Journal of Neurochemistry
Raven Press, Ltd., New York

(+)-[^{3}H]MK-801 Binding Sites in Postmortem Human Brain

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Abstract: The pharmacological specificity and the regional distribution of the *N*-methyl-D-aspartate receptor-associated 5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate (MK-801) binding sites in human postmortem brain tissue were determined by binding studies using (+)-[^{3}H]MK-801. Scatchard analysis revealed a high-affinity (K_D = 0.9 ± 0.2 n*M*, B_{max} = 499 ± 33 fmol/mg of protein) and a low-affinity (K_D = 3.6 ± 0.9 n*M*, B_{max} = 194 ± 44 fmol/mg of protein) binding site. The high-affinity site showed a different regional distribution of receptor density (cortex > hippocampus > striatum) compared to the low-affinity binding site (cerebellum > brainstem). The rank order pharmacological specificity and stereoselectivity of the high-(cortex) and low-(cerebellar) affinity binding sites were identical. However, all compounds tested showed greater potency at the high-affinity site in cortex. The results indicate that (+)-[^{3}H]MK-801 binding in human postmortem brain tissue shows pharmacological and regional specificity. **Key Words:** *N*-Methyl-D-aspartate—MK-801—Receptor—Human—Phencyclidine. **Quarum M. L. et al.** (+)-[^{3}H]MK-801 binding sites in postmortem human brain. *J. Neurochem.* **54,** 1163–1168 (1990).

Phencyclidine (PCP) was first used as an anesthetic (Greifenstein et al., 1958), but due to its psychotomimetic effects and its abuse potential, its use was discontinued. It has been shown in numerous studies that specific binding sites for [^{3}H]PCP (Vincent et al., 1979; Zukin and Zukin, 1979) and its analogue, [^{3}H]thienylphencyclidine ([^{3}H]TCP) (Vignon et al., 1983), exist in mammalian brain tissue. Electrophysiological and radioligand binding studies have demonstrated that PCP receptors are coupled with the *N*-methyl-D-aspartate (NMDA) subtype of L-glutamate receptors (Anis et al., 1983). Compounds that bind to PCP receptors are classified as noncompetitive NMDA antagonists, based on their ability to block the NMDA cation channel and to antagonize NMDA responses. PCP and most compounds that interact with PCP receptors also cross-react with σ-receptors (Sonders et al., 1988). One notable exception is the novel anticonvulsant, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]-cyclohepten-5,10-imine maleate [(+)-MK-801] (Clineschmidt et al., 1982), which is also the most potent PCP receptor ligand as yet identified (Wong et al., 1986).

Two binding sites for [^{3}H]PCP (Blaustein and Ickowicz, 1983) and [^{3}H]TCP (Vignon et al., 1986) have been demonstrated in rat brain. We recently have synthesized tritium-labelled (+)-MK-801 of high specific activity and have described its binding properties in guinea pig brain (Keana et al., 1988). A preliminary report has described racemic [^{3}H]MK-801 binding in postmortem human frontal cortex (Tam and Zhang, 1988). Two binding sites for (+)-[^{3}H]MK-801 were reported in a different region of postmortem human frontal cortex using (+)-MK-801 to define nonspecific binding (Kornhuber et al., 1989). In this article, we describe the presence of a high- and low-affinity binding site for (+)-[^{3}H]MK-801, along with pharmacological specificities and regional distributions.

MATERIALS AND METHODS

Materials

(+)-[^{3}H]MK-801 (sp. act. 97 Ci/mmol) was synthesized as previously described (Keana et al., 1988). The diaryl guanidine derivatives, *N,N'*-di-*o*-iodophenylguanidine (di-iodo-PG) and *N,N'*-di-*m*-tolylguanidine (di-m-TG), were also synthe-

Received May 17, 1989; revised manuscript received August 14, 1989; accepted August 15, 1989.

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Abbreviations used: di-iodo-PG, *N,N'*-di-*o*-iodophenylguanidine; di-m-TG, *N,N'*-di-*m*-tolylguanidine; DTG, 1,3-di(2-tolyl)guanidine; MK-801, 5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; (+)-PPP, (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; SKF 10,047, *N*-allylnormetazocine; TCP, thienylphencyclidine.

sized by one of us (J.F.W.K.). (+)-MK-801 was the generous gift of Merck, Sharpe and Dohme (Rahway, NJ, U.S.A.). All other chemicals and drugs used were of the highest chemical purity available from commercial sources.

Human autopsy tissue

Human postmortem tissue from five men and two women was obtained at autopsy. There was no history of long-term psychiatric or neurological disease and no chronic or recent use of antipsychotic or anticonvulsant drugs. The subjects' ages ranged from 56 to 80 years old. The interval between death and freezing of the tissue was between 8 and 36 h. Brain regions were dissected at the time of autopsy, frozen in liquid nitrogen, and stored at −70°C.

Preparation of brain membranes

A crude membrane preparation was obtained by homogenizing human postmortem tissue in 10 volumes (wt/vol) of ice-cold 0.32 *M* sucrose. The homogenate was centrifuged at 1,000 *g* for 10 min at 4°C. The nuclear pellet was discarded, and the supernatant was centrifuged at 20,000 *g* for 20 min. The pellet was resuspended in 5 m*M* Tris-HCl, pH 7.6, and again centrifuged at 20,000 *g* for 20 min. The resulting crude membranes were resuspended in assay buffer (5 m*M* Tris-HCl, pH 7.6) to a final protein concentration of 1 mg/ml and stored at −70°C.

Radioligand binding assay

Aliquots of frozen membranes were thawed and diluted to obtain a final assay protein concentration of 150 μg/ml (800 μg/ml for cerebellum) of membrane proteins with assay buffer. The membrane suspensions, consisting of 0.8 ml of membranes, 0.1 ml of (+)-[^{3}H]MK-801 (final concentration 2 n*M*), and 0.1 ml of buffer or unlabelled drug, were incubated for 8 h at room temperature and then rapidly filtered under vacuum through Whatman GF/B glass fiber filters using a Brandel 48-well cell harvester (Brandel, Gaithersburg, MD, U.S.A.). Filters were presoaked in 0.05% polyethylenimine. The filters were washed three times with 5 ml of cold assay buffer. Each filter was dissolved in 20 ml of Cytoscint (ICN Biomedicals, Costa Mesa, CA, U.S.A.), and radioactivity was measured by liquid scintillation spectrometry at a counting efficiency of 50%.

All binding assays were performed at room temperature. Nonspecific binding was defined as that remaining in the presence of 100 μ*M* PCP. Saturation data were evaluated by Scatchard analysis using both EBDA (McPherson, 1983) and LIGAND (Munson and Rodbard, 1980) data analysis programs. IC_{50} values were determined by plotting displacement curves on semilogarithmic graph paper, followed by interpolation. K_i values were calculated from the IC_{50} values and ligand concentration using the Cheng–Prusoff transformation (Yamamura et al., 1985).

RESULTS

Receptor binding and kinetics

(+)-[^{3}H]MK-801 bound with high affinity, selectivity, and saturability to PCP/NMDA receptors in membrane preparations from human postmortem tissue. Binding was linear between 50 μg/ml and 1 mg/ml (data not shown). A protein concentration of 150 μg/ml was used in all experiments, except for the cerebellum experiments, in which 800 μg/ml was the final assay protein concentration. Specific binding of (+)-[^{3}H]MK-801 to human frontal cortex reached equilibrium in 6 h and was stable for up to 24 h (Fig. 1A). The dissociation rate constant (4.22×10^{-3} min^{-1}) and the k_{obs} (1.29×10^{-2} *M* min^{-1}) were calculated with KINETIC (McPherson, 1985) for the cortex binding site. We could only get a one-site fit from KINETIC. The association rate constant (4.34×10^{-6} *M* min^{-1}) was calculated using the k_{obs} and K_D from KINETIC and the radioligand concentration. A K_D value of 972 p*M* was calculated by dividing the dissociation rate constant by the association rate constant.

Receptor pharmacology

Scatchard analysis of saturation binding experiments using EBDA (McPherson, 1983) and LIGAND (Munson and Rodbard, 1980) revealed a single population of binding sites in cortex (Fig. 2A), cerebellum (Fig. 2B), and all other brain regions examined. The binding of (+)-[^{3}H]MK-801 (30 p*M* to 10 μ*M*) to cerebellum (K_D = 3.6 ± 0.9 n*M*, B_{max} = 194 ± 44 fmol/mg, n = 7) was distinctly different from binding in cortex (K_D = 0.9 + 0.1 n*M*, B_{max} = 391 fmol/mg, n = 3). These differences prompted us to use these tissues to characterize the pharmacological specificity of the high- (cortex) and low-(cerebellum) affinity binding sites. The results of competition binding experiments are summarized in Table 1, and select drug displacement curves are shown in Fig. 3. These results indicate that the rank order of potency for compounds able to displace (+)-

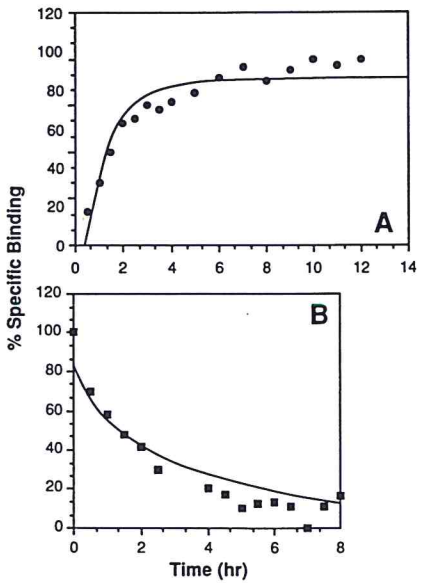

FIG. 1. Association and dissociation kinetic studies of (+)-[^{3}H]MK-801 in human postmortem frontal cortex. **A:** Association kinetics. Binding was initiated by addition of 1 n*M* (+)-[^{3}H]MK-801 to membrane preparations at given time intervals. The binding reaction was terminated by simultaneous filtration using a Brandel 48-well cell harvester as described in Materials and Methods. **B:** Dissociation kinetics. After incubation of (+)-[^{3}H]MK-801 with membranes for 8 h at room temperature, 100 μ*M* PCP was added at specific time intervals and binding was terminated by rapid filtration. Each curve is one of two experiments.

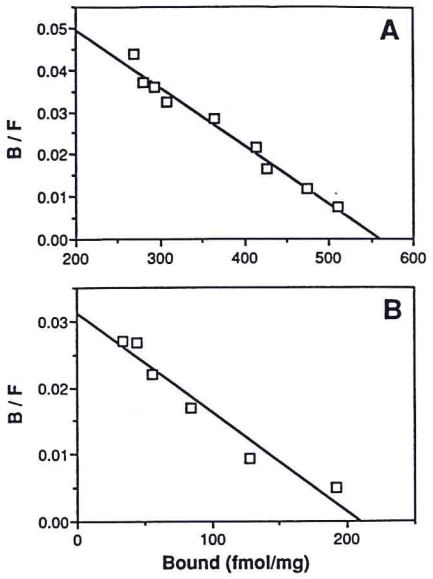

FIG. 2. Scatchard plots of (+)-[^{3}H]MK-801 binding in human frontal cortex (A) and cerebellum (B). Shown are representative experiments that have been replicated at least three times. Analysis using EBDA (McPherson, 1983) and LIGAND (Munson and Rodbard, 1980) reveals that (+)-[^{3}H]MK-801 binding in both cortex and cerebellum is to a single binding site.

[^{3}H]MK-801 is the same in frontal cortex and cerebellum, and show a rank order of potency consistent with what has been seen previously at the PCP/NMDA receptor complex (Keana et al., 1989). Furthermore, when the respective K_i values are compared between cortex and cerebellum, a correlation coefficient of 0.97 is obtained (Fig. 4). However, the K_i values for all drugs were lower in the cerebellum compared to the cortex. The stereoselectivity of the binding is preserved in both cerebellum and cortex. The (+) stereoisomers of MK-801, *N*-allylnormetazocine (SKF 10,047), and ketamine have increased potencies compared to (−) stereoisomers. The largest difference in the stereoselectivity of the binding is exhibited by dexoxadrol and levoxadrol, the latter having no ability to displace (+)-[^{3}H]MK-801. The diaryl guanidine derivatives, di-iodo-PG and di-m-TG, show binding affinity at the PCP/NMDA site (Table 1), confirming earlier studies in rodents (Keana et al., 1989).

TABLE 1. *Potencies of various drugs to compete with (+)-[^{3}H]MK-801 binding*

Drug	K_i (nM) against (+)-[^{3}H]MK-801	
	Human cortex	Human cerebellum
1. (+)-MK-801	0.90 ± 0.15[a]	3.6 ± 0.9[a]
2. (−)-MK-801	4.1 ± 0.7	15 ± 2
3. TCP	8.1 ± 0.4	27 ± 5
4. Dexoxadrol	9.9 ± 1.4	38 ± 3
5. (−)-Cyclazocine	20 ± 2	47 ± 10
6. PCP	25 ± 4	58 ± 3
7. (+)-SKF 10,047	108 ± 14	238 ± 18
8. (+)-Cyclazocine	113 ± 6	323 ± 35
9. Di-iodo-PG	123 ± 11	448 ± 5
10. (−)-SKF 10,047	199 ± 12	395 ± 21
11. D-Ketamine	252 ± 35	440 ± 35
12. Di-m-TG	276 ± 35	782 ± 104
13. L-Ketamine	1,318 ± 163	2,250 ± 825
14. Levoxadrol	>10 μM	>10 μM

[a] Correlation coefficient = 0.97.

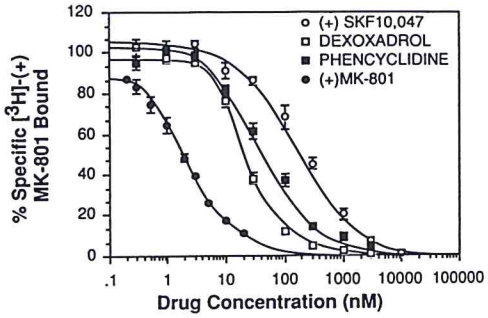

FIG. 3. Competition (+)-[^{3}H]MK-801 binding studies using frontal cortex membranes in the presence of various drugs. The data points are means ± SEM of three experiments.

Effect of NMDA agonists

Because L-glutamate and glycine have been shown to enhance (+)-[^{3}H]MK-801 binding (Foster and Wong, 1987; Reynolds et al., 1987), we questioned whether the decreased affinity of (+)-[^{3}H]MK-801 binding in cerebellum was due to suboptimum concentrations of these compounds. Binding was measured in cortex and cerebellum membrane preparations in the presence of 50 μM glycine, 100 μM L-glutamate, and 50 μM glycine plus 100 μM glutamate and in the absence of these compounds. No difference in total, specific, or nonspecific binding was observed in either cortex or cerebellum (Fig. 5), suggesting that in these

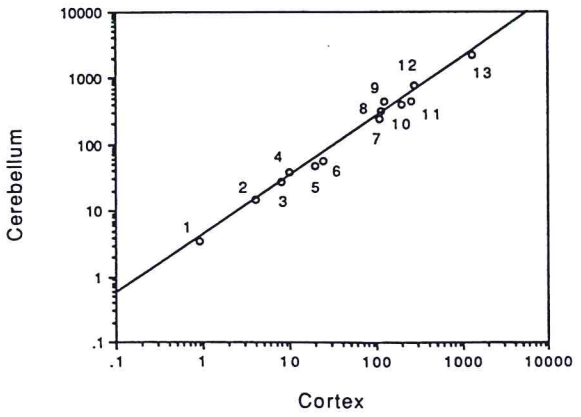

FIG. 4. Comparison of K_i values of various drugs in cortex (abscissa) and cerebellum (ordinate) (+)-[^{3}H]MK-801 binding assays. K_i values are the means of three experiments. A correlation coefficient of 0.97 was obtained. The number of each point corresponds to the drug listed in Table 1.

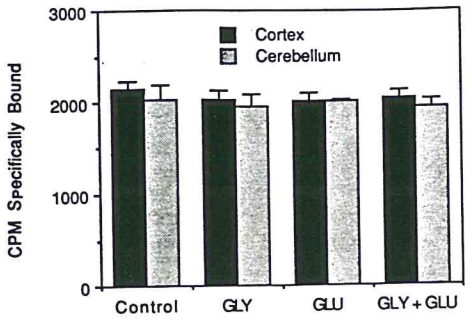

FIG. 5. Effect of L-glutamate (GLU) and glycine (GLY) on (+)-[^{3}H]MK-801 binding to human cortex (150 μg/ml protein) and cerebellar (800 μg/ml protein) membranes. Results shown are the means ± SEM of three experiments. L-Glutamate and glycine were added at saturating concentrations of 100 μM and 50 μM, respectively.

(unwashed) membranes the concentration of these compounds is already saturating.

Regional distribution of (+)-[^{3}H]MK-801 binding

An examination of six different brain regions revealed (+)-[^{3}H]MK-801 specific binding levels ranging from 44 to 499 fmol/mg of protein (Table 2). The lowest density of (+)-[^{3}H]MK-801 binding sites was found in brainstem and hypothalamus, and the highest density of sites was found in cortex and hippocampus. Cerebellum and striatum showed intermediate levels of binding. The affinity of (+)-[^{3}H]MK-801 binding in cerebellum was significantly different from the affinity observed in cortex ($p < 0.001$), hippocampus ($p < 0.001$), and striatum ($p < 0.002$), but not significantly different from brainstem using Student's t test (Student, 1907). The Hill coefficients of binding observed in all regions were close to 1, suggesting little cooperativity in (+)-[^{3}H]MK-801 binding. There was no significant binding in the hypothalamus.

Stability of (+)-[^{3}H]MK-801 binding

There were no significant differences in binding affinity or receptor density when comparing tissue prepared 8 h or 36 h after death. Furthermore, the binding of (+)-[^{3}H]MK-801 to human postmortem brain tissue showed excellent stability after up to 24 h of incubation at room temperature (data not shown).

DISCUSSION

This study demonstrates specific binding of (+)-[^{3}H]MK-801 to human postmortem brain tissue. High- and low-affinity binding sites have been characterized. The affinity of the low-affinity site is much different from the 166 nM K_D low-affinity site previously reported for human frontal cortex (Kornhuber et al., 1989). There are major differences which may account for the discrepancy. First, rostral frontal cortex was used here, and Brodmann areas 8 and 9 were used by Kornhuber et al. (1989). Secondly, 100 μM PCP was used to define nonspecific binding in this study as opposed to 100 μM MK-801 in the other study. The presence of two binding sites in mammalian brain is also consistent with previous studies using [^{3}H]PCP (Blaustein and Ickowicz, 1983; Mendelsohn et al., 1984) and [^{3}H]TCP (Vignon et al., 1986) as NMDA-associated receptor ligands.

Analysis of association and dissociation kinetic experiments in cortex reveals a K_D value (972 pM) for (+)-[^{3}H]MK-801 binding that is in excellent agreement with the K_D value (900 pM) calculated from saturation experiments. It should be noted that if both the high- and low-affinity sites were present in the same tissue, then Scatchard analysis would not be able to distinguish them because of their similar K_D values (less than fourfold). The apparent poor fit in the kinetic curves may be the result of a small amount of the low-affinity site in the frontal cortex. The long association and dissociation time (≥6 h) we report in this study agrees with the association time (4–6 h) we have observed using (+)-[^{3}H]MK-801 to label PCP/NMDA receptors in whole guinea pig brain crude membrane preparations (Keana et al., 1988). Others have observed a much shorter association and dissociation time using racemic [^{3}H]MK-801 and rat brain membrane preparations (Foster and Wong, 1987). This suggests that the binding kinetics of racemic [^{3}H]MK-801 and (+)-[^{3}H]MK-801 may be different, or there may be species differences between rat and guinea pig MK-801 binding sites.

The pharmacological specificity of (+)-[^{3}H]MK-801 binding in this study using human cortex and cerebellum membranes is consistent with our observations using whole guinea pig brain (Keana et al., 1988). This specificity is also consistent with previous studies using racemic [^{3}H]MK-801 (Wong et al., 1988), [^{3}H]PCP (Mendelsohn et al., 1984), and [^{3}H]TCP (Largent et al., 1986) in rat brain preparations.

Two binding sites for [^{3}H]PCP (Blaustein and Ickowicz, 1983; Mendelsohn et al., 1984) and [^{3}H]TCP (Vignon et al., 1986) in mammalian brain preparations have already been described. Two binding sites were also reported for (+)-[^{3}H]MK-801 in human frontal cortex, but with a much different low-affinity site in a slightly different region of frontal cortex (Kornhuber et al., 1989). The Scatchard plot of (+)-[^{3}H]MK-801 binding in cortex, cerebellum, and all other regions tested reveals only one class of receptor sites in each

TABLE 2. *Regional distribution of [^{3}H]MK-801 binding in postmortem human brain tissue*

Tissue	K_D (pM)	B_{max} (fmol/mg)	Hill coefficient
Cortex	900 ± 150	499 ± 33	1.03 ± 0.02
Cerebellum	3,600 ± 900	194 ± 44	0.99 ± 0.03
Hippocampus	650 ± 40	366 ± 68	0.96 ± 0.03
Striatum	866 ± 204	192 ± 7	0.83 ± 0.2
Brainstem	2,600 ± 1,300	44 ± 10	0.81 ± 0.1
Hypothalamus		No significant binding	

region examined; this was also shown in previous studies using other PCP receptor ligands (Mendelsohn et al., 1984; Vignon et al., 1986). However, in one study, low-affinity binding sites were also identified in rat brain hypothalamus and hippocampus using [^{3}H]TCP (Haring et al., 1987).

Because the primary distinction between these two (+)-[^{3}H]MK-801 binding sites is affinity for compounds that are active at the PCP receptor, and given the observations that glycine and L-glutamate enhance racemic [^{3}H]MK-801 binding by increasing the affinity of the ligand with little change in receptor density (Reynolds et al., 1987; Foster and Wong, 1987), we hypothesized that the low-affinity receptor was submaximally stimulated by these endogenous NMDA agonists. Our observations that additions of 50 μM glycine, 100 μM glutamate, and a combination of these compounds had no effect on binding suggests that glycine and L-glutamate are already present in our crude membrane preparations at supramaximal concentrations. Given the numerous other possible endogenous modulatory compounds for the PCP/NMDA receptor, one still cannot rule out the possibility that these two sites are the same receptor in different states. The regional distribution of (+)-[^{3}H]MK-801 binding in the human brain is similar to what has been reported using other NMDA ligands in rat brain (Sircar and Zukin, 1985; Gundlach et al., 1986; Maragos et al., 1988), including racemic [^{3}H]MK-801 (Bowery et al., 1988), with one exception: in human brain, the density of binding sites appears to be highest in frontal cortex, but in rat brain the highest density of binding is observed in hippocampus. This is in agreement with a previous study using [^{3}H]PCP to label human postmortem brain regions (Sircar and Zukin, 1983). Quantitative autoradiographic visualization of (+)-[^{3}H]MK-801 binding sites using human brain sections is needed to verify and extend these findings.

The inability of the σ-specific ligands 1,3-di(2-tolyl)guanidine (DTG), haloperidol, and (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine [(+)-PPP] to displace (+)-[^{3}H]MK-801 binding, coupled with the different pharmacological specificity and regional distribution of binding sites for these ligands in human brain (Largent et al., 1986), suggests that (+)-[^{3}H]MK-801 and σ-specific ligands are acting at distinctly different sites. The two diaryl guanidine derivatives (di-iodo-PG and di-m-TG) tested in this study have neuroprotective properties similar to those of other noncompetitive NMDA ligands in a model of glutamate toxicity (Keana et al., 1989). Besides having no activity at the NMDA site, the σ-specific ligands, DTG, (+)-PPP, and haloperidol, have no neuroprotective properties (Keana et al., 1989).

In summary, using postmortem human brain tissue, we have characterized high- (K_D = 0.90 M) and low- (K_D = 3.6 nM) affinity binding sites for (+)-[^{3}H]MK-801. The highest density of the high-affinity site is found in frontal cortex, and the highest density of the low-affinity binding site is found in cerebellum. (+)-[^{3}H]MK-801 binding to each of these sites is to a single class of receptors and shows saturability and reversibility. The pharmacological specificity of each of these sites is identical. Unlike rat brain, human frontal cortex shows an increased density of binding sites compared to hippocampus. Binding to postmortem human brain tissue in (+)-[^{3}H]MK-801 radioligand binding studies may better aid our understanding of the role of NMDA receptors in various behavioral and neuropathological processes.

Acknowledgment: This study was supported NIMH grants MH40303 and MH42608 and a grant from Cambridge Neurosciences Inc. M.L.Q. was supported by an NIMH NRSA postdoctoral research fellowship.

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