ELSEVIER

European Journal of Pharmacology 394 (2000) 211–219

ejp

www.elsevier.nl/locate/ejphar

[^{3}H]ifenprodil binding to NMDA receptors in porcine hippocampal brain membranes

Georg Höfner [1], Klaus Th. Wanner *

Institut für Pharmazie, Zentrum für Pharmaforschung, Ludwig-Maximilians-Universität München, Butenandtstr. 5-13, D-81377 München, Germany

Received 15 December 1999; received in revised form 20 January 2000; accepted 25 January 2000

Abstract

(±)-2-(4-Benzylpiperidino)-1-(4-hydroxyphenyl)propan-1-ol ([^{3}H]ifenprodil) binding to a subcellular fraction of porcine hippocampus, which was obtained by centrifugation on a discontinuous sucrose gradient, was investigated with the objective to label selectively the ifenprodil recognition site of native NMDA receptors. Saturation experiments revealed high-affinity sites for [^{3}H]ifenprodil in this membrane fraction which could be characterised by a K_d value of 23.0 ± 1.8 nM using a one-site model. Calculation of saturation isotherms on the basis of a two-site model yielded a K_{d1} value of 10.4 ± 2.4 nM and a K_{d2} value of 2200 ± 1300 nM, respectively. Inhibition of [^{3}H]ifenprodil binding by NR2B subunit-selective NMDA receptor antagonists, by polyamines, by σ receptor ligands, by a variety of ligands acting at different NMDA receptor recognition sites and by several cations was studied and compared with the effects of these compounds on (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine ([^{3}H]MK-801) binding under non-equilibrium conditions. It turned out that σ receptor ligands such as 1,3-di(2-tolyl)-guanidine (DTG), (+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine (*R*)-3-PPP, (*S*)-3-PPP and (1-{2-[bis(4-fluorophenyl)methoxy]ethyl})(-4-[3-phenylpropyl]piperazine) (GBR-12909) did not affect [^{3}H]ifenprodil binding in the nanomolar range or only slightly. In contrast, ifenprodil, eliprodil, nylidrin and haloperidol inhibited [^{3}H]ifenprodil binding in the nanomolar range and in the same rank order and with the same potency as observed for the inhibition of the high-affinity fraction of [^{3}H]MK-801 binding. The polyamines, which activate NMDA receptors, inhibited [^{3}H]ifenprodil binding in a biphasic manner. Their potency to inhibit the high-affinity fraction of [^{3}H]ifenprodil binding was found to be in the same range as their potency to enhance [^{3}H]MK-801 binding. In the presence of 10 μM spermine a significantly enhanced ($P = 0.0097$) rate of dissociation of [^{3}H]ifenprodil binding was found, suggesting that inhibition of [^{3}H]ifenprodil binding by spermine is not, or at least not exclusively mediated by a competitive interaction.

Keywords: NMDA receptor; σ site; [^{3}H]Ifenprodil; [^{3}H]MK-801; Hippocampal brain membrane, porcine

1. Introduction

Antagonists acting at different recognition sites of NMDA receptors exert neuroprotective effects in various ischemia models (Muir and Lees, 1995). (±)-2-(4-Benzylpiperidino)-1-(4-hydroxyphenyl)propan1-ol (Ifenprodil) and (±)-1-(4-chlorophenyl)-2-[4-(4-fluorobenzyl)-piperidino]ethanol (eliprodil) do not share the psychostimulant, amnesic, and neurotoxic effects observed for many other types of NMDA receptor antagonists (Scatton et al., 1994). Although ifenprodil and eliprodil have been shown not to interact exclusively with NMDA receptors, several lines of evidence have been presented that their neuroprotective effects are mediated at a distinct polyamine-sensitive regulatory site of an NMDA receptor subtype containing NR1 and NR2B subunits (Scatton et al., 1994). Unfortunately, [^{3}H]ifenprodil — up to now the only commercially available tritiated ligand with selectivity for NMDA receptors containing NR2B subunits — labels various other receptors and channels in the central nervous system with high-affinity (Chenard et al., 1995; Avenet et al., 1996). Labelling of σ sites in brain membrane fractions, an inherent problem when [^{3}H]ifenprodil is used, has been

* Corresponding author. Tel.: +49-89-2180-7277 / +49-89-2180-7249; fax: +49-89-2180-7247.

E-mail addresses: ghoef@cup.uni-muenchen.de (G. Höfner), klaus.wanner@cup.uni-muenchen.de (K.Th. Wanner).

[1] Also corresponding author. Tel.: +49-89-2180-7277 / +49-89-2180-7249; fax: +49-89-2180-7247.

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00084-4

circumvented by the addition of σ receptor ligands such as 1-{2-[bis(4-fluorophenyl)methoxy]ethyl}4-[3-phenylpropyl]piperazine (GBR-12909), 1,3-di(2-tolyl)-guanidine (DTG), (+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine or (+)-pentazocine ((*R*)-3-PPP) to mask these sites (Dana et al., 1991; Carter et al., 1992; Segal and Skolnick, 1999). Recent studies, however, revealed that σ receptor ligands such as haloperidol and trifluperidol selectively inhibit NMDA receptors, comprised of NR1A and NR2B subunits, with their binding possibly overlapping with the binding site of ifenprodil (Coughenour and Cordon, 1997; Whittemore et al., 1997b; Gallagher et al., 1998), and that σ receptor ligands appear to inhibit NMDA receptors indirectly (Yamamoto et al., 1995).

Previous investigations of the regional and the subcellular distribution of [^{3}H]ifenprodil binding in rat brain demonstrated that the density of [^{3}H]ifenprodil binding ascribed to NMDA receptors is high in several forebrain regions such as the hippocampus (Dana et al., 1991, Hashimoto et al., 1994; Nicolas and Carter, 1994), whereas the regional distribution of [^{3}H]ifenprodil binding ascribed to σ sites is almost homogenous. Finally, it was also found that the ratio of NMDA receptors to σ site labelling is highest in favour of the NMDA receptor in density gradient fractions termed synaptosomal and mitochondrial fractions (Knight et al., 1991; Cagnotto et al., 1994; Hashimoto et al., 1994). Therefore we undertook a study to characterise [^{3}H]ifenprodil binding to subcellular fractions from porcine hippocampal brain membranes, with the intention to uncover whether the NMDA receptor can be selectively labelled by [^{3}H]ifenprodil in a native receptor source in the absence of any ligands masking σ sites.

2. Materials and methods

2.1. Membrane preparation and subcellular fractionation

The hippocampi from 40 pig brains (from the local slaughterhouse) kept on ice were pooled after a post-mortem delay of 2–3 h, suspended in 0.32 M sucrose, 5 mM Tris AcOH pH 7.4 (about 103 g tissue in 1030 ml), homogenised with a potter (PotterS Braun, 800 rpm, 12 up-and-down strokes), and centrifuged at $1000 \times g$ for 5 min at 4°C following a literature procedure (Gordon-Weeks, 1987). The resulting pellet was resuspended in the same volume 0.32 M sucrose, 5 mM Tris AcOH pH 7.4 and centrifuged at $1000 \times g$ for 5 min at 4°C again. The supernatants (S_1 and S_1') were combined and centrifuged at $12\,000 \times g$ for 20 min at 4°C to yield P_2. The whiter portion of P_2 was centrifuged twice again as described above and yielded a pellet (P_2'') which was resuspended in 300 ml 0.32 M sucrose, 5 mM Tris AcOH pH 7.4 and frozen in liquid nitrogen. Aliquots of 50 ml P_2'' were thawed and centrifuged in six 38-ml tubes on a discontinuous sucrose gradient (10 ml 0.85 M sucrose, 10 ml 1.0 M sucrose, 10 ml 1.2 M sucrose) at $85\,000 \times g_{av}$ for 120 min at 4°C. The 0.32/0.85 M interface (P_{2A}), the 0.85/1.0 M interface (P_{2B}), the 1.0/1.2 M interface (P_{2C}) and the pellet under the gradient (P_{2D}) were collected, each resuspended in 80 ml 5 mM Tris AcOH pH 7.4 and centrifuged at $48\,000 \times g$ for 20 min at 4°C. Since preliminary experiments revealed that P_{2C} and P_{2D} were most suited for our purposes, these fractions were combined, resuspended in 225 ml 5 mM Tris AcOH pH 7.4, and frozen in liquid nitrogen (about 300 mg protein in P_{2CD} from 40 brains) after measurement of their protein concentration (Bradford, 1976) with bovine serum albumin as standard.

2.2. Binding of [^{3}H]MK-801 and [^{3}H]DTG to the subcellular fractions P_{2A-D}

[^{3}H]MK-801 ((5*R*, 10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine) binding: Aliquots of the subcellular fractions were incubated in the presence of 1 nM [^{3}H]MK-801, 100 μM L-glutamate, 30 μM glycine, 5 mM Tris AcOH pH 7.4 in a total volume of 500 μl at 25°C for 4 h as previously described (Höfner and Wanner, 1996). Non-specific binding was determined in the presence of 10 μM PCP.

[^{3}H]DTG binding: Aliquots of the subcellular fractions were incubated in the presence of 4 nM [^{3}H]DTG, 50 mM Tris HCl pH 7.4 in a total volume of 500 μl at 25°C for 90 min as previously described (Weber et al., 1986). Non-specific binding was determined in the presence of 10 μM haloperidol.

2.3. [^{3}H]ifenprodil binding

Aliquots of about 30 μg protein (P_{2CD}) were incubated in the presence of [^{3}H]ifenprodil, 5 mM Tris AcOH pH 7.4 in a total volume of 250 μl at 4°C for 2 h. Saturation experiments were performed with 8–10 concentrations of [^{3}H]ifenprodil in the range 0.2–125 nM, and competition experiments were performed with about 2 nM [^{3}H]ifenprodil and 6–12 concentrations of the competing drug. In dissociation experiments aliquots of about 60 μg

Table 1
Specific [^{3}H]MK-801 and [^{3}H]DTG binding to subcellular membrane fractions of porcine hippocampus

Fraction	[^{3}H]MK-801 bound (fmol/mg protein)	[^{3}H]DTG bound (fmol/mg protein)
P_{2A}	55 ± 9	111 ± 15
P_{2B}	378 ± 45	94 ± 14
P_{2C}	3660 ± 215	87 ± 20
P_{2D}	1920 ± 294	109 ± 27

Experiments were performed as described in Section 2. The results are given as means ± S.E.M., $n = 3$.

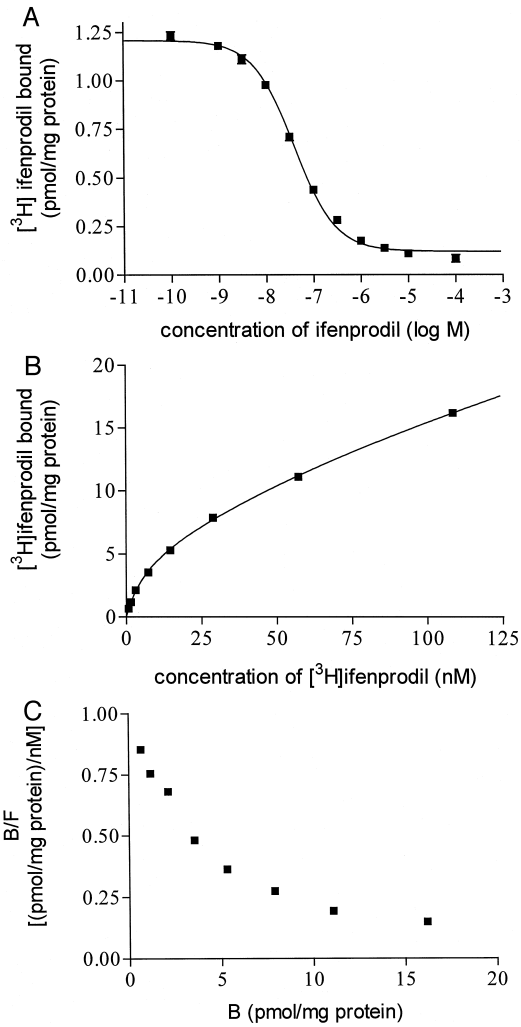

Fig. 1. (A) One representative competition experiment (out of three experiments) with [^{3}H]ifenprodil and unlabelled ifenprodil in the P_{2CD} fraction of porcine hippocampal brain membranes performed as described in Section 2, but without subtraction of non-specific binding (mean ± S.E.M from triplicates). (B) Specific [^{3}H]ifenprodil binding (expressed as difference between total binding and non-specific binding determined in the presence of 10 μM ifenprodil) to the P_{2CD} fraction of porcine hippocampal brain membranes in one representative saturation experiment (out of three) performed as described in Materials and methods. (C) Scatchard plot derived from the saturation experiment shown in Fig. 1B.

protein (P_{2CD}) were incubated in the presence of 2 nM [^{3}H]ifenprodil, 5 mM Tris AcOH pH 7.4 in a total volume of 250 μl at 4°C for 2 h. After addition of 20 μl 135 μM ifenprodil (control) or 10 μl 270 μM ifenprodil and 10 μl 270 μM spermine, the dissociation was stopped at time points from 5 up to 160 min. In all experiments incubation was terminated by filtration through Whatman GF/B filters pre-soaked for 1 h in 0.5% polyethylenimine, using a Brandel M-24R harvester. The filters were rapidly rinsed with cold buffer (4 × 2 ml) and counted in 3 ml Rotiszint Eco Plus using a Packard TriCarb 1600 liquid scintillation counter. Non-specific binding was determined in the presence of 10 μM ifenprodil.

2.4. [^{3}H]MK-801 binding

Aliquots of about 50–60 μg protein (P_{2CD}) were incubated in the presence of 2.8 nM [^{3}H]MK-801, 100 μM L-glutamate, 30 μM glycine, 5 mM Tris AcOH pH 7.4 and 6–12 concentrations of the test compound in a total volume of 500 μl at 25°C for 15 min as previously described (Höfner and Wanner, 1996).

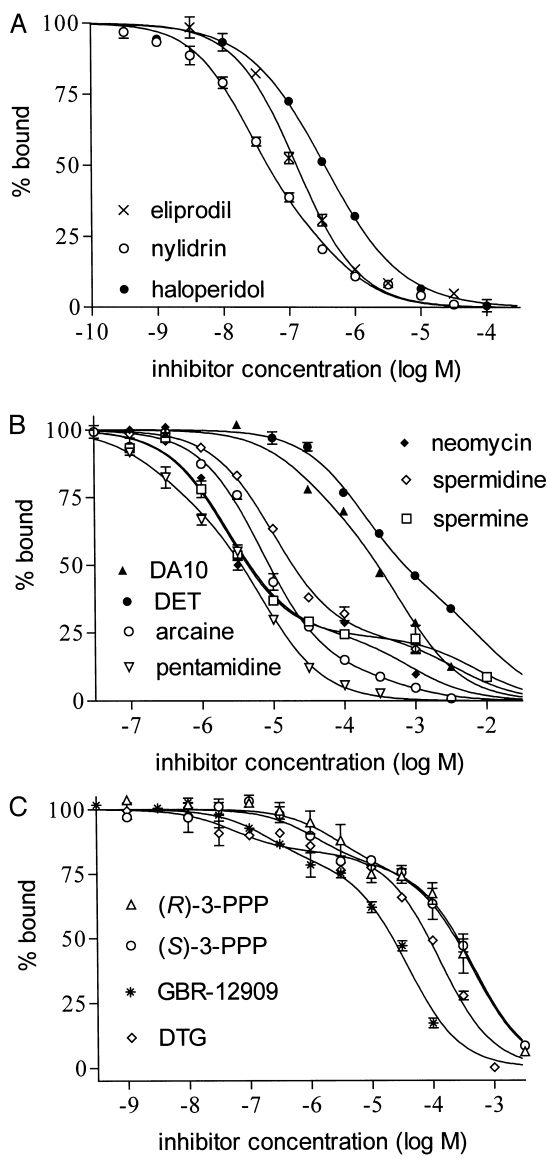

Fig. 2. One representative competition experiment (out of a number of experiments specified in brackets) with [^{3}H]ifenprodil and (A) eliprodil ($n = 4$), nylidrin ($n = 3$) and haloperidol ($n = 3$); (B) spermine ($n = 5$), spermidine ($n = 3$), neomycin ($n = 3$), DA10 (1,10-diaminodecane) ($n = 3$), DET (diethylentriamine) ($n = 4$), arcaine ($n = 3$) and pentamidine ($n = 3$); (C) DTG ($n = 4$), (*R*)-3-PPP ($n = 3$), (*S*)-3-PPP ($n = 3$) and GBR-12909 ($n = 4$) in the P_{2CD} fraction of porcine hippocampal brain membranes performed as described in Section 2 (mean ± S.E.M. from triplicates).

2.5. Data analysis

The dissociation constant (K_d) and the density of binding sites (B_{max}) were calculated from saturation isotherms by means of the non-linear curvefitting program Radlig/Kell (Cambridge, UK). The concentration of competing drugs that inhibits 50% of specific [^{3}H]ifenprodil binding (IC_{50}), the concentration of compounds producing half-maximal enhancement or inhibition of [^{3}H]MK-801 binding (EC_{50}) and the dissociation rate constant (k_{off}) were calculated using built-in equations of Prism (GraphPad Software, San Diego, CA, USA) for sigmoidal dose-response curves with variable Hill slopes, for one- and two-site competition as well as for one-phase exponential decay. Statistical analyses were performed using the *F*-test to determine whether data could be described by a single- or a two-site model. Results based on a two-site model are only specified when the *F*-test significantly ($P < 0.05$) favoured the two-site model in each case, if not stated otherwise. All data are expressed as means ± S.E.M. of at least three separate experiments, each carried out in triplicate.

2.6. Materials

[^{3}H]ifenprodil, [^{3}H]MK-801 and [^{3}H]DTG were purchased from NEN (Brussels, Belgium); GBR-12909, (*R*)-3-PPP, (*S*)-3-PPP, DTG, MK-801, and PCP from RBI (Natick, MA, USA); CPP ((*RS*)-4-(3-phosphonopropyl)-piperazine-2-carboxylic acid), DCKA (5,7-dichlorokynurenic acid), DNQX (6,7-dinitroquinoxaline-2,3-dione), AP-5 ((*RS*)-2-amino-5-phosphonopentanoic acid), and ifenprodil from Tocris Cookson (Bristol, UK); Tris, polyethylenimine from ICN (Eschwege, Germany), spermidine trihydrochloride from Sigma (Oberhaching, Germany); 1,10-diaminodeane, sucrose, spermine tetrahydrochloride, diethylenetriamine, L-glutamate, and glycine from Merck (Darmstadt, Germany); NMDA and D-serine from Fluka (Neu-Ulm, Germany). Eliprodil was prepared by J. Pabel at the Institute for Pharmacy of the LMU in Munich (Pabel et al., 1999; Bioorg. Med. Chem. Lett., submitted).

3. Results

Comparison of the subcellular fractions P_{2A-D} of porcine hippocampal brain membranes with respect to [^{3}H]MK-801 binding as a marker for NMDA receptors and [^{3}H]DTG binding as a marker for σ sites in preliminary experiments showed that the highest density of NMDA receptors was found in P_{2C} and P_{2D}, whereas [^{3}H]DTG-labelled σ sites were hardly detected in P_{2C} and P_{2D} in comparison to [^{3}H]MK-801-labelled sites (Table 1). Therefore the combined P_{2CD} fraction was used in all the following experiments. Incubation of the P_{2CD} fraction at 4°C with 2 nM [^{3}H]ifenprodil showed that specific binding increased linearly with added protein (8–70 μg/assay tube), that [^{3}H]ifenprodil binding seemed to reach equilibrium within 2 h and that binding did not notably differ in frozen and thawed membranes from fresh preparations (data not shown). Homologous competition experiments with 2 nM [^{3}H]ifenprodil (Fig. 1A) revealed that competition was apparently monophasic, that the affinity (K_d) of [^{3}H]ifenprodil was in the range of 10–100 nM and that 12.8 ± 2.2% [^{3}H]ifenprodil binding remained in the presence of 10 μM ifenprodil. In the following experiments

Table 2
Inhibition of [^{3}H]ifenprodil binding

Compound	IC_{50} (μM)	n_H	IC_{50high} (μM)	IC_{50low} (μM)	% High-affinity	*n*
Ifenprodil	0.0377 ± 0.0042	−0.91 ± 0.05				5
Eliprodil	0.110 ± 0.010	−0.94 ± 0.05				4
Haloperidol	0.452 ± 0.044	−0.90 ± 0.06				3
Nylidrin	0.0662 ± 0.0118	−0.68 ± 0.05	0.0211 ± 0.0140	1.33 ± 1.68	61	3
Spermine	6.62 ± 1.55	−0.49 ± 0.02	4.00 ± 0.77	> 1000	78	5
Spermidine	17.9 ± 2.9	−0.54 ± 0.01	7.51 ± 1.39	> 1000	76	3
Neomycin	4.30 ± 0.60	−0.57 ± 0.02	1.81 ± 0.14	586 ± 113	77	3
Arcaine	13.6 ± 4.5	−0.70 ± 0.11	6.63 ± 1.52	832 ± 803	81	3
DET[a]	662 ± 127	−0.61 ± 0.06	135 ± 25	> 1000	55	4
DA10[b]	282 ± 77	−0.83 ± 0.07				3
Pentamidine	4.23 ± 0.77	−0.81 ± 0.04				3
DTG	52.6 ± 6.8	−0.59 ± 0.05	0.164 ± 0.067	117 ± 12	19	4
(*R*)-3-PPP	181 ± 5	−0.58 ± 0.05	2.17 ± 0.66	423 ± 35	22	3
(*S*)-3-PPP	234 ± 77	−0.52 ± 0.05	0.697 ± 0.557	519 ± 119	19	3
GBR-12909	36.3 ± 7.4	−0.42 ± 0.05	0.117 ± 0.033	> 100[c]	24	4
PCP	1340 ± 220	−0.83 ± 0.20				3

Experiments were performed as described in Section 2. The results are given as means ± S.E.M.
[a] Diethylentriamine.
[b] 1,10 Diaminodecane.
[c] Insoluble at concentrations higher than 100 μM.

Table 3
Inhibition of [^{3}H]ifenprodil binding

Compound	Specific binding (%) at a single concentration
L-glutamate	102 ± 3% (10 μM)
NMDA	97 ± 1% (10 μM)
CPP	61 ± 1% (10 μM)
AP-5	86 ± 1% (10 μM)
Glycine	91 ± 1% (10 μM)
D-serine	97 ± 1% (10 μM)
DCKA	117 ± 6% (10 μM)
DNQX	101 ± 3% (10 μM)
Na^+	97 ± 3% (1 mM)
K^+	98 ± 3% (1 mM)
Zn^{2+}	52 ± 1% (100 μM)
Ca^{2+}	73 ± 1% (100 μM)
Mg^{2+}	68 ± 2% (100 μM)

Experiments were performed as described in Section 2. The results are given as means ± S.E.M., $n = 3$.

non-specific binding was therefore defined with 10 μM ifenprodil. Increasing the [^{3}H]ifenprodil concentration up to 125 nM did not achieve saturation of binding sites (Fig. 1B,C). Non-linear least-squares fitting of the saturation isotherms derived from these experiments with a one-site model yielded a K_d value of 23.0 ± 1.8 nM and a B_{max} value of 15.6 ± 0.8 pmol/mg protein ($r^2 \geq 0.89$), whereas with a two-site model a K_{d1} value of 10.4 ± 2.4 nM and a B_{max1} value of 13.7 ± 7.4 pmol/mg protein for the high-affinity sites and a K_{d2} value of 2200 ± 1300 nM and a B_{max2} value of 282 ± 165 pmol/mg protein for the low-affinity sites ($r^2 \geq 0.99$) were calculated, respectively. The two-site model fitted the saturation isotherms in two of three experiments significantly better, as indicated by the *F*-test ($P < 0.05$).

Various NMDA and σ receptor ligands as well as several cations were investigated for their ability to inhibit 2 nM [^{3}H]ifenprodil binding at porcine hippocampal brain membranes in the P_{2CD} fraction (Fig. 2A–C and Tables 2 and 3). Only the compounds proposed to be NR2B-selective NMDA receptor antagonists, ifenprodil, eliprodil, nylidrin and haloperidol, inhibited [^{3}H]ifenprodil binding in the nanomolar range. Ifenprodil, eliprodil and haloperidol inhibited [^{3}H]ifenprodil binding in an apparently monophasic fashion, as indicated by n_H values close to unity whereas the inhibition elicited by nylidrin was characterised by an n_H value of −0.68. For these compounds a rank order of potency ifenprodil > nylidrin > eliprodil > haloperidol was observed. The NMDA receptor-activating polyamines spermine, spermidine and neomycin produced biphasic curves for inhibition of [^{3}H]ifenprodil binding

About 80% of total inhibition could be attributed to the high-affinity fraction characterised by IC_{50} values in the low micromolar range whereas the low-affinity fraction was characterised by IC_{50} values in the high micromolar range to the millimolar range, respectively.

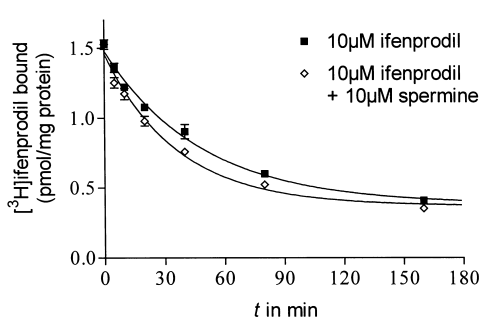

Fig. 3. Dissociation of [^{3}H]ifenprodil binding initiated by addition of 10 μM ifenprodil alone (control) and 10 μM ifenprodil + 10 μM spermine from one representative experiment (out of four experiments) performed as described in Section 2 (mean ± S.E.M. from triplicates).

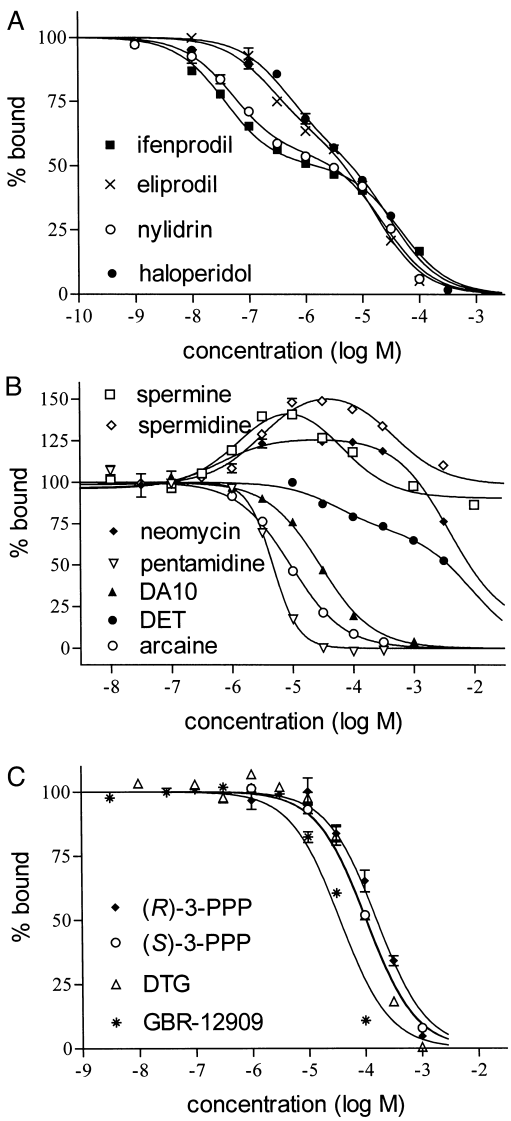

Fig. 4. One representative experiment (out of a number of experiments specified in brackets) showing inhibition or enhancement of [^{3}H]MK-801 binding by (A) ifenprodil ($n = 4$), eliprodil ($n = 3$), nylidrin ($n = 3$) and haloperidol ($n = 4$); (B) spermine ($n = 3$), spermidine ($n = 5$), neomycin ($n = 3$), DA10 (1,10-diaminodecane) ($n = 3$), DET (diethylenetriamine) ($n = 3$), arcaine ($n = 3$) and pentamidine ($n = 3$); (C) DTG($n = 3$), (*R*)-3-PPP ($n = 3$), (*S*)-3-PPP ($n = 3$) and GBR-12909 ($n = 4$) in the P_{2CD} fraction of porcine hippocampal brain membranes. The experiment was performed as described in Section 2 (mean ± S.E.M. from triplicates).

Table 4
Enhancement or inhibition of [^{3}H]MK-801 binding

Compound	EC_{50} (μM)	n_H	EC_{50high} (μM)	EC_{50low} (μM)	% High-affinity	n
Ifenprodil	4.19 ± 0.45[a]	−0.36 ± 0.03	0.0608 ± 0.0076	21.8 ± 2.6	49	4
Eliprodil	2.33 ± 0.56[a]	−0.60 ± 0.07	0.396 ± 0.096	12.9 ± 2.0	56	3
Haloperidol	7.12 ± 1.29[a]	−0.58 ± 0.04	0.971 ± 0.194	48.4 ± 7.0	51	4
Nylidrin	2.48 ± 0.39[a]	−0.48 ± 0.03	0.0657 ± 0.0053	47.4 ± 12.2	43	3
Spermine			0.788 ± 0.143[b]	113 ± 18[a]		3
Spermidine			2.77 ± 0.33[b]	736 ± 217[a]		5
Neomycin			0.883 ± 0.189[b]	452 ± 219[a]		3
Arcaine	9.33 ± 1.10[a]	−1.06 ± 0.04				3
DET[c]	> 1000	−0.36 ± 0.03	29.1 ± 8.7	> 1000	26	3
DA10[d]	30.4 ± 7.0[a]	−0.82 ± 0.05				3
Pentamidine	4.19 ± 0.46[a]	−1.85 ± 0.03				3
DTG	15.6 ± 0.75[a]	−1.09 ± 0.04				3
(*R*)-3-PPP	211 ± 2[a]	−1.23 ± 0.06				3
(*S*)-3-PPP	104 ± 1[a]	−1.10 ± 0.06				3
GBR-12909	24.6 ± 3.6[a]	−1.46 ± 0.20				4

[^{3}H]MK-801 binding under non-equilibrium conditions was performed as described in Section 2. The results are given as means ± S.E.M.
[a] Inhibition of [^{3}H]MK-801 binding.
[b] Enhancement of [^{3}H]MK-801 binding.
[c] Diethylentriamine.
[d] 1,10 Diaminodecane.

Dissociation experiments revealed that 10 μM spermine significantly ($P = 0.0097$, paired t-test, $n = 4$) enhanced k_{off} of [^{3}H]ifenprodil binding (0.028 ± 0.03 min^{-1}, mean ± S.E.M.) in comparison to control (0.021 ± 0.02 min^{-1}, mean ± S.E.M., $n = 4$, Fig. 3).

All the polyamine ligands with ascribed antagonist effects at NMDA receptors reduced [^{3}H]ifenprodil binding in the micromolar range too, showing a rank order of potency pentamidine > arcaine > 1,10-diaminodeane > diethylenetriamine, but revealing differences regarding their n_H values. Of the other NMDA receptor ligands and cations, only the divalent cations Zn^{2+}, Mg^{2+}, Ca^{2+} and the glutamate antagonists CPP and AP-5 were unequivocally able to decrease [^{3}H]ifenprodil binding in the micromolar range. The glycine antagonists DCKA slightly increased whereas DNQX hardly did not affect [^{3}H]ifenprodil binding at a concentration of 10 μM. The inhibition of [^{3}H]ifenprodil binding to the P_{2CD} fraction of porcine hippocampal brain membranes by the σ receptor ligands DTG, (*R*)-3-PPP, (*S*)-3-PPP and GBR-12909 was characterised by IC_{50} values in the medium or in the high micromolar range. Fitting the competition data for these ligands to a two-site model was superior over fitting to a one-site model and revealed a high-affinity fraction of about 20% characterised by IC_{50h} values of 117 ± 33 nM for GBR-12909, 164 ± 67 nM for DTG, 2170 ± 660 nM for (*R*)-3-PPP and 697 ± 557 nM for (*S*)-3-PPP.

The NR2B subunit-selective compounds as well as the polyamine- and the σ receptor ligands were evaluated for their potency to influence NMDA receptor function as recorded by [^{3}H]MK-801 binding to the P_{2CD} fraction of porcine hippocampal brain membranes in the presence of 100 μM glutamate and 30 μM glycine for 15 min (Fig. 4A–C and Table 4). The ligands proposed to be NR2B-selective NMDA receptor antagonists, namely, ifenprodil, nylidrin, eliprodil and haloperidol, inhibited [^{3}H]MK-801 binding in a biphasic way. For these compounds IC_{50} values for the high-affinity site, which amounted to about 50% of total inhibition, in the nM range were determined. The rank order of potency at the high-affinity site was again ifenprodil > nylidrin > eliprodil > haloperidol, as observed for [^{3}H]ifenprodil binding, and eliprodil > ifenprodil > haloperidol ≅ nylidrin for the low-affinity site, respectively. The polyamines spermine, spermidine and neomycin induced an enhancement of [^{3}H]MK-801 binding at low micromolar concentrations and reduced [^{3}H]MK-801 binding in the high micromolar range. The putative polyamine ligands arcaine, 1,10-diaminodeane and pentamidine inhibited [^{3}H]MK-801 binding in an apparently monophasic manner with IC_{50} values in the low micromolar range and with a rank order of potency pentamidine > arcaine > 1,10-diaminodeane. Diethylene triamine reduced a small portion (26%) of [^{3}H]MK-801 binding in the micromolar range and the major portion (74%) in the millimolar range, as calculated from the flat inhibition curves using a two-site model. For the σ receptor ligands apparently monophasic inhibition of [^{3}H]MK-801 binding in the micromolar range with a rank order of potency DTG > GBR-12909 > (*S*)-3-PPP > (*R*)-3-PPP was observed.

4. Discussion

In the present study we identified a high-affinity site for [^{3}H]ifenprodil in porcine hippocampal brain membranes that was characterised by a K_d value of 23.0 ± 1.8 nM based on a one-site analysis or by a K_{d1} value of 10.4 ± 2.4

nM based on a two-site analysis, respectively. These results are consistent with results derived from experiments using rat brain membranes in the presence of DTG or GBR-12909 to mask σ sites (Schoemaker et al., 1990; Hashimoto et al., 1994). An additionally existing low-affinity site for [^{3}H]ifenprodil in the micromolar range may be deduced from the saturation isotherms. For competition experiments performed with 2 nM [^{3}H]ifenprodil, however, notable labelling of low-affinity sites can be excluded as indicated by the homologous competition experiments.

Previous investigations of rat brain proposed that high-affinity [^{3}H]ifenprodil binding to NMDA receptors may be separated from high-affinity [^{3}H]ifenprodil binding to σ sites by the use of selected brain regions such as the hippocampus and by the use of selected subcellular fractions such as synaptosomal and mitochondrial fractions (Knight et al., 1991; Cagnotto et al., 1994; Hashimoto et al., 1994; Nicolas and Carter, 1994). This study demonstrated that our P_{2CD} porcine hippocampal membrane fraction enabled almost selective labelling of NMDA receptors by ifenprodil on a large scale without the need to mask σ_1 or σ_2 sites.

Of the inhibitors investigated, only ifenprodil, eliprodil, haloperidol and nylidrin inhibited [^{3}H]ifenprodil binding to the P_{2CD} porcine hippocampal membrane fraction in the nanomolar range. Electrophysiological investigations with recombinant subunit combinations demonstrated that these compounds possess a high selectivity for heterodimers containing the NR2B subunit and yielded IC_{50} values of 0.18 μM for nylidrin (Whittemore et al., 1997a), 0.31 μM for ifenprodil and 1.0 μM for eliprodil (Whittemore et al., 1997b) as well as 3.1 μM for haloperidol (Ilyin et al., 1996).

In order to prove that [^{3}H]ifenprodil binding to the P_{2CD} porcine hippocampal membrane fraction reflects binding to NMDA receptors, we studied [^{3}H]MK-801 binding to the same membrane preparation by incubating the samples in the presence of saturating concentrations of L-glutamate and glycine for 15 min. These conditions have been shown to reflect [^{3}H]MK-801 binding under non-equilibrium conditions and are appropriate to record effects of ifenprodil and polyamine ligands on NMDA receptors (Höfner and Wanner, 1996). We observed a markedly biphasic inhibition of [^{3}H]MK-801 binding to brain membranes with ifenprodil, eliprodil, and haloperidol, as described in studies with [^{3}H]MK-801 or [^{3}H]TCP (Reynolds and Miller, 1989; Scatton et al., 1994; Coughenour and Cordon, 1997), and with nylidrin. The almost exact correlation of the potencies of ifenprodil, eliprodil, haloperidol and nylidrin for inhibition of [^{3}H]ifenprodil binding with the potencies to inhibit the high-affinity fraction of [^{3}H]MK-801 binding strongly indicates that [^{3}H]ifenprodil labels NMDA receptors in the P_{2CD} porcine hippocampal membrane fraction. The n_H values deduced from [^{3}H]ifenprodil inhibition curves deviated slightly but consistently from unity for ifenprodil, eliprodil and haloperidol and deviated distinctly from unity for nylidrin but this may not be in contradiction to this suggestion since different potencies for ifenprodil at NMDA receptors containing NR2B subunits but differing in stoichiometry have been reported (Hess et al., 1996). Additionally, it should be noted that ifenprodil as well as nylidrin and eliprodil are composed of both enantiomers, which possibly interact with definite NMDA receptor subtypes with different affinity, as demonstrated for ifenprodil (Avenet et al., 1996).

In adult mouse forebrains the binary NR1/NR2A and NR1/NR2B receptors are found in amounts of 37% and 40%, respectively (Chazot and Stephenson, 1997). In our study the fraction of [^{3}H]MK-801 binding inhibited with high- and low-affinity by the NR2B-subunit-selective compounds was almost equal. Therefore it could be assumed that the [^{3}H]MK-801 binding inhibited with high-affinity by the NR2B-subunit-selective compounds reflects binding to NMDA receptors containing the high-affinity ifenprodil recognition site, whereas the [^{3}H]MK-801 binding inhibited with low affinity by the NR2B subunit-selective compounds reflects binding to NMDA receptors lacking the high-affinity ifenprodil recognition site and may be mediated by competition with the radioligand as suggested previously (Carter et al., 1997; Whittemore et al., 1997b).

Inhibition of NMDA receptors by ifenprodil has been shown to be polyamine-sensitive (Scatton et al., 1994). The NMDA-receptor-stimulating polyamines spermine, spermidine and neomycin inhibited [^{3}H]ifenprodil binding biphasically. A high-affinity fraction of nearly 80% of [^{3}H]ifenprodil binding was characterised by IC_{50} values in the low micromolar range. Interestingly, these IC_{50} values correlated with the EC_{50} values calculated for stimulation of [^{3}H]MK-801 binding to the P_{2CD} fraction of porcine hippocampal brain membranes. Stimulation of [^{3}H]MK-801 binding by neomycin, spermine and spermidine in the presence of 30 μM glycine should be glycine-independent (Berger, 1995). Polyamine recognition sites mediating glycine-independent stimulation as well as high-affinity ifenprodil recognition sites — regarding heterodimers — have been shown to be restricted to the same (NR2B subunit containing) NMDA receptor subtypes (Johnson, 1996; Williams, 1997). In our experiments, spermine enhanced dissociation of [^{3}H]ifenprodil binding, suggesting that [^{3}H]ifenprodil labels not or not only those polyamine recognition sites mediating glycine-independent stimulation of [^{3}H]MK-801 binding. This result is in line with the suggestion that spermine reduces the affinity of ifenprodil at NMDA receptors (Kew and Kemp, 1998).

Pentamidine has been characterised as an NMDA receptor antagonist that does not act directly at the L-glutamate, at the glycine, or at the PCP recognition site but which is able to modulate the effect of spermidine on channel opening (Reynolds and Aizenman, 1992). Pentamidine inhibited [^{3}H]ifenprodil and [^{3}H]MK-801 binding almost equally. However, pentamidine exhibited no selectivity for ifenprodil-sensitive NMDA receptors, as indicated by the

monophasic inhibition of [^{3}H]MK-801 binding. Of the other polyamines which reduced [^{3}H]MK-801 binding in our assay, only arcaine inhibited the high-affinity fraction of [^{3}H]ifenprodil binding in the low micromolar range and with almost equal potency as observed for [^{3}H]MK-801 binding. 1,10-Diaminodeane, which was originally described as an inverse agonist at the polyamine site (Williams et al., 1991) but which was later classified as a voltage-dependent open channel blocker (Rock and Macdonald, 1992; Subramaniam et al., 1992), showed an IC_{50} value for inhibition of [^{3}H]ifenprodil binding about one order of magnitude higher than the EC_{50} value for inhibition of [^{3}H]MK-801 binding, which supports the later hypothesis.

For DTG and GBR-12909, σ receptor ligands with ascribed K_i values distinctly below 100 nM for σ_1 and σ_2 sites (Hashimoto and London, 1993; Cagnotto et al., 1994; Hellewell et al., 1994; Whittemore et al., 1997b), the low-affinity phase amounted to about 80% of total inhibition of [^{3}H]ifenprodil binding and was characterised by IC_{50} values in the high micromolar range. (*R*)-3-PPP, which has reported K_i values about or below 100 nM for σ_1 and σ_2 sites (Hashimoto and London, 1993; Hellewell et al., 1994; Whittemore et al., 1997b), did not inhibit [^{3}H]ifenprodil binding to the P_{2CD} membrane fraction of porcine hippocampus substantially in the nanomolar range. Haloperidol has been shown to label σ_1 and σ_2 sites in the low nanomolar range (Hellewell et al., 1994; Whittemore et al., 1997b). In our assay, however, haloperidol inhibited [^{3}H]ifenprodil binding in an apparently monophasic manner with an IC_{50} value of 452 nM. There was apparently no such high-affinity fraction for [^{3}H]ifenprodil labelled sites. This finding, together with the corresponding potency of haloperidol to inhibit [^{3}H]ifenprodil binding and the high-affinity fraction of [^{3}H]MK-801 binding, indicates that [^{3}H]ifenprodil predominantly labels NMDA receptors and not σ sites. Our results showing that ifenprodil and haloperidol label the NR2B subunit are in accord with a recently performed study which demonstrated that the NMDA antagonism mediated by the ifenprodil analog CP101,606 ((1*S*,2*S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol) and haloperidol is dependent on a single amino acid substitution in the NR2B subunit (Brimecombie et al., 1998).

In summary, it could be demonstrated that [^{3}H]ifenprodil labels high-affinity recognition sites at NMDA receptors in the P_{2CD} porcine hippocampal brain membrane preparation. Although some of the results presented here raise the possibility that the [^{3}H]ifenprodil binding sites are not a completely homologous population, σ_1 and σ_2 sites appear not to be labelled to a discernible extent.

Acknowledgements

We thank E. Armbrust, S. Duensing-Kropp and S. Lukassen for technical assistance. Financial support of this work by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie is gratefully acknowledged.

References

Avenet, P., Leonardon, J., Besnard, F., Graham, D., Frost, J., Depoortere, H., Langer, S.Z., Scatton, B., 1996. Antagonist properties of the stereoisomers of ifenprodil at NR1A/NR2A and NR1A/NR2B subtypes of the NMDA receptor expressed in Xenopus oocytes. Eur. J. Pharmacol. 296, 209–213.

Berger, M.L., 1995. On the true affinity of glycine for ist binding site at the NMDA receptor complex. J. Pharmacol. Methods 34, 79–88.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brimecombie, J.C., Gallagher, M.J., Lynch, D.R., Aizenman, E., 1998. An NR2B point mutation affecting haloperidol and CP101,606 sensitivity of single recombinant *N*-Methyl-D-Aspartate receptors. J. Pharmacol. Exp. Ther. 286, 627–634.

Cagnotto, A., Bastone, A., Mennini, T., 1994. [^{3}H](+)-Pentazocine binding to rat brain σ1-receptors. Eur. J. Pharmacol. 266, 131–138.

Carter, C., Avenet, P., Benavides, J., Besnard, F., Biton, B., Cudennec, A., Duverger, D., Frost, J., Giroux, C., Graham, D., Langer, S.Z., Nowicki, J.P., Oblin, A., Perault, G., Pigasse, S., Rosen, P., Sanger, D., Schoemaker, H., Thenot, J.P., Scatton, B., 1997. Ifenprodil and eliprodil: neuroprotective NMDA receptor antagonists and calcium channel blockers. In: Herling, P.L. (Ed.), Excitatory Amino Acids — Clinical Results with Antagonists. Academic Press, San Diego, pp. 57–151.

Carter, C.J., Minisclou, C., Schoemaker, H., Scatton, B., 1992. Polyamine-directed non-competitive NMDA antagonists. Mol. Neuropharmacol. 2, 107–112.

Chazot, P.L., Stephenson, F.A., 1997. Molecular dissection of native mammalian forebrain NMDA receptors containing the NR1 C2 exon: direct demonstration of NMDA receptors comprising NR1, NR2A, and NR2B subunits within the same complex. J. Neurochem. 69, 2138–2144.

Chenard, B.L., Bordner, J., Butler, T.W., Chambers, L.K., Collins, M.A., De Costa, D.L., Ducat, M.F., Dumont, M.L., Fox, C.B., Mena, E.E., Menniti, F.S., Nielsen, J., Pagnozzi, M.J., Richter, K.E.G., Ronau, R.T., Shalaby, I.A., Stemple, J.Z., White, W.F., 1995. (1*S*,2*S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol: a potent new neuroprotectant which blocks *N*-methyl-D-aspartate responses. J. Med. Chem. 38, 3138–3145.

Coughenour, L.L., Cordon, J.J., 1997. Characterisation of haloperidol and trifluperidol as subtype-selective *N*-methyl-D-aspartate (NMDA) receptor antagonists using [^{3}H]TCP and [^{3}H]ifenprodil binding in rat brain membranes. J. Pharmacol. Exp. Ther. 28, 584–592.

Dana, C., Benavides, J., Schoemaker, H., Scatton, B., 1991. Pharmacological characterisation and autoradiographic distribution of polyamine-sensitive [^{3}H]ifenprodil binding sites in the rat brain. Neurosci. Lett. 125, 45–48.

Gallagher, M.J., Huang, H., Lynch, D.R., 1998. Modulation of the *N*-methyl-D-aspartate receptor by haloperidol: NR2B-specific interactions. J. Neurochem. 70, 2120–2128.

Gordon Weeks, P.R., 1987. Isolation of synaptosomes, growth cones and their subcellular components. In: Turner, A.J., Bachelard, H.S. (Eds.), Neurochemistry a Practical Approach. IRL Press, Oxford, pp. 1–26.

Hashimoto, K., London, E.D., 1993. Further characterisation of [^{3}H]ifenprodil binding to σ receptors in rat brain. Eur. J. Pharmacol. 236, 159–163.

Hashimoto, K., Mantione, C.R., Spada, M.R., Neumeyer, J.L., London, E.D., 1994. Further characterisation of [^{3}H]ifenprodil binding in the rat brain. Eur. J. Pharmacol. 266, 67–77.

Hellewell, S., Bruce, A., Feinstein, G., Orringer, J., Williams, W., Bowen, W.D., 1994. Rat liver and kidney contain high densities of σ_1 and σ_2 receptors: characterisation by ligand binding and photoaffinity labeling. Eur. J. Pharmacol. 268, 9–18.

Hess, S.D., Daggett, L.P., Crona, J., Deal, C., Lu, C.-C., Urrutia, A., Chavez-Noriega, L., Ellis, S.B., Johnson, E.C., Velicelebi, G., 1996. Cloning and functional characterisation of human *N*-methyl-D-aspartate receptors. J. Pharmacol. Exp. Ther. 278, 808–816.

Höfner, G., Wanner, K.Th., 1996. Characterisation of [^{3}H]MK-801 binding and its cooperative modulation by pig brain membranes. J. Recept. Signal Transduction Res. 16, 297–313.

Ilyin, V.I., Whittemore, E.R., Guastella, J., Weber, E., Woodward, R.M., 1996. Subtype-selective inhibition of *N*-methyl-D-aspartate receptors by haloperidol. Mol. Pharmacol. 50, 1541–1550.

Johnson, T.D., 1996. Modulation of channel function by polyamines. Trends Pharmacol. Sci. 17, 22–27.

Kew, J.N.C., Kemp, J.A., 1998. An allosteric interaction between the NMDA receptor polyamine and ifenprodil sites in rat cultured cortical neurons. J. Physiol. 512, 17–28.

Knight, A.R., Noble, A., Wong, E.H.F., Middlemiss, D.N., 1991. The subcellular distribution and pharmacology of the sigma recognition site in the guinea-pig brain and liver. Mol. Neuropharmacol. 1, 71–75.

Muir, K.W., Lees, K.R., 1995. Clinical experience with excitatory amino acid antagonist drugs. Stroke 26, 503–513.

Nicolas, C., Carter, C., 1994. Autoradiographic distribution and characteristics of high- and low-affinity polyamine-sensitive [^{3}H]ifenprodil sites in the rat brain: possible relationship to NMDAR2B receptors and calmodulin. J. Neurochem. 63, 2248–2258.

Reynolds, I.J., Aizenman, E., 1992. Pentamidine is an *N*-methyl-D-aspartate receptor antagonist and is neuroprotective in vitro. J. Neurosci. 12, 970–975.

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.

Rock, D.M., MacDonald, R.L., 1992. The polyamine diaminodecane (DA10) produces a voltage-dependent flickery block of single NMDA receptor channels. Neurosci. Lett. 144, 111–115.

Scatton, B., Avenet, P., Benavides, J., Carter, C., Duverger, D., Oblin, A., Perault, G., Sanger, D., Schoemaker, H., 1994. Neuroprotective potential of the polyamine site-directed NMDA receptor antagonists — ifenprodil and eliprodil. In: Palfreyman, M.G., Reynolds, I.J., Skolnick, P. (Eds.), Direct and Allosteric Control of Glutamate Receptors. CRC Press, Boca Raton, pp. 139–154.

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

Segal, J.A., Skolnick, P., 1999. Polyamine like actions of aminoglycoside derivatives at NMDA receptors. Eur. J. Pharmacol. 347, 311–317.

Subramaniam, S., Doneva, S.D., Rogawski, M.A., 1992. Hydrophobic interactions of *n*-alkyl diamines with the *N*-methyl-D-aspartate receptor: voltage dependent and -independent blocking sites. Mol. Pharmacol. 45, 117–124.

Weber, E., Sonders, M., Quarum, M., McLean, S., Pou, S., Keana, J.F.W., 1986. 1,3,-Di(2-[5-^{3}H]tolyl)guanidine: a selective ligand that labels σ-type receptors for psychotomimetic opiates and antipsychotic drugs. Proc. Natl. Acad. Sci. USA 83, 8784–8788.

Whittemore, E.R., Ilyin, V.I., Konkoy, C.S., Woodward, R.M., 1997a. Subtype-selective antagonism of NMDA receptors by nylidrin. Eur. J. Pharmacol. 337, 197–208.

Whittemore, E.R., Ilyin, V.I., Woodward, R.M., 1997b. Antagonism of *N*-methyl-D-aspartate receptors by σ site ligands: potency, subtype-selectivity and mechanisms of inhibition. J. Pharmacol. Exp. Ther. 282, 326–338.

Williams, K., 1997. Interactions of polyamines with ion channels. Biochem. J. 325, 289–297.

Williams, K., Romano, C., Dichter, M.A., Molinoff, P.B., 1991. Modulation of the NMDA receptor by polyamines. Life Sci. 48, 469–498.

Yamamoto, H., Yamamoto, T., Sagi, N., Klenerova, V., Goji, K., Kawai, N., Baba, A., Takamori, E., Moroji, T., 1995. Sigma ligands indirectly modulate the NMDA receptor-ion channel complex on intact neuronal cells via σ1 site. J. Neurosci. 15, 731–736.