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REGIONAL DISTRIBUTION AND PROPERTIES OF [^{3}H]MK-801 BINDING SITES DETERMINED BY QUANTITATIVE AUTORADIOGRAPHY IN RAT BRAIN

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Abstract—[^{3}H]MK-801 binding in rat brain was characterized using a quantitative autoradiographic binding assay. [^{3}H]MK-801 binding (5 nM) reached equilibrium by 120 min at 23°C. [^{3}H]MK-801 appeared to label a single high affinity site with an affinity constant of approximately 11 nM. [^{3}H]MK-801 binding was heterogeneously distributed throughout the brain with the following order of binding densities: hippocampal formation > cortical areas > striatum > thalamus.

Competitive *N*-methyl-D-aspartate antagonists, DL-2-amino-5-phosphonopentanoic acid, DL-2-amino-7-phosphonoheptanoic acid, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, and *cis*-4-phosphonomethyl-2-piperidine carboxylic acid, inhibited [^{3}H]MK-801 binding. Glycine antagonists, 7-chlorokynurenic acid and kynurenic acid, also inhibited [^{3}H]MK-801 binding. Furthermore, the inhibition of [^{3}H]MK-801 binding by the quinoxalinedione compounds 6-cyano-7-nitroquinoxaline-2,3-dione and 6,7-dinitroquinoxaline-2,3-dione was reversed by glycine. [^{3}H]MK-801 binding was also inhibited by zinc ions. [^{3}H]MK-801 binding was enhanced by glycine or *N*-methyl-D-aspartate.

These results demonstrate that [^{3}H]MK-801 can be used in a quantitative autoradiographic assay as a functional probe for the *N*-methyl-D-aspartate receptor complex.

Glutamate is a major excitatory amino acid neurotransmitter mediating neuronal signalling within the mammalian central nervous system. Glutamate interacts with at least three receptors which have been classified on the basis of agonists that selectively activate them: quisqualate, kainate and *N*-methyl-D-aspartate (NMDA).[13,36,60] The NMDA receptor is the best characterized of the receptor subtypes due to the availability of selective antagonists.[36] Activation of NMDA receptors has been implicated in a number of physiological and pathological processes including long-term potentiation, learning and memory, hypoxic–ischemic neuronal damage, and chronic neural degeneration.[8,47]

The NMDA receptor is associated with a cation channel that is gated by magnesium in a voltage-dependent fashion.[40,54] The receptor–ion channel complex is modulated by several regulatory sites. Glycine, acting at a strychnine-insensitive site, increases NMDA receptor activation.[25] 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) and *cis*-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755) act as competitive antagonists at the NMDA recognition site.[19,32,37,38] Dissociative anesthetics such as ketamine, phencyclidine (PCP) and the novel drug, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801 or dizocilipine maleate), are non-competitive antagonists of the NMDA receptor.[2,21,55] Electrophysiological and biochemical studies indicate that these drugs act by interacting with a so-called PCP receptor within the ion channel as potent non-competitive antagonists of NMDA receptors.[42,44,45,55,57] Although autoradiographic binding studies with [^{3}H]MK-801 have been reported, these studies have not assessed the kinetics, pharmacology or regional effects of glycine, glutamate and other modulators on the binding of [^{3}H]MK-801 to tissue sections.[4,5] We have characterized in detail a quantitative autoradiographic binding assay for the PCP receptor using [^{3}H]MK-801 in rat brain.

EXPERIMENTAL PROCEDURES

[^{3}H]*MK*-801 *binding assay*

Male Sprague–Dawley rats (175–250 g, Charles River) were decapitated, the brains rapidly removed and mounted on microtome chucks in Lipshaw embedding matrix and frozen under powdered dry ice. Twenty-micrometer horizontal sections were cut on a Lipshaw cryostat and thaw-mounted onto gelatin-coated slides. Tissue sections were used immediately or stored at −20°C for less than 24 h. [^{3}H]MK-801 binding was not altered in tissue sections that were stored frozen. Sections were prewashed for 30 min in 50 mM Tris–acetate buffer (pH 7.4) at 4°C and blown dry

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Abbreviations: AMPA, [*RS*]-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP5, DL-2-amino-5-phosphonopentanoic acid; AP7, DL-2-amino-7-phosphonoheptanoic acid; CGS 19755, *cis*-4-phosphonomethyl-2-piperidine carboxylic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; DNQX, 6,7-dinitro-quinoxaline-2,3-dione; HA-966, 3-amino-1-hydroxypyrrolide-2-one; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; (+)PPP, (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; SMDG, stratum moleculare of the dentate gyrus; SR-CA1, stratum radiatum of CA1; TCP, *N*-(1-[2-thienyl]cyclohexyl)3,4-piperidine.

under a stream of room temperature air before performing the [^{3}H]MK-801 binding assay. In all [^{3}H]MK-801 binding experiments, triplicate tissue sections were incubated in 50 mM Tris–acetate buffer (pH 7.4 at room temperature) containing 5–20 nM [^{3}H]MK-801 at a final volume of 10 ml. Under these conditions less than 5% of total ligand was bound and "zone A" conditions[2a] (10% or less of total ligand in the incubation mixture is bound) are maintained. In order to assess the effects of temperature on the assay, [^{3}H]MK-801 binding was performed at 4°C, 23°C and 37°C. Optimal pH conditions were determined by varying the pH of the 50 mM Tris–acetate buffer in the incubation mixture between pH 5.0 and 9.0.

For regional distribution studies, tissue sections were incubated for 120 min in 50 mM Tris–acetate buffer (pH 7.4) containing 5 nM [^{3}H]MK-801 at room temperature. Non-specific binding was determined in the presence of 5 μM unlabeled MK-801. Following the incubation, sections were dipped quickly into 50 mM Tris–acetate buffer (pH 7.4 at 4°C), then rinsed for 80 min in 250 ml of cold buffer and blown dry under warm air. In kinetic studies of the association rate, sections were incubated in 50 mM Tris–acetate buffer (pH 7.4, room temperature) containing 5 nM [^{3}H]MK-801 for 11 time points between 2 and 360 min and were then rinsed for 80 min in cold buffer. For determination of the dissociation rate, sections were first incubated in 50 mM Tris–acetate buffer (pH 7.4, room temperature) containing 5 nM [^{3}H]MK-801 for 120 min and were then dipped quickly in buffer and placed in a large volume of buffer ("infinite dilution") for nine time points between 0 and 360 min at room temperature. In saturation studies, for the lower concentrations, sections were incubated for 120 min at room temperature in 50 mM Tris–acetate buffer containing concentrations of [^{3}H]MK-801 ranging from 1 to 20 nM. For the higher concentrations, sections were incubated for 120 min in 20 nM [^{3}H]MK-801 diluted with unlabeled MK-801 ranging from 20 to 300 nM. Non-specific binding was determined for each point in the presence of 20 μM MK-801.

In competition studies, unlabeled drugs were included in the incubation mixture. In separate experiments, tissue sections were prewashed for 30 min in 50 mM Tris–acetate buffer at 37°C (pH 7.4) and [^{3}H]MK-801 binding performed in the presence of NMDA (100 μM) or glycine (100 nM or 100 μM) added to the incubation mixture.

Dried sections were placed in X-ray cassettes with appropriate radioactive standards and apposed to either LKB Ultrofilm ^{3}H or Amersham Hyperfilm. Following a 3–4 week exposure at 4°C, films were developed in D-19 (Kodak), fixed and dried. All data presented were analysed from resultant autoradiographic images. Ten to twenty readings per area from triplicate sections were analysed using computer-assisted image analysis (Imaging Research, Inc., St Catherine's, Ontario). Data from kinetic studies were analysed using the non-linear regression program LIGAND.[36a] IC_{50} values for competitors were calculated by log–logit analysis and K_i values were determined by the method of Cheng and Prusoff[5a] using the formula $K_i = IC_{50}/(1 + ([L]/K_d))$.

Materials

[^{3}H]MK-801 (29.4 Ci/mmol) was obtained from Dupont New England Nuclear (Boston, MA). Unlabeled MK-801 was a gift from Dr L. L. Iversen, Merck, Sharpe & Dohme Research Laboratories (Essex, U.K.). *N*-(1-[2-Thienyl]cyclohexyl)3,4-piperidine (TCP) and (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine [(+)PPP] were gifts from Dr J. Woods, University of Michigan (Ann Arbor, MI). [*RS*]-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and CPP were purchased from Research Biochemicals (Natick, MA). CGS 19755 was a gift from Dr R. A. Lovell, Ciba-Geigy Corporation (Summit, NJ). Tetrahydroaminoacridine was purchased from Aldrich Chemicals (Milwaukee, WI). 3-Amino-1-hydroxypyrrolide-2-one (HA-966), 7-chlorokynurenic acid, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitro-quinoxaline-2,3-dione (DNQX), DL-2-amino-5-phosphonopentanoic acid (AP5), and DL-2-amino-7-phosphonoheptanoic acid (AP7) were obtained from Tocris Neuramin (Essex, U.K.). All other chemicals were purchased from Sigma (St Louis, MO).

RESULTS

Characterization of [^{3}H]*MK-801 binding*

In initial experiments, [^{3}H]MK-801 binding performed at 4°C, 23°C and 37°C indicated that the optimal incubation temperature was 23°C. At 4°C and 37°C, [^{3}H]MK-801 binding in stratum radiatum of the CA1 (SR-CA1) was reduced by 64% and 17%, respectively, compared to sections incubated at 23°C. Varying the Tris–acetate incubation buffer over a range of pH values from pH 5.0 to 9.0 revealed optimal [^{3}H]MK-801 binding at pH 7.4. At the lower pH range between 5.0 and 6.8 and pHs greater than 8.0, [^{3}H]MK-801 binding was reduced. In preliminary experiments, equilibrium was reached at 120 min at 23°C and the optimal rinse time was 80 min. At shorter rinse times specific binding was reduced slightly. These incubation and rinse times were used in subsequent saturation, pharmacological and regional distribution studies. Under these conditions, specific binding (total binding minus binding in the presence of 5μM MK-801) represented 95% of total binding.

Scatchard analyses of the saturation isotherms revealed that [^{3}H]MK-801 labeled an apparent single high affinity site. The maximal binding (B_{max}) of [^{3}H]MK-801 in layers I and II of frontal cortex was 2.53 ± 0.14 pmol/mg protein and the affinity (K_d) was 11.6 ± 0.5 nM (Fig. 1). B_{max} and similar K_d values were obtained for SR-CA1 and stratum moleculare of the dentate gyrus (SMDG). In SR-CA1, the K_d for [^{3}H]MK-801 binding was 14.3 ± 0.9 nM and the B_{max} was 4.0 ± 0.2 pmol/mg protein, while in the SMDG the K_d was 13.4 ± 1.4 nM and B_{max} was 3.1 ± 0.2 nM.

In 50 mM Tris–acetate buffer 5 nM [^{3}H]MK-801 binding reached equilibrium by 120 min at 23°C and remained at equilibrium until at least 360 min (Fig. 2). Specific binding (total binding minus binding in the

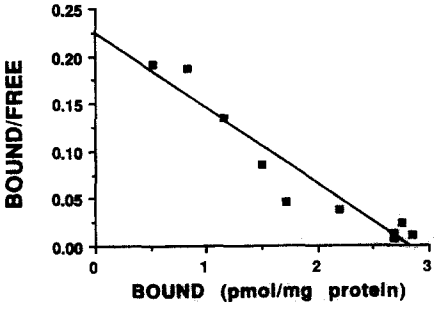

Fig. 1. Representative Scatchard plot of [^{3}H]MK-801 binding in layers I and II of frontal cortex. Rat brain sections were incubated in 1–20 nM [^{3}H]MK-801 and unlabeled MK-801 (20–300 nM) for 2 h at 25°C. Each point represents average specific binding in three sections (total binding minus binding in the presence of 20 μM MK-801). The data are representative of three separate experiments.

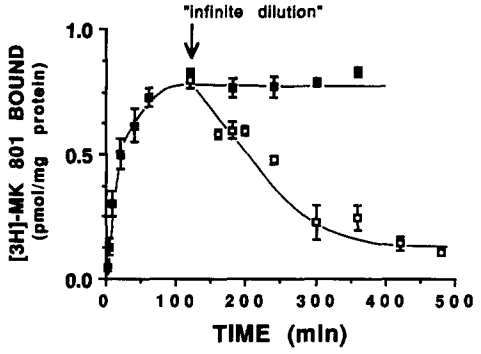

Fig. 2. Time-course of association and dissociation of [³H]MK-801 binding to layers I and II of frontal cortex. In association studies, rat brain sections were incubated in 5 nM [³H]MK-801 for time points indicated on the abscissa at 25°C and then rinsed for 80 min in 50 mM Tris–acetate buffer. In separate dissociation studies, sections were incubated in 5 nM [³H]MK-801 for 2 h and dissociation started by placing sections into a large volume of 50 mM Tris–acetate buffer ("infinite dilution") for time points up to 6 h. Data represent mean specific binding ± S.E.M. of three animals. The data are representative of three separate experiments.

presence of 5 μM MK-801) represented 95% of the total binding. The rate constants, k_{+1} and k_{-1} were calculated from association and dissociation data. The association and dissociation curves fit a single site kinetic model. The kinetically derived dissociation constant (K_d) for the frontal cortex layers I and II was 0.958 ± 0.21 nM (mean ± S.E.M.). Similar K_d values were observed in other regions. In SR-CA1 the K_d for [³H]MK-801 binding was 0.79 ± 0.25 nM while in the SMDG the K_d was 0.49 ± 0.08 nM.

Pharmacological profile of [³H]*MK-801 binding*

[³H]MK-801 binding was displaced by unlabeled MK-801 in a monophasic manner. In SR-CA1 MK-801 displaced [³H]MK-801 binding with a K_i of 7.3 ± 0.8 nM (mean ± S.E.M.) and a Hill coefficient (n_H) of 0.98 ± 0.06 (Fig. 3). In addition, TCP displaced [³H]MK-801 binding in SR-CA1 with a K_i of

Table 1. Inhibition of [³H]MK-801 binding to rat brain sections by various compounds

Compound	K_i	n_H	n
MK-801	7.3 ± 0.8 nM	0.98 ± 0.06	3
TCP	55 ± 1.8 nM	0.85 ± 0.02	3
CPP	7.0 ± 0.8 μM	1.7 ± 0.36	3
CGS 19755	3.7 ± 0.3 μM	1.4 ± 0.22	3
7-Chlorokynurenic acid	22.7 ± 6.0 μM	1.4 ± 0.13	4
Kynurenic acid	206 ± 54 μM	1.9 ± 0.34	4
CNQX	40 ± 3.2 μM	2.1 ± 0.2	4
DNQX	22 ± 2.6 μM	2.1 ± 0.3	4
Zinc	88 ± 23 μM	1.2 ± 0.3	3

Tissue sections were incubated with 5 nM [³H]MK-801 in the presence of the appropriate compound for 2 h at 25°C and rinsed for 80 min in 50 mM Tris–acetate buffer. Data are presented as mean ± S.E.M. K_i values were calculated from IC_{50} values using the Cheng–Prusoff equation.[5a]

55 ± 1.8 nM (mean ± S.E.M.) and a Hill coefficient (n_H) of 0.85 ± 0.02 (Fig. 3 and Table 1). Competitive NMDA antagonists inhibited [³H]MK-801 binding. In SR-CA1, CPP inhibited [³H]MK-801 binding with an apparent K_i of 7.0 ± 0.8 μM (mean ± S.E.M.) and n_H of 1.7 ± 0.36, while CGS 19755 inhibited binding with an apparent K_i of 3.7 ± 0.3 μM and n_H of 1.4 ± 0.22 (Fig. 4 and Table 1). AP7 (100 μM) and AP5 (100 μM) also inhibited [³H]MK-801 binding by 80% and 82%, respectively. The inhibition of [³H]MK-801 binding by CPP (10 μM) was reversed when 100 μM NMDA was included in the incubation mixture.

[³H]MK-801 binding was also inhibited by glycine antagonists (Fig. 5 and Table 1). Of the glycine antagonists, 7-chlorokynurenic acid was the most potent inhibitor of [³H]MK-801 binding with a K_i of 22.7 ± 6.0 μM and n_H of 1.4 ± 0.13, while HA-966 failed to displace [³H]MK-801 binding in SR-CA1. Kynurenic acid inhibited [³H]MK-801 binding with a K_i of 206 ± 54 μM and n_H of 1.9 ± 0.34. The quinoxalinedione compounds, CNQX and DNQX, also inhibited [³H]MK-801 binding in SR-CA1 with K_i values of 40 ± 3.2 and 22 ± 2.6 μM, respectively

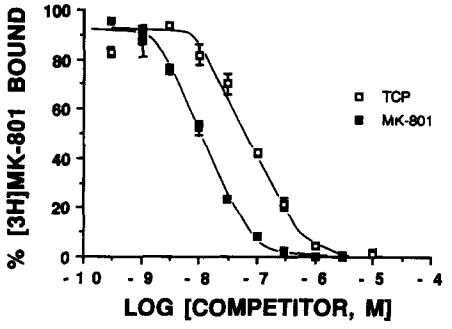

Fig. 3. Inhibition of [³H]MK-801 binding by TCP and MK-801 in stratum radiatum of the CA1 region of hippocampus. Rat brain sections were incubated in 5 nM [³H]MK-801 either in the presence of varying concentrations of TCP or MK-801. Each point represents mean specific binding ± S.E.M. of three animals. The data are representative of three separate experiments.

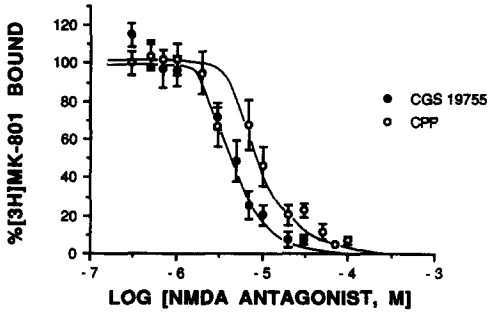

Fig. 4. Inhibition of [³H]MK-801 binding by NMDA antagonists in stratum radiatum of the CA1 region of hippocampus. Rat brain sections were incubated in 5 nM [³H]MK-801 in the presence of varying concentrations of CPP or CGS 19755. Each point represents mean specific binding ± S.E.M. of three animals. The data are representative of three separate experiments.

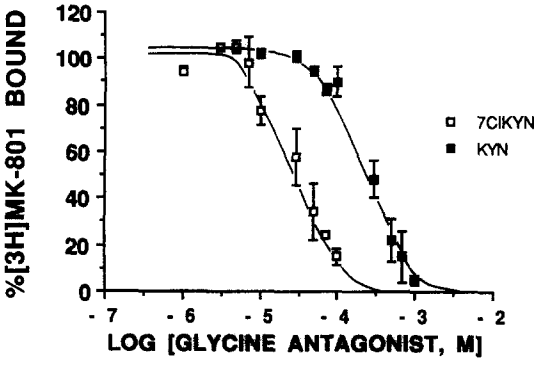

Fig. 5. Inhibition of [^{3}H]MK-801 binding by glycine antagonists in stratum radiatum of the CA1 region of hippocampus. Rat brain sections were incubated in 5 nM [^{3}H]MK-801 in the presence of varying concentrations of 7-chlorokynurenic acid or kynurenic acid. Each point represents mean specific binding ± S.E.M. of four animals. The data are representative of four separate experiments.

(Fig. 6 and Table 1). The Hill numbers for CNQX and DNQX were 2.1 ± 0.2 and 2.1 ± 0.3, respectively. CNQX (100 μM) reduced [^{3}H]MK-801 binding to 17% of control; however, the addition of 10 μM glycine restored [^{3}H]MK-801 binding to 90% of control. Similarly, glycine reversed the inhibition of [^{3}H]MK-801 binding by DNQX.

Zinc ions inhibited [^{3}H]MK-801 binding with a K_i of 88 ± 23 μM and n_H of 1.2 ± 0.3. Tetrahydroaminoacridine (100 μM) inhibited [^{3}H]MK-801 binding by 54% while 100 μM dextromethorphan inhibited binding by 92% in SR-CA1. When tested at 100 μM, (+)PPP and AMPA did not inhibit [^{3}H]MK-801 binding.

If 100 μM NMDA was included in the incubation mixture following a standard prewash, [^{3}H]MK-801 binding was enhanced slightly but not significantly in SR-CA1. When brain sections were prewashed in 50 mM Tris–acetate (pH 7.4) for 30 min at 37°C and then incubated with 5 nM [^{3}H]MK-801 for 2 h in the presence of 100 μM NMDA, binding in SR-CA1 and SMDG was significantly enhanced ($P < 0.004$) by 181% and 214%, respectively. In a separate experiment, the effect of glycine stimulation on [^{3}H]MK-801 binding in brain sections prewashed at 37°C was examined. The addition of 100 nM glycine and 100 μM glycine to the incubation mixture significantly increased [^{3}H]MK-801 binding in the SMDG by 214% and 255%, respectively ($P < 0.05$ and $P < 0.001$).

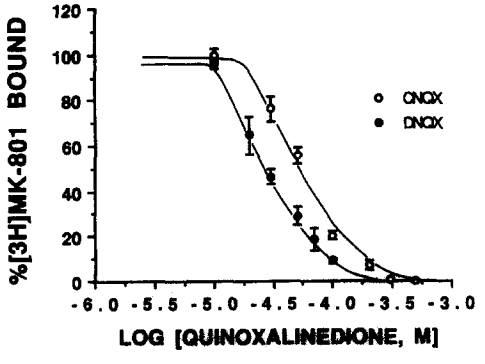

Fig. 6. Inhibition of [^{3}H]MK-801 binding by quinoxalinediones in stratum radiatum of the CA1 region of hippocampus. Rat brain sections were incubated in 5 nM [^{3}H]MK-801 in the presence of varying concentrations of CNQX or DNQX. Each point represents mean specific binding ± S.E.M. of four animals. The data are representative of four separate experiments.

Regional distribution of [^{3}H]*MK*-801 binding sites

[^{3}H]MK-801 binding in rat brain sections displayed regional heterogeneity. The regional distribution of [^{3}H]MK-801 binding is summarized in Table 2, in terms of both absolute amount of bound [^{3}H]MK-801 and the amount bound relative to SR-CA1. Representative autoradiographs of [^{3}H]MK-801 binding in horizontal sections of two levels in rat brain are shown in Fig. 7.

The amount of [^{3}H]MK-801 binding in the forebrain varied widely. A distinct laminar pattern of [^{3}H]MK-801 binding was present in the hippocampal formation. Within the hippocampus, the amount of binding was greatest in the CA1 region followed by CA3 and CA4. SR-CA1 displayed the highest amount of [^{3}H]MK-801 binding in the brain. SMDG also exhibited high amounts of binding relative to the SR-CA1.

Relatively high amounts of binding were present in the medial and lateral olfactory nuclei, primary olfactory cortex and external plexiform layer, while the internal granule layer of the olfactory tract and glomerular layers exhibited the least amount of binding. In the cortical areas, the distribution of [^{3}H]MK-801 binding varied between regions; however, there was more [^{3}H]MK-801 binding to the superficial cortical layers than to the deep layers. The highest amount of [^{3}H]MK-801 binding was present in the cingulate area, whereas the entorhinal region exhibited the least binding. Intermediate levels of binding were present in the frontoparietal and frontal cortical areas.

The basal ganglia also exhibited heterogeneous [^{3}H]MK-801 binding. Highest amounts of binding were present in caudate–putamen and nucleus accumbens, whereas globus pallidus and entopeduncular nucleus exhibited lower amounts. Of the basal forebrain structures, the lateral septum displayed the highest amount of binding while the binding in the ventral limb of the diagonal band and bed nucleus of the stria terminalis did not exceed background levels.

Heterogeneous [^{3}H]MK-801 binding was also observed in the thalamus. Binding in the habenula and nucleus reunions did not exceed background levels. All other thalamic nuclei displayed moderate amounts of binding.

[^{3}H]MK-801 binding was minimal in brainstem structures and cerebellum when the assay was performed using 5 nM [^{3}H]MK-801.

Table 2. Regional distribution of [³H]MK-801 binding to rat brain sections

Brain region	Abbreviation	[³H]MK-801 bound (pmol/mg protein) Mean ($n = 4$)	S.E.M.	Relative to stratum radiatum of CA1 (%)
Olfactory region				
Glomerular layer	GL	0.058	0.001	5
External plexiform layer	EPL	0.360	0.002	33
Internal granule layer	IGL	0.158	0.017	14
Medial anterior olfactory nucleus	Med AON	0.524	0.041	48
Lateral anterior olfactory nucleus	Lat AON	0.539	0.051	49
Primary olfactory cortex	POC	0.498	0.027	45
Cortex				
Entorhinal, layers I and II	1,2 ENT	0.341	0.017	31
Entorhinal, layer IV	4 ENT	0.318	0.016	29
Entorhinal, layers V and VI	5,6 ENT	0.241	0.006	22
Frontoparietal, layers I and II	1,2 FrPa	0.608	0.028	55
Frontoparietal, layer IV	4 FrPa	0.301	0.018	27
Frontoparietal, layers V and VI	5,6 FrPa	0.192	0.011	17
Frontal, layers I and II	1,2 FRCX	0.602	0.031	55
Frontal, layer IV	4 FRCX	0.340	0.016	31
Frontal, layers V and VI	5,6 FRCX	0.137	0.003	12
Cingulate, layers I and II	1,2 CICX	0.644	0.033	59
Cingulate, layers V and VI	5,6 CICX	0.498	0.028	45
Basal ganglia				
Accumbens nucleus	Acb	0.278	0.034	25
Entopeduncular nucleus	EPN	0.006	0.006	1
Caudate–putamen, medial	Med-CPu	0.284	0.011	26
Caudate–putamen, lateral	Lat-CPu	0.227	0.017	21
Caudate–putamen, anterior	Ant-CPu	0.450	0.037	41
Caudate–putamen, posterior	Post-CPu	0.574	0.048	52
Globus pallidus, anterior	Ant-GP	0.038	0.009	3
Globus pallidus, posterior	Post-GP	0.135	0.018	12
Basal forebrain				
Ventral limb diagonal band	VLDB	0.050	0.013	4
Bed nucleus stria terminalis	BNST	0.088	0.016	8
Lateral septum	LS	0.331	0.029	30
Thalamic nuclei				
Medial dorsal	MD-THAL	0.245	0.017	22
Anterior ventral	AV-THAL	0.235	0.019	21
Ventral lateral	VL-THAL	0.220	0.013	20
Ventral posterior medial	VPM-THAL	0.211	0.022	19
Habenula	HB-THAL	0.000	0.000	0
Lateral geniculate	LG	0.266	0.021	24
Medial geniculate	MG	0.250	0.018	23
Reunions	RE	0.030	0.007	3
Hippocampal formation				
CA1, stratum lacunosum moleculare	SLM-CA1	0.614	0.023	56
CA1, stratum radiatum	SR-CA1	1.090	0.045	100
CA1, stratum pyramidale	SP-CA1	0.513	0.017	47
CA1, stratum oriens	SO-CA1	0.946	0.041	86
CA3. stratum radiatum	SR-CA3	0.606	0.028	55
CA3, stratum pyramidale	SP-CA3	0.189	0.008	17
CA3, stratum oriens	SO-CA3	0.494	0.034	45
CA4	CA4	0.199	0.011	18
Dentate gyrus, stratum moleculare	SMDG	0.792	0.028	72
Brainstem				
Central gray	CG	0.000	0.000	0
Inferior colliculus	IC	0.011	0.001	1
Substantia nigra	SNR	0.019	0.011	2
Cerebellum				
Molecular layer	Mol-Cb	0.000	0.000	0
Granule layer	Gr-Cb	0.000	0.000	0

Data represent mean ± S.E.M. of four animals. Sections were incubated in 5 nM [³H]MK-801 for 2 h at 25°C and then rinsed for 80 min in 50 mM Tris–acetate buffer (pH 7.4 at 4°C). Quantification of autoradiograms was performed as described in the text.

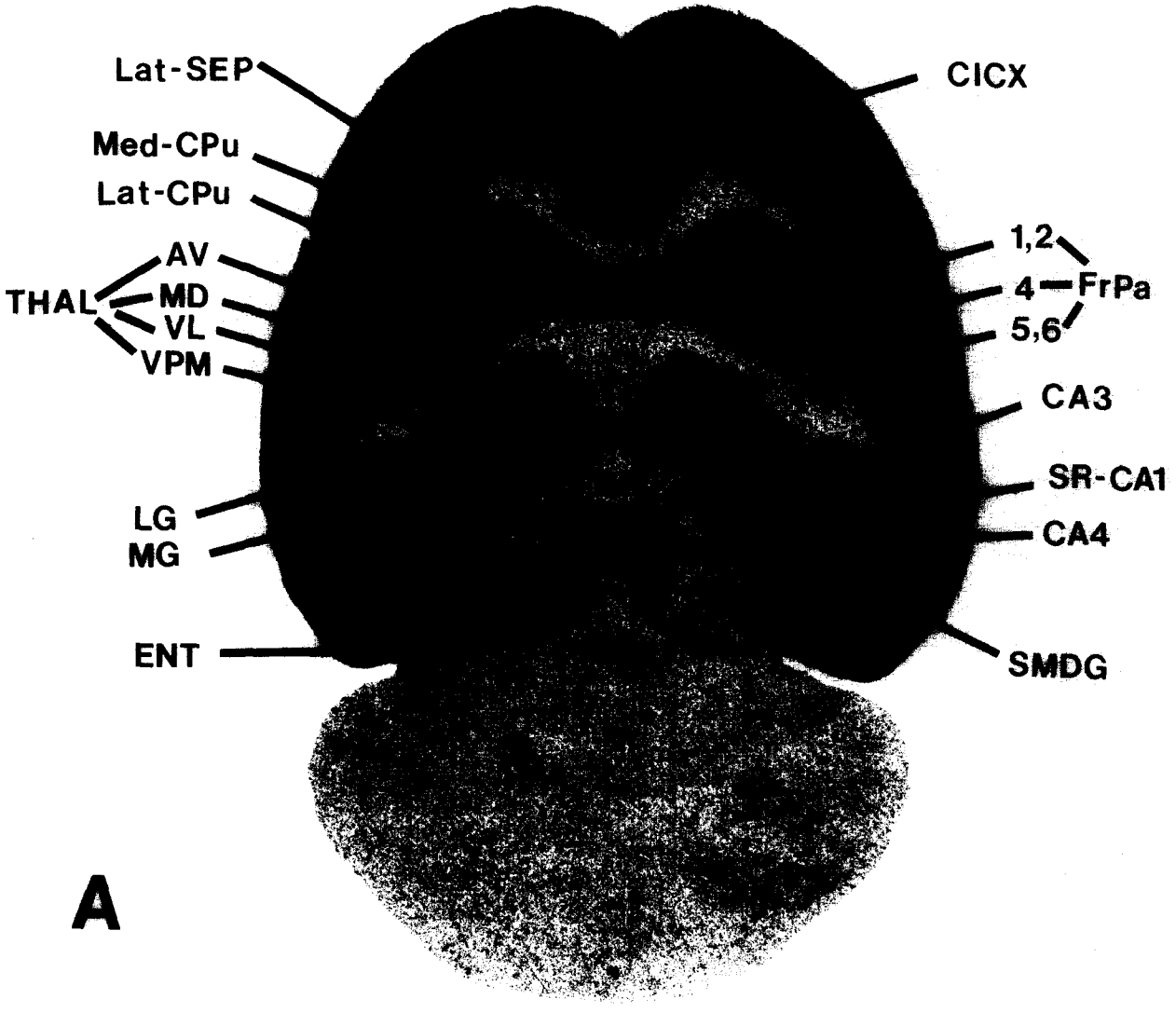

Fig. 7A.

DISCUSSION

We have characterized a quantitative autoradiographic assay of [^{3}H]MK-801 binding to rat brain tissue sections. The biochemical characteristics of this binding have similarities to those expected for membrane receptor binding sites; i.e., a pH optimum of approximately 7.4 in Tris–acetate buffer and a temperature optimum at 23°C. Although many receptor binding sites have a temperature optimum at 4°C, as discussed below, this ligand has a slow association and dissociation with its binding site. Such temperature optima have been observed for other high affinity ligands such as [^{3}H]spiroperidol and [^{3}H]quinuclidinylbenzilate.[39,46]

Scatchard analyses of [^{3}H]MK-801 saturation studies indicated a single population of high affinity binding sites with a Hill coefficient of 1. Previous studies in homogenates, however, have suggested the presence of both high and low affinity [^{3}H]MK-801 binding sites and [^{3}H]TCP binding sites.[17,24,53] Our assay conditions, however, were unfavorable for observing a low affinity binding sites because of the long rinse times and the emphasis on MK-801 concentrations below 50 nM in the saturation studies. Additional points at higher concentrations of [^{3}H]MK-801 and the use of shorter rinse times would be necessary to accurately assess a potential low affinity site. Interestingly, at 5 nM [^{3}H]MK-801, no binding was observed in the cerebellum; however, at 20 nM [^{3}H]MK-801, binding could be observed in the cerebellar granule cell layer, suggesting the possibility that a low affinity site might exist in this region (unpublished observations).

In homogenate preparations, kinetic studies of [^{3}H]TCP and [^{3}H]MK-801 binding have revealed very slow rates of association and dissociation.[23,27,28] These kinetic constants are influenced by the concentration of glutamate, glycine and divalent cations present during the incubation.[42,45,56] In our studies, [^{3}H]MK-

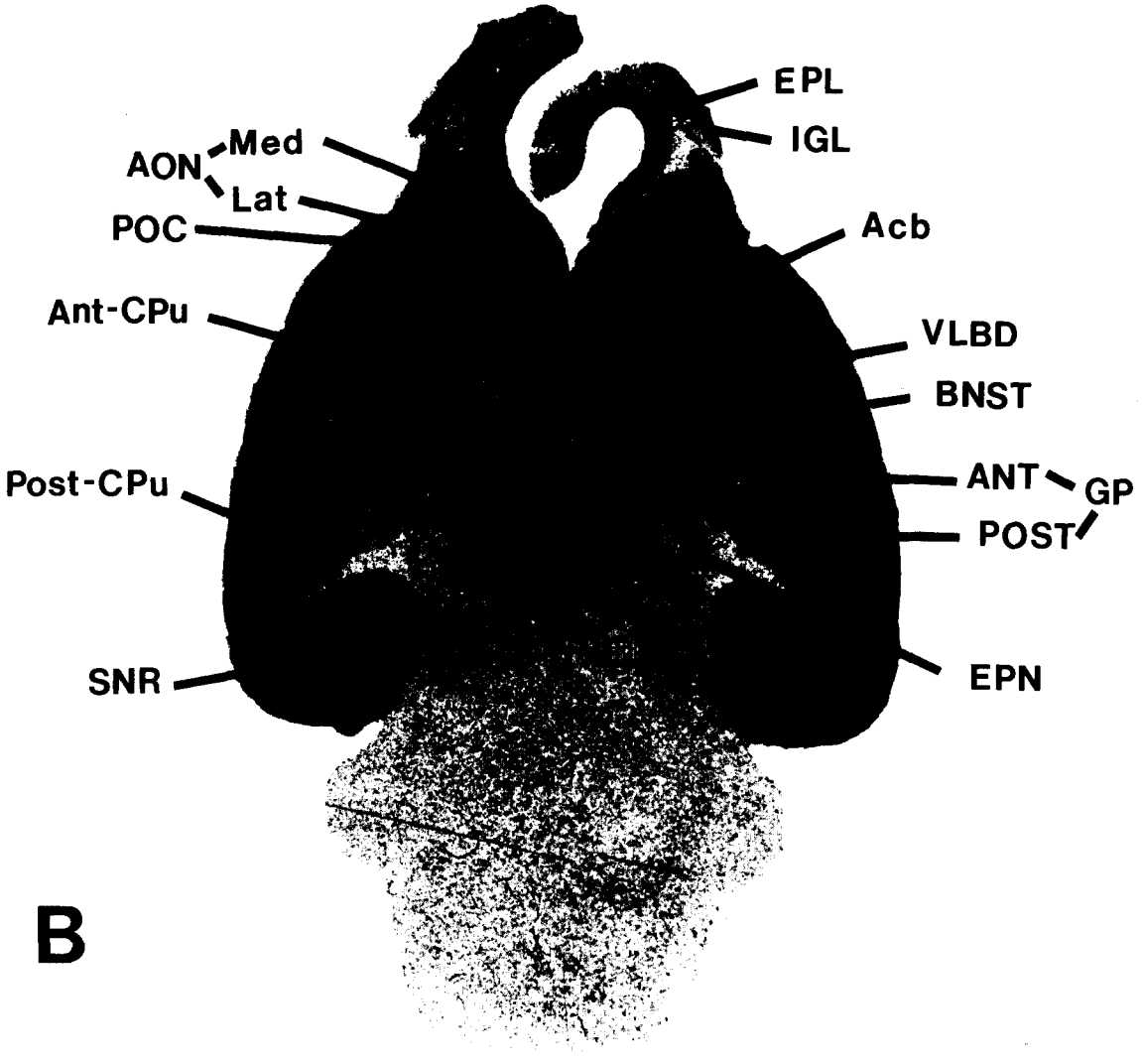

Fig. 7B.

Fig. 7. Autoradiographs of [³H]MK-801 binding to horizontal sections of rat brain (A, dorsal section; B, ventral section). Sections were incubated in 5 nM [³H]MK-801. For abbreviations see Table 2.

801 binding was found to associate very slowly, reaching equilibration by 2 h at room temperature. Dissociation experiments with tissue sections placed in "infinite dilution" and in the absence of added glutamate and glycine revealed very slow dissociation rates. Both association and dissociation curves fit a single site kinetic model. Considerably more data points would be necessary, however, to clearly distinguish two sites. Since such detailed studies have already been carried out in homogenates,[3a,23,27] it did not seem to be productive to repeat them using autoradiographic techniques. Calculation of the equilibrium dissociation constant, K_d, from the dissociation and association rate constants revealed K_d values less than 1 nM, which did not agree with the K_d values calculated from equilibrium saturation studies. The association rate constants observed in these experiments were similar to those observed by Javitt and Zukin[23] and Kloog *et al.*[27] in the presence of glutamate and glycine. The dissociation rates observed in our studies are considerably slower than those observed in the presence of glutamate and glycine in homogenate studies and probably reflect the fact that the dissociation of [³H]MK-801 and [³H]TCP is potently affected by the concentrations of glutamate and glycine in the dissociation buffer. In fact, our dissociation rates determined without glutamate or glycine in the rinse buffer were similar to those determined in homogenate studies in the absence of glutamate and glycine.[27] Thus, in routine quantitative autoradiography, association rates were fast because of the presence of endogenous glutamate and glycine and dissociation rates were slow because of their absence, leading to a calculated $K_d = k_{-1}/k_{+1}$ that is smaller than the equilibrium K_d. Association rates could be determined at several different ligand concentrations and the K_d determined directly from the data (see Javitt and Zukin[23]). Alternatively,

dissociation experiments in the presence of known concentrations of glutamate and glycine could be carried out. These experiments are currently being carried out in our laboratory.

The pharmacology of [^{3}H]MK-801 binding in rat brain sections is similar to that described in homogenate binding studies of the PCP site. MK-801 and TCP displaced [^{3}H]MK-801 binding with high affinity and with Hill coefficients of 1. [^{3}H]MK-801 binding is also inhibited by dextromethorphan which has been shown to interact with [^{3}H]TCP binding and which has been found to attentuate NMDA-induced neurotoxicity in cortical cell cultures.[6,15,48] In contrast, the sigma site compound, (+)PPP,[31] had no effect on [^{3}H]MK-801 binding. These data are consistent with the studies suggesting [^{3}H]MK-801 is a highly selective ligand for the channel associated with the NMDA receptor. Tetrahydroaminoacridine, a cholinesterase inhibitor reported to displace [^{3}H]TCP binding from rat brain membranes[1] and to selectively reduce NMDA receptor-mediated toxicity,[12] also inhibited [^{3}H]MK-801 binding.

The cation channel associated with the NMDA receptor is modulated by glutamate and glycine.[25,42,52] The presence of both compounds is necessary for optimal binding of the ligands to the PCP receptor and for optimal activation of the NMDA receptor complex as measured physiologically. In extensively washed membrane preparations, ligand binding to the PCP site is extremely slow. Both glutamate and glycine enhance binding of ligands to the PCP site and the two amino acids together appear to act synergistically.[3,45,50,51,56] In *Xenopus* oocyte preparations, glycine has been demonstrated to be necessary for the activation of the NMDA receptor complex by glutamate agonists.[35] Following our standard prewash, incubation with exogenously added NMDA or glycine did not enhance [^{3}H]MK-801 binding. It is likely that the concentrations of glutamate and glycine remaining in the tissue sections following a standard prewash were sufficient to fully activate the NMDA receptor–ion channel complex and permit binding of [^{3}H]MK-801. Prewashing the rat brain sections for 30 min at 37°C did apparently remove substantial amounts of endogenous amino acids because under these conditions [^{3}H]MK-801 binding following a 2-h incubation was stimulated significantly with the addition of exogenous amino acids. Detailed kinetic experiments to address the effects of glutamate and glycine on [^{3}H]MK-801 binding were not performed; however, glutamate and glycine stimulation of [^{3}H]MK-801 binding in tissue sections incubated at non-equilibrium conditions (a 10-min period) was much greater than following a 2-h incubation (data not shown). This observation is consistent with a report by Hosford *et al.*,[22a] which demonstrated that [^{3}H]TCP binding to rat brain sections was enhanced by glutamate and glycine at non-equilibrium conditions. Glutamate and glycine could be stimulating [^{3}H]MK-801 binding by increasing the affinity of [^{3}H]MK-801 for its binding site[14a,45] or the presence of these agonists could increase the accessibility of [^{3}H]MK-801 to its binding site by increasing the channel open time.[3a] Detailed kinetic studies using tissue sections that are subjected to rigorous prewashing (30 min in buffer at 37°C) would need to be performed to address the mechanism by which glutamate and glycine stimulate [^{3}H]MK-801 binding; however, the anatomical integrity of the sections is decreased with excessive prewashing and the degree of stimulation of [^{3}H]MK-801 binding is variable following such prewashes. Furthermore, because of the known regional variation in the endogenous glutamate and glycine concentrations in brain it is unlikely that reproducible methods will become available soon to adequately remove endogenous ligands from tissue sections for autoradiography. Thus, homogenate preparations may be more suitable for such studies.

NMDA and glycine antagonists, however, have potent effects on [^{3}H]MK-801 binding. The competitive NMDA antagonists CPP and CGS 19755 essentially eliminated all [^{3}H]MK-801 binding at concentrations of 100 μM. AP5 and AP7 had similar effects on [^{3}H]MK-801 binding. NMDA added back to the incubation buffer in the presence of CPP reversed this inhibition. CPP and CGS 19755 inhibited [^{3}H]MK-801 binding in a non-competitive manner; therefore only apparent K_i values can be calculated. Interestingly, the Hill coefficients for CPP and CGS 19755 inhibition of [^{3}H]MK-801 binding were significantly greater than 1, suggesting a positive allosteric interaction of these compounds with the [^{3}H]MK-801 binding site. Alternatively, these high Hill coefficients could reflect the fact that CPP and CGS 19755 slow the association rate of MK-801 binding and may therefore raise the apparent Hill coefficient. This effect of glutamate and glycine antagonists on association rate may also have influenced the calculation of their apparent K_i values. The 2-h incubation period used in our assay is longer than previously reported autoradiographic studies of [^{3}H]MK-801 binding;[4,5] thus the apparent K_i values calculated for the NMDA antagonists may appear less potent than K_i values obtained from [^{3}H]MK-801 binding performed at shorter incubation periods or directly from NMDA-sensitive [^{3}H]glutamate assays. Glycine antagonists kynurenic acid and 7-chlorokynurenic acid also potently inhibited [^{3}H]-MK-801 binding and virtually eliminated measurable binding. Glycine reversed this inhibition of [^{3}H]MK-801 binding. As with the glutamate antagonists, the Hill coefficients for kynurenic and 7-chlorokynurenic acid inhibition of [^{3}H]MK-801 binding were significantly greater than 1, suggesting again a positive allosteric interaction with the [^{3}H]MK-801 binding site. The rank order potency of both the NMDA and glycine antagonists are identical to those observed in previous homogenate studies.[10,49] In homogenate preparations, HA-966 produced only a partial decrease of [^{3}H]MK-801 binding.[9] Interestingly, in our studies HA-966 did not have any effect

on [^{3}H]MK-801 binding to rat brain tissue sections. This would be consistent with the recent report by Dansyz *et al.*[9] that suggested that HA-966 modulates the NMDA receptor complex differently than 7-chlorokynurenic acid. It is also possible that HA-966 only acts at a subset of glycine sites that is not measured in this assay.

The non-NMDA receptor agonist, AMPA,[29,30] had no effect on [^{3}H]MK-801 binding. The quinoxaline derivatives CNQX and DNQX, recently described as AMPA receptor antagonists,[14,22] however, did inhibit [^{3}H]MK-801 binding in our autoradiographic assay. CNQX and DNQX have been shown in prior studies to interact with the glycine site at high concentrations[20,26,33] and are likely to inhibit [^{3}H]MK-801 binding by interacting with that site. Consistent with this notion is the fact that glycine reverses CNQX antagonism of [^{3}H]MK-801 binding. As with the other NMDA and glycine antagonists, the Hill coefficients for CNQX and DNQX inhibition for [^{3}H]MK-801 binding were greater than 1, suggesting a positive allosteric interaction with the [^{3}H]MK-801 site.

In addition to regulation by glutamate and glycine, the NMDA receptor–ion channel activity appears to be modulated by zinc ions. In homogenate studies, zinc inhibited [^{3}H]MK-801 binding.[43,44] Furthermore, NMDA-evoked responses are antagonized by zinc.[7,41,54] At concentrations consistent with these physiological and biochemical studies, zinc inhibited [^{3}H]MK-801 binding in this assay with a Hill coefficient of 1.

[^{3}H]MK-801 binding was heterogeneously distributed throughout the rat brain with the following order of binding densities: hippocampal formation > cortical areas > striatum > thalamus. Interestingly, the brainstem and cerebellum did not reveal [^{3}H]MK-801 binding above background levels when assayed in the presence of 5 nM [^{3}H]MK-801. As discussed above, it is possible that a low affinity binding site in these areas may be present but is not detected using standard assay conditions. In fact, [^{3}H]TCP binding studies in membrane preparations have revealed the presence of low affinity PCP binding sites in the cerebellum.[17,18,53] Furthermore, in studies of rat cerebellar slices, an allosteric interaction between PCP binding sites and NMDA receptors has been reported.[11, 58,59]

CONCLUSION

The regional distribution of [^{3}H]MK-801 binding sites in rat brain sections reported here is similar to the pattern of NMDA receptors labeled with [^{3}H]glutamate or (±)[^{3}H]CPP.[16,38] There is a high degree of correlation between the regional binding densities of [^{3}H]MK-801 binding and strychnine-insensitive [^{3}H]-glycine binding in rat brain sections (S. Y. Sakurai and J. W. McDonald, unpublished observations). Furthermore, the distribution of [^{3}H]MK-801 binding and [^{3}H]TCP binding in rat brain sections is also highly correlated ($r^2 = 0.96$, data taken from Maragos *et al.*[34]). Thus, the [^{3}H]MK-801 binding to rat brain sections described in this paper appears to be labeling the NMDA receptor–ion channel complex.

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