional assays in parallel, although this is rarely attempted.

Binding sites which have the pharmacological characteristics expected of NMDA receptors have been identified by use of several radioligands and mammalian central nervous

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system (CNS) preparations. Thus, NMDA-sensitive binding sites for the agonist L-[³H]-glutamate have been demonstrated in synaptic plasma membranes, postsynaptic densities (PSD) and brain sections (Fagg & Matus, 1984; Monaghan & Cotman, 1986; Foster & Fagg, 1987a; Monahan & Michel, 1987), and pharmacologically-similar sites have been detected in crude PSD for the agonists D-[³H]-aspartate and [³H]-NMDA (Foster & Fagg, 1987a). The competitive antagonists D- (and DL-) [³H]-2-amino-5-phosphonovalerate (AP5), DL-[³H]-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate (CPP) and [³H]-*cis*-4-phosphonomethyl-2-piperidine carboxylate (CGS 19755) also label NMDA-sensitive sites in similar preparations (Olverman *et al.*, 1984; 1986; 1988a,b; Murphy *et al.*, 1987; 1988; Foster & Fagg, 1987a).

In the present study, we have obtained estimates of the affinity of compounds for NMDA receptors in rat cerebral cortex tissue using both radioligand binding and electrophysiological techniques. This was achieved by measuring the NMDA-sensitive binding of L-[³H]-glutamate to crude postsynaptic densities (PSD) from rat cerebral cortex and depolarizing responses in rat cortical slices evoked by NMDA receptor agonists. By comparing values obtained by these two approaches in the same brain region and species, we sought direct evidence that NMDA-sensitive L-[³H]-glutamate binding sites are the transmitter recognition sites of the NMDA receptor and to provide accurate affinity values for agonists and antagonists at cerebro-cortical NMDA receptors. A preliminary account of some of this work has appeared (Foster *et al.*, 1986).

Methods

Preparation of crude postsynaptic densities

Crude PSD from rat cerebral cortex were prepared by a modification of previously described methods (Cohen *et al.*, 1977; Matus & Taff-Jones, 1978; Fagg & Matus, 1984; Foster & Fagg, 1987a). For each preparation, 20 male Sprague-Dawley rats were killed and their whole cerebral cortices dissected and placed immediately in a solution of 0.32 M sucrose, 5 mM Tris-acetate buffer (pH 7.4) on ice. All further procedures were carried out at 4°C. The tissue was homogenized in 10 volumes (w/v) of 0.32 M sucrose, 5 mM Tris-acetate buffer (pH 7.4) and centrifuged at 1000g for 10 min. The supernatant (S₁) was collected, the pellet resuspended in a small volume of the sucrose solution and recentrifuged at 1000g for 10 min. The second supernatant was added to S₁, and the mixture centrifuged at 17000g for 20 min. The pellet (P₂) was suspended in 20 volumes (with respect to original tissue weight) of 5 mM Tris-acetate (pH 8.0) and stirred on ice for 60 min, then centrifuged at 48000g for 30 min. The pellet was suspended in 5 mM Tris-acetate (pH 7.0) and an equal volume of 2.4 M sucrose in 5 mM Tris-acetate (pH 7.0) added to give a final sucrose concentration of 1.2 M. An aliquot (15 ml) of this suspension was placed in the bottom of each of 6 Beckman Ultra Clear tubes, and overlaid with 15 ml of 0.9 M sucrose, 5 mM Tris-acetate (pH 7.0), and finally centrifuged in a Beckman SW 28.1 swing-out rotor for 90 min at 83000g in a Beckman L8-M Ultra centrifuge. The synaptic plasma membranes were collected from the 0.9–1.2 M sucrose interface, diluted to 80 ml with 5 mM Tris-acetate (pH 7.0) and stirred on ice; 20 ml of 2% Triton X-100 in 5 mM Tris-acetate (pH 7.0) was slowly added, the mixture stirred for a further 15 min and then 100 ml of 5 mM Tris-acetate (pH 7.0) added before centrifugation at 48000g for 60 min. The pellets were suspended in approximately 20 ml of 5 mM Tris-acetate buffer (pH 7.0) and frozen at –20°C for at least 15 h. After thawing, membranes were washed a further 3 times by resuspension in 5 mM Tris-acetate (pH 7.0) and centrifugation at 48000g for 30 min, and finally resuspended in a small volume of the same buffer (approx. 4 mg membrane protein ml^{–1}) and stored in aliquots at –80°C. Membrane

protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as a standard.

L-[³H]-glutamate binding assay

Assays were carried out in 1.5 ml polypropylene microfuge tubes, and the incubation mixture consisted of crude PSD (routinely 50 µg protein) and 50 nM L-[³H]-glutamate (except in the case of saturation analyses) in 50 mM Tris-acetate buffer (pH 7.0) in a final volume of 500 µl. Test compounds were added to the incubation tubes on ice before addition of the radioligand and 1 mM L-glutamate was used to determine non-specific binding. Tubes were incubated at 32°C for 30 min and then centrifuged for 4 min in an Eppendorf microfuge. The supernatant was aspirated, the pellets solubilized in 0.5 ml of 2% sodium dodecyl sulphate and radioactivity determined by scintillation counting.

Cortical slice preparation

For *in vitro* neurophysiological experiments, cerebro-cortical slices of rat brain were prepared in a manner similar to that described by Harrison & Simmonds (1985). Coronal sections (500 µm thick) through the level of the corpus callosum were cut in artificial cerebrospinal fluid (aCSF) at room temperature, with an Oxford Vibratome. The aCSF (composition (mM): NaCl 124, MgSO₄ 2, KCl 2, KH₂PO₄ 1.25, NaHCO₃ 25, CaCl₂ 2 and glucose 10) was gassed continuously with an O₂/CO₂ (95:5, vol/vol) mixture. Cortical wedges (≈1 mm wide) were cut from these slices and mounted in a two-compartment bath, with the ventral margin of the cortical tissue traversing a greased slot which separated the chambers, so that the cortical tissue lay almost entirely within one compartment, and the white matter entirely within the other.

The chamber containing the cortical tissue (volume 0.3 ml) was continuously perfused at a rate of 1.5–2 ml min^{–1} with Mg²⁺-free aCSF, containing 0.1 µM tetrodotoxin to prevent spontaneous depolarizing potentials. The d.c. potential between the two compartments was monitored via Ag/AgCl electrodes with a high-input impedance amplifier and continuously displayed on a chart recorder.

Data analysis

In the radioligand binding experiments, inhibition curves were analysed for a 1-site model by the equation %I = %I_{max}/1 + (IC₅₀/[L])^{nH} and for a 2 site model by the equation %I = (%I_{max}(1) × [L]/IC₅₀(1) + [L]) + (%I_{max}(2) × [L]/IC₅₀(2) + [L]). IC₅₀ is the concentration of ligand giving 50% inhibition of specific binding, [L] is the ligand (inhibitor) concentration and nH is the Hill coefficient. Calculations were performed with the data manipulation and analysis software RS1 (Bolt, Beranek and Newman, Inc.).

In the electrophysiological experiments, all of the competitive antagonists studied produced parallel shifts to the right of the NMDA concentration-response curve. Control responses to 5, 10 and 20 µM NMDA were obtained and then the antagonist was continuously perfused for at least 30 min, to allow it to reach equilibrium, before reapplying NMDA. The shift to the right of the concentration-response curve was usually measured at half the control 20 µM NMDA response level. Arunlakshana-Schild plots (Arunlakshana & Schild, 1959) were produced from the concentration-ratios obtained in this way. Linear regression lines were fitted by the method of least squares (RS1).

Materials

L-[³H]-glutamate (40–60 Ci mmol^{–1}) was obtained from Dupont-New England Nuclear. D- and L-glutamate, quinolinic acid, D-α-amino adipate (D-AA), D- and L-aspartate, D-aspartate-β-hydroxamate, L-glutamate-γ-hydroxamate, DL-threo-3-hydroxyaspartate, kainate and dihydrokainate were pur-

chased from Sigma. Quisqualate was obtained from Cambridge Research Biochemicals (Cambridge). All other drugs were obtained from Tocris Neuramin (Essex).

Results

L-[³H]-glutamate binding experiments

Saturation analyses Initial experiments using crude PSD from rat cerebral cortex indicated that the specific binding of *L*-[³H]-glutamate (50 nM) was linear with membrane protein over the range 20–200 µg/assay tube, had a broad pH optimum between pH 6.8 and 7.4 and had attained equilibrium by 30 min at 32°C (results not shown). Routine assay conditions chosen for all subsequent experiments were 50–100 µg membrane protein, pH 7.0 and incubation at 32°C for 30 min. In agreement with previous results obtained with crude PSD from rat whole forebrain (Foster & Fagg, 1987a), the specific binding of *L*-[³H]-glutamate at a radioligand concentration of 50 nM represented 70% of the total binding, and 76% of the specific binding was maximally displaced by NMDA (see Table 1).

Saturation analyses were carried out over an *L*-[³H]-glutamate concentration range of 20–1000 nM in the presence and absence of 100 µM NMDA, a concentration which gives maximal displacement of NMDA-sensitive *L*-[³H]-glutamate binding (Table 1). Figure 1 shows a typical saturation curve and values obtained from three separate experiments indicated the following kinetic values: specific binding: K_D (nM) = 201 (181, 222) (geometric mean, –s.e.mean, +s.e.mean); B_{max} (pmol mg^{–1} protein) = 21.2 ± 2.2 (mean $\pm$ s.e.mean); Hill coefficient = 1.0 ± 0.05 (mean $\pm$ s.e.mean). NMDA-sensitive binding: K_D = 120 (114, 126); B_{max} = 11.4 ± 0.8 ; Hill coefficient = 1.2 ± 0.17 . Thus, *L*-[³H]-glutamate bound with higher affinity to the NMDA-sensitive sites which comprised approximately 50% of the total number of specific binding sites.

Inhibition studies A detailed analysis was made of the ability of a number of acidic amino acids to inhibit *L*-[³H]-glutamate (50 nM) binding to crude PSD from rat cerebral cortex (Tables 1 and 2, Figure 2). In agreement with previous reports (Fagg & Matus, 1984; Foster & Fagg, 1987a), compounds known to be selective NMDA receptor ligands were unable to cause complete inhibition of specific *L*-[³H]-glutamate binding. Although saturation analyses indicated that the total number

Figure 1 Typical saturation curves for *L*-[³H]-glutamate binding to crude postsynaptic densities (PSD) from rat cerebral cortex. Assays were carried out over a range of radioligand concentrations from 20–1000 nM in the presence and absence of 100 µM N-methyl-D-aspartate (NMDA). (Δ) *L*-[³H]-glutamate binding inhibited by 1 mM *L*-glutamate (i.e. total specific binding), (○) *L*-[³H]-glutamate binding inhibited by 100 µM NMDA (i.e. NMDA-sensitive binding).

of NMDA-sensitive and -insensitive sites were equal (see above), *L*-[³H]-glutamate had a higher affinity for NMDA-sensitive sites and consequently these comprise the majority of sites labelled at a radioligand concentration of 50 nM. NMDA, N-methyl-L-aspartate (NMLA), D-aspartate, *trans*-2,3-piperidine dicarboxylate (*trans*-2,3-PDA), quinolinate, CPP, D-AP5, D-2-amino-7-phosphonoheptanoate (D-AP7), D-β-aspartylaminomethylphosphonate (D-β-AspAmp) and D-AA all gave maximal inhibition values of 65–90% (Tables 1 and 2). This is consistent with the idea that these compounds inhibit mainly the NMDA-sensitive component of *L*-[³H]-glutamate binding.

Compounds known to be 'broad spectrum' excitatory amino acid receptor ligands such as *L*- and D-glutamate and *cis*-2,3-PDA caused complete inhibition of *L*-[³H]-glutamate binding, indicating an interaction with both NMDA-sensitive and -insensitive populations of sites. For *cis*-2,3-PDA and D-

Table 1 Inhibition of *L*-[³H]-glutamate binding to crude postsynaptic densities (PSD) from rat cortex by acidic amino acid agonists

		IC_{50} (µM)	% I_{max}	nH
Glutamate	L	0.31 (0.27, 0.35)	99 ± 1	0.98 ± 0.04
	D	23.0 (18.1, 29.4)	98 ± 2	0.70 ± 0.03
Aspartate	L	1.91 (0.82, 1.62)	89 ± 2	0.75 ± 0.08
	D	1.18 (0.86, 1.57)	82 ± 2	0.90 ± 0.04
N-methyl-aspartate	L	14.5 (11.6, 18.1)	80 ± 5	0.94 ± 0.20
	D	3.68 (2.62, 5.20)	76 ± 6	0.67 ± 0.06
<i>trans</i> -2,3-PDA	DL	6.52 (4.77, 8.91)	77 ± 5	0.78 ± 0.07
<i>cis</i> -2,3-PDA	DL	89.6 (67.5, 119)	102 ± 5	0.64 ± 0.08
Aspartate-β-hydroxamate	L	143 (137, 150)	94 ± 5	0.85 ± 0.13
	D	42.2 (39.1, 45.4)	84 ± 4	1.07 ± 0.22
Glutamate-γ-hydroxamate threo-3-hydroxyaspartate	L	35.3 (29.5, 42.1)	101 ± 7	0.79 ± 0.11
	DL	14.7 (13.6, 15.9)	91 ± 7	0.81 ± 0.20
Quinolinate		153 (122, 193)	90 ± 6	0.77 ± 0.17
Quisqualate	DL (i)	0.04 (0.01, 0.07)	22 ± 2	—
	(ii)	59.6 (57.4, 61.9)	73 ± 2	—
Kainate	(i)	0.40 (0.20, 0.77)	22 ± 2	—
	(ii)	389 (299, 507)	58 ± 8	—
Dihydrokainate	(i)	6.0 (4.8, 7.4)	16 ± 2	—
	(ii)	448 (459, 497)	79 ± 1	—

IC_{50} values are the concentration of drug which inhibited 50% of specific *L*-[³H]-glutamate binding and are the geometric mean (–s.e.mean, +s.e.mean) of ≥ 3 separate experiments. % I_{max} is the percentage maximal inhibition of *L*-[³H]-glutamate binding and nH is the Hill coefficient, these are expressed as arithmetic mean $\pm$ s.e.mean.

Figure 2 Inhibition of L-[³H]-glutamate binding to crude postsynaptic densities (PSD) from rat cerebral cortex by acidic amino acids. Radioligand concentration was 50 nM. Inhibition curves for: (a) the agonists L-glutamate (●), NMDA (Δ), *trans*-2,3-PDA (□), NMLA (▽), quinolinate (○); (b) the antagonists D-AP5 (●), D-β-AspAmp (Δ), D-AP7 (□), D-GG (▽); (c) the agonists quisqualate (●), kainate (Δ) and dihydrokainate (□). Each curve was from a separate experiment which was repeated 2–3 times with similar results (see Tables 1 and 2 for affinity values). For graphs (a) and (b) percentage inhibition data were analysed using a 1-site fit model; for (c) shallow inhibition curves were best fitted by a 2-site model. Note that L-glutamate, quisqualate and dihydrokainate caused complete inhibition of L-[³H]-glutamate binding, indicating affinity at both NMDA and non-NMDA receptors. For abbreviations, see text.

glutamate, inhibition curves were shallow as reflected by the low Hill coefficients (Table 1). This was also the case with quisqualate, kainate and dihydrokainate, the inhibition curves for which could be resolved into 2 components (Figure 2c). For each of these compounds the low affinity component predominated, representing 58–79% of the binding sites, approximating the proportion expected of NMDA-sensitive sites (see above). The high-affinity component comprised 16–22% of the sites, approximating the proportion of NMDA-insensitive L-[³H]-glutamate binding sites (Table 1). α -Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) inhibited a minor component of L-[³H]-glutamate binding sites, giving $17.3 \pm 2.6\%$ inhibition ($n = 9$) at $100 \mu\text{M}$ and $29.3 \pm 12.2\%$ inhibition ($n = 3$) at $1000 \mu\text{M}$. Thus, AMPA appeared to inhibit selectively the NMDA-insensitive population of L-[³H]-glutamate binding sites. However, the small degree of inhibition observed precluded an accurate estimation of the affinity of AMPA.

Table 2 Inhibition of L-[³H]-glutamate binding to crude postsynaptic densities (PSD) from rat cortex by acidic amino acid antagonists

		IC_{50} (μM)	% I_{max}	nH
CPP	DL	1.57 (0.74, 3.21)	68 ± 9	0.81 ± 0.21
AP5	L	49.9 (37.4, 66.4)	80 ± 3	0.83 ± 0.14
	D	2.69 (2.27, 3.12)	84 ± 5	0.71 ± 1.12
	DL	5.10 (4.11, 6.38)	80 ± 3	0.85 ± 0.08
AP7	L	62.9 (50.2, 78.9)	82 ± 2	0.66 ± 0.12
	D	6.17 (3.46, 11.0)	65 ± 6	0.82 ± 0.08
	DL	11.6 (10.9, 12.5)	73 ± 5	0.93 ± 0.01
AA	D	9.8 (9.6, 10.6)	80 ± 1	0.63 ± 0.05
β -AspAmp	D	11.6 (9.07, 14.9)	85 ± 2	0.68 ± 0.06
GG	D	71.0 (54.9, 91.9)	69 ± 2	0.79 ± 0.18
% inhibition at $300 \mu\text{M}$				
AP4	DL		27.8 ± 13.9	
AP6	DL		32.1 ± 9.4	
AP8	DL		18.0 ± 3.9	
GAMS	D		15.4 ± 9.5	
AS	DL		39.5 ± 8.0	

IC_{50} , % I_{max} and nH values are as defined in Table 1 and are the means of ≥ 3 separate experiments. For abbreviations, see text.

L-Glutamate was the most potent inhibitor of L-[³H]-glutamate binding to crude PSD from rat cortex and was 74 times more active than D-glutamate. No stereoselectivity was apparent for aspartate, both isomers being potent inhibitors of the binding (Table 1). Amongst selective NMDA agonists, NMDA was most potent followed by *trans*-2,3-PDA, NMLA and quinolinate. Of the antagonists, CPP was the most potent, followed by D-AP5, D-AP7 and D-β-AspAmp; DL-2-amino-4-phosphonobutyric acid (DL-AP4), DL-2-amino-6-phosphonohexanoic acid (DL-AP6) and DL-amino-8-phosphonooctanoic acid (DL-AP8) were all poor inhibitors, as was D-γ-glutamylaminomethylsulphonate (D-GAMS). The L-isomers of AP5 and AP7 were weaker than the corresponding D-isomers. All of the compounds which selectively inhibited NMDA-sensitive binding sites had shallow inhibition curves as reflected by the low Hill coefficients. This was apparent for the agonists NMDA, *trans*-2,3-PDA and quinolinate as well as the competitive antagonists CPP, D-AP5, D-AP7 and D-β-AspAmp.

Four compounds which are inhibitors of glutamate and aspartate uptake were tested for their ability to inhibit L-[³H]-glutamate binding (Table 1). DL-Threo-3-hydroxyaspartate was the most active of these compounds having a potency comparable with NMLA. The maximal inhibition shown by D-aspartate-β-hydroxamate was suggestive of a selective interaction with NMDA-sensitive binding sites, whereas the remaining three compounds caused complete displacement of L-[³H]-glutamate binding.

Cortical slice experiments

Agonists It was not possible to construct full concentration-response curves for acidic amino acid agonists since exposure of cortical slices to high concentrations invariably led to a general reduction of responsiveness, presumably due to neurotoxic effects of the excitatory amino acids. Therefore, a comparison of agonist potencies was made relative to NMDA as a reference compound (Table 3). Amongst agonists thought to be selective for the NMDA receptor, *trans*-2,3-PDA was 2.5 times weaker than NMDA, whereas NMLA was 10 fold weaker and quinolinate 60 fold weaker. These relative potency values were similar to those determined from the receptor binding experiments (Table 3). The isomers of glutamate and aspartate, D-aspartate-β-hydroxamate and L-glutamate-γ-hydroxamate were considerably less potent than NMDA and their relative potency values versus NMDA were all much

Table 3 Relative potencies of acidic amino acid agonists in the rat cortical slice preparation

	Cortical slice Relative potency ¹	Inhibition of L-[³ H]-glutamate binding to crude PSD from rat cortex Relative potency ¹
NMDA	1	1
NMLA	9.9 ± 0.4	3.9
trans-2,3-PDA	2.8 ± 0.1	1.8
Quinolate	59.6 ± 4.1	41.6
L-Glutamate	63.1 ± 8.1	0.1
D-Glutamate	39.8 ± 3.0	6.3
L-Aspartate	75.7 ± 9.2	0.5
D-Aspartate	58.7 ± 7.1	0.3
D-Aspartate-β-hydroxamate	89.6 ± 3.0	11.5
L-Glutamate-γ-hydroxamate	139.4 ± 3.6	9.6

¹ NMDA = 1

Relative potencies in the cortical slice were calculated as described in the text from ≥3 separate experiments. Relative potencies in the L-[³H]-glutamate binding experiments were calculated from the mean values of IC₅₀ in Table 1.

greater than would be predicted from the binding assay results (Table 3).

Antagonists Antagonist affinities were obtained by Schild analysis (Arunlakshana & Schild, 1959) of experiments from

which concentration-response ratios were calculated from rightward shifts in the NMDA concentration-response curve caused by various concentrations of antagonist (Figure 3). The majority of antagonists tested blocked NMDA responses with

Figure 3 Inhibition of N-methyl-D-aspartate (NMDA) induced depolarizations in the rat cortical slice by D,L-AP7. (a) Trace of depolarizations produced by 1 min applications of NMDA (N) and quisqualate (Q) (numbers indicate concentration in μM). D,L-AP7 (100 μM, from arrow) reduced the responses to NMDA but not quisqualate. (b) Concentration-response curves to NMDA (triangles) and quisqualate (squares) in the absence (open symbols) and presence (filled symbols) of 100 μM D,L-AP7. D,L-AP7 antagonized the NMDA responses, resulting in a parallel shift of the concentration-response curve to the right, whilst it had no effect on the quisqualate responses. For abbreviations, see text.

Figure 4 Schild plots for the antagonism of N-methyl-D-aspartate (NMDA) depolarizations in rat cortical slices by CPP (●), D-AP5 (■), D-β-AspAmp (▲) and D-γ-glutamyl glycine (◆). Each point is the mean of at least four experiments. Slopes were not significantly different from 1, indicating a competitive antagonism of NMDA receptors. For abbreviations, see text.

Table 4 Inhibition values for antagonism of NMDA responses in the rat cortical slice by acidic amino acids

	<i>pA</i> ₂	Slope of Schild plot (± 95% confidence limits)
CPP	6.17	1.03 ± 0.08
D-AP5	5.62	0.95 ± 0.17
D-AP7	5.28	0.99 ± 0.16
DL-AP5	5.24	1.04 ± 0.11
DL-AP7	5.20	1.04 ± 0.13 ¹
D-β-AspAmp	5.00	0.97 ± 0.16
D-GG	4.43	1.06 ± 0.16
D-AA	3.97	1.14 ± 0.15
D-GAMS	< 4	—

¹ Slope for 10–100 μM, 100–300 μM slope = 0.51 ± 0.23. *pA*₂ values were derived from Schild analysis as described in the text. For abbreviations, see text.

Figure 5 Correlation of affinity values obtained in radioligand binding and electrophysiological experiments for competitive N-methyl-D-aspartate (NMDA) receptor antagonists. pIC_{50} values obtained from NMDA-sensitive L-[3H]-glutamate binding are compared to pA_2 values obtained using the cortical slice preparation. When D-AA was excluded from the analysis (see text) there was an excellent correlation with a correlation coefficient of 0.96. The dotted line indicates the 95% confidence limits. For abbreviations, see text.

no effect on those for quisqualate (Figure 3b) and gave Schild plots with slopes not significantly different from 1 (see Figure 4 and Table 4), indicating a selective, competitive antagonism of NMDA receptors. Table 4 summarizes the pA_2 and Schild plot values obtained. CPP was the most potent antagonist and the D-isomers of AP5 and AP7 were more potent than the racemates, in agreement with the stereoselectivity expected of the NMDA receptor. D- γ -Glutamylglycine (D-GG) was a weak NMDA antagonist which, at the concentrations tested, had little effect on quisqualate or kainate responses (concentration-ratios at $100\mu M$; quisqualate = 1.27 ± 0.02 ; kainate = 1.15 ± 0.10 , $n = 4$). D-GAMS was a very weak antagonist, having a similar effect at $100\mu M$ against NMDA, quisqualate and kainate responses, with respective concentration-ratios of 1.38 ± 0.17 , 1.62 ± 0.16 and 1.46 ± 0.17 ($n = 4$). DL- α -Aminosuberate (DL-AS) was also a weak NMDA receptor antagonist with an apparent pA_2 value of 4.14 ± 0.026 ($n = 4$), calculated from the shift in the NMDA concentration-response curve produced by $100\mu M$ DL-AS.

Affinity values for competitive NMDA receptor antagonists calculated from both electrophysiological and radioligand binding experiments are compared in Figure 5. When D-AA was excluded from the analysis, an excellent correlation was apparent between the two sets of data with a correlation coefficient of 0.96.

Discussion

This study was designed to investigate the pharmacology of NMDA recognition sites in the rat cerebral cortex by both radioligand binding and electrophysiological techniques. With this combined approach we have obtained convincing evidence that NMDA-sensitive L-[3H]-glutamate binding sites in crude PSD are the recognition sites of the NMDA receptor and have provided values of agonist and antagonist affinity at this receptor. In general, these values are in good agreement with previous results obtained with several radioligands (Foster & Fagg, 1987a; Lehmann *et al.*, 1987; Fagg & Baud, 1988; Murphy *et al.*, 1988; Olverman *et al.*, 1988a,b) and in electrophysiological experiments using several CNS regions (Evans *et al.*, 1979; 1982; Jones *et al.*, 1984; Harrison & Simmonds, 1985; Davies *et al.*, 1986).

In rat cortex PSD, L-[3H]-glutamate binds with high affinity to an NMDA-sensitive population of sites which comprise approximately 50% of the total number of L-[3H]-glutamate binding sites labelled under our assay conditions. The K_D of 120 nM for NMDA-sensitive L-[3H]-glutamate binding sites is a lower value (i.e. higher affinity) than those typically reported by other groups who have measured L-[3H]-glutamate binding under conditions where NMDA-sensitive sites predominate (K_D values range from 240–620 nM; Fagg & Matus, 1984; Monaghan & Cotman, 1986; Foster & Fagg, 1987; Monaghan & Michel, 1987). In these earlier studies, K_D and B_{max} determinations were made for total L-[3H]-glutamate binding sites with no attempt to differentiate NMDA-sensitive and -insensitive populations. It cannot be assumed, because NMDA inhibits the majority of L-[3H]-glutamate binding sites at 'routine' assay concentrations (usually well below the K_D value), that both K_D and B_{max} values obtained reflect predominantly those of NMDA-sensitive L-[3H]-glutamate binding sites. Indeed, in the present experiments L-[3H]-glutamate bound with higher affinity to the NMDA-sensitive population of sites and at a radioligand concentration of 50 nM, NMDA inhibited 76% of the total specific binding. However, saturation analyses over a range of radioligand concentrations indicated that the total number of NMDA-sensitive and -insensitive sites was equal. Our values for the K_D and B_{max} values of the NMDA-sensitive component of L-[3H]-glutamate binding agree closely with those recently reported by Thedinga *et al.* (1989) for NMDA-sensitive L-[3H]-glutamate binding sites in synaptic membranes from rat telencephalon.

The contention that NMDA-sensitive L-[3H]-glutamate binding sites are recognition sites of the NMDA receptor is based on a comparison of agonist and competitive antagonist potencies in radioligand binding and electrophysiological experiments. In general, estimation of agonist potencies in electrophysiological experiments using intact tissue is made difficult by such factors as uptake and metabolism of compounds, receptor desensitization, varying efficacy and neurotoxic effects. In the case of excitatory amino acids the uptake systems which transport these compounds into neurones and glia (see Fagg & Lane, 1979) are probably the major confounding factor (Ariel *et al.*, 1984; Garthwaite, 1985). In the present experiments, a good agreement between radioligand binding and electrophysiological data was obtained for NMDA, NMLA, *trans*-2,3-PDA and quinolinate, all of which are selective NMDA receptor agonists (Watkins & Evans, 1981; Davies *et al.*, 1982), and which are poor substrates of acidic amino acid transport systems (Balcar & Johnston, 1972; Foster *et al.*, 1983). This was not true, however, of the isomers of glutamate and aspartate, L-glutamate- γ -hydroxamate and D-aspartate- β -hydroxamate, all of which were considerably weaker as agonists on the cortical slice than in the radioligand binding experiments. This can be explained by the fact that all of these compounds are good substrates for acidic amino acid uptake systems in CNS tissue (Balcar & Johnston, 1972; Roberts & Watkins, 1975). Interestingly, D-glutamate was the least discrepant between the two assays and, of these compounds, is the poorest substrate for the high-affinity uptake system but is transported by a low affinity system (Balcar & Johnston, 1972; Benjamin & Quastel, 1976). Nevertheless, the 6 fold difference between the relative potencies of D-glutamate in the electrophysiological and binding experiments indicates, as suggested previously (Cox *et al.*, 1977; Lodge, 1979), that low affinity transport systems also exert a considerable influence on the potency of excitatory amino acids in intact CNS tissue. Thus, it is clear that the affinity of agonists for the NMDA receptor can best be determined by radioligand binding experiments or in situations where uptake systems cannot interfere e.g. concentration-jump experiments on isolated voltage-clamped neurones.

The determination of affinities for competitive antagonists provides a better means of comparing binding sites with receptors. By use of the Schild equation (Arunlakshana & Schild, 1959), affinity values can be derived from electrophysi-

ological data which are theoretically equivalent to affinity values obtained from inhibition data in radioligand binding experiments. An additional advantage for study of NMDA receptors is that the competitive antagonists available are generally poor substrates for acidic amino acid uptake systems (Cross *et al.*, 1986; Lehmann *et al.*, 1987). CPP, AP5, AP7, D-AA and D- β -AspAmp have all been reported as selective competitive NMDA antagonists (Evans *et al.*, 1979; 1982; Jones *et al.*, 1984; Davies *et al.*, 1986; Lehmann *et al.*, 1987). This was confirmed in the present electrophysiological experiments by the fact that these compounds had little or no effect on depolarizations induced by quisqualate or kainate and gave Schild plots with slopes of 1. D-GG was a less selective antagonist, but appeared to be competitive versus NMDA responses. The excellent correlation obtained by comparing the affinities of competitive NMDA receptor antagonists (with the exception of D-AA, see below) in radioligand binding and electrophysiological experiments strongly suggests that NMDA-sensitive L-[3 H]-glutamate binding sites are the NMDA recognition sites of the NMDA receptor. The essential structure-activity profile of these competitive NMDA antagonists in our experiments was a preference for D-isomers and longer-chain derivatives such that, amongst the series of α -amino- ω -phosphonocarboxylates, maximal activity was carried by the 5 and 7 carbon members (AP5 and AP7), features, which have been well-documented previously (Evans *et al.*, 1982).

D-AA was an atypical NMDA receptor antagonist having a 10 fold greater affinity in the radioligand binding experiments than in the cortical slice. This may be explained by uptake of D-AA into the slice (Charles & Chang, 1981), which would reduce the effective concentration at the receptor. However, a further anomaly was observed with the analogue DL-AS. In agreement with previous results with spinal cord slices (Evans *et al.*, 1979) this compound was more potent than D-AA as an NMDA antagonist in the cortical slice, but was a poor inhibitor of L-[3 H]-glutamate binding (Table 2, see also Fagg & Baud, 1988). Perhaps the antagonism of NMDA responses by DL-AS is mediated by more than one site within the receptor complex.

It is not clear whether the NMDA-insensitive binding sites for L-[3 H]-glutamate represent non-NMDA excitatory amino acid receptors. Since L-glutamate is a mixed agonist, and in autoradiographic experiments has been demonstrated to bind to sites which resemble each of the three excitatory amino acid receptor sub-types (Monaghan *et al.*, 1983), the NMDA-insensitive binding of L-[3 H]-glutamate may result from an interaction with AMPA and/or kainate receptors. Fagg & Matus (1984) proposed that NMDA-insensitive sites in PSD from rat brain were AMPA receptors based on their sensitivity to inhibition by this compound. The present data tend to support this idea since quisqualate was a good inhibitor of NMDA-insensitive sites ($IC_{50} = 40$ nM), and was more potent than kainate and dihydrokainate (see Table 1). AMPA itself appeared to be a selective inhibitor of NMDA-insensitive L-[3 H]-glutamate binding. Thus, the NMDA-insensitive binding of L-[3 H]-glutamate to PSD may represent an interaction with the AMPA receptor, although it should be noted that this population of sites may be heterogeneous and despite

the absence of added ions during the assay, could include chloride/calcium-dependent glutamate transport sites (Pin *et al.*, 1984), which are very sensitive to inhibition by quisqualate (Fagg *et al.*, 1983).

The majority of compounds with inhibition profiles suggestive of a selective inhibition of NMDA-sensitive binding sites had shallow inhibition curves as reflected by the low Hill coefficients. This was particularly evident for NMDA itself and the selective NMDA antagonists D-AP5 and D-AP7 (see Table 1). Interestingly, low Hill coefficients were observed for the inhibition of [3 H]-CPP binding by a number of acidic amino acids (Murphy *et al.*, 1987) and two components of [3 H]-CGS 19755 binding have been identified (Murphy *et al.*, 1988). On the basis of autoradiographic studies of L-[3 H]-glutamate and [3 H]-CPP binding, Monaghan *et al.* (1988) have suggested the existence of agonist and antagonist-preferring NMDA receptors which have a distinct anatomical localization. It is possible, therefore, that the shallow inhibition curves observed here reflect a heterogeneous population of NMDA receptor types or states in the cortical tissue used for the present studies. If this is the case, it is likely that the present results reflect mainly the agonist-preferring state of the receptor, since we have used L-[3 H]-glutamate as a radioligand. A further point of interest is that estimation of the affinity of competitive NMDA receptor antagonists in antagonist binding assays (Murphy *et al.*, 1987; 1988; Olverman *et al.*, 1988) yield values which are up to 10 fold lower (i.e. higher affinity) than those reported here or by other authors using L-[3 H]-glutamate as the radioligand. This may suggest that different forms of the NMDA recognition site predominate when labelled by an agonist or antagonist radioligand. If so, the close correspondence in absolute values for the affinities of competitive antagonists between radioligand binding and electrophysiological experiments reported here would suggest that it is the predominant form of NMDA receptor labelled by L-[3 H]-glutamate (agonist-preferring?) which is functionally the more important in the rat cerebral cortex.

All of the inhibitors of high-affinity L-glutamate/L-aspartate uptake tested in the present experiments also possess significant affinity for NMDA-sensitive L-[3 H]-glutamate binding sites. The most potent of these was DL-threo-3-hydroxyaspartate, which appeared to inhibit selectively NMDA-sensitive L-[3 H]-glutamate binding sites. Since these compounds inhibit glutamate transport with affinities in the low micromolar range (Roberts & Watkins, 1975; Benjamin & Quastel, 1976), they cannot be used as selective blockers of high-affinity L-glutamate/L-aspartate uptake.

In conclusion, by directly comparing results from radioligand binding and electrophysiological experiments we have demonstrated that NMDA-sensitive L-[3 H]-glutamate binding sites represent the NMDA recognition sites of the NMDA receptor. We have also provided affinity values for both agonists (from the binding experiments) and competitive antagonists (from both binding and electrophysiological experiments) at NMDA receptors in rat cerebral cortex.

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References

- ARIEL, M., LASATER, E.M., MANGEL, S.C. & DOWLING, J.E. (1984). On the sensitivity of H1 horizontal cells of the carp retina to glutamate, aspartate and their agonists. *Brain Res.*, **295**, 179–183.
- ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.
- BALCAR, V.J. & JOHNSTON, G.A.R. (1972). The structural specificity of the high affinity uptake of L-glutamate and L-aspartate by rat brain slices. *J. Neurochem.*, **19**, 2657–2666.
- BENJAMIN, A.M. & QUASTEL, J.H. (1976). Cerebral uptakes and exchange diffusion *in vitro* of L- and D-glutamates. *J. Neurochem.*, **26**, 431–441.
- CHARLES, A.K. & CHANG, Y-F. (1981). Uptake, release, and metabolism of D- and L- α -aminoadipate by rat cerebral cortex. *J. Neurochem.*, **36**, 1127–1136.
- COHEN, R.S., BLOMBERG, F., BERZINS, R. & SIEKEVITZ, P. (1977). The structure of postsynaptic densities isolated from dog cerebral cortex. *J. Cell. Biol.*, **74**, 181–203.
- COX, D.W.G., HEADLEY, M.H. & WATKINS, J.C. (1977). Actions of L- and D-homocysteate in rat C.N.S.: a correlation between low-affinity uptake and the time course of excitation by microelectrophoretically applied L-glutamate analogues. *J. Neurochem.*, **29**, 579–588.

- CROSS, A.J., SKAN, W.J. & SLATER, P. (1986). The association of [³H]D-aspartate binding and high-affinity glutamate uptake in the human brain. *Neurosci. Lett.*, **63**, 121–124.
- DAVIES, J., EVANS, R.H., HERRLING, P.L., JONES, A.W., OLVERMAN, H.J., POOK, P. & WATKINS, J.C. (1986). CPP, a new potent and selective NMDA antagonist. Depression of central neuron responses, affinity for [³H]D-AP5 binding sites on brain membranes and anticonvulsant activity. *Brain Res.*, **382**, 169–173.
- DAVIES, J., EVANS, R.H., JONES, A.W., SMITH, D.A.S. & WATKINS, J.C. (1982). Differential activation and blockade of excitatory amino acid receptors in the mammalian and amphibian central nervous systems. *Comp. Biochem. Physiol.*, **72**, 211–244.
- EVANS, R.H., FRANCIS, A.A., HUNT, K., OAKES, D.J. & WATKINS, J.C. (1979). Antagonism of excitatory amino acid-induced responses and of synaptic excitation in the isolated spinal cord of the frog. *Br. J. Pharmacol.*, **67**, 591–603.
- EVANS, R.H., FRANCIS, A.A., JONES, A.W., SMITH, D.A.S. & WATKINS, J.C. (1982). The effect of a series of ω -phosphonic- α -carboxylic amino acids on electrically evoked and excitant amino acid-induced responses in isolated spinal cord preparations. *Br. J. Pharmacol.*, **75**, 65–75.
- FAGG, G.E. & BAUD, J. (1988). Characterization of NMDA receptor-ionophore complexes in the brain. In *Excitatory Amino Acids in Health and Disease*. ed. Lodge, D. pp. 63–90. Chichester: John Wiley and Sons Ltd.
- FAGG, G.E., FOSTER, A.C. & GANONG, A.H. (1986). Excitatory amino acid synaptic mechanisms and neurological function. *Trends Pharmacol. Sci.*, **7**, 357–363.
- FAGG, G.E., FOSTER, A.C., MENA, E.E. & COTMAN, C.W. (1983). Chloride and calcium ions separate L-glutamate receptor populations in synaptic membranes. *Eur. J. Pharmacol.*, **88**, 105–110.
- FAGG, G.E. & LANE, J.D. (1979). The uptake and release of putative amino acid transmitters. *Neurosci.*, **4**, 1015–1036.
- FAGG, G.E. & MATUS, A. (1984). Selective association of N-methyl aspartate and quisqualate types of L-glutamate receptor with brain postsynaptic densities. *Proc. Natl. Acad. Sci., U.S.A.*, **81**, 6876–6880.
- FOSTER, A.C., COLLINS, J.F. & SCHWARCZ, R. (1983). On the excitotoxic properties of quinolinic acid, 2,3-piperidine dicarboxylic acids and structurally related compounds. *Neuropharmacol.*, **22**, 1331–1342.
- FOSTER, A.C. & FAGG, G.E. (1987a). Comparison of L-[³H]glutamate, D-[³H]aspartate, DL-[³H]AP5 and [³H]NMDA as ligands for NMDA receptors in crude postsynaptic densities from rat brain. *Eur. J. Pharmacol.*, **133**, 291–300.
- FOSTER, A.C. & FAGG, G.E. (1987b). Taking apart NMDA receptors. *Nature*, **329**, 395–396.
- FOSTER, A.C., GRIMWOOD, S. & KEMP, J.A. (1986). Electrophysiological and radioligand binding studies of N-methyl-D-aspartate receptors in rat cerebral cortex. *Br. J. Pharmacol.*, **89**, 869P.
- GARTHWAITE, J. (1985). Cellular uptake disguises action of L-glutamate on N-methyl-D-aspartate receptors. *Br. J. Pharmacol.*, **85**, 297–307.
- HARRISON, N.L. & SIMMONDS, M.A. (1985). Quantitative studies on some antagonists of N-methyl-D-aspartate in slices of rat cerebral cortex. *Br. J. Pharmacol.*, **84**, 381–391.
- JONES, A.W., SMITH, D.A.S. & WATKINS, J.C. (1984). Structure-activity relations of dipeptide antagonists of excitatory amino acids. *Neurosci.*, **13**, 573–581.
- LEHMANN, J., SCHNEIDER, J., McPHERSON, S., MURPHY, D.E., BERNARD, P., TSAI, C., BENNETT, D.A., PASTOR, G., STEEL, D.J., BOEHM, C., CHENEY, D.L., LIEBMAN, J.M., WILLIAMS, M. & WOOD, P.L. (1987). CPP, a selective N-methyl-D-aspartate (NMDA)-type receptor antagonist: characterization *in vitro* and *in vivo*. *J. Pharmacol. Exp. Ther.*, **240**, 737–746.
- LODGE, D. (1979). L-Histidine effects on sensitivity of cat spinal neurones to amino acids. *Neurosci. Lett.*, **14**, 171–176.
- LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- MATUS, A.I. & TAFF-JONES, D.H. (1978). Morphology and molecular composition of isolated postsynaptic junctional structures. *Proc. R. Soc. B.*, **203**, 135–151.
- MONAGHAN, D.T. & COTMAN, C.W. (1986). Identification and properties of N-methyl-D-aspartate receptors in rat brain synaptic plasma membranes. *Proc. Natl. Acad. Sci., U.S.A.*, **83**, 7532–7536.
- MONAGHAN, D.T., HOLETS, V.L., TOY, D.W. & COTMAN, C.W. (1983). Anatomical distribution of four pharmacologically distinct [³H]-L-glutamate binding sites. *Nature*, **306**, 176–179.
- MONAGHAN, D.T., OLVERMAN, H.J., NGUYEN, L., WATKINS, J.C. & COTMAN, C.W. (1988). Two classes of N-methyl-D-aspartate recognition sites: Differential distribution and differential regulation by glycine. *Proc. Natl. Acad. Sci., U.S.A.*, **85**, 9836–9840.
- MONAHAN, J.B. & MICHEL, J. (1987). Identification and characterization of an N-methyl-D-aspartate-specific L-[³H]glutamate recognition site in synaptic plasma membranes. *J. Neurochem.*, **48**, 1699–1708.
- MURPHY, D.E., HUTCHINSON, A.J., HURT, S.D., WILLIAMS, M. & SILLS, M.A. (1988). Characterization of the binding of [³H]-CGS 19755: a novel N-methyl-D-aspartate antagonist with nanomolar affinity in rat brain. *Br. J. Pharmacol.*, **95**, 932–938.
- MURPHY, D.E., SCHNEIDER, J., BOEHM, C., LEHMANN, J. & WILLIAMS, M. (1987). Binding of [³H]-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid to rat brain membranes: A selective, high-affinity ligand for N-methyl-D-aspartate receptors. *J. Pharmacol. Exp. Ther.*, **240**, 778–784.
- OLVERMAN, H.J., JONES, A.W., MEWETT, K.N. & WATKINS, J.C. (1988a). Structure/activity relations of N-methyl-D-aspartate receptor ligands as studied by their inhibition of [³H]D-2-amino-5-phosphonopentanoic acid binding in rat brain membranes. *Neurosci.*, **26**, 17–31.
- OLVERMAN, H.J., JONES, A.W. & WATKINS, J.C. (1984). L-Glutamate has higher affinity than other amino acids for [³H]-D-AP5 binding sites in rat brain membranes. *Nature*, **307**, 460–465.
- OLVERMAN, H.J., JONES, A.W. & WATKINS, J.C. (1988b). [³H]D-2-Amino-5-phosphonopentanoate as a ligand for N-methyl-D-aspartate receptors in the mammalian central nervous system. *Neurosci.*, **26**, 1–15.
- OLVERMAN, H.J., MONAGHAN, D.T., COTMAN, C.W. & WATKINS, J.C. (1986). [³H]CPP, a new competitive ligand for NMDA receptors. *Eur. J. Pharmacol.*, **131**, 161–162.
- PIN, J.P., BOCKAERT, T.J. & RECASENS, M. (1984). The Ca²⁺/Cl⁻-dependent L-[³H]glutamate binding: a new receptor or a particular transport process? *FEBS Lett.*, **175**, 31–36.
- ROBERTS, P.J. & WATKINS, J.C. (1975). Structural requirements for the inhibition for L-glutamate uptake by glia and nerve endings. *Brain Res.*, **85**, 120–125.
- THEDINGA, K.H., BENEDICT, M.S. & FAGG, G.E. (1989). The N-methyl-D-aspartate (NMDA) receptor complex: a stoichiometric analysis of radioligand binding domains. *Neurosci. Lett.*, **104**, 217–222.
- WATKINS, J.C. & COLLINGRIDGE, G.L. (1989). *The NMDA Receptor*. Oxford: IRL Press.
- WATKINS, J.C. & EVANS, R.H. (1981). Excitatory amino acid transmitters. *Annu. Rev. Pharmacol. Toxicol.*, **21**, 165–204.
- WATKINS, J.C. & OLVERMAN, H.J. (1987). Agonists and antagonists for excitatory amino acid receptors. *Trends Neuro. Sci.*, **10**, 265–272.

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