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Changes of Glutamate and Polyamine Levels of Hippocampal Microdialysates in Response to Occlusion of Both Carotid Arteries in Mongolian Gerbils

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ABSTRACT

Reversible brain ischemia was produced by occluding both common carotid arteries for 5 min, and the effects of aminoguanidine (AG), DL-α-difluoromethylornithine (DFMO), MK-801, and nimodipine (NM) on the ischemia induced changes of the polyamine, glutamate and acetylcholine levels in the hippocampus CA1 subfield and the specific [^{3}H] MK-801 binding to the hippocampus synaptosomal membranes were studied with a histological reference of the cresyl violet stained hippocampus. The basal putrescine level (PT: 74.4±8.8 nM) showed a rapid increase (up to 1.7 fold) for 5 min of ischemia, remained significantly increased for 6 h, and then resumed the further increase to amount gradually up to about 3 fold 96 h after recirculation. However, the level of spermidine was little changed, and the spermine level showed a transient increase during ischemia followed by a sustained decrease to about 40% of the preischemic level after recirculation. The increase of PT level induced by brain ischemia was enhanced with AG or MK-801, but it was reduced by DFMO or NM. The basal glutamate level (GT: 0.90±0.14 μM) rapidly increased to a peak level of 8.19±1.14 μM within 5 min after onset of the ischemia and then decreased to the preischemic level in about 25 min after recirculation. And NM reduced the ischemia induced increase of GT level by about 25%, but AG, DFMO and MK-801 did not affect the GT increase. The basal acetylcholine level (ACh: 118.0±10.5 μM) did little change during/after brain ischemia and was little affected by AG or NM. But DFMO and MK-801, respectively, produced the moderate decrease of ACh level. The specific [^{3}H] MK-801 binding to the hippocampus synaptosomal membrane was little affected by brain ischemia for 5 min. The control value (78.9 fmole/mg protein) was moderately decreased by AG and MK-801, respectively but was little changed by DFMO or NM. The microscopic findings of the brains extirpated on day 7 after ischemia showed severe neuronal damage of the hippocampus, particularly CA1 subfield. NM and AG moderately attenuated the delayed neuronal damage, and DFMO, on the contrary, aggravated the ischemia induced damage. However, MK-801 did not protect the hippocampus from ischemic damage. These results suggest that unlike to the mode of anti-ischemic action of NM, AG might protect the hippocampus from ischemic injury as being negatively regulatory on the N-methyl-D-aspartate (NMDA) receptor function in the hippocampus.

Key Words: Brain ischemia, Polyamine, Glutamate, NMDA receptor, Mongolian gerbil

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INTRODUCTION

The occlusion of both common carotid arteries for 5 min of ischemia results in delayed neuronal death of hippocampal CA1 pyramidal neurons in gerbils (Kirino, 1982). The CA1 neuronal damage appears to be in part linked to elevated extracellular glutamate levels in the hippocampus during and shortly after ischemia. The excitotoxic effect of glutamate on the hippocampus via activation of N-methyl-D-aspartate (NMDA) receptors has been known to contribute a cause in the brain damage associated with hypoxia (Somjen *et al.*, 1993; Vornov and Coyle, 1991), stroke (Buchan *et al.*, 1992), and hypoglycemia (Tasker *et al.*, 1992; Zhang *et al.*, 1990; Choi, 1993). The NMDA receptor is a postsynaptic ligand-gated cation channel complex which is related with Ca^{2+} influx (Jahr, 1992; Kral *et al.*, 1993). The NMDA receptors has multiple regulatory sites. The agonist site is sensitive to excitatory amino acids such as glutamate. Glycine has a potentiatory role in the glutamate binding to NMDA receptor by binding to the strychnine-insensitive site (Lazarewicz *et al.*, 1992; Lehmann *et al.*, 1991). Recently, it has been demonstrated that polyamines stimulate NMDA receptor activation in a fashion that is additive to the effects of glutamate and glycine (Nussenzveig *et al.*, 1991; Williams *et al.*, 1991; Romano *et al.*, 1991).

Polyamines such as putrescine, spermidine and spermine plays a key role in cellular processes including cell proliferation, differentiation, neonatal growth and neoplastic growth (Pegg, 1986; Tabor and Tabor, 1984). Ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis, is markedly activated in different pathologic states of the brain such as seizure (Martinez *et al.*, 1991), excitotoxic conditions (Gardiner *et al.*, 1992; Porcella *et al.*, 1992; Reed and de Belleroche, 1990) and ischemia (Paschen, 1992; Dempsey et *et al.*, 1988). Putrescine has been shown to activate calcium influx (Komulainen and Bondy, 1987) and release of excitotoxic amino acids from nerve endings (Reed and de Belleroche, 1990), and thus it has been known to be putatively neurotoxic when high levels of this compound are present in nerve endings (Paschen *et al.*, 1993). Also, the injection of spermine and spermidine into the rat striatum produced a dose related loss of the neuronal markers glutamate decarboxylase and choline acetyltransferase (Bourdiol *et al.*, 1992), which was not blocked by MK-801, a potent and selective noncompetitive NMDA receptor antagonist (Wong *et al.*, 1986). However, the intrastriatal infusion of NMDA via a dialysis cannula in anesthetized rats resulted in a marked and rapid increase in the concentrations of spermine and spermidine recovered in the dialysate. The effects of NMDA were blocked by the previous systemic injection of MK-801 (Fage *et al.*, 1992). Introduction of the excitotoxins kainate or ibotenate into the nucleus basalis results in ODC activation and degeneration of the cholinergic cells which may be prevented with DFMO, the irreversible ODC inhibitor (Reed and de Belleroche, 1990), or by repeated injections of nimodipine (Gardiner and de Belleroche, 1990).

Current efforts to block this ischemic neuronal damage have focused primarily on the direct antagonism of glutamate receptors. Although this approach is effective experimentaly, there are at least four additional reasons to develop other neuroprotective methods. First, receptor antagonists may interfere with normal excitatory synaptic function (Favaron *et al.*, 1988). Consequent disturbances of brain function results in a serious side effect. Second, if treatment must be delayed, downstream approaches may still work when receptor stimulation has ceased and receptor antagonists are no longer effective (Choi *et al.*, 1991). Third, the final destructive cascades that mediate glutamate-induced neuronal injury also may mediate the neuronal injury triggered by other mechanisms (Chan *et al.*, 1985; Murphy *et al.*, 1989; Siesjö and Katsura, 1992; Siesjö, 1989). Thus in theory, downstream approaches may permit a greater neuro-protective efficacy than possible even with saturating concentrations of receptor antagonists. Finally, investigation of downstream approaches may yield valuable insights into the fundamental mechanisms underlying excitotoxic injury. In the present study, the effects of

aminoguanidine (AG), DL-α-difluoromethylornithine (DFMO), MK-801 and nimodipine (NM) on the ischemia-induced changes of extracellular glutamate, polyamine and acetylcholine concentrations in the hippocampal CA1 sector of Mongolian gerbil were studied with references of specific [^{3}H]MK-801 binding and histologic findings of hippocampus.

MATERIALS AND METHODS

Materials

Male Mongolian gerbils *(Meriones unguiculatus)*, weighing 60~80 g, were supplied from Korea Experimental Animal Laboratory Company. 5 gerbils were kept in a cage under a light-dark cycle with light on from 07:00 to 19:00h and received food and water ad libitum for one week before being studied. Acetylcholine, acetylcholinesterase (purified from E. electricus), choline oxidase, horseradish peroxidase (type II, HRP), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), aminoguanidine, and the hydrochlorides of putrescine, spermidine and spermine were purchased from Sigma (St. Louis, MO, USA). 1,8-Diaminooctane and 4-fluoro-3-nitrobenzotrifluoride (FNBT) were obtained from Aldrich (Milwaukee, WI, USA). (+)-[3-^{3}H]MK-801 (22.5 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, MA, USA). Unlabeled MK-801 was obtained from Research Biochemicals International (Natick, MA, USA). And DFMO was generously provided by Merrel Dow Laboratories (Cincinnati, OH, USA). Other chemicals were analytical or high performance liquid chromatography (HPLC) grade.

Animals and surgery

Mongolian gerbils were anesthetized with chloral hydrate (360 mg/kg, i.p.). Through a ventral middle cervical incision, both common carotid arteries were exposed and dissected free of surrounding tissues, and a 4-0 silk suture was looped around each artery. Body temperature was maintained at 37℃ with a heating pad. The head of animal was fixed to a stereotaxic apparatus (Stoelting, Chicago, IL, USA). The skull was exposed and a small burr hole for insertion of a microdialysis probe was drilled. Dura was carefully removed to avoid damage to the underlying cortex. The dialysis probe (1 mm long dialysis membrane, 0.22 mm o.d.; CMA 11, Carnegie Medicin AB, Stockholm, Sweden) was implanted perpendicularly into hippocampus (2.2 mm ventral to the cortical surface, 2.0 mm posterior to bregma, and 2.0 mm lateral to the midline suture). The 1 mm-long dialysis membrane was situated in the CA1 subfield of hippocampus. Subsequently, it was perfused with Ringer solution (NaCl 144 mM, KCl 4.8 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.7 mM, pH 6.7) at a flow rate of 2 μl/min by a microinjection pump (CMA/100, Carnegie Medicin AB, Stockholm, Sweden). Dialysate samples (10 μl) were collected every 5 min until a 1 h stable baseline was obtained such that the amino acid levels in the dialysates differed by no more than 5 % between samples. The suture around the two common carotid arteries were pulled by 10 g weights to occlude the circulation (Mitani *et al.*, 1989; Suzuki *et al.*, 1983). Following 5 min of ischemia, the sutures were cut and removed to restore the blood flow.

For *in vivo* microdialysis study, MK-801 (1 mg /kg) and NM (2 mg/kg) were respectively administered intraperitoneally 10 min before the bilateral occlusion of carotid arteries. AG (100 mg/kg) and DFMO (250 mg/kg) were respectively given intraperitoneally 12 hours and 3 hours before the carotid occlusion. For [^{3}H]MK-801 binding and histopathological study, each drugs administered according to the above schedule and injected intraperitoneally every 12 hours for 7 days before sacrifice.

Polyamine assay

A 10 μl-aliquot of each dialysate sample was mixed with diaminooctane (100 μg) as an internal standard and was evaporated to dryness with speed vac concentrator (SVC 200, Savant, NY, USA). For derivatization with FNBT (Spragg and Hutchings, 1983), the dry residue obtained was dissolved in 100 μl of 1 M sodium carbonate. This solution was reacted with 300 μl of FNBT reagent (10 μl FNBT/ml dimethyl sulfoxide), and after mixing, the reaction was allowed to proceed at 60℃ for 20 min. At the

end of this time, 40 μl of 1 M histidine in 1 M sodium carbonate was added to the reaction mixture and the incubation continued for a further 5 min. After cooling the mixture, the N-2′-nitro-4′-trifluoromethylphenyl polyamine (NPT-polyamine) derivatives were extracted twice with 2 ml of 2-methylbutane. After centrifuge at 1,500 g for 5 min, the organic phase was evaporated to dryness in a conical centrifuge tube with speed vac concentrator and the residue was reconstituted with 1 ml of HPLC-grade methanol. 20 μl of the methanol solution was applied to the HPLC analysis. The 20 μl of the methanol solution obtained above was applied on a reversed-phase HPLC system equipped with a ODS column (3 μm: 6×100 mm, ERMA, Japan), and then the separation of NPT-polyamines was completely accomplished by an isocratic elution of acetonitrile-water (80:20) mobile phase at the rate of 1.2 ml/min within 20 min. The absorbance of effluent at 242 nm was monitored on a 2-channel chart recorder.

Glutamate assay

A 10 μl-aliquot of each dialysate sample was assayed for amino acids by HPLC with electrochemical detection after the precolumn derivatization with o-phthaldialdehyde as previously described (Donzanti and Yamamoto, 1988). The derivatization reagent was prepared by dissolving 27 mg of o-phthaldialdehyde in 1 ml of 100 % methanol and 9 ml of 0.1 M sodium tetraborate (pH 9.4). This stock solution was then diluted 1:3 with the sodium tetraborate, after which 10 μl of β-mer-captoethanol was added. A 5 μl aliquot of this reagent solution was then added to the 10 μl sample dialysate or standard amino acid solution. After a 2-min reaction time 10 μl of the sample was injected into a BAS HPLC apparatus via a Rheodyne 7125 injector. Standard amino acid solutions were prepared by initially dissolving the amino acids in 100% methanol and diluting with Ringer solution to yield a composite standard containing 1 ng of each amino acid. Derivatives of amino acids were separated on a C18 reversed-phase column (3 μm: 6×100 mm, ERMA, Japan) and eluted with a 0.1 M sodium phosphate buffer (pH 6.4) containing 28% methanol and 50 mg/L of EDTA. Detection was at a glassy carbon electrode maintained at +0.7 V by an LC-4B am-perometric detector (Bioanalytical Systems, Inc., West Lafayette, IN, USA). Flow rate was 1.0 ml/min. From a standard chromatogram, concentrations of amino acids in dialysates were calculated from peak heights in the individual chromatograms.

Acetylcholine assay

The 100 μl of the dialysate was washed 5 times with 1.0 ml of chilled water-saturated diethyl ether and was exposed to nitrogen gas stream for the removal of the residual ether. And then, 50 μl of the remained was added with 10 μl of 0.5% sodium metaperiodate to eliminate the reducing substances which interfere with the chemiluminescent reaction. The reaction medium were at first, consist of 0.4 ml of 200 mM sodium phosphate buffer, 15 μl of an assay chemiluminescent mixture and 10 μl of the extract, and finally added with 100 μl of acetylcholinesterase (AchE) reagent. And then the luminescent count was integrated for 60 sec using a Berthold Biolumat LB 2500C. The recovery rate was about 72.1%. Just before the acetylcholine determination, the assay luminescent mixture (250 μl) was prepared with 25 unit choline oxidase (in 100 μl deionized water), 10 unit HRP (in 50 μl deionized water) and 18 μg luminol (in 100 μl of 0.2 M Tris buffer, pH 8.6). And the AchE reagents were prepared as following: a 250 μl of AchE (1,000 unit per ml) in cold deionized water was passed through a coarse Sephadex G-50 column of 5 ml volume equilibrated in deionized water, and the column was further eluted with 1.45 ml deionized water before collecting the enzyme elute of about 0.7 ml, which was stored below −40℃. Immediately before the assay, the working reagent was prepared by dilution of the stock solution with 19 volumes of 0.2 M sodium phosphate buffer, pH 8.6.

[^{3}H]MK-801 receptor bindings

The hippocampal tissues from Mongolian gerbils were dissected and homogenized in 0.32 M sucrose (10% wt/vol) using a glass-teflon homogenizer (10 strokes at 500 rpm). The

homogenate was centrifuged at 1,000 g for 10 min. The supernatant was removed and centrifuged at 20,000 g for 20 min. The resulting pellet was resuspended and homogenized using a ultrasonic processor (Cole-Parmer, Neles, IL, USA) in 10 volumes of 5 mM Tris-HCl (pH 7.7 at 4℃) followed by centrifugation at 8,000 g for 20 min. The resulting supernatant and upper buffy coat layer of the pellet were combined and centrifuged at 50,000 g for 20 min. The pellet was resuspended in 10 volumes of 5 mM Tris, homogenized with the ultrasonic processor, and centrifuged at 50,000 g for 20 min. This wash step was repeated two additional times and the final pellet was stored at −80℃ for at least 18 h and no more than 2 weeks before use. On the day of assay, membrane pellets were resuspended in 20 volumes of 5 mM Tris, homogenized with the ultrasonic processor, and centrifuged at 50,000 g for 20 min. This wash step was repeated four more times and the final pellet was resuspended in assay buffer (5 mM Tris HCl, pH 7.5 at 23℃). [^{3}H]MK-801 binding assays were performed at 23℃ in an incubation volume of 1 ml with approximately 200~300 g of membrane protein per tube. The concentration of [^{3}H]MK-801 was 2 nM. [^{3}H] MK-801 and duplicate samples were incubated at 23℃ for 1 h. Nonspecific binding was determined using 100 μM MK-801. Incubation was terminated by rapid filtration through Whatman GF/B filters, which were washed immediately with two 5-ml portions of ice-cold assay buffer in a ten-place filter manifold (Hoeffer Scientific Instruments, San Francisco, CA, USA). The time required for the complete filtration and washing procedure was less than 10 sec. Radioactivity was measured using Luma Gel (Lumac-LSC, Groningen, The Netherlands) scintillation cocktail and a β-scintillation counter (LKB 1214, RackBeta) at an efficiency of 40 ~50% for [^{3}H]. Protein concentrations were determined according to the method of Peterson (1977).

Histopathological study

Seven days after the ischemic insult, the animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). and perfused transcardially with heparinized saline and then with 10% formalin in 0.1 M phosphate buffer (pH 7.4). Two hours after perfusion fixation, the brains were removed, divided into coronal sections (about 0.5 cm in thickness) and kept in the same fixative overnight at 4℃. After fixation, each tissue block was dehydrated in ethanol and embedded in paraffin. Serial coronal sections were cut at 6 μm by a microtome and mounted on gelatine-coated slides and were stained with cresyl violet. Stained sections were examined with a light microscope.

Data analysis

The statistical significance of differences in data between control and ischemic animals was assessed by two-tailed Student's *t*-test. Statistical significance for all tests was defined as $p < 0.05$.

RESULTS

Extracellular polyamine concentrations of hippocampus

The basal extracellular putrescine concentration in the dialysate was 74.4±8.8 nM. Immediately after ischemia, extracellular concentration of putrescine increased to 126.8±10.8 nM. This elevated extracellular putrescine concentration was maintained at 1.7-fold basal for at least 4 h after ischemia, and a further sustained increase in putrescine concentration was noted by 6 h after ischemia. There was no consistent alteration in the level of spermidine following ischemia. But extracellular spermine concentration appeared to increase transiently during ischemia followed by a sustained decrease to a maximum of 40 % below the basal output for 4 h after ischemia, and returned to the initial baseline by 6 h after ischemia (Fig. 1).

The extracellular putrescine concentration showed a sustained increase for 96 h after ischemia. To determine if the later increase of putrescine concentrations could be affected by AG, DFMO, MK-801 or NM, the putrescine concentration was measured for 96 h after ischemia. Both AG and MK-801 produced increases in putrescine concentration, representing a 20.2 % and 24.4 % increase respectively, com-

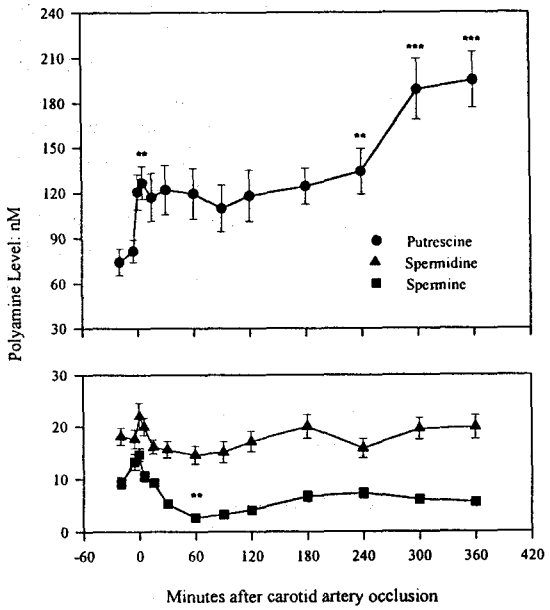

Fig. 1. Time course changes of the polyamine levels of the dialysates obtained from the hippocampus of gerbils subjected to bilateral carotid occlusion for 5 min. The dialysates were collected in 5 min fractions during microdialysis with a flow rate of 2 μl/min. The values were presented as mean±S.E.M. of 5 or 6 data. ** and *** indicate $p<0.02$ and $p<0.01$ as compared to the preischemic values.

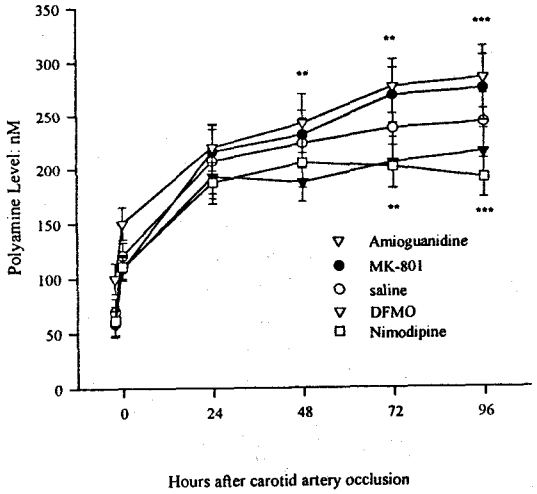

Fig. 2. Time course changes of the putrescine level of the dialysates obtained from the hippocampus of gerbils subjected to bilateral carotid artery occlusion for 5 min. MK-801 (1 mg/kg) and nimodipine (2 mg/kg) were respectively given 10 min before the carotid artery occlusion. Aminoguanidine (100 mg/kg) and DFMO (250 mg/kg) were respectively given 12 hours and 3 hours before the carotid artery occlusion. The dialysate collection and the data were processed as described in Fig. 1. ** and *** indicate $p<0.02$ and $p<0.01$ as compared to the preischemic values.

pared to those of saline control in 96 h after ischemia. In contrast, pretreatment with DFMO or NM appeared to reduce the ischemia-induced increase in putrescine concentration (Fig. 2).

Extracellular glutamate concentration of hippocampus

Basal extracellular glutamate concentration ($0.90\pm0.14\,\mu$M) was rapidly increased, reaching a peak of approximately $8.19\pm1.14\,\mu$M within 5 min after ischemia, followed by a decrease to baseline values by 25 min after reperfusion. AG, DFMO, or MK-801 had no effects on the ischemia-induced increase in glutamate concentration, but NM reduced the peak by about 20 ~30 % (Fig. 3).

Extracellular acetylcholine concentration of hippocampus

Basal extracellular acetylcholine concentration ($118.0\pm10.5\,\mu$M) did not change significantly during the ischemia period. Preischemic acetylcholine concentrations from DFMO or MK-801 treated animals were significantly lower than corresponding samples from saline treated animals by 33.1 and 24.6%, respectively and remained depressed for the remainder of the experiment (Fig. 4).

[^{3}H]MK-801 binding

The specific [^{3}H]MK-801 binding to the hippocampus synaptosomal membrane was little affected by brain ischemia for 5 min. Specific

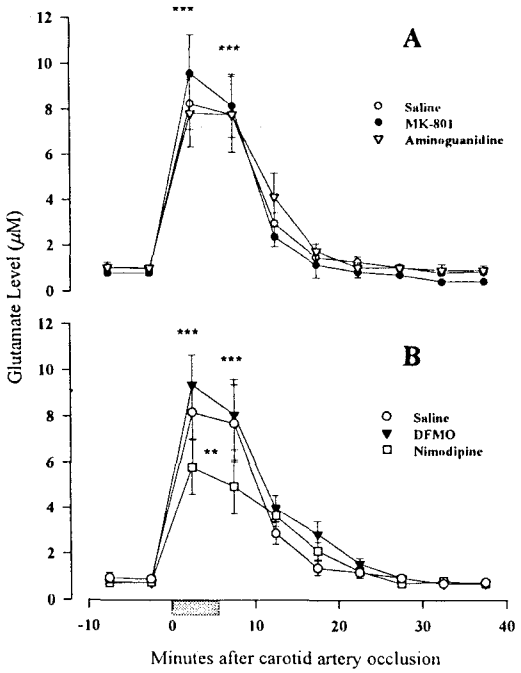

Fig. 3. Time course changes in the dialysate level of glutamate sampled from the hippocampus of gerbils subjected to bilateral carotid occlusion for 5 min. MK-801, nimodipine, aminoguanidine and DFMO were respectively given as described in Fig. 2. The dialysate collection and the data were processed as described in Fig. 1. ** and *** indicate $p<0.02$ and $p<0.01$ as compared to the preischemic values.

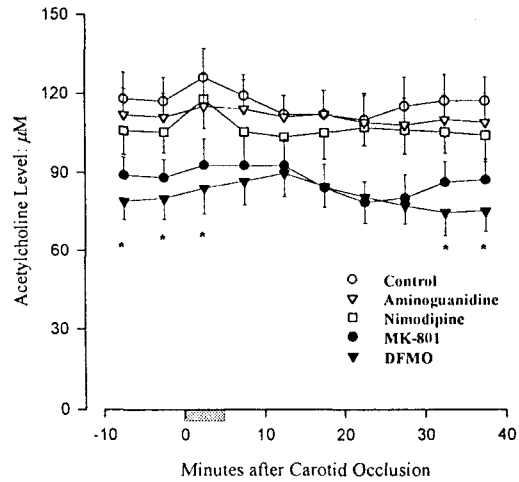

Fig. 4. Time course changes of the acetylcholine level of the dialysate obtained from the hippocampus of gerbils subjected to bilateral carotid occlusion for 5 min. MK-801, nimodipine, aminoguanidine and DFMO were respectively given as described in Fig. 2. The dialysate collection and the data were processed as described in Fig. 1. * indicates $p<0.05$ as compared to the values of the control group.

[^{3}H]MK-801 binding of hippocampal synaptosome (78.9 fmole/mg protein) was not affected by a treatment with either DFMO or NM, whereas was significantly decreased with AG and MK-801, respectively, by 39.0 and 46.0 % (Fig. 5).

Histopathology

Sham-operated gerbils showed no neuronal damage throughout the brain. Gerbils subjected to 5-min ischemia revealed severe neuronal damage in the brain. The most frequently affected regions were the hippocampal CA1 subfield, followed by hippocampal CA3 subfield. MK-801 did not attenuate the delayed neuronal death of hippocampal pyramidal cells 7 days after ischemia significantly, and AG and NM moderately protect against neuronal damage. However, DFMO aggravated the loss of hippocampal pyramidal cells in gerbils subjected to transient ischemia (Fig. 6).

DISCUSSION

The lack of posterior communicating arteries in the Mongolian gerbil (Levine and Sohn, 1969) renders it susceptible to high-grade bilateral forebrain ischemia following bilateral common carotid artery occlusion, and the Mongolian gerbil has become a very useful small animal model for brain ischemia. Reversible occlusion of both carotid arteries for 5 or more min results in delayed neuronal death of hippocampal CA1 pyramidal neurons in gerbils (Kirino, 1982). In this experiment, the effects of AG, DFMO, MK-801 and nimodipine on the is-

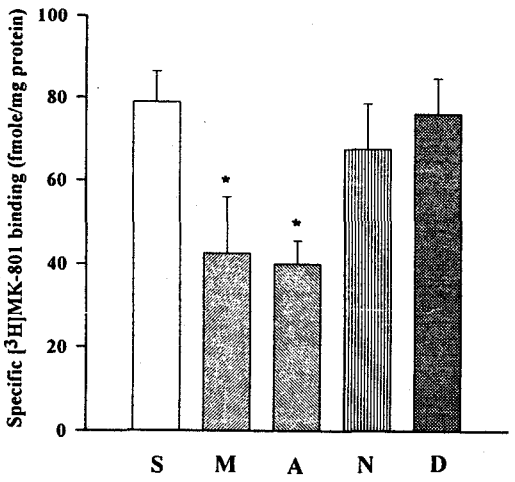

Fig. 5. Effects of MK-801, nimodipine, aminoguanidine and DFMO on the total specific binding of [^{3}H]MK-801 to the synaptosomal membrane fractions prepared from the hippocampus of gerbils decapitated on the 7th day after occlusion of both carotid arteries for 5 min. Treatments: Saline (S), MK-801 (M: 1 mg/kg) and nimodipine (N: 2 mg/kg) were respectively given 10 min before and for 7 days after both carotid artery occlusion. After the occlusion, each drug were given at 12 h intervals for 7 days from 12 h after the occlusion till 12 h before decapitation of gerbils. * indicates $p<0.05$ as compared to the value of the saline treated group.

chemia-induced changes of extracellular glutamate, polyamine and acetylcholine concentrations in the hippocampal CA1 sector of Mongolian gerbil with [^{3}H]MK-801 binding were studied with a histologic reference of the cresyl violet stained hippocampus.

It has been found by in vivo microdialysis of the hippocampus that the extracellular glutamate concentration increases significantly during 10 min of transient cerebral ischemia (Benveniste *et al.*, 1984; Globus *et al.*, 1991; Silverstein *et al.*, 1991). Glutamate could be released from neurotransmitter pools in neurons depolarized by the elevation of extracellular potassium seen under ischemia (Hansen, 1985). Release of glutamate from this transmitter pool is most probably dependent on calcium-influx (Kish and Ueda, 1991) via N-channel activation (Meyer *et al.*, 1989) and the release process is arrested within a few minutes after start of ischemia, showing ATP requirment (Diemer *et al.*, 1993). Another explanation could be that glutamate could diffuse out from the metabolic pools in neurons and astroglia due to altered permeabilities of cell membranes, possibly induced under ischemia (Drejer *et al.*, 1985). Alternatively, the elevation of extracellular glutamate seen under ischemia could be caused by impairment of the high-affinity uptake into presynaptic terminals and astrocytes, which under normal conditions appears quantitatively to be the major mechanism for removal of neuronally released glutamate. Approximately 2 min after induction of ischemia extracellular [K^+] increases steeply up to 60~80 mM with accompanying decrease in both extracellular sodium and chloride (Hansen, 1985). This drastic change of the [K^+]/[Na^+] ratio reverses the glutamate uptake carriers in astrocytes and presynaptic terminals (Globus *et al.*, 1988; Nicholls and Atwell, 1990). In the synaptosomes isolated from rat cerebral cortex, cyanide-induced hypoxia inhibits the Ca^{2+}-dependent release of glutamate when the intrasynaptosomal ATP/ADP ratio decreases below 1.7 and also induces a continuous glutamate efflux through a Ca^{2+}-independent pathway that accounts for 25% of the total intrasynaptosomal glutamate released in 5 min (Kauppinen *et al.*, 1988; Sanchez-Prieto and Gonzaales, 1988). Changes during ischemia may involve not only neuronal cells, but glia as well. Swelling of astrocytes is an early event in experimental brain ischemia models, and leads to membrane depolarization and neurotransmitter release (Kimelberg *et al.*, 1990; Kimelberg *et al.*, 1989). In this experiment, basal extracellular glutamate concentration (0.90±0.14 μM) was rapidly increased, reaching a peak of approximately 8.19±1.14 μM within 5 min after ischemia, followed by a decrease to baseline values during over 25 min after ischemia. AG, DFMO, or MK-801 had no effects on the ischemia-induced increase in glutamate concentration, but NM reduced the peak by about 20~30 %. The finding that NM reduced the ischemia-induced increase in glutamate concen-

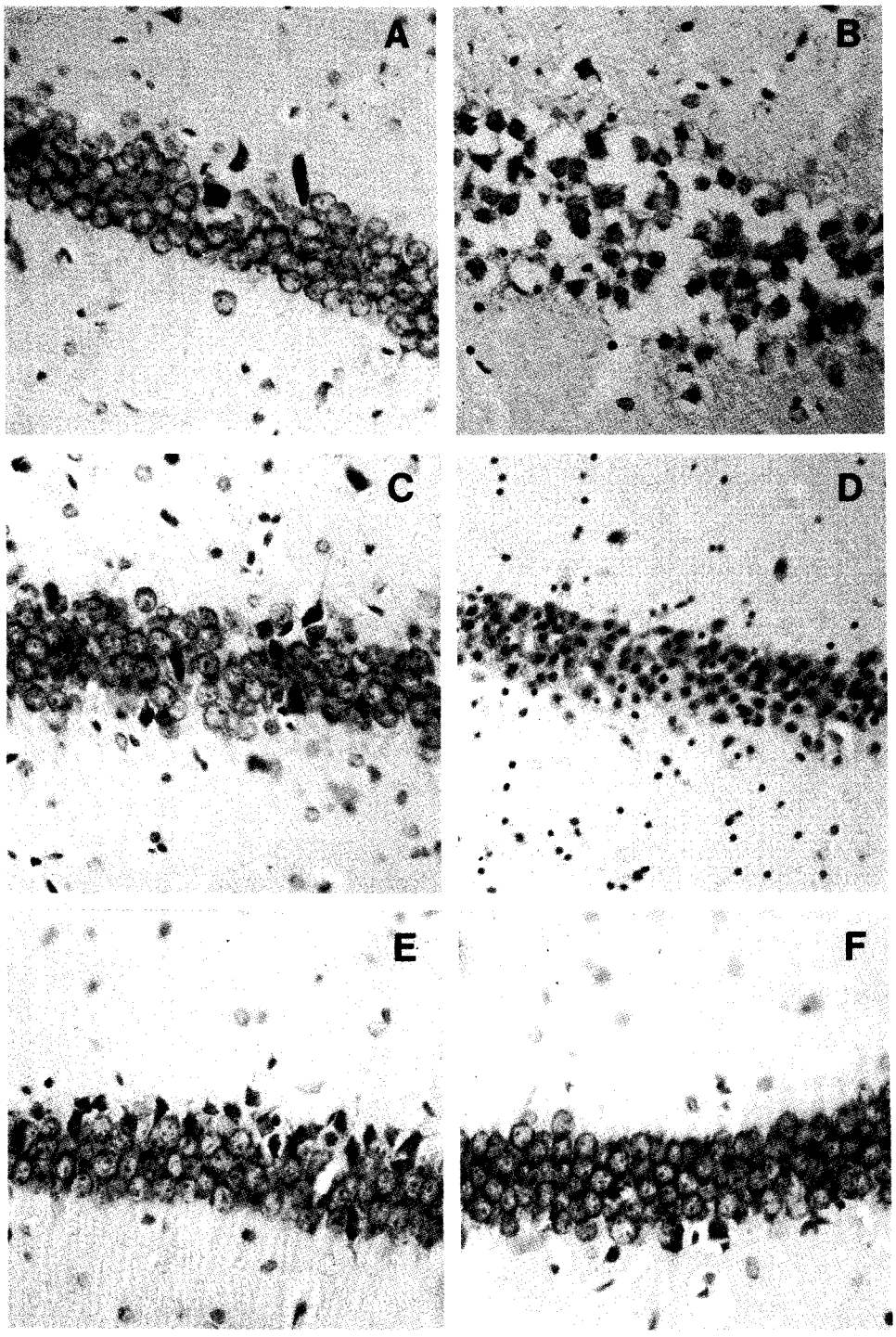

Fig. 6. Light microscopic findings of coronal sections stained with cresyl violet, showing morphological changes of pyramidal cells in CA1 area in the hippocampus on 7 days after 5 min transient cerebral ischemia. A: normal-sham operated, B: control-saline treated, C: aminoguanidine (100 mg/kg), D: DFMO (250 mg/kg), E: MK-801 (1 mg/kg), F: nimodipine (2 mg/kg)

tration is not consistent with those of Gemba *et al* (1993) that nimodipine did not inhibit the ischemic glutamate release even at a dose of 10 mg/kg in the hippocampal CA1 region of stroke-prone spontaneously hypertensive rats. Nimodipine is a L-type calcium channel blocker and was lipophilic enough to permeate into the brain tissue and to attain pharmacologically active levels at CNS receptor site (Krieglstein, 1990). Nimodipine-sensitive L-type channels may not be very common in nerve terminals (Lu *et al.*, 1991), and activation of presynaptic (N-type) calcium channels in nerve terminals is a primary cause of excessive neurotransmitter release in brain ischemia (McBurney *et al.*, 1992). However, the contribution made by changes in neuronal efflux to the overall increase in extracellular glutamate seen in ischemia is relatively minor (Collard and Menon-Johansson, 1993) and there is some evidence that exocytosis of glutamate from rat hippocampal synaptosome may be mediated by multiple types of calcium channels and NM inhibits Ca^{2+}-independent release of endogenous glutamate from intact synaptosomes (Terrian *et al.*, 1990).

Polyamine synthesis plays the key roles in cellular processes including neonatal growth, cell proliferation and differentiation as well as neoplastic growth (Pegg, 1986; Tabor and Tabor, 1984). Synthesis of the polyamines, putrescine, spermidine, and spermine is controlled by the activity of the rate-limiting enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC). Beside their function in cellular growth processes, polyamines, particularly putrescine has been shown to activate an influx of calcium ions (Komulainen and Bondy, 1987) and a release of excitotoxic amino acids from nerve endings (Reed and de Belleroche, 1990), and, if present in the extracellular compartment, modulate the activity of the NMDA receptor (spermidine and spermine) (Suzuki *et al.*, 1993; Enomoto *et al.*, 1993; Ransom and Stec, 1988). And they also modulate the calcium-buffering capacity of mitochondria (spermine) (Jensen *et al.*, 1987; Rottenberg and Marbach, 1990). Reversible cerebral ischemia triggers pathological disturbances in hippocampal polyamine metabolism that are characterized by a sharp increase of ODC synthesis and a marked suppression of SAMDC synthesis in parallel with the inhibition of overall protein synthesis. In the hippocampal CA1 subfield, however, putrescine levels remained high for several days after cerebral ischemia (Paschen *et al.*, 1987). ODC immunohistochemistry has revealed that the observed changes are neuronal responses to reversible ischemia (Müller *et al.*, 1991). In addition, evidence is accumulating that polyamines may be released from the cell during ischemia and after prolonged recirculation at a time when cell necrosis is apparent (Paschen, 1992). In the present study, post-ischemic elevated extracellular putrescine concentration was maintained at 1.7-fold basal for at least 4 h after ischemia, and a further sustained increase in putrescine concentration was noted by 6 h after ischemia. There was no consistent alteration in the level of spermidine following ischemia. But spermine concentration showed transient increase during ischemia followed by a sustained decrease down to about 40% below the basal output for 4 h after ischemia. These time-based changes of polyamines occured after ischemia support the studies of Koenig *et al* (1990), who observed early and delayed increases in putrescine level commenced 6 h after recirculation and Paschen *et al* (1987) who reported spermidine and spermine levels did not increase during recirculation. However, in contrast to the report the pretreatment with DFMO or MK-801 abolished all early and delayed increases in ODC activity and polyamine levels (Koenig *et al*, 1990). The present study demonstrated that the extracellular putrescine level in response to ischemia was only slightly decreased by the pretreatment with DFMO or MK-801. DFMO completely suppressed the rise in ODC activity following ischemia but little affected the ischemia-induced increase putrescine levels suggesting that the observed overshoot in the putrescine formation following ischemia was only partly caused by activation of ODC (Paschen *et al.*, 1988). This finding is further supported by the evidence that 30 min occlusion of both carotid and vertebral arteries produced a severe depression in SAMDC activity for several days in the rat (Dienel *et al.*, 1985). Postischemic treatment of gerbils

with NM significantly reduced the putrescine content in the lateral striatum, but it had no significant effect on polyamine synthesis in the hippocampal CA1 subfield (Paschen *et al.*, 1988). The NM effect on the post-ischemic increase in putrescine level was not readily apparent in the present study. MK-801 had no effect in gerbils on the ischemia-induced activation of ODC gene expression (Dempsey *et al.*, 1988) but suppressed the post-ischemic increases in ODC activity and putrescine level almost completely (Koenig *et al.*, 1990). Also it was known that introduction of the excitotoxins, kainate or ibotenate into the nucleus basalis results in a massive and rapid induction of ODC activity, and this induction of ODC was shown to be prevented by treatment of rats with MK-801, indicating the involvement of NMDA receptors (Reed and de Belleroche, 1990). The present results seem to support that there were some evidences that pathways other than NMDA receptor may be involved with the ischemic changes of ODC activaty and polyamine metabolism (Gardiner *et al.*, 1992; Gardiner and de Belleroche, 1990).

The binding of [^{3}H]MK-801 to NMDA receptors is enhanced in the presence of NMDA receptor agonists and are by competitive NMDA receptor antagonists (Foster and Wong, 1987). So, it appears that [^{3}H]MK-801 binds specifically to a site within the ion channel of the NMDA receptor complex under the activated state of the receptor. Also, the results of recent biochemical and electrophysiological studies have suggested that a recognition site for polyamines exists as a part of the NMDA receptor complex (Williams *et al.*, 1989). This site appears to be distinct from previously described binding sites for glutamate, glycine, Mg^{2+}, Zn^{2+}, and open channel blockers such as MK-801. In the presence of both glutamate and glycine, spermine and spermidine enhanced [^{3}H]MK-801 binding to the soluble receptor preparation (Ransom and Stec, 1988), whereas putrescine and cadaverine gave the binding inhibition of small degree (Bakker *et al.*, 1991). When spermine and spermidine were tested under the conditions where [^{3}H]MK-801 binding approached equilibrium, their abilities to enhance [^{3}H]MK-801 binding were much reduced, suggesting that the polyamines increase the rate to equilibrium (Bakker *et al.*, 1991; Romano *et al.*, 1991; Steele *et al.*, 1990). Putrescine are selectively inhibitory on the effect of polyamines that enhance the binding of [^{3}H]MK-801 (Williams *et al.*, 1989). So it is proposed that the polyamine site is merely modulatory, but neither necessary nor sufficient, for NMDA channel opening (Lehmann *et al.*, 1991; Williams *et al.*, 1991). Wallace *et al* (1992) observed that the enhancement of [^{3}H]HK-801 binding was related with elevated extracellular glutamate concetration in the occlusion of the middle cerebral artery of the rat. In the present study, [^{3}H]MK-801 binding of hippocampal synaptosome (78.9 fmole/mg protein) was not affected by the treatment with DFMO or NM, but was significantly decreased with AG or MK-801 by 39.0 and 46.0 %, respectively. These results may reflect that the postischemic increase in putrescine level may inhibit [^{3}H]MK-801 binding and reduce the NMDA receptor activation in response to ischemia. However, the decrease of [^{3}H]MK-801 binding does not always support the protection of the hippocampal pyramidal cells from ischemic injury, since a progressive loss of [^{3}H]MK-801 binding may be due to neuronal loss (Araki *et al.*, 1993; Araki *et al.*, 1992). But there was no clear correlation between neuronal necrosis and reduction in NMDA receptor (Araki *et al.*, 1993) and significant neuronal damage was not apparent in MK-801 or AG-treated groups in this study.

In the histopathological study, MK-801 did not significantly attenuate the delayed neuronal death of hippocampal cells 7 days after ischemia significantly, and AG and NM moderately attenuated the neuronal damage. However, DFMO aggravated the loss of hippocampal pyramidal cells in gerbils subjected to transient ischemia. The protective effects of MK-801 against ischemic cell damage has been controversial (Warner *et al.*, 1991; Von Lubitz *et al.*, 1993; Murase *et al.*, 1993). The present results seem to be a line with the studies showing that (Buchan *et al.*, 1991; Buchan and Pulsinelli, 1990; Buchan, 1992) treatment with MK-801 failed to protect CA1 hippocampal damage (Buchan *et al.*, 1991; Buchan and Pulsinelli, 1990; Buchan, 1992), and that neuroprotective

activity of MK-801 against transient global ischemia appears to be largely a consequence of postischemic hypothermia rather than a direct action on NMDA receptor complex (Hayward *et al.*, 1993). These results suggest that MK-801 is a relatively poor neuroprotective agent against a transient forebrain ischemia. Several reports have provided many eridences on the protective effects of NM against the ischemic damage of brain tissue (Krieglstein, 1990; Teasdale *et al.*, 1990; Jacewicz *et al.*, 1990). However, it was not clearly clarified that calcium antagonists actually reduce the cytosolic $[Ca^{2+}]i$ of the brain caused by ischemia in vivo, even if the drugs were administered preischemically. And the protective effects of NM in this study has been mainly explained by its effect on the cerebrovascular system (Teasdale *et al.*, 1990; Schmidt and Waldemar, 1990; Lo *et al.*, 1991). In Mongolian gerbils, NM exerted a full protective effect on CA1 pyramidal neurons only after repeated application extending over 24 hours after the ischemia (Mossakowski and Gadamski, 1990). Also, Hara *et al* (1990) reported that NM administered immediately after 3 h of transient unilateral middle cerebral artery occlusion significantly attenuated the post-ischemic increase of tissue water content. The findings in the present study that DFMO little affected the ischemia-induced increase of putrescine concentration is contrast with the protective DFMO effect against NMDA toxicity in tissue culture (Porcella *et al.*, 1992; Markwell *et al.*, 1990). However, the relationship between the extent of postischemic changes in putrescine level and density of cellular injury remains to be clarigied (Paschen *et al.*, 1988). There are four different mechanisms of polyamine-dependent cell injury that may be drawed from this study results: (1) an overactivation of calcium fluxes and neurotransmitter release in areas due to an overshoot in putrescine formation; (2) disturbances of the calcium homeostasis resulting from an impairment of the calcium buffering capacity of mitochondria in regions in which spermine levels are reduced; (3) an overactivation of the NMDA receptor complex caused by a release of polyamines into the extracellular space during and after ischemia; (4) an overproduction of hydrogen peroxide resulting from an activation of the interconversion of spermidine into putrescine via the enzymes spermidine N-acetyltransferase and polyamine oxidase (Persson and Pegg, 1984). The reason that DFMO aggravated the loss of hippocampal pyramidal cells in gerbils subjected to transient ischemia remains to be studied.

These results suggest that unlike to the mode of anti-ischemic action of NM, AG might protect the hippocampus from ischemic injury as being negatively regulatory on the NMDA receptor function in the hippocampus.

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=국문초록=

뇌허혈 손상에 있어서 해마-세포외액내 Glutamate와 Polyamine 농도의 변동에 관한 연구

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뇌-허혈후 나타나는 신경세포의 손상에 glutamate의 과다한 유리와 그의 N-methyl-D-aspartate (NMDA) 수용체: calcium 통로 활성작용 및 polyamine중 putrescine의 증가로 인한 신경세포내 [Ca^{2+}]의 상승과 관련 있다는 보고들이 있다. 본 연구에서는 Mongolian gerbil에서 5분간 경동맥을 차단하여 뇌-허혈을 가한후 재관류시 해마의 세포외액내 polyamine, glutamate, acetylcholine농도, 해마의 [^{3}H]MK-801 결합능의 변동 및 해마조직소견의 변동에 미치는 비가역성 ornithine decarboxylase (ODC) 억제제인 difluoromethylornithine (DFMO), diamine oxidase (DAO) 억제제인 aminoguanidine (AG), NMDA 수용체 길항제인 MK-801 및 calcium 통로 차단제인 nimodipine (NM)의 효과를 비교-검색하였다. 해마 세포외액내 polyamine, glutamate 및 acetylcholine은 microdialysis probe를 해마의 CA1부위에 위치시킨 후 나온 분취액을 HPLC와 luminometer를 사용하여 측정하였고, 해마조직에서 신경세포의 손상은 cresyl-violet 염색법으로 관찰하였다. 허혈후 해마 세포외액내 putrescine농도는 5분이내에 급속히 증가하여 뇌-허혈후 96시간까지 증가되는 경향을 보였으며 AG과 MK-801 처치시 saline 처치군에 비하여 증가정도가 상승되었으나 NM과 DFMO 처치로 putrescine의 증가는 감소되는 경향을 보였다. 해마 세포외액내 glutamate의 농도는 허혈후 5분 이내에 9배이상 유의하게 증가한 후 급격히 감소되어 25분후에는 정상치로 회복되었으나, 이같은 변동은 AG, DFMO 및 MK-801 처치로 영향을 받지 않았고 NM 처치로는 glutamate의 증가가 둔화되는 경향을 보였다. 해마 세포외액내 acetylcholine 농도는 허혈에 의하여 큰변동이 없었으나 허혈전 acetylcholine농도는 DFMO나 MK-801처치로 감소되는 경향을 보였다. 해마-synaptosome막의 [^{3}H]MK-801 결합능은 saline 처치군에 비하여 AG과 MK-801 처치로 유의하게 감소되었다. 해마의 조직소견상 AG과 NM은 허혈후의 신경세포손상을 억제하고, MK-801은 손상의 예방에 별 영향을 주지 못하였으나 DFMO는 허혈에 의한 신경세포의 손상을 더욱 악화시키는 경향을 보였다. 이상의 결과로 미루어 NM과 다른기전으로 AG은 해마신경세포의 손상을 NMDA-수용체: calcium 통로의 활성화를 조절하여 허혈성 뇌손상을 억제할 수 있으리라 사료된다.