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

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Abstract—[3H]MK-801 binding in rat brain was characterized using a quantitative autoradiographic binding assay. [3H]MK-801 binding (5 nM) reached equilibrium by 120 min at 23°C. [3H]MK-801 appeared to label a single high affinity site with an affinity constant of approximately 11 nM. [3H]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, 3-(2-amino-5-phosphonopentanoic acid, NMDA receptor activation. 19 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, and cis-4-phosphonomethyl-2-piperidine carboxylic acid, inhibited [3H]MK-801 binding. Glycine antagonists, 7-chlorokynurenic acid and kynurenic acid, also inhibited [3H]MK-801 binding. Furthermore, the inhibition of [3H]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. [3H]MK-801 binding was also inhibited by zinc ions. [3H]MK-801 binding was enhanced by glycine or N-methyl-D-aspartate receptor complex.

These results demonstrate that [3H]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). The NMDA receptor is associated with a cation channel that is gated by magnesium in a voltage-dependent fashion. ° The receptor-ion channel complex is modulated by several regulatory sites. Glycine, acting at a strychnine-insensitive site, increases NMDA receptor activation. 19 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 receptor 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 dizocilpine maleate), are non-competitive antagonists of the NMDA receptor. 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. 42,44,55,57 Although autoradiographic binding studies with [3H]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 [3H]MK-801 to tissue sections. 4,5 We have characterized in detail a quantitative autoradiographic binding assay for the PCP receptor using [3H]MK-801 in rat brain.

EXPERIMENTAL PROCEDURES

[3H]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. [3H]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
under a stream of room temperature air before performing the [H]MK-801 binding assay. In all [H]MK-801 binding experiments, triplicate tissue sections were incubated in 50 mM Tris-acetate buffer (pH 7.4 at room temperature) containing 5 nM [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 (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, [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 [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 [H]MK-801 for 11 times 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 [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 [H]MK-801 ranging from 1 to 20 nM. For the higher concentrations, sections were incubated for 120 min in 20 nM [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 [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 exposed to either LKB Ultrfilm 3H or Amersham Hyperfilm. Following a 3-4 week exposure at 4°C, films were developed in D-19 (Kodak), fixed in buffer at 37°C (pH 7.4) and [3H]MK-801 binding performed in the incubation mixture. In separate experiments, tissue sections were incubated for 120 min in 50 mM Tris-acetate buffer (pH 7.4 at room temperature) containing 5 nM [3H]MK-801 at 4°C, 23°C and 37°C. [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 [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, [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 [H]MK-801 labeled an apparent single high affinity site. The maximal binding (Bma x) of [H]MK-801 in layers I and II of frontal cortex was 2.53 ± 0.14 pmol/mg protein and the affinity (Kd) was 11.6 ± 0.5 nM (Fig. 1). Bma x and similar Kd values were obtained for SR-CA1 and stratum moleculare of the dentate gyrus (SMDG). In SR-CA1, the Kd for [H]MK-801 binding was 14.3 ± 0.9 nM and the Bma x was 4.0 ± 0.2 pmol/mg protein, while in the SMDG the Kd was 13.4 ± 1.4 nM and Bma x was 3.1 ± 0.2 nM.

In 50 mM Tris-acetate buffer 5 nM [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 presence of 20 μM MK-801) varied significantly between regions.

### Characterization of [H]MK-801 Binding

In initial experiments, [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, [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 [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, [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.

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### Results

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[\textsuperscript{3}H]MK-801 binding sites in rat brain

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK-801</td>
<td>7.3 ± 0.8</td>
<td>0.98 ± 0.06</td>
<td>3</td>
</tr>
<tr>
<td>TCP</td>
<td>55 ± 1.8</td>
<td>0.85 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>CPP</td>
<td>7.0 ± 0.8</td>
<td>1.70 ± 0.36</td>
<td>3</td>
</tr>
<tr>
<td>CGS 19755</td>
<td>3.7 ± 0.3</td>
<td>1.4 ± 0.22</td>
<td>3</td>
</tr>
<tr>
<td>7-Chlorokynurenic acid</td>
<td>22.7 ± 6.0</td>
<td>1.4 ± 0.13</td>
<td>3</td>
</tr>
<tr>
<td>Kynurenic acid</td>
<td>206 ± 54</td>
<td>1.9 ± 0.34</td>
<td>4</td>
</tr>
<tr>
<td>CNQX</td>
<td>40 ± 3.2</td>
<td>2.1 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>DNQX</td>
<td>22 ± 2.6</td>
<td>2.1 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>Zinc</td>
<td>88 ± 23</td>
<td>1.2 ± 0.3</td>
<td>3</td>
</tr>
</tbody>
</table>

Tissue sections were incubated with 5 nM [\textsuperscript{3}H]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.\textsuperscript{a}

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 [\textsuperscript{3}H]MK-801 binding. In SR-CA1, CPP inhibited [\textsuperscript{3}H]MK-801 binding with an apparent $K_i$ of 7.0 ± 0.8 nM (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 [\textsuperscript{3}H]MK-801 binding by 80% and 82%, respectively. The inhibition of [\textsuperscript{3}H]MK-801 binding by CPP (10 μM) was reversed when 100 μM NMDA was included in the incubation mixture.

[\textsuperscript{3}H]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 [\textsuperscript{3}H]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 [\textsuperscript{3}H]MK-801 binding in SR-CA1. Kynurenic acid inhibited [\textsuperscript{3}H]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 [\textsuperscript{3}H]MK-801 binding in SR-CA1 with $K_i$ values of 40 ± 3.2 and 22 ± 2.6 μM, respectively.

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\textsuperscript{a} Tissue sections were incubated with 5 nM [\textsuperscript{3}H]MK-801 in the presence of the appropriate compound for 2 h at 25°C and then 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.
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.

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

If 100 \(\mu\)M 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 \(\mu\)M 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 \(\mu\)M glycine to the incubation mixture significantly increased \[^{3}H\]MK-801 binding in the SMDG by 214% and 255%, respectively (\(P < 0.005\) and \(P < 0.001\)).

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 reuniens 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

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Abbreviation</th>
<th>Mean (n = 4)</th>
<th>S.E.M.</th>
<th>Relative to stratum radiatum of CA1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory region</td>
<td></td>
<td></td>
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<tr>
<td>Glomerular layer</td>
<td>GL</td>
<td>0.058</td>
<td>0.001</td>
<td>5</td>
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<tr>
<td>External plexiform layer</td>
<td>EPL</td>
<td>0.360</td>
<td>0.002</td>
<td>33</td>
</tr>
<tr>
<td>Internal granule layer</td>
<td>IGL</td>
<td>0.158</td>
<td>0.017</td>
<td>14</td>
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<tr>
<td>Medial anterior olfactory nucleus</td>
<td>Med AON</td>
<td>0.524</td>
<td>0.041</td>
<td>48</td>
</tr>
<tr>
<td>Lateral anterior olfactory nucleus</td>
<td>Lat AON</td>
<td>0.539</td>
<td>0.051</td>
<td>49</td>
</tr>
<tr>
<td>Primary olfactory cortex</td>
<td>POC</td>
<td>0.498</td>
<td>0.027</td>
<td>45</td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Entorhinal, layers I and II</td>
<td>1,2 ENT</td>
<td>0.341</td>
<td>0.017</td>
<td>31</td>
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<tr>
<td>Entorhinal, layer IV</td>
<td>4 ENT</td>
<td>0.318</td>
<td>0.016</td>
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<td>Entorhinal, layers V and VI</td>
<td>5,6 ENT</td>
<td>0.241</td>
<td>0.006</td>
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<td>Frontoparietal, layers I and II</td>
<td>1,2 FrPa</td>
<td>0.608</td>
<td>0.028</td>
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<tr>
<td>Frontoparietal, layer IV</td>
<td>4 FrPa</td>
<td>0.301</td>
<td>0.018</td>
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<tr>
<td>Frontoparietal, layers V and VI</td>
<td>5,6 FrPa</td>
<td>0.192</td>
<td>0.011</td>
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<tr>
<td>Frontal, layers I and II</td>
<td>1,2 FRCCX</td>
<td>0.602</td>
<td>0.031</td>
<td>55</td>
</tr>
<tr>
<td>Frontal, layer IV</td>
<td>4 FRCCX</td>
<td>0.340</td>
<td>0.016</td>
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<tr>
<td>Frontal, layers V and VI</td>
<td>5,6 FRCCX</td>
<td>0.137</td>
<td>0.003</td>
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<td>Cingulate, layers I and II</td>
<td>1,2 CICX</td>
<td>0.644</td>
<td>0.033</td>
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<tr>
<td>Cingulate, layers V and VI</td>
<td>5,6 CICX</td>
<td>0.498</td>
<td>0.028</td>
<td>45</td>
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<tr>
<td>Basal ganglia</td>
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<tr>
<td>Accumbens nucleus</td>
<td>Acb</td>
<td>0.278</td>
<td>0.034</td>
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<tr>
<td>Entopeduncular nucleus</td>
<td>EPN</td>
<td>0.006</td>
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<tr>
<td>Caudate–putamen, medial</td>
<td>Med-CPu</td>
<td>0.264</td>
<td>0.011</td>
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<td>Caudate–putamen, lateral</td>
<td>Lat-CPu</td>
<td>0.227</td>
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<tr>
<td>Caudate–putamen, anterior</td>
<td>Ant-CPu</td>
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<tr>
<td>Caudate–putamen, posterior</td>
<td>Post-CPu</td>
<td>0.574</td>
<td>0.048</td>
<td>52</td>
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<tr>
<td>Globus pallidus, anterior</td>
<td>Ant-GP</td>
<td>0.038</td>
<td>0.009</td>
<td>3</td>
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<tr>
<td>Globus pallidus, posterior</td>
<td>Post-GP</td>
<td>0.135</td>
<td>0.018</td>
<td>12</td>
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<tr>
<td>Basal forebrain</td>
<td></td>
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<tr>
<td>Ventral limb diagonal band</td>
<td>VLDB</td>
<td>0.050</td>
<td>0.013</td>
<td>4</td>
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<tr>
<td>Bed nucleus stria terminalis</td>
<td>BNST</td>
<td>0.088</td>
<td>0.016</td>
<td>8</td>
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<tr>
<td>Lateral septum</td>
<td>LS</td>
<td>0.331</td>
<td>0.029</td>
<td>30</td>
</tr>
<tr>
<td>Thalamic nuclei</td>
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<tr>
<td>Medial dorsal</td>
<td>MD-THAL</td>
<td>0.245</td>
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<tr>
<td>Anterior ventral</td>
<td>AV-THAL</td>
<td>0.235</td>
<td>0.019</td>
<td>21</td>
</tr>
<tr>
<td>Ventral lateral</td>
<td>VL-THAL</td>
<td>0.220</td>
<td>0.013</td>
<td>20</td>
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<tr>
<td>Ventral posterior medial</td>
<td>VPM-THAL</td>
<td>0.211</td>
<td>0.022</td>
<td>19</td>
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<tr>
<td>Habenula</td>
<td>HB-THAL</td>
<td>0.000</td>
<td>0.000</td>
<td>0</td>
</tr>
<tr>
<td>Lateral geniculate</td>
<td>LG</td>
<td>0.265</td>
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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.
DISCUSSION

We have characterized a quantitative autoradiographic assay of \(^{3H}\)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 \(^{3H}\)spiroperidol and \(^{3H}\)quinuclidinylbenzilate.

Scatchard analyses of \(^{3H}\)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 \(^{3H}\)MK-801 binding sites and \(^{3H}\)TCP binding sites. 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 \(^{3H}\)MK-801 and the use of shorter rinse times would be necessary to accurately assess a potential low affinity site. Interestingly, at 5 nM \(^{3H}\)MK-801, no binding was observed in the cerebellum; however, at 20 nM \(^{3H}\)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 \(^{3H}\)TCP and \(^{3H}\)MK-801 binding have revealed very slow rates of association and dissociation. These kinetic constants are influenced by the concentration of glutamate, glycine and divalent cations present during the incubation. In our studies, \(^{3H}\)MK-
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, 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 and Kloog et al. 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. 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). 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 \([\text{H}]\text{MK-801}\) binding in rat brain sections is similar to that described in homogenate binding studies of the PCP site. MK-801 and TCP displaced \([\text{H}]\text{MK-801}\) binding with high affinity and with Hill coefficients of 1. \([\text{H}]\text{MK-801}\) binding is also inhibited by dextromethorphan which has been shown to interact with \([\text{H}]\text{TCP}\) binding and which has been found to attenuate NMDA-induced neurotoxicity in cortical cell cultures. In contrast, the sigma site compound, (+)PPP, had no effect on \([\text{H}]\text{MK-801}\) binding. These data are consistent with the studies suggesting \([\text{H}]\text{MK-801}\) is a highly selective ligand for the channel associated with the NMDA receptor. Tetrahydroaminoacridine, a cholinesterase inhibitor reported to displace \([\text{H}]\text{TCP}\) binding from rat brain membranes and to selectively reduce NMDA receptor-mediated toxicity, also inhibited \([\text{H}]\text{MK-801}\) binding.

The cation channel associated with the NMDA receptor is modulated by glutamate and glycine. Following our standard prewash, incubation with exogenously added NMDA or glycine did not enhance \([\text{H}]\text{MK-801}\) 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 \([\text{H}]\text{MK-801}\). Prewashing the rat brain sections for 30 min at 37°C did apparently remove substantial amounts of endogenous amino acids because under these conditions \([\text{H}]\text{MK-801}\) 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 \([\text{H}]\text{MK-801}\) binding were performed; however, glutamate and glycine stimulation of \([\text{H}]\text{MK-801}\) 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., which demonstrated that \([\text{H}]\text{TCP}\) binding to rat brain sections was enhanced by glutamate and glycine at non-equilibrium conditions. Glutamate and glycine could be stimulating \([\text{H}]\text{MK-801}\) binding by increasing the affinity of \([\text{H}]\text{MK-801}\) for its binding site or the presence of these agonists could increase the accessibility of \([\text{H}]\text{MK-801}\) to its binding site by increasing the channel open time. 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 \([\text{H}]\text{MK-801}\) binding; however, the anatomical integrity of the sections is decreased with excessive prewashing and the degree of stimulation of \([\text{H}]\text{MK-801}\) 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 \([\text{H}]\text{MK-801}\) binding. The competitive NMDA antagonists CPP and CGS 19755 essentially eliminated all \([\text{H}]\text{MK-801}\) binding at concentrations of 100 μM. AP5 and AP7 had similar effects on \([\text{H}]\text{MK-801}\) binding. NMDA added back to the incubation buffer in the presence of CPP reversed this inhibition. CPP and CGS 19755 inhibited \([\text{H}]\text{MK-801}\) binding in a non-competitive manner, therefore only apparent Ki values can be calculated. Interestingly, the Hill coefficients for CPP and CGS 19755 inhibition of \([\text{H}]\text{MK-801}\) binding were significantly greater than 1, suggesting a positive allosteric interaction of these compounds with the \([\text{H}]\text{MK-801}\) 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 Ki values. The 2-h incubation period used in our assay is longer than previously reported autoradiographic studies of \([\text{H}]\text{MK-801}\) binding, thus the apparent Ki values calculated for the NMDA antagonists may appear less potent than Ki values obtained from \([\text{H}]\text{MK-801}\) binding performed at shorter incubation periods or directly from NMDA-sensitive \([\text{H}]\text{glutamate}\) assays. Glycine antagonists kynurenic acid and 7-chlorokynurenic acid also potently inhibited \([\text{H}]\text{MK-801}\) binding and virtually eliminated measurable binding. Glycine reversed this inhibition of \([\text{H}]\text{MK-801}\) binding. As with the glutamate antagonists, the Hill coefficients for kynurenic and 7-chlorokynurenic acid inhibition of \([\text{H}]\text{MK-801}\) binding were significantly greater than 1, suggesting again a positive allosteric interaction with the \([\text{H}]\text{MK-801}\) binding site. The rank order potency of both the NMDA and glycine antagonists are identical to those observed in previous homogenate studies. In homogenate preparations, HA-966 produced only a partial decrease of \([\text{H}]\text{MK-801}\) binding. Interestingly, in our studies HA-966 did not have any effect.
on [3H]MK-801 binding to rat brain tissue sections. This would be consistent with the recent report by Danszy et al.\(^1\) 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 [3H]MK-801 binding. The quinoxaline derivatives CNQX and DNQX, recently described as AMPA receptor antagonists,\(^14,22\) however, did inhibit [3H]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 [3H]MK-801 binding by interacting with that site. Consistent with this notion is the fact that glycine reverses CNQX antagonism of [3H]MK-801 binding. As with the other NMDA and glycine antagonists, the Hill coefficients for [3H]MK-801 binding were greater than 1, suggesting a positive allosteric interaction with the [3H]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 [3H]MK-801 binding.\(^5,44\) Furthermore, NMDA-evoked responses are antagonized by zinc\(^7,41,54\). At concentrations consistent with these physiological and biochemical studies, zinc inhibited [3H]MK-801 binding in this assay with a Hill coefficient of 1.

[3H]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 [3H]MK-801 binding above background levels when assayed in the presence of 5 nM [3H]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, [3H]TCP binding studies in membrane preparations have revealed the presence of low affinity PCP binding sites in the cerebellum.\(^7,18,33\) Furthermore, in studies of rat cerebellar slices, an allosteric interaction between PCP binding sites and NMDA receptors has been reported.\(^11,38,39\)

**CONCLUSION**

The regional distribution of [3H]MK-801 binding sites in rat brain sections reported here is similar to the pattern of NMDA receptors labeled with [3H]glutamate or [3H]CPP.\(^16,38\) There is a high degree of correlation between the regional binding densities of [3H]MK-801 binding and strychnine-insensitive [3H]glycine binding in rat brain sections (S. Y. Sakurai and J. W. McDonald, unpublished observations). Furthermore, the distribution of [3H]MK-801 binding and [3H]TCP binding in rat brain sections is also highly correlated \((r^2 = 0.96, \text{data taken from Maragos et al.})^4\). Thus, the [3H]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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**REFERENCES**


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