Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 7104–7108, September 1986
Neurobiology

The anticonvulsant MK-801 is a potent *N*-methyl-D-aspartate antagonist

(excitatory amino acid receptors/neurodegenerative diseases/phencyclidine/σ opioid)

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Communicated by Maurice R. Hilleman, June 2, 1986

ABSTRACT **The compound MK-801 {(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate)} is a potent anticonvulsant that is active after oral administration and whose mechanism of action is unknown. We have detected high-affinity (K_d = 37.2 ± 2.7 nM) binding sites for [^{3}H]MK-801 in rat brain membranes. These sites are heat-labile, stereoselective, and regionally specific, with the hippocampus showing the highest density of sites, followed by cerebral cortex, corpus striatum, and medulla–pons. There was no detectable binding in the cerebellum. MK-801 binding sites exhibited a novel pharmacological profile, since none of the major neurotransmitter candidates were active at these sites. The only compounds that were able to compete for [^{3}H]MK-801 binding sites were substances known to block the responses of excitatory amino acids mediated by the *N*-methyl-D-aspartate (*N*-Me-D-Asp) receptor subtype. These comprised the dissociative anesthetics phencyclidine and ketamine and the σ-type opioid *N*-allylnormetazocine (SKF 10,047). Neurophysiological studies *in vitro*, using a rat cortical-slice preparation, demonstrated a potent, selective, and noncompetitive antagonistic action of MK-801 on depolarizing responses to *N*-Me-D-Asp but not to kainate or quisqualate. The potencies of phencyclidine, ketamine, SKF 10,047, and the enantiomers of MK-801 as *N*-Me-D-Asp antagonists correlated closely (r = 0.99) with their potencies as inhibitors of [^{3}H]MK-801 binding. This suggests that the MK-801 binding sites are associated with *N*-Me-D-Asp receptors and provides an explanation for the mechanism of action of MK-801 as an anticonvulsant.**

The excitatory amino acids L-glutamate and L-aspartate are thought to act as the principal excitatory neurotransmitters in mammalian brain. The receptors mediating their actions are generally divided into the major subtypes *N*-methyl-D-aspartate (*N*-Me-D-Asp, sometimes referred to as "NMDA"), quisqualate, and kainate, based on their activation by these selective agonists (1–5). There is considerable interest in the development of pharmacological agents that might act to block these receptors. Selective competitive antagonists of *N*-Me-D-Asp receptors have been developed, such as D-2-amino-5-phosphonovaleric acid and D-2-amino-7-phosphonoheptanoic acid (4, 5), but these zwitterionic compounds do not penetrate readily into the central nervous system. Nevertheless, when administered intracerebrally, *N*-Me-D-Asp antagonists are potent anticonvulsants (6) and have been shown to possess a remarkable ability to protect against permanent neuronal damage in animal models of cerebral ischemia and hypoglycemia (7, 8). In the present studies we describe a noncompetitive *N*-Me-D-Asp antagonist known from previous studies to penetrate readily into the central nervous system. The compound is MK-801 {(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate} (Fig. 1), a substance previously reported to possess an unusual spectrum of pharmacological activities as a potent anticonvulsant that exhibits both anxiolytic and sympathomimetic properties (9–11).

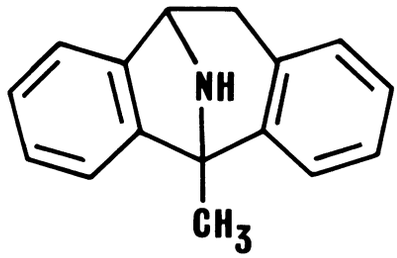

FIG. 1. Structure of MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine.

We used [^{3}H]MK-801 of high specific activity to identify a population of high-affinity binding sites in rat brain. Neurophysiological experiments showed that MK-801 selectively and noncompetitively antagonized the excitatory actions of *N*-Me-D-Asp in rat brain.

MATERIALS AND METHODS

[^{3}H]MK-801 (22.3 Ci/mmol; 1 Ci = 37 GBq) was prepared from the 7-bromo analog of MK-801 by tritium–halogen exchange. This precursor, together with samples of MK-801 and its (−) enantiomer, was kindly provided by P. Anderson (Merck Sharp & Dohme Research Laboratories, West Point, PA). Samples of (±)-*N*-allylnormetazocine (SKF 10,047) and phencyclidine were provided by The National Institute on Drug Abuse (Baltimore, MD). All other reagents were obtained from commercial sources.

For *in vitro* binding assays, cerebral cortices from male Sprague–Dawley rats (200–300 g) were homogenized in 9 volumes of ice-cold 0.32 M sucrose by nine strokes with a Teflon/glass homogenizer at 500 rpm. The homogenate was centrifuged for 10 min at 1000 × *g*, and the supernatant was recentrifuged at 10,000 × *g* for 20 min at 4°C. The pellet was suspended in assay buffer (118 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/5 mM $NaHCO_3$/20 mM Hepes/1.2 mM KH_2PO_4/2.5 mM $CaCl_2$/11 mM glucose, pH 7.4) and incubated at 23°C for 20 min prior to final centrifugation at 10,000 × *g* for 20 min at 4°C. The pellet was resuspended in assay buffer (70 ml per gram of original tissue). Binding of [^{3}H]MK-801 was measured by incubating 750-μl duplicate aliquots of this crude membrane suspension (≈0.75 mg of protein) with 100 μl of buffer containing displacer or of buffer alone (total

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Abbreviations: *N*-Me-D-Asp, *N*-methyl-D-aspartate; aCSF, artificial cerebrospinal fluid.

binding), 100 μl of 50 nM [^{3}H]MK-801, and 50 μl of buffer for 60 min at 23°C. Nonspecific binding was defined by 100 μM (final concentration) unlabeled MK-801.

Incubation was terminated by rapid filtration through Whatman GF/B filters, which were washed immediately with two 5-ml portions of ice-cold assay buffer in a Brandel M 24-R cell harvester. The time required for the complete filtration and washing procedure was less than 10 sec. Radioactivity on the filters was determined by liquid scintillation counting in standard vials with 10 ml of Hydrofluor (National Diagnostics, Somerville, NJ) at 41% counting efficiency. Protein concentrations were determined according to the method of Lowry *et al.* (12).

For *in vitro* neurophysiological experiments, cortical slices of rat brain were prepared in a manner similar to that described by Harrison and Simmonds (13). Coronal sections (500 μm thick) through the level of the corpus callosum were cut in artificial cerebrospinal fluid (aCSF) at room temperature, using an Oxford Vibratome. The aCSF (124 mM NaCl/2 mM $MgSO_4$/2 mM KCl/1.25 mM KH_2PO_4/25 mM $NaHCO_3$/2 mM $CaCl_2$/10 mM glucose) was gassed continuously with an oxygen/carbon dioxide (95:5, vol/vol) mixture. Cortical wedges (≈1 mm wide) were cut from these slices and mounted in a two-compartment bath, with the ventral margin of the cortical tissue traversing a greased slot which separated the chambers, so that the cortical tissue lay almost entirely within one compartment, and the white matter entirely within the other.

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

RESULTS

[^{3}H]MK-801 labeled high-affinity binding sites in rat cerebral cortical membranes in a saturable manner (Fig. 2*A*). The binding of 5 nM [^{3}H]MK-801 attained equilibrium after a 30-min incubation at 23°C in a near-physiological solution (assay buffer, see *Materials and Methods*). The "specific" binding accounted for 80% of the total binding and was abolished by pretreating the membranes at 80°C for 10 min. This binding was fully reversible in the presence of 100 μM unlabeled MK-801 ($t_{1/2}$ for dissociation = 3 min) and exhibited a linear relationship with protein concentration between 0.2 and 1.2 mg per assay. Scatchard analysis of the saturation data indicated the presence of a single population of sites (Fig. 2*B*). Data from six experiments gave apparent affinity (K_d) and site density (B_{max}) values (mean ± SEM) of 37.2 ± 2.7 nM and 0.825 ± 0.102 pmol/mg of protein, respectively.

Studies of [^{3}H]MK-801 binding in membrane homogenates from different rat brain regions indicated a clear regional specificity (Table 1). The hippocampus exhibited the highest density of binding sites, followed by the cerebral cortex, striatum, and medulla–pons, whereas no specific binding of [^{3}H]MK-801 was detected in membranes prepared from rat cerebellum.

Investigations of the pharmacological specificity of the [^{3}H]MK-801 site in the cerebral cortex indicated stereoselectivity, with the (−) isomer of MK-801 being one-seventh as potent as MK-801 [the (+) isomer] (Fig. 3). Excitatory amino acid receptor ligands and a large number of compounds related to other neurotransmitter systems failed to show any displacement activity at concentrations up to 100 μM (Table 2). Compounds showing activity at less than 100 μM included the dissociative anesthetics phencyclidine and (±)-ketamine and the σ-opioid receptor ligand (±)-SKF

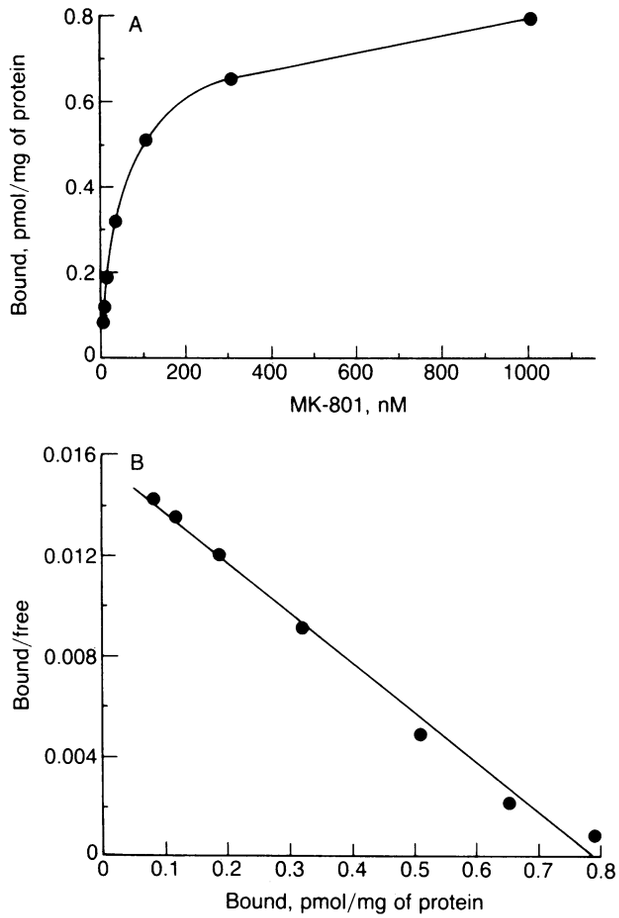

FIG. 2. (*A*) Saturation analysis of specific [^{3}H]MK-801 binding to rat cortical membranes. Membranes were incubated with 5 nM [^{3}H]MK-801 in the presence of increasing concentrations of unlabeled MK-801 (0.03–1.0 μM). Data are from a single experiment (which was repeated nine times, with similar results) and values are the means of triplicate determinations. (*B*) Scatchard plot of the specific binding data in *A*.

10,047, although these were 30–250 times less potent than MK-801 itself (Fig. 3 and Table 2). Analysis of these displacement curves by a computer-assisted iterative curve-fitting program (implemented by A. Richardson, based on RS1 by Bolt, Beranek and Newman, Boston, MA, 1985) indicated mass-action profiles with Hill coefficients near unity, consistent with the recognition of a single population of sites. Studies of [^{3}H]MK-801 binding sites in hippocampus and striatum indicated that they possessed similar kinetic properties and pharmacological specificity (data not shown).

In rat cortical-slice preparations, exposure to MK-801 produced a potent blockade of depolarizing responses to *N*-Me-D-Asp (Figs. 4 and 5*A*). The threshold concentration of MK-801 for this effect was ≈75 nM, which produced an

Table 1. Regional distribution of [^{3}H]MK-801 binding in rat brain membranes

	K_d, nM	B_{max}, pmol/mg of protein	*n*
Hippocampus	37.6 ± 4.4	1.143 ± 0.103	5
Cortex	37.2 ± 2.7	0.825 ± 0.102	6
Striatum	40.0 ± 3.5	0.654 ± 0.037	4
Medulla–pons	56.2 ± 2.9	0.180 ± 0.007	3

Membranes from various brain regions were incubated with [^{3}H]MK-801 (5 nM) as described in *Materials and Methods* and the legend of Fig. 1. Results are mean values ± SEM of *n* experiments.

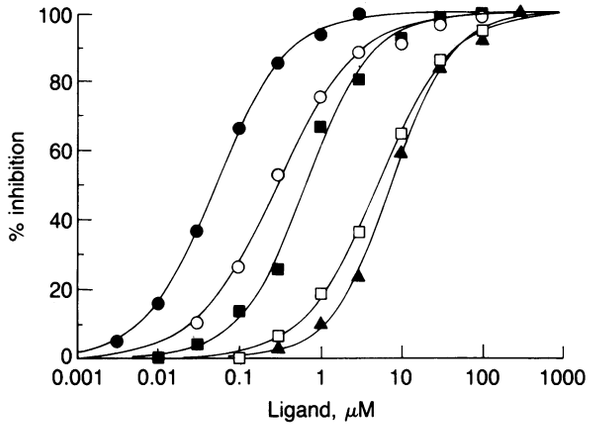

FIG. 3. Inhibition of the binding of [^{3}H]MK-801 (5 nM) in rat cortical membranes by MK-801 (●), the (−) enantiomer of MK-801 (○), phencyclidine (■), (±)-ketamine (□), and (±)-SKF 10,047 (▲). Results are from single experiments, which were all repeated at least four times (see Table 2).

insurmountable blockade of *N*-Me-D-Asp responses (Fig. 4). This antagonism was highly selective for *N*-Me-D-Asp, as MK-801 in the highest concentrations tested had no effect on responses to quisqualate (30 μM MK-801) or kainate (1 μM MK-801) (Figs. 4 and 5*A*). The blockade of *N*-Me-D-Asp responses by MK-801 appeared to develop slowly, reaching a maximum only after 90–120 min of continuous superfusion with MK-801 (Fig. 4). The effect of MK-801 was persistent, with only partial recovery after a 3-hr wash-out period. In Mg^{2+}-free aCSF and in the absence of tetrodotoxin, spontaneous paroxysmal depolarizing shifts were present in the majority of slices (13), and this epileptiform activity was abolished by MK-801 at concentrations that blocked *N*-Me-D-Asp responses.

One of the actions of dissociative anesthetics and σ opioids is to antagonize *N*-Me-D-Asp responses in a noncompetitive manner (13, 15, 16). We compared the potencies of phencyclidine, (±)-ketamine, (±)-SKF 10,047, and the enantiomers of MK-801 as antagonists of *N*-Me-D-Asp responses in the rat cortical slice and as inhibitors of [^{3}H]MK-801 binding to rat cortical membranes. With rat cortical

Table 2. Inhibition of [^{3}H]MK-801 binding to rat cortical membranes

	K_i, nM	Hill coefficient	*n*
MK-801	30.5 ± 1.5	0.92 ± 0.03	10
(−)-MK-801	211.7 ± 25.4	0.91 ± 0.04	5
Phencyclidine	875.2 ± 37.7	0.89 ± 0.02	9
(±)-Ketamine	4916 ± 528	0.88 ± 0.01	5
(±)-SKF 10,047	7522 ± 721	0.99 ± 0.05	7

Membranes were incubated with [^{3}H]MK-801 (5 nM) as described in *Materials and Methods*. Results are presented as mean ± SEM of determinations. IC_{50} values and Hill coefficients were measured from data obtained using at least six concentrations of drug (in duplicates), by computer-assisted iterative curve fitting. K_i values were calculated from IC_{50} values based on the Cheng–Prusoff equation (14) using a K_d value of 38 nM for [^{3}H]MK-801. The following drugs gave no inhibition of [^{3}H]MK-801 binding when tested at concentrations up to 100 μM: L-glutamate, *N*-Me-D-Asp, D-aspartate, kainate, quisqualate, DL-2-amino-5-phosphonovalerate, γ-aminobutyrate, kojic amine, (−)-baclofen, clonazepam, picrotoxin, pentobarbital, avermectin B_{1a}, glycine, strychnine, taurine, phenytoin, sodium valproate, ethosuximide, carbamazepine, γ-butyrolactone, 5-hydroxytryptamine, dopamine, haloperidol, (+)- and (−)-butaclamol, norepinephrine, methamphetamine, cocaine, prazosin, phentolamine, carbachol, atropine, morphine, etorphine, naloxone, verapamil, diltiazem, 8-phenyltheophylline, ouabain, and amantadine.

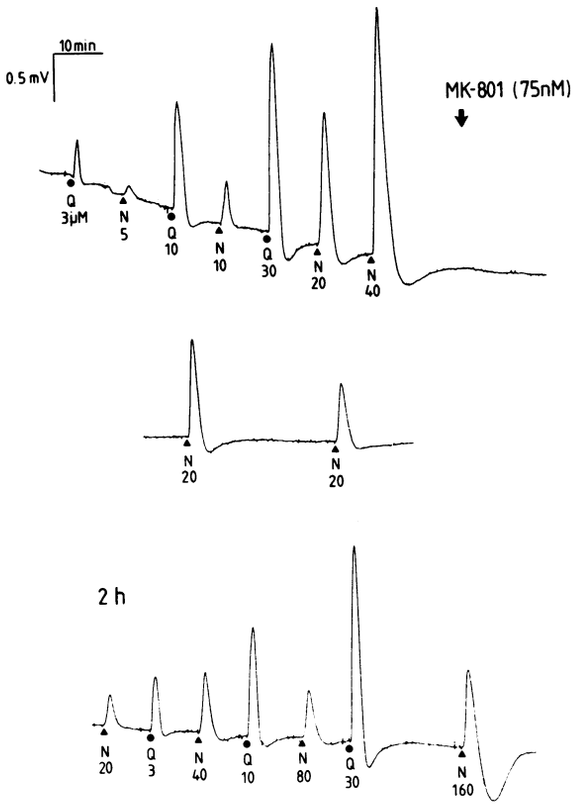

FIG. 4. Depolarizations of rat cerebral cortical tissue, relative to the white matter, produced by 1-min applications of *N*-Me-D-Asp (N, ▲) and quisqualate (Q, ●); the numbers indicate test concentrations in μM. MK-801 (75 nM) was continuously perfused from the time indicated by the arrow and *N*-Me-D-Asp (20 μM) was applied every half hour starting 1 hr later. After 2 hr of continuous perfusion with MK-801, various test concentrations of *N*-Me-D-Asp and quisqualate were repeated; note the insurmountable block of *N*-Me-D-Asp responses by MK-801.

slices, phencyclidine, (±)-ketamine, and (±)-SKF 10,047, although all weaker than MK-801, also produced a selective blockade of *N*-Me-D-Asp responses. However, unlike MK-801, they shifted the *N*-Me-D-Asp dose–response curve to the right in an apparently parallel manner. An example of the effect of phencyclidine is illustrated in Fig. 5*B*. There was excellent correlation (r = 0.99) between the potencies of these compounds as antagonists of *N*-Me-D-Asp responses and as inhibitors of [^{3}H]MK-801 binding (Fig. 6).

DISCUSSION

In the present study, we used biochemical and electrophysiological techniques to identify the mechanism of action of the potent anticonvulsant MK-801 (9). The use of [^{3}H]MK-801 enabled the identification of high-affinity saturable binding sites with kinetic properties consistent with those of a drug receptor (Figs. 2*A* and 3). MK-801 binding sites have an unusual specificity; the only compounds studied that displayed any appreciable affinity for these sites were the dissociative anesthetics phencyclidine and (±)-ketamine and the σ opioid (±)-SKF 10,047. The close correlation between the potencies of these compounds and the enantiomers of MK-801 as antagonists of *N*-Me-D-Asp responses and as inhibitors of [^{3}H]MK-801 binding (Fig. 6) suggests that the site labeled by [^{3}H]MK-801 is associated with *N*-Me-D-Asp receptors. This site is clearly different from the *N*-Me-D-Asp recognition site, since neither *N*-Me-D-Asp itself nor any other excitatory amino acid analogs tested had any affinity for the MK-801 binding site (Table 2). However, preliminary

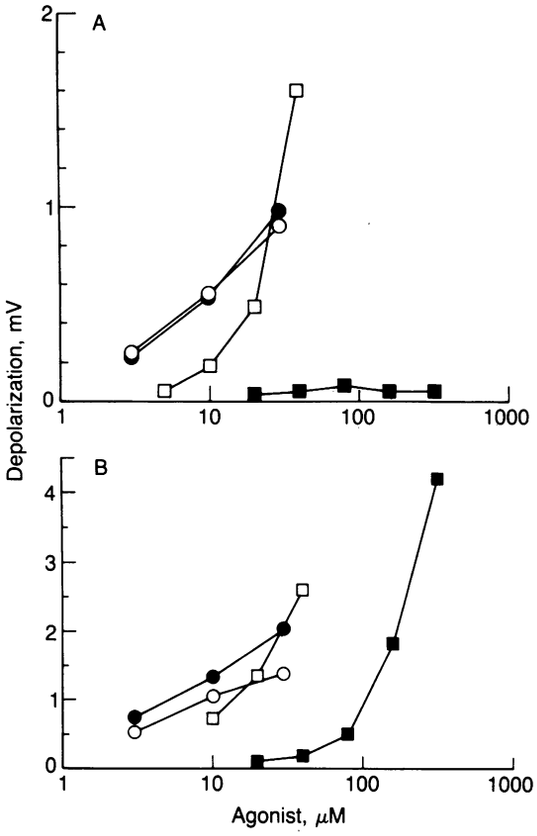

FIG. 5. Dose–response curves obtained from rat cerebral cortical slices for *N*-Me-D-Asp (□, ■) and quisqualate (○, ●) before (open symbols) and following (filled symbols) 2 hr of continuous perfusion with MK-801 (235 nM, *A*) or phencyclidine (5 μM, *B*). MK-801 completely abolished depolarizing responses to *N*-Me-D-Asp but had no effect on those to quisqualate. The responses to *N*-Me-D-Asp were antagonized by phencyclidine and the dose–response curve was shifted to the right, whereas responses to quisqualate were slightly increased in the presence of phencyclidine.

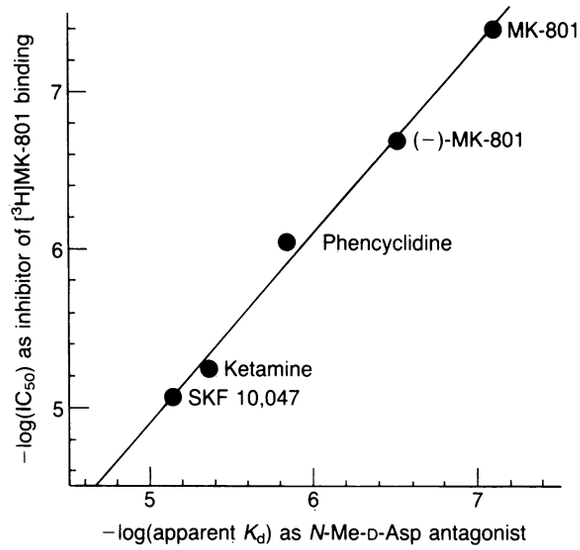

FIG. 6. Correlation between the potencies of MK-801, the (−) enantiomer of MK-801, phencyclidine, (±)-ketamine, and (±)-SKF 10,047 as inhibitors of [^{3}H]MK-801 binding and antagonists of *N*-Me-D-Asp responses in the rat cortical-slice preparation. The apparent potencies for phencyclidine (5 and 10 μM), (±)-ketamine (100 μM), and (±)-SKF 10,047 (5 and 30 μM) as *N*-Me-D-Asp antagonists were calculated from the dose-ratios (DR) they produced at the concentrations shown, using the equation $DR - 1 = B/K_d$, where B is the antagonist concentration ($n = 4$ or more slice preparations at each concentration). For MK-801 and its (−) enantiomer, the potency value was taken as the threshold dose that produced a significant reduction in *N*-Me-D-Asp responses, as it was impossible to estimate a dose-ratio because of the marked flattening of the dose–response relationship.

biochemical experiments have shown that *N*-Me-D-Asp-receptor ligands can modulate [^{3}H]MK-801 binding properties under certain conditions, suggesting a close association between the two sites (data not shown). It has recently been suggested that ketamine and phencyclidine may block the ion channel operated by activation of the *N*-Me-D-Asp receptor (16), and thus it is possible that the MK-801 binding site may be associated with this channel. This is consistent with the noncompetitive nature of the antagonism observed with MK-801 and the similarities between the regional distribution of [^{3}H]MK-801 binding sites, *N*-Me-D-Asp-sensitive L-glutamate binding sites (17), and those labeled by the competitive *N*-Me-D-Asp antagonist D-2-amino-5-phosphono[^{3}H]valerate (18).

The slow time course of the block of *N*-Me-D-Asp depolarizing responses by MK-801 was intriguing. However, we have found subsequently that the development of the antagonism does not simply relate to the length of time the tissue is exposed to MK-801 but rather to repeated additions of the agonist, *N*-Me-D-Asp. A possible use-dependency of the MK-801 antagonism would be in keeping with an open channel-blocking mechanism of action.

While phencyclidine, (±)-ketamine, and (±)-SKF 10,047 have modest affinities for the MK-801 site, they are also known to interact with σ-opioid receptors (19–23, 25). Initial biochemical studies strongly suggest that the MK-801 site is not identical to the σ-opioid binding site, as MK-801 and its (−) isomer were found to possess only very low potencies in displacing the σ-opioid ligand (+)-[^{3}H]SKF 10,047 from binding sites in rat brainstem. Unlike the MK-801 site (Table 2), the pharmacological specificity of the σ site differs in that haloperidol is more potent than SKF 10,047, followed by phencyclidine and ketamine (21, 22). Furthermore, the σ site, as labeled by tritiated (+)-SKF 10,047 and (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine, occurs in highest density in the brainstem and cerebellum and is present at only low densities in the hippocampus and cortex (19–22).

Our results provide an explanation for the mechanism of action of the orally active anticonvulsant MK-801. This compound acts at a functionally distinct site in mammalian brain that is related to the *N*-Me-D-Asp subtype of excitatory amino acid receptors. These findings have important implications for the potential future use of MK-801 in the treatment of epilepsy and as a protective agent in neurodegenerative disorders (24).

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