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Regionally Distinct *N*-Methyl-D-Aspartate Receptors Distinguished by Quantitative Autoradiography of [^{3}H]MK-801 Binding in Rat Brain

Sharin Y. Sakurai, *John B. Penney, and *Anne B. Young

*Department of Neurology, University of Michigan, Ann Arbor, Michigan; and *Department of Neurology, Massachusetts General Hospital, Boston, Massachusetts, U.S.A.*

Abstract: Quantitative autoradiography of [^{3}H]MK-801 binding was used to characterize regional differences in *N*-methyl-D-aspartate (NMDA) receptor pharmacology in rat CNS. Regionally distinct populations of NMDA receptors were distinguished on the basis of regulation of [^{3}H]MK-801 binding by the NMDA antagonist 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP). CPP inhibited [^{3}H]MK-801 binding in outer cortex (OC) and medial cortex (MC) with apparent K_i values of 0.32–0.48 μM, whereas in the medial striatum (MS), lateral striatum (LS), CA1, and dentate gyrus (DG) of hippocampus, apparent K_i values were 1.1–1.6 μM. In medial thalamus (MT) and lateral thalamus (LT) the apparent K_i values were 0.78 μM. In the presence of added glutamate (3 μM), the relative differences in apparent K_i values between regions maintained a similar relationship with the exception of the OC. Inhibition of [^{3}H]-MK-801 binding by the glycine site antagonist 7-chlorokynurenic acid (7-ClKyn) distinguished at least two populations of NMDA receptors that differed from populations defined by CPP displacement. 7-ClKyn inhibited [^{3}H]MK-801 binding in OC, MC, MS, and LS with apparent K_i values of 6.3–8.6 μM, whereas in CA1, DG, LT, and MT, K_i values were 11.4–13.6 μM. In the presence of added glycine (1 μM), the relative differences in apparent K_i values were maintained. Under conditions of differential receptor activation, regional differences in NMDA receptor pharmacology can be detected using [^{3}H]MK-801 binding. **Key Words:** 3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid—Glutamate—7-Chlorokynurenic acid—MK-801—Phencyclidine—*N*-Methyl-D-aspartate receptor. **Sakurai S. Y. et al.** Regionally distinct *N*-methyl-D-aspartate receptors distinguished by quantitative autoradiography of [^{3}H]-MK-801 binding in rat brain. *J. Neurochem.* **60,** 1344–1353 (1993).

Excitatory amino acids such as glutamate or aspartate are the predominant neurotransmitters mediating excitatory synaptic transmission at a majority of synapses in mammalian CNS. Based on electrophysiological and biochemical studies, excitatory amino acid receptors have been divided into the following scheme: *N*-methyl-D-aspartate (NMDA) receptors, (*R,S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, kainate receptors, and metabotropic receptors linked to phosphoinositide turnover (Dingledine et al., 1988; Monaghan et al., 1989; Young and Fagg, 1990).

The NMDA receptor is found throughout the mammalian CNS and is involved in both physiological and pathophysiological processes. Activation of the NMDA receptor is believed to play a role in the establishment of long-term potentiation, learning, memory, synaptic plasticity, and development (Collingridge and Bliss, 1987; Cotman and Iversen, 1987; Collingridge and Singer, 1990). Overactivation of NMDA receptors is thought to contribute to neuronal damage in epilepsy, hypoxia–ischemia, and neurodegenerative disorders (Maragos et al., 1987; Rothman and Olney, 1987; Choi, 1988; Greenamyre and Young, 1990).

The NMDA receptor is a ligand-gated ion channel that is gated by magnesium in a voltage-dependent manner (Nowak et al., 1984). Several different modu-

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Address correspondence and reprint requests to Dr. A. B. Young at Neurology Service, Massachusetts General Hospital, Fruit Street, Boston, MA 02114, U.S.A.

Abbreviations used: ANOVA, analysis of variance; 7-ClKyn, 7-chlorokynurenic acid; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; DG, dentate gyrus; LS, lateral striatum; LT, lateral thalamus; MC, medial cortex; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate; MS, medial striatum; MT, medial thalamus; NMDA, *N*-methyl-D-aspartate; OC, outer cortex; PCP, phencyclidine; PLSD, protected least significant difference.

latory sites on the NMDA receptor complex regulate channel activity. Glycine, acting at a strychnine-insensitive glycine binding site, enhances receptor activation and is necessary for maximal activity (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). The dissociative anesthetics ketamine, phencyclidine (PCP), and (+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801; dizocilpine maleate) are noncompetitive antagonists of the NMDA receptor that bind to the so-called PCP site within the ion channel and prevent the passage of Na^+ and Ca^{2+} through the channel (Anis et al., 1983; Honey et al., 1985; Reynolds et al., 1987; Wong et al., 1987). Finally, Zn^{2+} and polyamines, acting at separate sites, also modulate NMDA receptor activity (Peters et al., 1987; Westbrook and Mayer, 1987; Ransom and Stec, 1988; Reynolds and Miller, 1988*a*,*b*, 1989; Christine and Choi, 1990).

Although NMDA receptors have been classified as a homogeneous population of receptors, some studies indicate the existence of subtypes of the NMDA receptor (Perkins and Stone, 1983; Maragos et al., 1988; Monaghan et al., 1988; Honoré et al., 1989; McDonald et al., 1990). Electrophysiological and radioligand binding studies indicate that one population of NMDA receptors, localized in the hindbrain and cerebellum, is insensitive to quinolinate, whereas quinolinate effectively depolarizes and potently displaces binding to forebrain NMDA receptors (Perkins and Stone, 1983; Stone and Burton, 1988; Monaghan and Beaton, 1991). Receptor binding studies using ligands that label the PCP site have revealed low-affinity PCP binding sites in the cerebellum (Vignon et al., 1986; Ebert et al., 1991). Throughout the forebrain the ratio of glycine to NMDA and PCP binding sites varies between brain structures (McDonald et al., 1990). A striking example of variant stoichiometry among NMDA, PCP, and glycine binding sites is found in the cerebellar granule cell layer, where the density of NMDA and glycine binding sites is relatively high but that of PCP binding sites is low (Maragos et al., 1988; Young and Fagg, 1990). The presence of a unique NMDA receptor subtype in the cerebellum is supported by the recent discovery of an NMDA receptor RNA that is localized specifically in the cerebellum and not in the forebrain (Kutsuwada et al., 1992; Monyer et al., 1992).

Monaghan et al. (1988) have proposed the existence of agonist- and antagonist-preferring forms of the NMDA receptor based on binding studies using [^{3}H]glutamate and 3-(2-[^{3}H]carboxypiperazin-4-yl)propyl-1-phosphonic acid ([^{3}H]CPP), an NMDA antagonist. Comparing agonist and antagonist displacement of [^{3}H]glutamate versus [^{3}H]CPP in rat brain, these researchers reported that agonists are more potent inhibitors of [^{3}H]glutamate binding than of [^{3}H]CPP binding, whereas antagonists are more potent displacers of [^{3}H]CPP binding than of [^{3}H]-glutamate binding. Furthermore, the relative proportions of agonist- and antagonist-preferring sites varied across regions.

Because binding of NMDA-receptor channel ligands such as [^{3}H]MK-801 is dependent on channel activation, [^{3}H]MK-801 can be used not only as a label for NMDA receptors, but also as a marker for channel activation (Young and Fagg, 1990). To determine if regional differences in NMDA receptor pharmacology exist in rat CNS, we have investigated the effects of differential activation of the NMDA receptor on quantitative autoradiographic [^{3}H]MK-801 binding.

EXPERIMENTAL PROCEDURES

Tissue preparation

Male Sprague–Dawley rats (weighing 175–250 g; Charles River) were decapitated, and brains were removed rapidly, coated with Lipshaw embedding matrix, mounted on microtome chucks, and frozen under powdered dry ice. Horizontal sections 20 μm thick were cut on a Lipshaw cryostat and thaw-mounted onto gelatin-coated slides. Sections were stored at −20°C for <24 h.

Autoradiography

A detailed description of the method for quantitative autoradiography of [^{3}H]MK-801 binding has been published (Sakurai et al., 1991). In brief, tissue sections were prewashed in 50 m*M* Tris-acetate buffer (pH 7.4) for 30 min at 4°C and blown-dry under a stream of cool air before the [^{3}H]MK-801 binding assay was performed. In all experiments, quadruplicate tissue sections were incubated for 2 or 4 h in 50 m*M* Tris-acetate (pH 7.4) at room temperature containing 5 n*M* [^{3}H]MK-801 at a final volume of 10 ml. Nonspecific binding was determined in the presence of 5 μM unlabeled MK-801. For control conditions in CPP competition studies, tissue sections were incubated with CPP in the presence of 1 μM glycine. In separate experiments assessing CPP inhibition of [^{3}H]MK-801 binding under maximal stimulated conditions, glutamate (3 μM) was included in the incubation mixture containing CPP and 1 μM glycine. For control conditions in 7-chlorokynurenic acid (7-ClKyn) competition studies, tissue sections were incubated with 7-ClKyn in the presence of 3 μM glutamate. In separate experiments, glycine (1 μM) was present in the incubation mixture containing 7-ClKyn and glutamate (3 μM).

Following the incubation, sections were dipped quickly into 50 m*M* Tris-acetate buffer (pH 7.4) at 4°C, rinsed in 250 ml of cold buffer for 80 min, and blown-dry under a stream of warm air. Dried tissue sections were placed in x-ray cassettes with appropriate radioactive standards and apposed to Hyperfilm (Amersham). Following a 3–4-week exposure period at 4°C, films were developed in D-19 (Kodak), fixed, and dried. All data presented were analyzed from resultant autoradiograms using computer-assisted image analysis (Imaging Research, Inc., St. Catharines, Ontario, Canada). Optical densities from autoradiograms were converted to binding densities using a polynomial regression curve derived from the calibrated radioactive standards. Ten to 20 readings per brain area from each of the quadruplicate sections were analyzed. Specific binding was calculated as the difference between total binding and nonspecific binding. IC_{50} values for competitors of [^{3}H]MK-801

binding were calculated by log–logit analysis. Apparent K_i values for competitors of [^{3}H]MK-801 binding were determined by the method of Cheng and Prusoff (1973) using the formula $K_i = IC_{50}/[1 + ([L]/K_d)]$. Previously established K_d values for [^{3}H]MK-801 binding were used to determine apparent K_i values in this study (Sakurai et al., 1991). Because CPP and 7-ClKyn are known to act at sites distinct from the [^{3}H]MK-801 binding site, the apparent K_i values do not represent K_i values for the competitors at the glutamate recognition site or the glycine modulatory site but rather an estimate of the potency of these agents at interacting allosterically with the [^{3}H]MK-801 binding site. The apparent K_i values are mean ± SEM values from four animals. Statistical comparison of regional apparent K_i values was determined by one-way analysis of variance (ANOVA), followed by Fisher's PLSD test for post hoc comparisons using the Statview II program (Abacus Concepts, Berkeley, CA, U.S.A.). Hill coefficients were compared by unpaired two-tailed Student's t test.

Materials

[^{3}H]MK-801 was obtained from Dupont, NEN (Boston, MA, U.S.A.). Nonradioactive MK-801 was a gift from Dr. L. L. Iversen (Merck, Sharpe and Dohme Research Laboratories, Essex, U.K.). CPP was purchased from Cambridge Research Biochemicals (Wilmington, DE, U.S.A.). 7-ClKyn was purchased from Tocris Neuramin (Essex). All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

RESULTS

CPP inhibition of [^{3}H]MK-801 binding (2-h incubation)

Under control conditions (no added glutamate, 1 μM glycine), equilibrium was reached at 100 min and remained stable for up to 6 h. The addition of as much as 100 μM glutamate and 30 μM glycine did not increase binding above control conditions and did not accelerate association. Under control conditions, CPP inhibited [^{3}H]MK-801 binding in a regionally distinct manner (Table 1). In cortical layers I–III [outer cortex (OC)] and cortical layers IV–VI [medial cortex (MC)], [^{3}H]MK-801 binding was displaced with apparent K_i values of 0.48 ± 0.02 and 0.32 ± 0.02 μM (mean ± SEM), respectively, and Hill coefficient (n_H) values of 0.98 ± 0.02 and 1.1 ± 0.04, respectively. The apparent K_i value of OC was significantly different from the K_i value of MC under control conditions ($p < 0.02$ by unpaired two-tailed Student's t test). Regional variation in CPP inhibition of [^{3}H]MK-801 binding was observed in other brain areas. In medial striatum (MS), lateral striatum (LS), stratum radiatum of CA1 (CA1), and stratum moleculare of dentate gyrus (DG) of hippocampus, apparent K_i values ranged from 1.1 to 1.6 μM, and n_H values ranged from 0.91 to 1.0. [^{3}H]MK-801 binding was displaced in medial thalamus (MT) and lateral thalamus (LT) with an apparent K_i of 0.78 μM and an n_H of 1.0 ± 0.1 and 1.3 ± 0.12, respectively. The K_i values for MS, LS, CA1, DG, MT, and LT were significantly different from those of OC and MC ($p < 0.05$ by ANOVA and Fisher's PLSD).

In the presence of added glutamate (3 μM), the CPP displacement curves for [^{3}H]MK-801 binding shifted rightward (Fig. 1). The apparent K_i values between regions maintained a similar relationship with the exception of OC, where the apparent K_i value increased 2.5 times more than that in the other areas (Table 1). In the presence of added glutamate (3 μM), CPP inhibition of [^{3}H]MK-801 binding in MC (6.5 ± 1.6 μM)

TABLE 1. *Regional variations in CPP inhibition of [^{3}H]MK-801 binding in rat brain*

	Control		Glutamate (3 μM)	
Region	K_i (μM)	n_H	K_i (μM)	n_H
OC	0.48 ± 0.02[a]	0.98 ± 0.02	23.9 ± 1.7[b]	1.9 ± 0.19[c]
MC	0.32 ± 0.02[a,d]	1.1 ± 0.04	6.51 ± 1.6[e,f]	1.3 ± 0.23[c]
MS	1.56 ± 0.11[g]	1.0 ± 0.05	34.0 ± 1.1	1.9 ± 0.05[c]
LS	1.25 ± 0.08[h]	1.0 ± 0.05	25.4 ± 1.4	1.9 ± 0.12[c]
CA1	1.42 ± 0.07[i]	0.91 ± 0.01	22.3 ± 2.0	1.8 ± 0.12[c]
DG	1.10 ± 0.02[i]	0.99 ± 0.07	22.1 ± 2.0	1.8 ± 0.12[c]
LT	0.78 ± 0.09	1.0 ± 0.10	14.5 ± 0.57[j]	1.9 ± 0.13[c]
MT	0.78 ± 0.07	1.3 ± 0.12	14.6 ± 0.53[j]	1.9 ± 0.13[c]

Apparent K_i values for each brain region were determined by quantitative autoradiography and are mean ± SEM values from four animals. Differences in K_i values between brain regions were significant at $p < 0.05$ by ANOVA and Fisher's PLSD as indicated.

[a] Significantly different from MS, LS, CA1, DG, MT, and LT.
[b] Significantly different from MC, LT, and MT.
[c] Significantly >1 ($p < 0.0001$ by Student's t test).
[d] Significantly different from OC, $p < 0.02$ by unpaired two-tailed Student's t test.
[e] Significantly different from OC, MS, LS, CA1, DG, LT, and MT.
[f] Significantly different from OC, $p < 0.05$ by unpaired two-tailed Student's t test.
[g] Significantly different from DG, LT, and MT.
[h] Significantly different from LT and MT.
[i] Significantly different from LT and MT.
[j] Significantly different from MS, LS, CA1, and DG.

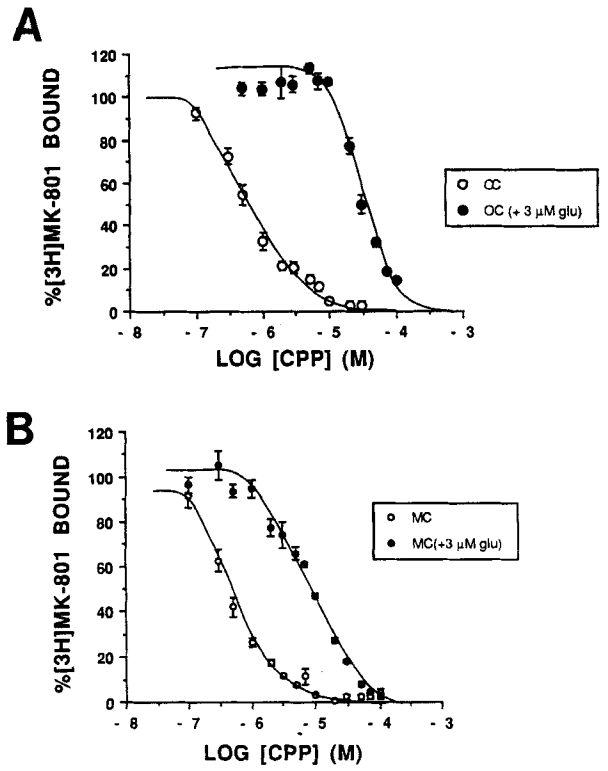

FIG. 1. Inhibition of [^{3}H]MK-801 binding by CPP (**A**) in outer cortical layers I-III (OC) under control and stimulated conditions and (**B**) in cortical layers IV–VI (MC) under control and stimulated conditions. In control CPP displacement experiments (○), sections were incubated in the presence of added glycine (1 μM). In CPP displacement studies under stimulated conditions (●), sections were incubated in the presence of added glycine (1 μM) and added glutamate (3 μM). Data are mean ± SEM (bars) values from four animals.

was significantly more potent than in OC (23.9 ± 1.1 μM; $p < 0.05$). The apparent K_i values for MS, LS, CA1, and DG ranged from 22 to 34 μM, whereas in MT and LT, CPP inhibited [^{3}H]MK-801 binding with an apparent K_i of 14.5 μM. With glutamate stimulation, the n_H values for CPP inhibition of [^{3}H]MK-801 ranged from 1.3 to 1.9 and were significantly >1 ($p < 0.0001$). Figure 2 shows representative autoradiograms depicting the anatomically distinct pattern of CPP displacement of [^{3}H]MK-801 binding.

CPP inhibition of [^{3}H]MK-801 binding (4-h incubation)

In addition to the standard 2-h incubation period, CPP inhibition of [^{3}H]MK-801 binding under control conditions (presence of 1 μM glycine) and under stimulated conditions (presence of 1 μM glycine and 3 μM glutamate) was examined using a 4-h incubation. CPP displacement curves for [^{3}H]MK-801 binding following a 4-h incubation were shifted (five- to eightfold) to the right of CPP displacement curves obtained from 2-h incubations (Fig. 3). Under control conditions, the apparent K_i values between regions maintained a similar relationship to apparent K_i values from 2-h incubations (Table 2). The apparent K_i values for OC (3.8 ± 0.3 μM) and MC (2.0 ± 0.4 μM) were significantly different from each other ($p < 0.04$ by unpaired two-tailed Student's t test). In MS, LS, CA1, and DG, the apparent K_i values ranged from 5.8 to 8.5 μM, whereas the apparent K_i values for LT and MT were 4.1 and 4.3 μM, respectively. The apparent K_i values for MS, LS, CA1, DG, LT, and MT differed significantly from those for OC and MC ($p < 0.05$ by ANOVA and Fisher's PLSD). The Hill coefficients for CPP displacement of [^{3}H]MK-801 ranged from 1.1 to 1.6.

Following a 4-h incubation in the presence of added glycine (1 μM) and glutamate (3 μM), CPP displacement curves for [^{3}H]MK-801 binding were also shifted (two- to threefold) to the right of CPP curves obtained from 2-h incubations. CPP inhibited [^{3}H]-MK-801 binding in OC with an apparent K_i of 63.2 ± 3.65 μM. In MC, CPP was a more potent displacer of [^{3}H]MK-801 binding, with an apparent K_i of 25.5 ± 0.85 μM.

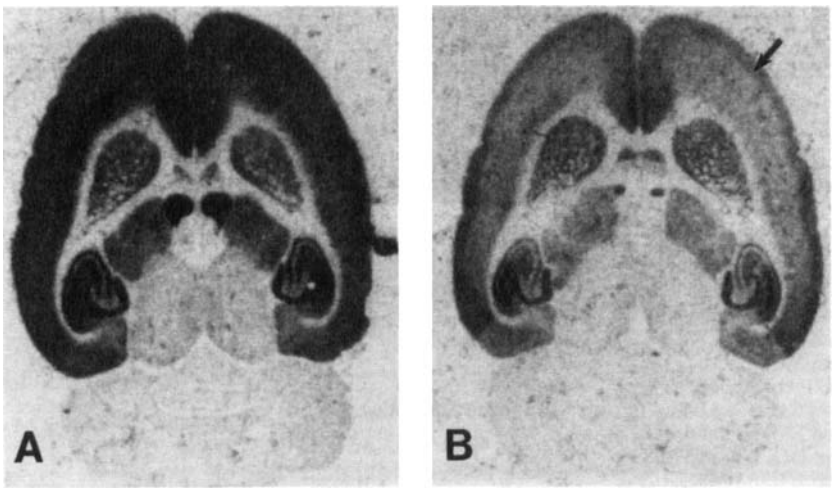

FIG. 2. Representative autoradiograms of [^{3}H]MK-801 binding in horizontal rat brain sections. **A:** Total [^{3}H]MK-801 binding. **B:** CPP (2 μM) inhibition of [^{3}H]MK-801 binding under stimulated conditions (in the presence of 1 μM glycine and 3 μM glutamate). The arrow indicates cortical layers I–III.

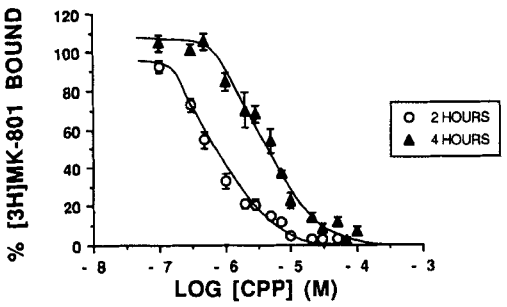

FIG. 3. Inhibition of [^{3}H]MK-801 binding by CPP following a 2- or 4-h incubation under control conditions (in the presence of 1 μM glycine) in cortical layers I–III (OC). Data are mean ± SEM (bars) values from four animals.

7-ClKyn inhibition of [^{3}H]MK-801 binding

Inhibition of [^{3}H]MK-801 binding by 7-ClKyn distinguished a regionally distinct pattern of binding. Under control conditions, 7-ClKyn inhibited [^{3}H]-MK-801 binding in OC, MC, MS, and LS with apparent K_i values of 6.3–8.6 μM, whereas in CA1, DG, LT, and MT, significantly different apparent K_i values ranged from 11 to 13 μM ($p < 0.05$ by ANOVA; Fig. 4 and Table 3). Hill coefficients ranged from 1.7 to 2.9 and were all significantly >1 ($p < 0.0001$).

In the presence of added glycine (1 μM), the apparent K_i values for 7-ClKyn inhibition of [^{3}H]MK-801 binding shifted (five- to sixfold) in all brain regions with the exception of OC, which shifted by ninefold (Table 3). 7-ClKyn inhibited [^{3}H]MK-801 binding in OC, CA1, and DG with apparent K_i values ranging from 70 to 75 μM. In MC, MS, and LS, the apparent K_i values of 30.0–48.0 μM differed significantly from OC, CA1, and DG ($p < 0.05$ by ANOVA and Fisher's PLSD), whereas in LT and MT the apparent K_i values were 60.2 ± 2.6 and 56.4 ± 1.6 μM, respectively. Hill coefficients ranging from 1.4 to 2.6, were significantly >1 ($p < 0.0001$). Figure 5 shows representative autoradiograms of 7-ClKyn inhibition of [^{3}H]MK-801 binding in rat brain.

DISCUSSION

To assess the distribution and modulation of the NMDA receptor channel, [^{3}H]MK-801 binding was used to label NMDA receptors in rat brain sections under conditions that differentially activated the receptor. In well-washed homogenate preparations, [^{3}H]MK-801 binding to the PCP site within the ion channel is highly regulated by glutamate and glycine, and the presence of both is necessary to measure binding (Bonhaus and McNamara, 1988; Kloog et al., 1988*a,b*; Javitt and Zukin, 1989*a,b*). NMDA and glycine antagonists, such as CPP and 7-ClKyn, respectively, inhibit [^{3}H]MK-801 binding (Foster and Wong, 1987; Kloog et al., 1988*a,b*; Ransom and Stec, 1988; Sircar et al., 1989; Sakurai et al., 1991). Following our standard prewash, enough endogenous glutamate and glycine are present in the tissue sections to activate NMDA receptors and allow [^{3}H]MK-801 to bind specifically (Sakurai et al., 1991). In this study, CPP inhibition of [^{3}H]MK-801 binding was examined under control conditions, that is, in the presence of a known amount of glycine, and under maximally stimulated conditions, that is, in the presence of both added glutamate and glycine. Similarly, 7-ClKyn inhibition of [^{3}H]MK-801 binding was assessed under control conditions (presence of added glutamate) and under maximally stimulated conditions (presence of both added glutamate and added glycine).

TABLE 2. *Regional variations in CPP inhibition of [^{3}H]MK-801 binding in rat brain following a 4-h incubation*

	Control		Glutamate (3 μM)	
Region	K_i (μM)	n_H	K_i (μM)	n_H
OC	3.76 ± 0.27[a]	1.3 ± 0.05	63.3 ± 3.6[b]	2.2 ± 0.58[c]
MC	2.03 ± 0.45[a,d]	1.1 ± 0.06	25.5 ± 0.85	1.3 ± 0.16
MS	8.51 ± 1.1[e]	1.6 ± 0.11	—	—
LS	8.34 ± 1.1[f]	1.4 ± 0.15	—	—
CA1	7.42 ± 1.1[g]	1.3 ± 0.13	—	—
DG	5.87 ± 0.47[g]	1.1 ± 0.09	—	—
LT	4.17 ± 0.49	1.1 ± 0.14	—	—
MT	4.34 ± 0.59	1.1 ± 0.06	—	—

Apparent K_i values for each brain region were determined by quantitative autoradiography and are mean ± SEM values from four animals. Differences in K_i values between brain regions were significant at $p < 0.05$ by ANOVA and Fisher's PLSD as indicated.

[a] Significantly different from MS, LS, CA1, DG, MT, and LT.
[b] Significantly different from MC.
[c] Significantly >1 ($p < 0.0001$ by Student's t test).
[d] Significantly different from OC, $p < 0.04$ by unpaired two-tailed Student's t test.
[e] Significantly different from DG, LT, and MT.
[f] Significantly different from DG, LT, and MT.
[g] Significantly different from LT and MT.

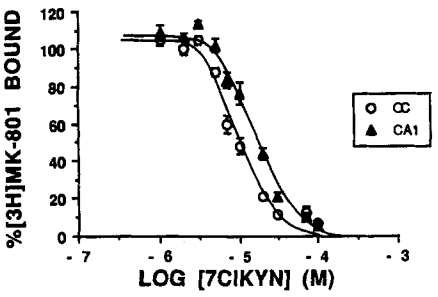

FIG. 4. Inhibition of [^{3}H]MK-801 binding by 7-ClKyn in outer cortical layers I–III (OC) or stratum radiatum of hippocampus (CA1) under control conditions (presence of 3 μM glutamate). Data are mean ± SEM (bars) values from four animals.

Regionally distinct populations of NMDA receptors were distinguished on the basis of inhibition of [^{3}H]MK-801 binding by CPP. Under control conditions the apparent K_i values for CPP inhibition of [^{3}H]-MK-801 binding varied significantly across brain regions. CPP inhibited [^{3}H]MK-801 binding most potently in OC and MC. In LT, MT, MS, LS, CA1, and DG, CPP inhibited [^{3}H]MK-801 binding with significantly different affinities than in OC and MC. In the presence of glutamate (3 μM), the potency of CPP to displace [^{3}H]MK-801 binding maintained a similar rank order of differences between brain regions. A notable difference, however, was the dramatic 50-fold rightward shift in the apparent K_i for CPP inhibition of [^{3}H]MK-801 binding in OC. In other regions, the affinities changed by 20-fold. Furthermore, in the presence of glutamate, the apparent K_i for CPP inhibition of [^{3}H]MK-801 binding in OC was significantly greater than inhibition in MC but was now equipotent to that in LS, CA1, and DG. Based on direct labeling of the agonist and antagonist sites by [^{3}H]-glutamate and [^{3}H]CPP, respectively, Monaghan et al. (1988) demonstrated that MS consisted of high levels of agonist-preferring sites, whereas LT and cortical layers contained higher levels of antagonist binding sites. The results of this study are consistent with these findings in that CPP inhibition of [^{3}H]MK-801 binding was most potent in LT/MT and in cortical layers and least potent in MS/LS.

Monaghan (1991) reported differential stimulation of [^{3}H]MK-801 binding to NMDA receptors by L-glutamate in cortical layers as well as different brain regions. [^{3}H]MK-801 binding was enhanced by low glutamate concentrations in the outer cortical layers (agonist-preferring NMDA receptors), whereas binding in the inner cortical layers was enhanced by high glutamate concentrations (antagonist-preferring NMDA receptors) (Monaghan, 1991). In the present study inhibition of [^{3}H]MK-801 binding in OC and MC displayed differential sensitivities to CPP under control conditions and in the presence of glutamate. Under control and maximally stimulated conditions, [^{3}H]-MK-801 binding was more potently inhibited by CPP in the MC. These findings would be consistent with a predominance of antagonist-preferring NMDA receptors in MC. Furthermore, Monaghan (1991) reported that MS was most sensitive to L-glutamate stimulation, suggesting that NMDA receptors in this area were agonist preferring. In the present study, [^{3}H]-MK-801 binding in MS displayed the lowest affinity for CPP, suggesting the presence of the agonist-preferring state of the NMDA receptor.

Dansyz et al. (1989) demonstrated that agonist- and antagonist-preferring sites on the NMDA receptor

TABLE 3. *Regional variations in 7-ClKyn inhibition of [^{3}H]MK-801 binding in rat brain*

	Control		Glycine (1 μM)	
Region	K_i (μM)	n_H	K_i (μM)	n_H
OC	7.86 ± 0.65[a]	1.9 ± 0.16[b]	70.1 ± 4.5[c]	2.0 ± 0.21[b]
MC	6.34 ± 0.36[a]	1.8 ± 0.01[b]	30.0 ± 1.9[d]	1.4 ± 0.13[b]
MS	7.2 ± 0.36[a]	2.2 ± 0.08[b]	48.0 ± 3.4[e]	2.1 ± 0.15[b]
LS	7.18 ± 0.36[a]	1.8 ± 0.13[b]	38.2 ± 2.6[f]	2.3 ± 0.21[b]
CA1	12.0 ± 0.94	2.2 ± 0.26[b]	72.9 ± 0.85[g]	2.1 ± 0.02[b]
DG	12.53 ± 0.81	1.7 ± 0.14[b]	75.0 ± 3.1[a]	2.2 ± 0.10[b]
LT	13.22 ± 1.3	2.6 ± 0.36[b]	60.2 ± 2.6	2.6 ± 0.15[b]
MT	11.42 ± 0.87	2.9 ± 0.29[b]	56.4 ± 1.6	2.4 ± 0.03[b]

Apparent K_i values for each brain region were determined by quantitative autoradiography and are mean ± SEM values from four animals. Differences in K_i values between brain regions were significant at $p < 0.05$ by ANOVA and Fisher's PLSD as indicated.

[a] Significantly different from CA1, DG, MT, and LT.
[b] Significantly >1 ($p < 0.0001$ by Student's t test).
[c] Significantly different from MC, MS, and LS.
[d] Significantly different from MS, LS, CA1, DG, LT, and MT.
[e] Significantly different from LS, CA1, DG, LT, and MT.
[f] Significantly different from CA1, DG, LT, and MT.
[g] Significantly different from MT and LT.

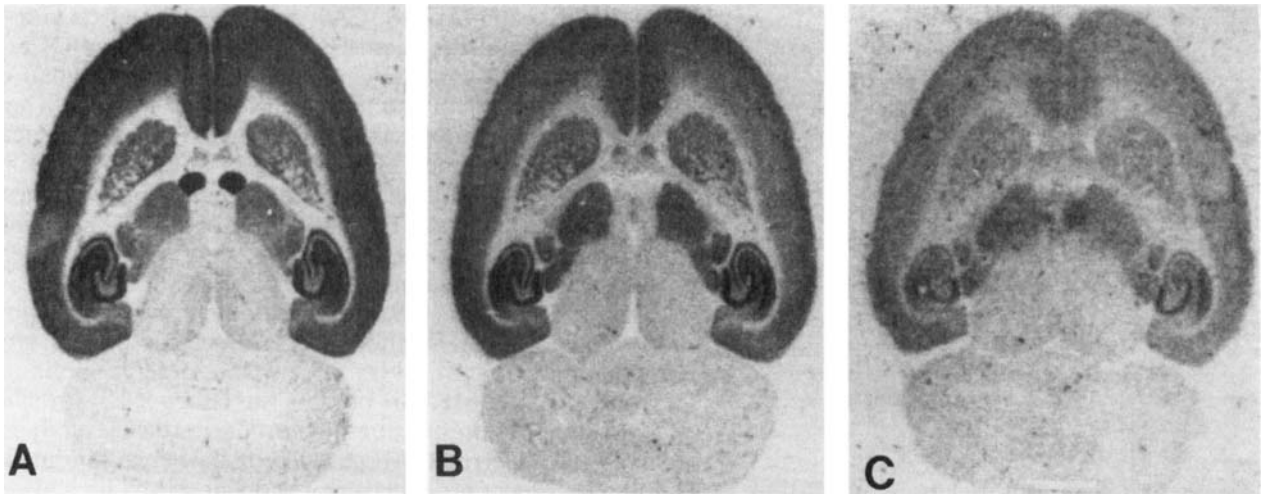

FIG. 5. Representative autoradiograms of [^{3}H]MK-801 binding in horizontal rat brain sections. **A:** Total [^{3}H]MK-801 binding. **B:** 7-ClKyn (10 μM) inhibition of [^{3}H]MK-801 binding under stimulated conditions (in the presence of 3 μM glutamate and 1 μM glycine). **C:** 7-ClKyn (20 μM) inhibition of [^{3}H]MK-801 binding under stimulated conditions (in the presence of 3 μM glutamate and 1 μM glycine).

complex were independently regulated by glycine, 7-ClKyn, and HA-966. Furthermore, in homogenate studies, O'Shea et al. (1991) described regional heterogeneity of the glycine binding site and three different mechanisms by which the glycine domain on the NMDA receptor may differentially modulate channel activity. The present study demonstrates that NMDA receptor populations can also be distinguished by regulation at the strychnine-insensitive glycine binding site in an autoradiographic assay. 7-ClKyn inhibition of [^{3}H]MK-801 binding distinguished regional differences in NMDA receptor pharmacology that differed from the regional differences defined by CPP displacements. Under control conditions (no added glycine, 3 μM glutamate), [^{3}H]MK-801 binding in OC, MC, MS, and LS was inhibited by 7-ClKyn with similar potencies, whereas 7-ClKyn had a lower affinity for inhibiting [^{3}H]MK-801 binding in LT, MT, DG, and CA1. In the presence of added glycine, the apparent K_i values for the regions shifted rightward, and additional differences between regions became apparent. The potency of 7-ClKyn to inhibit [^{3}H]MK-801 binding in the MC was significantly greater than in OC in the presence of glycine, whereas in control conditions the affinities were similar. Unlike the dramatic increases in apparent K_i values obtained for CPP inhibition of [^{3}H]MK-801 binding in the presence of added glutamate, the increases in the apparent K_i values for 7-ClKyn inhibition in the presence of glycine were only five- to ninefold across brain areas. The present study demonstrates regionally distinct regulation of [^{3}H]MK-801 binding by the NMDA agonist site and the strychnine-insensitive glycine binding site.

The presence of glutamate and glycine enhances NMDA receptor activity and increases the association rate of [^{3}H]MK-801, but CPP slows the rate of association (Kloog et al., 1988*a,b*). We have demonstrated previously that in our autoradiographic assay [^{3}H]-MK-801 binding is at equilibrium by 2 h (Sakurai et al., 1991). In the displacement assays performed in this study, however, at high concentrations of CPP the association rate of [^{3}H]MK-801 binding may have been slowed enough such that binding was at nonequilibrium conditions. Furthermore, regional differences in endogenous glutamate concentrations could also cause apparent regional differences in K_i values for CPP inhibition of [^{3}H]MK-801 binding. To address these issues, CPP inhibition of [^{3}H]MK-801 binding was examined following a 4-h incubation period under control conditions (presence of 1 μM glycine) and under stimulated conditions (presence of 1 μM glycine and 3 μM glutamate).

Following a 4-h incubation period, under control conditions (presence of 1 μM glycine) the apparent K_i values increased in all brain regions examined. The differences between apparent K_i values for the various regions following a 4-h incubation, however, maintained a similar relationship in comparison to the apparent K_i values obtained after a 2-h incubation. As the rank order of differences in apparent K_i values between regions was maintained at the longer incubation period, this suggests that the differences in apparent K_i values between regions were not due to the presence of varying amounts of endogenous glutamate in the tissue. Because the apparent K_i values for CPP inhibition of [^{3}H]MK-801 binding did increase following a 4-h incubation, it appears that in the presence of CPP [^{3}H]MK-801 binding may be at nonequilibrium conditions. As the rank order of regional differences in apparent K_i values was maintained at the longer incubation period, the regional differences in apparent K_i values are probably due to differential activation of the NMDA receptor–ion channel complex between regions. Even if binding was not at equi-

librium, the results of this study suggest that [^{3}H]MK-801 binding can be used to demonstrate regional heterogeneity of binding under conditions of differential activation. Nonequilibrium binding of another PCP ligand, *N*-(1-[2-thienyl]cyclohexyl)-3,4-[^{3}H]piperidine, ([^{3}H]TCP) has also been shown to be a reliable marker of the activated state of the NMDA receptor complex (Hosford et al., 1990).

Glutamate and glycine antagonists may slow the association rate of [^{3}H]MK-801 binding and thereby artificially result in high Hill coefficients (Sakurai et al., 1991). In the present study under control conditions, the apparent Hill coefficients were not significantly different than 1 for CPP inhibition of [^{3}H]MK-801 binding. The presence of glycine (1 μM) in the control condition for CPP inhibition of [^{3}H]MK-801 binding may have been sufficient enough to allow equilibrium to be reached even at the highest CPP concentrations. In the presence of glutamate, however, the Hill coefficients for CPP inhibition of [^{3}H]-MK-801 binding were significantly >1. Because higher concentrations of CPP were required to inhibit [^{3}H]MK-801 binding in the presence of glutamate, association rates at the higher concentrations of CPP may have been slowed, thereby artificially raising the Hill coefficients. Even after 4-h incubations, however, the Hill coefficients in the presence of glutamate were >1. The Hill coefficients for 7-ClKyn inhibition of [^{3}H]MK-801 binding were significantly >1 under both control and stimulated conditions. These high Hill coefficients may also reflect the effect of antagonists on the association rate for [^{3}H]MK-801 binding. Alternatively, the presence of added glutamate and glycine may reveal more than one population of binding sites, which would also be reflected in Hill coefficients of >1. The allosteric interaction between sites also may be altered under conditions that differentially activate the NMDA receptor complex, thereby resulting in higher Hill coefficients.

On the basis of our study, it appears that regional heterogeneity of NMDA receptor pharmacology can be demonstrated by NMDA agonist site and strychnine-insensitive glycine binding site regulation of [^{3}H]MK-801 binding in rat forebrain. One population of NMDA receptors, distributed in the MC, exhibited a higher affinity for CPP than in the outer cortical layers. Another population of NMDA receptor subtypes in MT and LT had a slightly lower affinity for CPP, whereas NMDA receptors distributed in the MS and LS as well as in the DG and CA1 region of the hippocampus exhibited the lowest affinity for CPP. Regulation of the NMDA receptor complex at the strychnine-insensitive glycine binding site revealed the presence of at least two populations of NMDA receptors. One population of receptors, distributed in the OC and MC and the LS and MS, had a higher affinity for the glycine antagonist 7-ClKyn than NMDA receptors distributed in the MT and LT and the DG and CA1 region of the hippocampus.

The results of our study suggest that the regional heterogeneity of [^{3}H]MK-801 binding in rat forebrain under certain conditions may be due to the presence of at least two or more subtypes of the NMDA receptors that are differentially regulated by the glutamate and glycine binding sites. Alternatively, two or three receptor subtypes may be expressed in different proportions within a region, resulting in heterogeneity of [^{3}H]MK-801 binding under any given conditions. Our study focused on the forebrain because under our standard assay conditions, [^{3}H]MK-801 binding is not present in the cerebellum (Sakurai et al., 1991). Electrophysiological and binding studies suggest, however, that a pharmacologically distinct NMDA receptor is present in the cerebellum (Perkins and Stone, 1983; Stone and Burton, 1988; Monaghan and Beaton, 1991).

The heterogeneity of [^{3}H]MK-801 binding under conditions that differentially activate the NMDA receptor in the forebrain may be due to the regionally specific expression of different NMDA receptor proteins. Now that the NMDA receptor has been cloned (Kumar et al., 1991; Moriyoshi et al., 1991), characterization of the heterogeneity of the NMDA receptor at the molecular level will be possible. In fact, Nakanishi and co-workers have reported discrepancies between the distribution of NMDA receptor ligand binding sites and NMDA receptor mRNA expression sites, which may reflect the presence of multiple NMDA receptor subtypes (Moriyoshi et al., 1991). More recently, three additional NMDA receptor subunits have been cloned (Meguro et al., 1992; Monyer et al., 1992). These subunits are expressed selectively throughout the brain, and different combinations of these subunits exhibit functional diversity when characterized electrophysiologically (Meguro et al., 1992; Monyer et al., 1992). The molecular diversity of the NMDA receptor subunits and the posttranslational processing of receptor proteins are likely to contribute to the pharmacological heterogeneity of the NMDA receptor complex.

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