Autoradiographic analysis of $^3$H-MK-801 (dizocilpine) in vivo uptake and in vitro binding after focal cerebral ischemia in the rat

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The clinical utility of N-methyl-D-aspartate (NMDA) receptor antagonists is now being assessed in ischemic brain injury in humans. The uptake and retention of NMDA receptor antagonists in ischemic tissue will influence the design of clinical trials. The effects of permanent occlusion of the middle cerebral artery, induced 15 minutes prior to isotope administration, on the uptake of $^3$H-MK-801 (dizocilpine) have been assessed in the rat with quantitative autoradiography. In a group of three rats at 15 minutes after the intravenous administration of $^3$H-MK-801, the level (mean ± standard error of the mean) of isotopic tracer in the ischemic cortex and striatum was markedly less than that in the contralateral hemisphere (ipsilateral vs. contralateral caudate nucleus: $22 ± 4$ vs. $84 ± 11$ pmol/gm, $p < 0.01$). In contrast, in a group of five rats at 60 minutes after the intravenous administration of $^3$H-MK-801, the level of isotopic tracer in the ischemic cortex and striatum was greater than that in the contralateral hemisphere (ipsilateral vs. contralateral caudate nucleus: $52 ± 8$ vs. $32 ± 4$ pmol/gm, $p < 0.05$). There were no significant alterations in the specific binding of $^3$H-MK-801 in vitro in ischemic tissue at equivalent times. The early uptake of $^3$H-MK-801 into the central nervous system is dominated by the level of cerebral blood flow, whereas at later times after administration enhancement of MK-801 binding by elevated extracellular glutamate concentrations appears to be more important in determining the level of the drug in ischemic tissue.

Key Words • ischemia • autoradiography • dizocilpine • NMDA receptor • excitatory amino acid antagonist • rat

There is increasing evidence that excitatory amino acids, such as glutamate and aspartate, contribute to the development of ischemic brain damage. Blockade of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor can markedly reduce the amount of ischemic brain damage in a variety of animal models. There are convincing data that competitive and noncompetitive NMDA antagonists ameliorate ischemic brain injury in models of experimental focal cerebral ischemia, central nervous system (CNS) trauma, and subdural hematoma; in contrast, in models of global ischemia, no consensus has yet emerged concerning the neuroprotective effects of NMDA antagonists. A number of competitive and noncompetitive NMDA receptor antagonists are presently entering the early stages of clinical evaluation.

In investigations of experimental ischemia in animals, the protocol can be readily tailored to optimize drug dose (generally to define maximum effect rather than the threshold dose) and the timing of drug treatment (pretreatment or treatment initiated immediately after the ischemic insult). In contrast, in humans, the design of clinical trials with new drugs, such as NMDA receptor antagonists, is constrained by the need to employ the lowest effective dose in order to minimize any adverse reactions to the drug and by the timing of medical care (the elapsed time between onset of ischemia and availability of patients for treatment). Considerable emphasis is now being placed on precisely defining the plasma concentrations of various NMDA receptor antagonists at which anti-ischemic efficacy is observed. However, the extent of neuroprotection is determined by drug concentrations not in plasma but in the regions vulnerable to ischemic injury at critical times during the ischemic period. The aim of the present study was to assess the uptake and retention of the potent noncompetitive NMDA receptor antagonist $^3$H-
animal in the lateral position, the left side of the cra-

were made to the ventilator to maintain PaCO

nium was shaved, and an incision was made perpendic-

ular to the midpoint of the zygoma. The temporalis

muscle was incised and retracted to allow a craniotomy

sutured closed.

The MCA was permanently occluded via a subtempo-

ar approach, as described previously.31A2 With the

animals prior to sacrifice. Each brain was removed

rapidly and frozen in isopentane at −42°C. Multiple

20-μm coronal sections were prepared semiserially on

a cryostat and allowed to air-dry at room temperature.

MK-801 (dizocilpine)4422 into the CNS using quantita-

tive autoradiography in rats with acute permanent oc-

cclusion of a middle cerebral artery (MCA).

The MCA was identified, coagulated, and transected proxi-

mally into both femoral arteries and one femoral vein to allow the continuous monitoring of mean arterial blood pressure (MABP), the sampling of arterial blood, and the intravenous injection of radioactive tracers (when appropriate). Arterial blood gas status was monitored intermittently and adjustments were made to the ventilator to maintain PaCO2 between 32 and 36 mm Hg. A rectal probe was used to monitor body temperature and a heating lamp was used to maintain body temperature at approximately 37°C.

The MCA was permanently occluded via a subtemporal approach, as described previously.31,41 With the animal in the lateral position, the left side of the cranium was shaved, and an incision was made perpendicular to the midpoint of the zygoma. The temporalis muscle was incised and retracted to allow a craniotomy to be performed in the inferior temporal bone. Under the operating microscope, the dura was opened and the MCA was identified, coagulated, and transected proximal to the lenticulostriate branches. The wound was sutured closed.

In Vivo Uptake of 3H-MK-801

Fifteen minutes after transection of the MCA, 3H-

MK-801 (100 μCi, specific activity 29.4 Ci/mmol) was injected via the femoral venous cannula. Serial sampling of arterial blood was carried out at 0, 0.25, 0.5, 0.75, 1, 2, 3, 5, 7.5, 10, and 17 minutes, and when appropriate, 30, 45, and 60 minutes after radioisotope administration. The arterial samples were immediately centrifuged and the plasma radioisotope concentrations determined by liquid scintillation analysis. Immediately prior to sacrifice of the rats, a few drops of arterial blood were collected on a preweighed filter disc to assess the whole-blood concentration of the isotope. Arterial sampling was performed to confirm that the plasma pharmacokinetics were similar in all animals and to provide data for use with mathematical modeling of brain pharmacokinetics.

The animals were killed by decapitation, three rats at 15 minutes after the intravenous injection of 3H-

MK-801 and five rats at 60 minutes after injection. Each brain was removed promptly and frozen in isopentane at −42°C. Multiple 20-μm coronal sections were prepared semiserially on a cryostat. The sections were dried rapidly on a hot-plate at 60°C. The sections were then exposed to tritium-sensitive film with precalibrated plastic standards for 12 weeks. Local concentations of tritium were determined by quantitative densitometry.* Densitometric measurements of local isotope concentrations were made at defined neuroanatomical sites using 0.1-sq mm regions of interest. Measurements of caudate nucleus were restricted to the lateral portion of the nucleus, where consistent reductions in cerebral blood flow (CBF) are observed with MCA occlusion;42 measurements of the caudate nucleus were made rostrally at the maximum extent of the nucleus and caudally at the level of the globus pallidus.

In Vitro Binding of 3H-MK-801

To establish whether focal cerebral ischemia influenced the in vitro binding of 3H-MK-801 to its recognition site, five rats were sacrificed at 15 minutes after MCA occlusion and five rats at 60 minutes after occlusion. No radioactive tracer was administered to these animals prior to sacrifice. Each brain was removed rapidly and frozen in isopentane at −42°C. Multiple 20-μm coronal sections were prepared semiserially on a cryostat and allowed to air-dry at room temperature.

The in vitro binding of 3H-MK-801 was performed essentially as described previously.2 Sections were washed twice for 30 seconds in 50 mM Tris HCl buffer (pH 7.4, at 24°C) and then preincubated for 30 minutes in the same buffer. Following air-drying, the sections were incubated in 10 nM 3H-MK-801 and 50 mM Tris HCl for 45 minutes at room temperature. Nonspecific binding was determined in the presence of 10 μM MK-801. Sections were washed twice for 30 seconds in cold (4°C) distilled water and allowed to air-dry. Local concentrations were determined in the same regions of interest as in the in vivo uptake investigations by quantitative densitometry. Specific in vitro binding of 3H-MK-801 was calculated as the difference between total and nonspecific binding.

Results

In Vivo Uptake of 3H-MK-801

The group of rats sacrificed at 15 minutes after 3H-

MK-801 administration was similar to the group of rats sacrificed at 60 minutes in regard to MABP (mean values ± standard error of the mean at time of isotope administration: 91 ± 7 mm Hg in the group sacrificed at 15 minutes vs. 90 ± 7 mm Hg in the group sacrificed at 60 minutes), respiratory blood gas status, and plasma radioisotope levels during the first 15 minutes after administration.

At 15 minutes after the intravenous injection of 3H-

MK-801, the levels of radioisotope in regions supplied by the occluded MCA, such as the motor cortex and

Uptake and retention of $^3$H-MK-801 in ischemia

Fig. 1. Autoradiographs showing the in vivo uptake of $^3$H-MK-801 administered in rats intravenously 15 minutes after middle cerebral artery (MCA) occlusion, at the level of the rostral caudate nucleus (left) and globus pallidus (right). The coronal sections were prepared from rats sacrificed at 15 minutes (upper) or 60 minutes (lower) after administration of $^3$H-MK-801. The hemisphere ipsilateral to the MCA occlusion is on the left. The relative levels of isotope in each brain were related directly to relative optical density. Fifteen minutes after $^3$H-MK-801 administration, the levels of isotope were markedly less in the territory of the occluded MCA than in the equivalent areas in the contralateral hemisphere. Sixty minutes after $^3$H-MK-801 administration, the level of isotope was greater in the territory of the occluded MCA (most prominently in the lateral caudate nucleus) than in the equivalent areas in the contralateral hemisphere.

In Vitro Binding of $^3$H-MK801

There was no significant interhemispheric asymmetry in specific $^3$H-MK-801 binding in vitro in the rats sacrificed at either 15 or 60 minutes after MCA occlusion in regions within the territory of the occluded MCA or elsewhere in the CNS except at the site of the caudate nucleus, were significantly less (by up to 75%) than in the equivalent regions in the contralateral hemisphere (Figs. 1 and 2, Table 1). At 60 minutes after the intravenous injection of $^3$H-MK-801, the levels of radioisotope in regions supplied by the occluded MCA were greater (by at least 60%) than in the equivalent areas of the contralateral hemisphere (Figs. 1 and 2, Table 1). The only region with a markedly reduced radioisotope level was a circumscribed region at the rhinal fissure, close to the point of MCA occlusion (Fig. 1). In regions outside the territory of the occluded MCA, such as the anterior cingulate cortex, the dentate gyrus, and the cerebellum, radioisotope levels were similar in both hemispheres in animals sacrificed at either 15 or 60 minutes after $^3$H-MK-801 administration (Table 1).

### TABLE 1

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Group 1: Initial Uptake (pmol/gm)</th>
<th>Group 2: Late Uptake (pmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ipsi-lateral</td>
<td>Contra-lateral</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ipsi-lateral</td>
</tr>
<tr>
<td>caudate nucleus</td>
<td>22 ± 4†</td>
<td>84 ± 11</td>
</tr>
<tr>
<td>(caudal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>caudate nucleus</td>
<td>31 ± 6†</td>
<td>79 ± 15</td>
</tr>
<tr>
<td>(rostral)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>motor cortex</td>
<td>35 ± 6†</td>
<td>75 ± 9</td>
</tr>
<tr>
<td>frontal cortex</td>
<td>31 ± 6†</td>
<td>85 ± 14</td>
</tr>
<tr>
<td>sensory cortex</td>
<td>63 ± 12</td>
<td>81 ± 13</td>
</tr>
<tr>
<td>anterior cingulate cortex</td>
<td>75 ± 16</td>
<td>86 ± 17</td>
</tr>
<tr>
<td>dentate gyrus</td>
<td>65 ± 9</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>cerebellum</td>
<td>67 ± 5</td>
<td>66 ± 4</td>
</tr>
</tbody>
</table>

*Group 1 = three rats sacrificed at 15 minutes after intravenous $^3$H-MK-801 (dizocilpine) administration; Group 2 = five rats sacrificed at 60 minutes after $^3$H-MK-801 administration. Values are expressed as means ± standard error of the means. Significance of difference (for the comparison between the hemispheres ipsilateral and contralateral to middle cerebral artery (MCA) occlusion. Student's paired t-test): † = p < 0.05; †† = p < 0.01.
FIG. 2. Graph showing in vivo uptake of $^3$H-MK-801 in the rats sacrificed at 15 minutes (left) or 60 minutes (right) after intravenous administration of the isotope tracer. Data are presented as means ± standard error of the means. Statistical significance for the comparison between the hemispheres ipsilateral and contralateral to the middle cerebral artery occlusion (Student's paired t-test) are indicated by asterisks ($p < 0.05$) and double asterisks ($p < 0.01$). Data for the caudate nucleus relate to rostral portions of the nucleus.

MCA occlusion adjacent to the rhinal fissure (Fig. 3, Table 2). The focal reduction in in vitro binding of $^3$H-MK-801 and in vivo uptake in the perirhinal areas is consistent with mechanical and/or thermal damage to the adjacent cerebral tissue during MCA occlusion.

**Discussion**

**Sites of NMDA Receptor Blockade**

Blockade of the NMDA receptor can be achieved at a number of distinct sites within the NMDA receptor complex, such as the neurotransmitter recognition site, the ion channel site, and the polyamine site. In focal cerebral ischemia, blockade of the NMDA receptor produces marked reductions in ischemic damage; the degree of neuroprotection is similar irrespective of whether drug administration achieves NMDA receptor blockade via the recognition site, the ion channel site, or the polyamine site. The markedly varied chemistry and pharmacology of the different classes of NMDA antagonists (for example, competitive and noncompetitive antagonists) will have a considerable influence on their ultimate clinical utility in the treatment of cerebral ischemia in humans. Competitive NMDA antagonists such as CGS-19755 and D(-)E-4-(3 phosphonoprop-2-enyl)-piperazine-2-carboxylic acid act at the glutamate recognition site within the NMDA receptor complex; these drugs are highly charged hydrophilic molecules as a consequence of their phosphonic and carboxylic acid moieties. The presence of increased levels of glutamate reduces the in vitro binding of competitive NMDA antagonists to the agonist recognition site. Noncompetitive antagonists, such as MK-801, act at a site within the ion channel of the NMDA receptor complex and are highly lipophilic molecules. The presence of increased levels of glutamate markedly increases the in vitro binding of noncompetitive NMDA antagonists to the receptor complex by opening the ion channel.

**Drug Uptake in Cerebral Ischemia**

Cerebral ischemia is, by definition, associated with a profound reduction in CBF. During cerebral ischemia, there is a marked increase in the extracellular concentration of glutamate, irrespective of the nature and primary cause of the ischemic episode, including global ischemia, focal ischemia, subdural hemorrhage, and head trauma. These two events (reduction in CBF and increase in extracellular glutamate) may account for the modification of the uptake of $^3$H-MK-801 into ischemic areas described in the present study. For lipophilic molecules (such as MK-801) that enter the CNS with no diffusion restriction, their initial uptake into the brain should be dominated by their rate of delivery to the tissue. Thus, the autoradiograph of $^3$H-MK-801 uptake obtained 15 minutes after administration is similar to the CBF autoradiographs obtained after MCA occlusion using $^{14}$C-iodoantipyrine as the tracer.

The present study indicated that in a model of focal ischemia, the concentration of noncompetitive NMDA antagonists will be markedly lower in tissue at risk of ischemic damage for a period of time, even after adequate plasma levels of the drug have been established. Tissue dissection data from a study with a design similar to the present study indicated that in a model of focal ischemia, the concentration of noncompetitive NMDA antagonists will be markedly lower in tissue at risk of ischemic damage for a period of time, even after adequate plasma levels of the drug have been established.

**Table 2**

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Group 1: Binding (pmol/gm)</th>
<th>Group 2: Binding (pmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ipsi-lateral</td>
<td>Contra-lateral</td>
</tr>
<tr>
<td>caudate nucleus</td>
<td>58 ± 23</td>
<td>56 ± 11</td>
</tr>
<tr>
<td>(caudal)</td>
<td>59 ± 5</td>
<td>44 ± 9</td>
</tr>
<tr>
<td>motor cortex</td>
<td>124 ± 14</td>
<td>90 ± 20</td>
</tr>
<tr>
<td>frontal cortex</td>
<td>80 ± 15</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>sensory cortex</td>
<td>111 ± 11</td>
<td>99 ± 10</td>
</tr>
<tr>
<td>anterior cingulate</td>
<td>72 ± 10</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dentate gyrus</td>
<td>128 ± 15</td>
<td>179 ± 19</td>
</tr>
<tr>
<td>cerebellum</td>
<td>49 ± 21</td>
<td>46 ± 25</td>
</tr>
</tbody>
</table>

*Group 1 = five rats sacrificed 15 minutes after middle cerebral artery (MCA) occlusion; Group 2 = five rats sacrificed 60 minutes after MCA occlusion. Values are expressed as means ± standard error of the means. NA = no sections available for binding.
Uptake and retention of $^3$H-MK-801 in ischemia

Fig. 3. Autoradiographs showing the in vitro total binding of $^3$H-MK-801 in rats at the level of rostral caudate nucleus (left) and globus pallidus (right). The coronal sections were prepared from rats sacrificed at 15 minutes (left) or 60 minutes (right) after middle cerebral artery occlusion. The hemisphere ipsilateral to the occlusion is on the left. The $^3$H-MK-801 binding was symmetrical in both hemispheres.

to the present suggests that $^3$H-MK-801 concentrations in the ischemic and nonischemic cortex are dissimilar until 45 minutes after administration of the tracer. In experimental cerebral ischemia in animals, the delay in reaching neuroprotective levels of MK-801 after its administration can be circumvented by pretreatment (when CBF is normal) or by the use of large doses of the drug. In most projected clinical trials of noncompetitive NMDA antagonists, neither of these options is feasible because of the increased likelihood of adverse effects with elevated concentrations. It should be noted that uptake of the hydrophilic competitive NMDA antagonists is minimally influenced by the low CBF of ischemic regions. Competitive NMDA antagonists have very low blood-brain barrier permeability and, consequently, their CNS uptake is essentially time-dependent (and much slower than the uptake of MK-801 into even highly ischemic areas).

Ligand Binding to the NMDA Receptor

The increased level of $^3$H-MK-801 in ischemic areas is consistent with the well-known effect of glutamate on MK-801 binding to the NMDA receptor complex. Extracellular glutamate in the micromolar concentration range (concentration achieved in vivo during ischemia) produces a 600% increase in $^3$H-MK-801 binding to washed synaptosomes in vitro. A similar mechanism in vivo would account for the elevated $^3$H-MK-801 in the ischemic area at 60 minutes after its administration. In vitro studies of the binding of $^3$H-MK-801 to cerebral tissue after focal cerebral ischemia in the present study and elsewhere suggest that the level of NMDA receptor does not change for many hours after permanent MCA occlusion. Thus the increased binding of $^3$H-MK-801 in ischemic tissue in vivo does not reflect a change in the absolute number of binding sites. Compartmental modeling suggests that the higher levels of $^3$H-MK-801 in the area of low CBF/high glutamate cannot be attributed to differential elution of the tracer because of the different levels of CBF. The absence of enhanced $^3$H-MK-801 levels in white matter within the MCA territory supports such a view because these areas of white matter have a similar low CBF but lack NMDA receptors. Metabolic degradation of the tracer is equally unlikely to account for the observed effects because the major metabolites of MK-801 do not enter the CNS.

The amount of $^3$H-MK-801 that binds to the ion channel of the NMDA receptor is determined by the regional density of NMDA receptors and the local extracellular concentrations of glutamate and glycine. With in vitro ligand-binding autoradiography, the concentrations of glutamate and glycine are generally held constant, and the autoradiographs reflect the regional heterogeneity in the number of NMDA receptors.

In vitro binding studies have clearly indicated an extremely heterogeneous anatomical distribution of NMDA receptors, with the greatest concentration being observed within the hippocampal formation and the
outer layers of the neocortex (Fig. 3). The density of NMDA receptor binding in vitro does not determine the uptake and retention of $^3$H-MK-801 in vivo, even in the absence of cerebral ischemia; an example of this is the laminar homogeneity of isotope concentrations in the cerebral cortex contralateral to MCA occlusion (Fig. 1). The uptake and retention of $^3$H-MK-801 at 60 minutes after its administration reflect the level of activation of NMDA receptors rather than their number. In ischemic tissue, NMDA receptor activation is determined by the markedly elevated extracellular concentration of glutamate.

The failure of in vivo retention of $^3$H-MK-801 to reflect regional NMDA density, even in the contralateral hemisphere, suggests that factors other than receptor density (lipophilicity) determine local drug levels when extracellular glutamate levels are in their normal range.

Conclusions

The present study suggests that the uptake of MK-801 into ischemic tissue will initially be slower than uptake into cerebral tissue with normal CBF; however, subsequently the levels of MK-801 in ischemic tissue will be greater than in tissue with normal levels of extracellular glutamate. These data relating to the brain pharmacokinetics of MK-801 in focal cerebral ischemia facilitate the design of treatment regimens for humans that provide brain levels of the drug adequate for neuroprotection which minimize adverse effects on normal CNS function.

References


M. C. Wallace, G. M. Teasdale, and J. McCulloch
Uptake and retention of $^3$H-MK-801 in ischemia


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