Effects of Hypoxia-Ischemia and MK-801 Treatment on the Binding of a Phencyclidine Analogue in the Developing Rat Brain

Faye S. Silverstein, MD, John W. McDonald III, BS, Michael Bommarito, BS, and Michael V. Johnston, MD

The phencyclidine analogue [3H](1-[2-thienyl]cyclohexyl)piperidine (3H-TCP) binds to the ion channel associated with the N-methyl-D-aspartate receptor channel complex. In vitro autoradiography indicates that the distribution of 3H-TCP binding in brain closely parallels that of [3H]glutamate binding to the N-methyl-D-aspartate receptor. In nine 7-day-old rats, an acute focal hypoxic-ischemic insult produced by unilateral carotid artery ligation and subsequent exposure to 8% oxygen acutely reduced 3H-TCP binding ipsilateral to the ligation by 30% in the CA1, by 27% in the CA3, by 26% in the dentate gyrus, and by 17% in the striatum compared with values from the contralateral hemisphere. In 10 littermates that received 1 mg/kg of the neuroprotective noncompetitive N-methyl-D-aspartate antagonist MK-801 immediately before hypoxic exposure, the regional distribution of 3H-TCP binding in hypoxic-ischemic brain was relatively preserved and there were no interhemispheric asymmetries in 3H-TCP binding densities. In addition, in three unoperated rats decapitated 24 hours after MK-801 treatment, 3H-TCP binding was reduced by 15-35%; similar bilateral suppression of 3H-TCP binding was detected in MK-801-treated ligates. Our data indicate that 3H-TCP autoradiography can be used to assay the efficacy of neuroprotective agents in this experimental model of perinatal stroke. (Stroke 1990;21:310-315)

A large body of experimental evidence indicates that overactivation of the N-methyl-D-aspartate (NMDA)-type glutamate receptor contributes to the pathogenesis of hypoxic-ischemic neuronal injury. The NMDA receptor channel complex includes a glutamate recognition site, an associated cation channel, and additional regulatory sites. The dissociative anesthetic ketamine, phencyclidine, and the closely related drug MK-801 all selectively antagonize NMDA's actions in a noncompetitive fashion. Administration of both competitive and noncompetitive antagonists of NMDA can attenuate hypoxia-ischemia-induced neuronal injury in vivo.

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Supported by United States Public Health Service grants NS 01171 and 26142 (to F.S.S.), NS 19613 (to M.V.J.), and Medical Science Training Program grant ST32 6 MO7863-07 (to J.W.M.). MK-801 was provided by Merck Sharp & Dohme, West Point, Pennsylvania.

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Received July 14, 1989; accepted September 19, 1989.

Recent data indicate that these pathophysiologic mechanisms are also relevant in immature brain. In 7-day-old rat pups, unilateral carotid artery ligation followed by 2-3 hours of exposure to 8% oxygen (the balance nitrogen) results in ipsilateral forebrain injury; the striatum, hippocampus, and overlying cortex are damaged. Early after injury, the distribution of [3H]glutamate binding is disrupted; 24 hours after the ligation, [3H]glutamate binding is selectively reduced in brain regions destined for irreversible injury. In this experimental model, pretreatment with the noncompetitive NMDA antagonist MK-801 reduces the severity of tissue damage; the excitatory amino acid antagonist kynurenic acid is also neuroprotective in neonatal rats. A recent study in a closely related animal model confirmed that MK-801 reduces ischemia-induced histologic damage in immature brain.

[3H](1-[2-thienyl]-cyclohexyl)piperidine (3H-TCP), a phencyclidine analogue, is a ligand for the ion channel associated with the NMDA receptor. We used in vitro autoradiography to quantify 3H-TCP binding in rats decapitated 24 hours after right carotid artery ligation and hypoxic exposure following pretreatment with 1 mg/kg MK-801 or an equal volume of saline. Based on previous
observations of focal reductions in \[^{3}H\]glutamate binding, we anticipated that \[^{3}H\]TCP binding would be selectively reduced ipsilateral to the ligation. The major goals of our study were to determine if treatment with neuroprotective doses of MK-801 attenuated the acute loss of specific \(^{3}H\)-TCP binding and if \(^{3}H\)-TCP binding could be used to quantitatively assess neuroprotection.

**Materials and Methods**

All experiments were done in postnatal day (PND) 7 or 8 Sprague-Dawley rats. We included results from four groups: three PND 8 untreated unoperated rat pups, six rat pups that received 1 mg/kg MK-801 2 hours before sacrifice but no ligation, nine saline-pretreated pups that underwent right carotid artery ligation followed by exposure to 8% oxygen for 2.5 hours, and 10 MK-801-pretreated pups that underwent right carotid artery ligation followed by exposure to 8% oxygen for 2.5 hours.

Pups that underwent surgery were anesthetized with ether; the right carotid artery was then exposed and ligated in <5 minutes. After recovery from anesthesia, the pups were returned to their dams to feed for 1.5–2 hours. Within 10 minutes before hypoxic exposure, the pups received a 50-µl intraperitoneal injection of saline or 1 mg/kg MK-801 dissolved in saline. The pups were then placed in covered plastic chambers, warmed in a water bath, and exposed to 8% oxygen for 2.5 hours as previously described.\(^{10,11}\) The pups were returned to their dams and decapitated 24 hours later. The surgical protocol was approved by the University of Michigan Committee on Care and Use of Animals.

To visualize the regional distribution of \(^{3}H\)-TCP binding, in vitro autoradiography was done.\(^{13}\) Horizontal 20-µm frozen brain sections were incubated with 30 nM \(^{3}H\)-TCP (55 Ci/mmol, New England Nuclear, Boston, Massachusetts) in 50 mM Tris acetate with 1 mM Mg\(^{2+}\), pH 7.4. Nonspecific binding was assayed in the presence of 20 µM TCP.

Adjacent sections from selected brains were assayed for \(^{3}H\)glutamate binding to the NMDA site.\(^{17}\) Twenty-micron sections were incubated with 40 nM \(^{3}H\)glutamate (59 Ci/mmol, Amersham, Arlington Heights, Illinois) in 50 mM Tris acetate, pH 7.2, with 2.5 µM quisqualic acid. Nonspecific binding was determined in the presence of 1 mM L-glutamate.

Sections and carbon-14 standards (American Radiolabeled Chemicals, Inc., St. Louis, Missouri) were apposed to tritium-sensitive film (LKB-Ultrafilm, Gaithersburg, Maryland) for up to 6 weeks to generate the autoradiograms. Quantitative analysis of regional binding was done using a video-based image analysis system (MCID, St. Catharines, Canada). Optical density was measured bilaterally in three regions of the hippocampus (CA1, CA3, and the dentate gyrus), in the striatum, in the cingulum, and in the fornix; the latter two are typically spared from hypoxic-ischemic injury (five readings per region per section, four sections per brain).

Specific binding values were derived from polynomial regression analysis of optical density values in the standards calibrated against brain paste with known amounts of tritium and expressed as mean±SEM femtomoles per milligram protein. Side-to-side differences in hypoxic-ischemic pups were compared using paired t tests. The effects of MK-801 on binding in unoperated controls were assessed by analysis of variance using a microcomputer-based statistical package (SYSTAT, Evanston, Illinois).

**Results**

Regionally selective specific \(^{3}H\)-TCP binding was consistently detected in autoradiograms prepared...
from untreated PND 8 rat brain (Figure 1, left); binding was highest in the hippocampus. In contrast, virtually no specific binding was present if the pups were treated with 1 mg/kg MK-801 2 hours before decapitation (Figure 1, right; Figure 2).

Figure 2 compares 3H-TCP binding in six brain regions of PND 8 unoperated untreated controls and in unoperated rats treated with 1 mg/kg MK-801 2 or 24 hours before decapitation. Binding in all brain regions was suppressed by 75–82% 2 hours and by 15–35% 24 hours after MK-801 administration (p<0.001).

Right carotid artery ligation followed by exposure to 8% oxygen for 2.5 hours produces ipsilateral forebrain injury, characterized by atrophy and gliosis in the striatum, hippocampus, and cortex in approximately two thirds of PND 7 rats13; in the hippocampus, damage is relatively diffuse, extending to CA1, CA3, and the dentate gyrus.10,11 Representative coronal brain sections (Figure 3) demonstrate the extent of injury commonly observed in saline- and MK-801–treated pups killed on PND 12, 5 days later. MK-801 treatment reduced the extent of tissue loss in all brain regions.

In PND 8 rats decapitated 24 hours after right carotid artery ligation and 2.5 hours' exposure to 8% oxygen, ipsilateral 3H-TCP binding was reduced (Figure 4, left); anatomic detail in the hippocampus, which can be detected in autoradiograms from unoperated untreated PND 8 rat brain, is obscured. In CA1, CA3, the dentate gyrus, and the striatum, 3H-TCP binding was significantly reduced by 17–30% ipsilateral to the ligation (Table 1). In contrast, in autoradiograms from littermates pretreated with 1 mg/kg MK-801, only subtle reductions in ipsilateral 3H-TCP binding in the hippocampus and striatum are discernible (Figure 4, right). In the MK-801–treated pups, there were no significant side-to-side differences in 3H-TCP binding (Table 1). However, values in both hemispheres were lower than in age-matched untreated unoperated controls.
The density of NMDA-type [3H]glutamate binding sites is considerably higher than that of 3H-TCP binding sites in PND 7–8 rat brains (unpublished observations). Thus, at this developmental stage, the anatomic resolution is considerably greater in autoradiograms prepared to visualize NMDA binding sites (with [3H]glutamate) than in those prepared to visualize the associated ion channel (with 3H-TCP). To confirm preservation of the anatomic integrity of receptor binding in MK-801–treated rat pups observed with 3H-TCP, NMDA-type [3H]glutamate binding was also assayed. In pups decapitated 24 hours after right carotid artery ligation and exposure to 2.5 hours 8% oxygen, NMDA-type [3H]glutamate binding was reduced most prominently in the ipsilateral hippocampus and striatum (Figure 5, top left). Autoradiograms prepared from MK-801–treated hypoxic-ischemic rats (Figure 5, top right, bottom left, and bottom right) demonstrate varying degrees of preservation of NMDA-type [3H]glutamate binding ipsilateral to the ligation; this variability corresponds well with the observed range of MK-801’s neuroprotective effects.13

Discussion

Our goal was to determine if a redistribution of specific binding of 3H-TCP, a ligand for the NMDA receptor–associated ion channel which could potentially be administered in vivo, identified hypoxic-ischemic brain injury. The regionally selective binding of 3H-TCP was acutely disrupted in this experimental model of perinatal stroke. Ipsilateral to the carotid ligation, loss of 3H-TCP binding was most pronounced in the hippocampus and striatum (Figure 5, top left). Autoradiograms prepared from MK-801–treated hypoxic-ischemic rats (Figure 5, top right, bottom left, and bottom right) demonstrate varying degrees of preservation of NMDA-type [3H]glutamate binding.

### Table 1. 3H-TCP Binding in Brains of 8-Day-Old Rats Decapitated 24 Hours After Hypoxia-Ischemia

<table>
<thead>
<tr>
<th>Region</th>
<th>Unoperated controls (n=3)</th>
<th>Hyoxic-ischemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contralateral Ipsilateral</td>
<td>Difference (%)</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>157.3±9.0 110.0±10.0*</td>
<td>-30.2±4.9</td>
</tr>
<tr>
<td>Cingulum</td>
<td>212.7±12.6 82.1±5.8</td>
<td>-16.6±4.0</td>
</tr>
<tr>
<td>Striatum</td>
<td>61.8±2.4 87.2±8.3</td>
<td>25.4±5.6</td>
</tr>
</tbody>
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>3H-TCP, [3H][1-[2-triphenyl]cyclohexyl]piperidine. Binding assayed in 30 nM 3H-TCP; values expressed as mean±SEM fmol/mg protein from 4 sections/brain, 5 readings/region. In controls values from left and right hemispheres are combined. Differences calculated as (ipsilateral–contralateral)/contralateral.

*Fp<0.05, 0.01, respectively, different from contralateral by paired t test.
The reductions in $^3$H-TCP binding were similar to the pattern of disruption in total [$^3$H]glutamate binding previously reported in this model. These findings, together with the results from the autoradiograms prepared under conditions favoring [$^3$H]glutamate binding to the NMDA site, indicate that there is no preferential loss of this receptor subtype acutely with hypoxic-ischemic injury. The corresponding preservation of NMDA binding in the MK-801-treated group suggests that the pattern of $^3$H-TCP binding does indeed reflect neuroprotection.

In these experiments, a single concentration of ligand was used and we could not determine if reductions in binding reflected loss of binding sites or change in receptor affinity. Based on examination of corresponding histologic sections at this time interval that reveal loss of Nissl staining in lesioned regions and minimal edema, it appears likely that the reductions reflect a loss of binding sites. A decrease in the number of binding sites could reflect reversible membrane dysfunction, suppression of protein synthesis, hypoxia-ischemia--induced receptor downregulation, or irreversible membrane damage. Although we could not address the issue of one-to-one correspondence of reductions in $^3$H-TCP binding with irreversible neuronal injury, available information about the evolution of neuropathology in this model suggests that the focal reductions in binding ipsilateral to the ligation reflect, in large part, irreversible damage.

Although pretreatment with the noncompetitive NMDA antagonist MK-801 preserved both tissue integrity and the normal regional distribution of $^3$H-TCP binding, the binding density was suppressed bilaterally. Similar reductions in $^3$H-TCP binding were observed in unoperated controls decapitated 2 or 24 hours after MK-801 administration. The suppression of $^3$H-TCP binding could reflect persistent receptor occupation by MK-801 or receptor downregulation; our experiments did not address this issue directly. Of note, the behavioral effects of MK-801 in vivo (e.g., sedation) are long-lasting (up to 24 hours). Jarvis et al reported decreases in $^3$H-TCP binding in gerbil forebrain 4 days after an episode of transient ischemia in animals treated with the competitive NMDA antagonist CGS 19755. They interpreted their data as possibly reflecting ischemia-induced receptor desensitization; however, their observation could also reflect a direct effect of the NMDA antagonist on $^3$H-TCP binding.

Acute and chronic changes in binding of ligands for glutamate receptors have been reported in models of ischemic brain injury in mature animals. Leach et al found that 1 week after bilateral carotid artery occlusion in adult gerbils $^3$H-TCP binding assayed in tissue homogenates was significantly decreased. Westerberg et al reported that after an ischemic insult a decrease in the binding of $^3$H-AMPA, a ligand for a subset of quisqualate-type glutamate receptors, preceded cell death in CA1. In a subsequent study, these same authors suggested that postischemic receptor changes, especially decreased NMDA binding acutely, did not necessarily predict neuronal necrosis. It is uncertain to what extent results obtained in perinatal animals can be extrapolated to the adult brain. The cellular mechanisms that lead to irreversible neuronal injury in the immature brain may differ in part from pathophysiologic mechanisms in adult animals, as may the pharmacologic response to drugs such as NMDA antagonists. The susceptibility to NMDA-induced neurotoxicity peaks in the early postnatal period. As well, there is a transient early developmental peak in glutamate receptor expression. Although overactivation of NMDA receptors contributes to irreversible ischemia-induced neuronal injury at both developmental stages, some components of the response to injury may, in fact, be enhanced during the perinatal period.

In this experimental model, acute disruption of $^3$H-TCP binding occurs in brain regions susceptible to hypoxic-ischemic injury. Despite the phar-
macologic interactions that complicated our interpretation of these data, it appeared feasible to use this approach to assay the efficacy of neuroprotective agents. In particular, in vitro autoradiography (and possibly in vivo imaging) with appropriate ligands may provide useful information about the temporal evolution of irreversible injury and regional differences in the efficacy of neuroprotective agents. Furthermore, analysis of alterations in neurotransmitter ligand binding may delineate subtle functional changes in receptor organization that evolve after injury.

References


Key Words: anoxia • MK-801 • N-methyl-D-aspartate • rats
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Stroke. 1990;21:310-315
doi: 10.1161/01.STR.21.2.310

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