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Modulation of [^{3}H]MK-801 binding to NMDA receptors in vivo and in vitro

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Abstract

[^{3}H]MK-801 binding in vivo was used to determine the occupancy of NMDA receptor ligands shown to allosterically modulate binding in vitro. ED_{50} values (mg/kg) were obtained for the channel blockers (+)-5-methyl-10,11-dihydro-5,4-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate ((+)-MK-801, 0.2), 1-(1-phenylcyclohexyl)piperidine (phencyclidine, PCP, 1.7) and ketamine (4.4). Antagonists at the glutamate (DL-(2-carboxypiperazine-4-yl)propyl-1-phosphonate (DL-CPP, 5.7)) and glycine site (7-Chloro-4-hydroxy-3-(3-phenoxy)-phenyl-2(*H*)quinolinone (L-701,324, 14.1), 3*R*(+)*cis*-4-methyl-pyrrollid-2-one (L-687,414, 15.1)) inhibited [^{3}H]MK-801 binding in vivo to varying maximum levels (69%, 103% and 45%, respectively). NR2B subunit-selective compounds acting at the ifenprodil site inhibited [^{3}H]MK-801 in vivo by a maximum of 52–72% and gave ED_{50} values (mg/kg) of: (±)-(1*S**,2*S**)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol ((±)CP-101,606), 1.9; (±)-(3*R*,4*S*)-3-[4-(4-fluorophenyl)-4-hydroxypiperidin-1-yl]chroman-4,7-diol ((±)CP-283,097), 1.8; (±)-(*R**,*S**)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidine propanol ((±)Ro 25-6981), 1.0; ifenprodil, 6.0. The glycine site agonist D-serine stimulated binding to 151% of control with an ED_{50} of 1.7 mg/kg. Results show that [^{3}H]MK-801 binding in vivo may be used to measure receptor occupancy of ligands acting not only within the ion channel but also at modulatory sites on the NMDA receptor complex.

Keywords: NMDA receptor; Receptor occupancy, in vivo; NR2B subunit; MK-801; Radioligand binding

1. Introduction

The NMDA receptor is one of the three families of ionotropic glutamate receptors and is probably the most extensively characterised due to the availability of a wide variety of ligands acting at the receptor. The receptor complex is comprised of several modulatory domains including those for glutamate, glycine, phencyclidine (PCP), polyamines, zinc and protons (see Ozawa et al., 1998 for review). Interest in the NMDA receptor has also increased in recent years with the cloning of cDNAs encoding various NMDA receptor subunits (Ikeda et al., 1992; Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993). The receptor channel complex is comprised of two families of subunit; the NR1 subunit, of which there are eight splice variants (a–h) and the NR2 subunit, which has four subtypes (A–D), each encoded by distinct genes. The NR1 subunit is widely distributed throughout the brain. The four NR2 subunits have a more discrete distribution and one or more of these combine with NR1 subunits to form functional receptors, with different heteromeric subunit combinations having different pharmacological properties (Laurie and Seeburg, 1994; Buller et al., 1994; Priestley et al., 1995; Grimwood et al., 1996a). The NR2A subunit is fairly widely distributed in the central nervous system (CNS) with the NR2B subunit being found primar-

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ily in forebrain structures. NR2C is confined mainly to the cerebellum and the NR2D subunit is found in diencephalon, mesencephalon and brain stem (Wenzel et al., 1995). These discoveries, in conjunction with a proposed role for NMDA receptors in various neuropsychiatric disorders including cerebral ischaemia (Albers, 1990), schizophrenia (Heresco-Levy and Javitt, 1998), pain (Wiesenfeld-Hallin, 1998) and Parkinson's disease (Blandini et al., 1996), have led to the search for subtype-selective compounds with the view that selectively modifying a subpopulation of NMDA receptors will give a more specific approach to the treatment of these disorders. Several subtype-selective compounds have been identified including ifenprodil and the ifenprodil-like compounds eliprodil, CP-101,606, CP-283,097 and Ro 25-6981 (Carter et al., 1989; Chenard et al., 1995; Butler et al., 1998; Fischer et al., 1997). These all bind to the same recognition site (the ifenprodil site) and are selective for NR2B subunit-containing receptors.

The NMDA receptor has been extensively characterised in vitro using a variety of ligands acting at various modulatory sites on the receptor complex. Since [^{3}H]MK-801 binds to the activated state of the NMDA receptor complex, it has been used as a functional assay in vitro to investigate modulation by ion channel blockers (Wong et al., 1988), glutamate and glycine recognition site ligands (Wong et al., 1987; Foster and Wong, 1987; Foster et al., 1992) and polyamines (Williams et al., 1989). The NMDA receptor is unique in that it requires both glutamate and glycine (described as a co-agonist) to bind to their recognition sites for channel opening to occur. [^{3}H]MK-801 binding assay protocols are generally biased towards the investigation of a particular modulatory site, for example, using low levels of glycine for the glycine site (Foster et al., 1992) or high concentrations of glutamate and glycine for the polyamine site (Williams et al., 1989). In this study, we have utilised a [^{3}H]MK-801 binding protocol that demonstrates modulation through any of the receptor complex ligand binding sites, including the less well-characterised ifenprodil site.

While providing extensive information regarding intrinsic activities and affinities of ligands, [^{3}H]MK-801 binding in vitro gives no indication as to the in vivo occupation of the NMDA receptor by ligands as no account is taken of the pharmacodynamic properties of the drug. Price et al. (1988) developed an in vivo [^{3}H]MK-801 binding assay to measure occupancy of compounds acting at the ion channel site of the NMDA receptor, including MK-801 and thienylcyclohexylpiperidine (TCP). This assay provides an index of receptor occupancy given that a 50% reduction in receptor binding corresponds to 50% of receptors being occupied. We have used this same methodology to investigate whether the binding of [^{3}H]MK-801 to the ion channel can be modulated by ligands acting at the other sites on the receptor complex. A preliminary report of some of this work has been published (Murray et al., 1998).

2. Materials and methods

2.1. Non-equilibrium [^{3}H]MK-801 binding in vitro

[^{3}H]MK-801 (22.3 Ci/mmol; NEN) binding to well-washed P_2 membranes prepared from rat cortex/hippocampus was performed using a modified protocol to that described by Foster et al. (1992). Concentration–response curves of drugs were obtained using a radioligand concentration of 2 nM with a 60-min incubation at 22°C in the presence of 100 nM L-glutamate.

2.2. [^{3}H]MK-801 binding in vivo

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines.

The method used was essentially as described by Price et al. (1988). Male BKTO mice (20–25 g; Bantin and Kingman, UK) were housed four per cage on a 12-h light/dark cycle (lights on at 0700 h) with food and water available ad libitum. Animals were injected with either vehicle or test compound followed 15 min later by an injection of [^{3}H]MK-801 (200 μCi/kg intravenously (i.v.)) administered via the tail vein. (+)-5-Methyl-10,11-dihydro-5,4-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate ((+)-MK-801), 1-(1-phenylcyclohexyl)piperidine HCl (PCP), ketamine, DL-(2-carboxypiperazine-4-yl)propyl-1-phosphonate (DL-CPP) and D-serine were dissolved in 0.9% NaCl and injected intraperitoneally (i.p.) in a volume of 10 ml/kg. 7-Chloro-4-hydroxy-3-(3-phenoxy)-phenyl-2(*H*)quinolinone (L-701,324), 3*R*(+)*cis*-4-methyl-pyrrolid-2-one (L-687,414), (±)-(1*S**,2*S**)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol ((±)CP-101,606), (±)-(3*R*,4*S*)-3-[4-(4-fluorophenyl)-4-hydroxypiperidin-1-yl]chroman-4,7-diol ((±)CP-283,097), (±)-(*R**,*S**)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidine propanol ((±)Ro 25-6981) and ifenprodil were dissolved in 25% PEG300. L-701,324 and L-687,414 were administered i.v., (±)CP-283,097 was administered i.p. and (±)CP-101,606 and (±)Ro 25-6981 were administered subcutaneously (s.c.) in a volume of 10 ml/kg. Animals were humanely killed 10 min after injection of the tracer and the forebrain rapidly removed, weighed and homogenised in 40 volumes of 5 mM Tris–acetate buffer, pH 7 at 4°C using a polytron homogeniser at setting 8 for 5 s. Each drug was tested at five or six doses with two to four mice being used at each dose. A 500-μl aliquot was immediately filtered through Whatman GF/B filters presoaked in ice-cold buffer using a manifold filtration system followed by two 5-ml washes of ice-cold buffer. Filter and homogenate samples were left to soak overnight in 10 ml scintillation fluid (Hydrofluor, National Diagnostics) before radioactivity was determined using liquid scintillation counting. Total binding was determined

by measuring radioactivity in brains of vehicle treated animals while non-specific binding was determined using animals treated with MK-801 (3 mg/kg, s.c.) with the difference between the two defining the amount of specific binding.

2.3. Data analyses

2.3.1. In vitro studies

Using Grafit, inhibition and stimulation curves were analysed for a one-site model using the equation $\%E = \%E_{max}/[1 + (EC_{50}/[L])^{n_H}]$, where E is the effect (enhancement or inhibition), EC_{50} is the concentration of ligand giving 50% inhibition or enhancement of specific binding, [L] is the ligand (modulator) concentration, and n_H is the Hill coefficient.

2.3.2. In vivo studies

Occupancy was calculated from the following equations:

% accumulation = (filter d.p.m./homogenate d.p.m.) × 100.

% specific accumulation = mean % accumulation (total) − mean % accumulation (non-specific).

% inhibition = (mean % accumulation (total) − % accumulation (sample))/(% specific accumulation) × 100.

where % accumulation is % accumulation of label, % specific accumulation is percentage specific accumulation, % inhibition is the percentage inhibition of binding and mean % accumulation (total) and (non-specific) are the arithmetic means of the percentage accumulation in the vehicle and MK-801 treated groups, respectively.

2.4. Materials

(+)-MK-801 and L-701,324 were obtained from Tocris Cookson, PCP, ifenprodil and DL-CPP from RBI and D-serine and ketamine from Sigma. L-687,414, (±)-CP-101,606, (±)CP-283,097, (±)Ro 25-6981 were synthesised by the Medicinal Chemistry Department, Neuroscience Research Centre, Merck Sharp and Dohme Laboratories, UK.

3. Results

3.1. Non-equilibrium [^{3}H]MK-801 binding in vitro to measure receptor affinity

[^{3}H]MK-801 binding to rat brain membranes in vitro was performed under non-equilibrium conditions. Through careful control of the levels of glutamate and glycine within the assay, the binding conditions chosen enabled

Table 1
Modulation of [^{3}H]MK-801 binding in vitro and in vivo by NMDA receptor ligands
Data shown are the arithmetic means ± s.e.m. or geometric mean (−s.e.m., +s.e.m.) of three experiments. Routes of administration of drugs are indicated in parentheses after compound names. Maximum change is the maximum percent change in specific binding. Arrows indicate either inhibition (↓) or stimulation (↑) of [^{3}H]MK-801 binding. EC_{50} = concentration (nM) giving 50% of the maximum change ($n = 3$). ED_{50} = dose (mg/kg) giving 50% of the maximal change from three separate experiments, with two to four mice per dose.

	In vitro binding studies		In vivo binding studies	
	Maximum change (%)	EC_{50} (nM)	Maximum change (%)	ED_{50} (mg/kg)
Ion channel site				
MK-801(i.p.)	↓ 104 ± 8	8.59 (5.88, 12.6)	↓ 100 ± 1	0.18 (0.15, 0.21)
PCP (i.p.)	↓ 97 ± 7	97.4 (62.1, 153)	↓ 102 ± 2	1.74 (1.28, 2.38)
Ketamine (i.p.)	↓ 93 ± 2	759 (413, 1400)	↓ 99 ± 1	4.39 (3.88, 4.97)
Glycine site				
L-701,324 (i.v.)	↓ 66 ± 5	4.08 (3.86, 4.31)	↓ 103 ± 3	14.1 (12.8, 15.6)
L-687,414 (i.v.)	↓ 60 ± 11	1730 (1230, 2430)	↓ 45 ± 5	15.1 (10.4, 22.0)
D-Serine (i.p.)	↑ 188 ± 23	99.9 (73.2, 136)	↑ 51 ± 10	1.73 (0.73, 4.10)
Glutamate site				
DL-CPP (i.p.)	↓ 65 ± 5	2170 (1790, 2630)	↓ 69 ± 7	5.71 (3.69, 8.84)
Ifenprodil site				
Ifenprodil (s.c.)	↓ 62 ± 9	14.2 (7.28, 27.8)	↓ 52 ± 3	6.02 (4.54, 7.99)
(±)Ro 25-6981 (s.c.)	↓ 58 ± 3	3.41 (2.93, 3.96)	↓ 72 ± 2	0.98 (0.58, 1.64)
(±)CP-101,606 (s.c.)	↓ 52 ± 8	6.12 (4.54, 8.24)	↓ 57 ± 9	1.91 (1.38, 2.64)
(±)CP-283,097 (i.p.)	↓ 45 ± 5	7.01 (5.56, 8.86)	↓ 58 ± 12	1.83 (1.23, 2.72)

investigation of the effects of NMDA receptor ligands acting through a number of distinct modulatory sites on the NMDA receptor complex. NMDA receptor antagonists and the partial agonist, L-687,414, inhibited [^{3}H]MK-801 binding, while the glycine site agonist, D-serine, stimulated [^{3}H]MK-801 binding (Table 1; Fig. 1a). Complete inhibition of [^{3}H]MK-801 binding was observed with the ion channel blockers, MK-801, PCP and ketamine, while inhibition by antagonists of the amino acid recognition sites and the ifenprodil site was partial (~60% inhibition; Table 1; Fig. 1). Affinity values obtained (Table 1) were similar to those previously reported for inhibition of radioligands specific for each modulatory site (see Discussion).

3.2. *In vivo [^{3}H]MK-801 binding to measure receptor occupancy*

[^{3}H]MK-801 binding in vivo gave approximately 1500 and 300 d.p.m. for total and non-specific filters, respectively, with homogenates containing around 8000 d.p.m.. These values usually resulted in a % specific accumulation of around 10–15%. [^{3}H]MK-801 binding in vitro provided affinity values for ligands acting in an allosteric manner, which were similar to those obtained for binding to their distinct sites, therefore, [^{3}H]MK-801 binding was performed in vivo to investigate whether occupancy of NMDA ligands could also be determined. Table 1 shows ED_{50} doses and maximum inhibition values for all NMDA receptor antagonists tested. MK-801, PCP and ketamine dose dependently displaced [^{3}H]MK-801 binding, completely inhibiting binding at the highest dose tested (Fig. 2a). The competitive glutamate site antagonist DL-CPP also dose-dependently displaced [^{3}H]MK-801 binding, but was unable to completely displace binding even at the highest dose tested (80% inhibition at 300 mg/kg i.p.; Fig. 2b). Similarly, results also showed that the NR2B subtype-selective compounds (±)CP-101,606, (±)CP-283,097, (±)Ro 25-6981 and ifenprodil, while dose dependently inhibiting binding, failed to completely displace [^{3}H]MK-801 and only achieved 60–70% inhibition at the highest dose (Fig. 2c). When given i.p. or s.c., glycine site antagonists showed very little inhibition of [^{3}H]MK-801 binding (data not shown) and so these compounds were administered i.v.

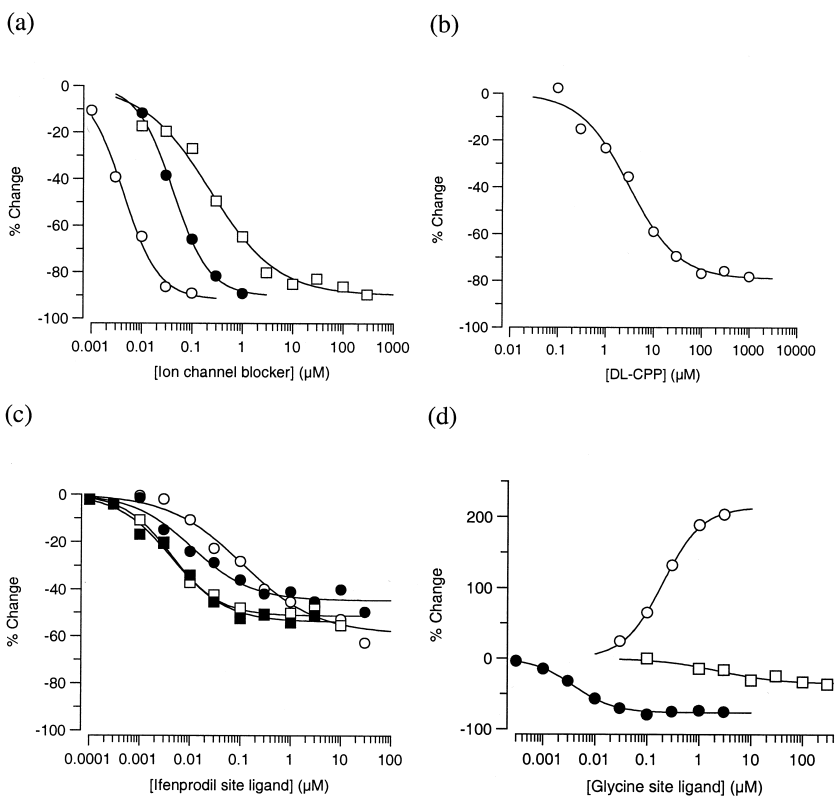

Fig. 1. Modulation of non-equilibrium [^{3}H]MK-801 binding to rat cortex/hippocampus membranes by NMDA receptor ligands. (a) Inhibition by the ion channel blockers (+)-MK-801 (○), PCP (●) and ketamine (□). (b) Inhibition by the glutamate site antagonist DL-CPP. (c) Inhibition by the NR2B subtype-selective ifenprodil site antagonists ifenprodil (○), (±)CP-101,606 (●), (±)CP-283,097 (■) and (±)Ro 25-6981 (□). (d) Inhibition by the glycine site full antagonist L-701,324 (●) and partial agonist L-687,414 (□) and stimulation by the agonist D-serine (○). Curves shown are from representative experiments with each data point representing the arithmetic mean of two determinations for which the standard error of the mean values never exceeded 5% of the respective mean.

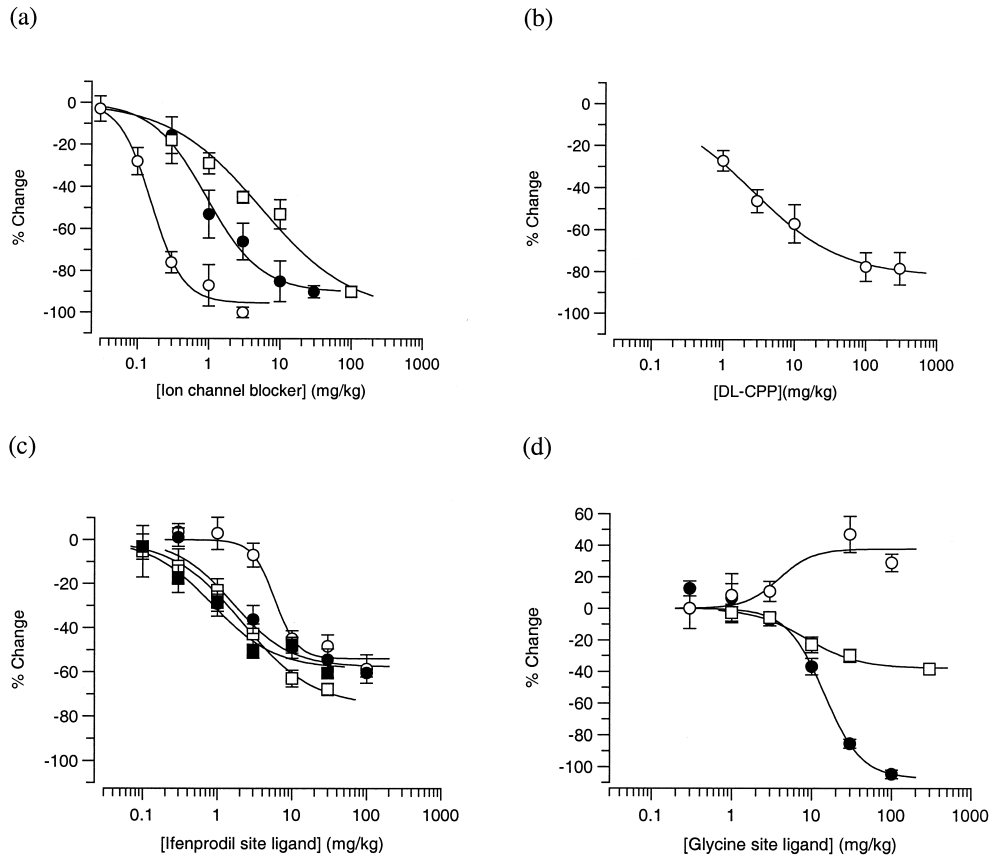

Fig. 2. Modulation of [^{3}H]MK-801 binding in vivo by NMDA receptor ligands. (a) Inhibition by the ion channel blockers MK-801 (○), PCP (●) and ketamine (□). (b) Inhibition by the glutamate site antagonist DL-CPP. (c) Inhibition by the NR2B subtype-selective ifenprodil site antagonists ifenprodil (○), (±)CP-101,606 (●), (±)CP-283,097 (■) and (±)Ro 25-6981 (□). (d) Inhibition by the glycine site full antagonist L-701,324 (●) and partial agonist L-687,414 (□) and stimulation by the agonist D-serine (○). Curves shown are representative of the data summarised in Table 1.

The full antagonist L-701,324 dose-dependently inhibited binding and fully displaced [^{3}H]MK-801 at the highest dose tested (Fig. 2d). The partial agonist L-687,414 also dose-dependently inhibited binding to a maximum inhibition of 45%, while the glycine site agonist D-serine stimulated binding in a dose-dependent manner to a maximum of 151% of control (Fig. 2d).

4. Discussion

Prior to addressing whether [^{3}H]MK-801 binding in vivo could be used to measure NMDA receptor ligand occupancy, we evaluated if modulation of [^{3}H]MK-801 binding in vitro could be detected by ligands acting at various sites on the receptor complex under a single assay protocol. [^{3}H]MK-801 binding in vitro was performed using well-washed brain membranes in the presence of 100 nM added glutamate. Under these non-equilibrium conditions, stimulation of binding was observed by a glycine site agonist and partial and full inhibition, respectively, were observed by a partial agonist and full antagonist at the glycine site. Affinity values obtained (Table 1) were similar to those previously reported using either the same, or similar, rat brain membrane preparations for inhibition of [^{3}H]MK-801 binding (under equilibrium conditions) for the ion channel blockers (Grimwood et al., 1996b), [^{3}H]L-689,560 binding by glycine site ligands (Grimwood et al., 1992, 1995), L-[^{3}H]glutamate binding by DL-CPP (Grimwood et al., 1991) and inhibition of NMDA receptor-specific [^{3}H]ifenprodil binding (Richards et al., 1998), [^{3}H]CP-101,606 binding (Butler et al., 1998) and [^{3}H]Ro 25-6981 binding (Mutel et al., 1998) by the ifenprodil site ligands. The percentage maximum change observed with these ligands (Table 1) was consistent with full non-subtype selective antagonism by the ion channel blockers, partial agonism by L-687,414 and an agonist action of D-serine. Incomplete inhibition by L-701,324 and DL-CPP was probably due to the inability of these compounds to completely overcome the endogenous levels of glycine and glutamate, respectively, present. Although this explanation is contradictory to L-701,324 completely inhibiting [^{3}H]MK-801 binding in vivo, where the concentration of

glycine present would predictably be higher. The partial inhibition observed with L-687,414 could also be due to the presence of endogenous glycine, although this compound is reported to be a partial agonist at the NMDA receptor glycine site (Priestley et al., 1998). The partial (45–62%) inhibition of [^{3}H]MK-801 binding by ifenprodil site ligands is similar to that observed in a previous [^{3}H]MK-801 binding study with NR2B subtype-selective ligands (Fischer et al., 1997) and is consistent with the ifenprodil site being located on NR2B subunit-containing receptors (Williams, 1993). Saturation analyses of [^{3}H]MK-801 and [^{3}H]ifenprodil binding to rat cortex/hippocampus membranes revealed B_{max} values of 4.75 and 2.45 pmol/mg protein, respectively (Grimwood et al., 1996b; Richards et al., 1998), consistent with NR2B subunit-containing receptors accounting for ~50% of this tissue preparation.

Data obtained using [^{3}H]MK-801 binding in vitro indicated that this radioligand could be used to investigate a number of modulatory sites on the receptor complex, therefore, [^{3}H]MK-801 binding in vivo was used to determine receptor occupancy. Although the % specific accumulation achieved with this methodology at 10–15% was relatively low compared with other in vivo occupancy assays, it was sufficient to allow investigation of drugs acting at the various modulatory sites on the NMDA receptor complex. The ED_{50} values obtained for the compounds acting at the ion channel site are consistent with those previously obtained using [^{3}H]TCP to label NMDA receptors in vivo (Maurice and Vignon, 1990) as well as those reported for inhibition of [^{3}H]MK-801 binding in vivo and NMDLA induced seizures (Price et al., 1988), substantiating this assay as a reliable estimate of in vivo occupancy for ion channel blockers of the NMDA receptor channel complex. This is not surprising given that this class of compound compete for the same binding site as the radioligand.

The dose of the competitive glutamate site antagonist DL-CPP occupying 50% of available receptors (5.7 mg/kg) corresponds to previously published ED_{50} values for inhibition of harmaline induced increase in cerebellar cGMP (Rao et al., 1991), an assay commonly used as a measure of in vivo receptor occupancy, indicating that occupancy of compounds acting at the glutamate recognition site can be measured by inhibition of in vivo [^{3}H]MK-801 binding, assuming that they are readily brain penetrant (a limiting factor with many compounds in this class). The maximum occupancy of 72% observed with DL-CPP may be due to the inability of this compound to overcome endogenous glutamate that is bound to this site in vivo, thus making complete displacement of the tracer impossible, a feature also seen with the in vitro studies described here.

The glycine site antagonist L-701,324 seemed to be considerably weaker (approximately fivefold) in this assay than would have been predicted from previously reported behavioural studies (Bristow et al., 1996). This class of compound had to be administered i.v., as when given i.p. or s.c. the inhibition of binding obtained was so low that curve fitting was not possible (data not shown). This may be due to the very high level of protein binding seen with this class of compound (Rowley et al., 1997) and may explain why the less protein bound glycine site partial agonist L-687,414 gave an ED_{50} that was closer to that previously reported using behavioural assays (Tricklebank et al., 1994; Boyce et al., 1999). The increase in [^{3}H]MK-801 binding observed with the glycine site agonist D-serine provides further evidence that the glycine site is not saturated in vivo (see Wood, 1995). The ifenprodil site compounds examined gave similar ED_{50} values to those obtained using a rat assay of neuropathic pain (Boyce et al., 1999). Ifenprodil, (±)CP-101,606 and (±)CP-283,097 maximally inhibited [^{3}H]MK-801 binding in vivo by 52–58%. This maximal effect was similar to that observed in the in vitro binding experiments and presumably reflects the proportion of NR2B subunit-containing receptors in mouse forebrain. It is unclear why (±)Ro 25-6981, the highest affinity NR2B subunit-selective compound tested, inhibited a higher proportion (72%) of [^{3}H]MK-801 binding.

While the efficacy of NMDA receptor antagonists can be determined using behavioural studies, these values are not necessarily a true indication of receptor occupancy, since only partial occupancy may be required to achieve a full behavioural effect. [^{3}H]MK-801 binding in vivo provides a more direct measurement of NMDA receptor occupation, and in this study, we have demonstrated that it is possible to obtain occupancy values for compounds acting at the various modulatory sites on the receptor complex, in addition to the ion channel binding site. Furthermore, we have shown that the rank order of potency of most compounds tested corresponds between the in vitro binding assay and the in vivo occupancy assay, suggesting a one-to-one relationship between occupancy and pharmacological action.

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