Combination Therapy Protects Ischemic Brain in Rats
A Glutamate Antagonist Plus a γ-Aminobutyric Acid Agonist

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Background and Purpose The excitotoxic effects of glutamate can be blocked almost completely with γ-aminobutyric acid (GABA), an inhibitory neurotransmitter, in cell culture, tissue slices, and in some animal models. After stroke in rats, we showed previously that an agonist of GABA, muscimol, was as neuroprotective as MK-801, an antagonist of glutamate. To obtain further neuroprotection and to avoid the side effects associated with high doses of MK-801, we wanted to assess the efficacy of the two agents in combination.

Methods Treatment was administered 5 minutes after embolic cerebral ischemia in Sprague-Dawley rats. The subjects were rated using a neurological evaluation 48 hours later. Visual-spatial learning was measured 8 to 10 weeks after stroke, after which we measured the volume of each cerebral hemisphere and several large cerebral compartments. Treatment groups included saline (n=27), MK-801 1.0 mg/kg (n=23), muscimol 1.0 mg/kg (n=17), and both agents together using a dose of 0.5 mg/kg each (n=25).

Results A probit analysis of the neurological ratings revealed a protective effect of muscimol used alone (MK-801 potency ratio, 2.0; P=.NS; muscimol potency ratio, 4.0; P<.05) and a protective effect of the combination (potency ratio, 5.0; P<.05). Focal ischemia caused a moderate to severe delay in the acquisition of visual-spatial information, which was completely eliminated by the combination treatment but only partially ameliorated with MK-801 or muscimol alone. Ischemia reduced the cerebral hemisphere volume from 0.42 mm$^3$ to 0.34 mm$^3$ (P<.0001), the volume density of cortex from 22% to 17% of total cerebral volume (P<.01), and that of hippocampus from 4.3% to 3.0% (P<.05). Only the combination was neuroprotective, as measured by the ratio of the lesioned to the contralateral hemisphere volume (P=.013). The combination treatment and MK-801 protected the hemisphere volume, the cortex, and the hippocampus and reduced the size of visible infarction.

Conclusions Combination therapy, using a glutamate antagonist and a GABA-A agonist, appeared to protect the brain and ameliorate a defect in learning behavior after stroke. The combination may have been more effective than either agent used alone, although further study of higher doses is needed. (Stroke. 1994;25:189-196.)

Key Words • embolism • GABA • glutamates • neuroprotection • rats

The excitotoxic hypothesis of ischemic injury is that glutamate receptor activation causes depolarization and voltage-gated entry of cations and water. Consistent with the hypothesis is the evidence that antagonists of the N-methyl-D-aspartate (NMDA) glutamate receptor block the excitotoxic sequelae of ischemia in tissue culture, slices, and animal models of cerebral ischemia. In response to these data, several trials of glutamate antagonists for neuronal protection have been organized. Clinical trials of NMDA antagonists for stroke are progressing slowly, however, because the agents cause unwanted behavioral side effects. Further, the NMDA receptor antagonist MK-801 appears to cause toxic microvacuolization in the cytoplasm of some neurons of the cingulate and retrosplenial cortex. These vacuoles are of unknown significance but may be prevented by anticholinergic agents.

In searching for an alternative way to block the toxicity of glutamate without side effects, we noted that the physiological and pathological consequences of excitotoxicity can be partially blocked with inhibitory neurotransmitters. The inhibitory neurotransmitter γ-aminobutyric acid (GABA) is ubiquitous in the brain, with very high concentrations in cortex and striatum. GABA application to cells completely blocks the effects of glutamate, including depolarization and voltage-gated calcium influx. GABAergic neurons are particularly resistant to ischemia in culture and in tissue slices. Recently we reported that the GABA-A agonist muscimol was very effective as a neuroprotectant during ischemia, and others have found similar results with other GABA-mimetic agents.

In the present study we sought to find a more potent treatment by using a combination of muscimol and MK-801. The combination ought to be more protective than either agent alone because both drugs block voltage-gated calcium entry through complementary mechanisms. We also sought to study the effect of the combination treatment on infarction volume. Infarction volume is difficult to measure from serial sections, shows considerable interindividual variance, and may reflect only damage to tissue at the central core of the ischemic territory, ignoring any effect in the penumbra. We consequently developed a method for accurately measuring the volume of discrete infarction.
cerebral compartments, such as cortex, hippocampus, and white matter.

Materials and Methods

The experimental protocol was approved by the University of California at San Diego Animal Research Committee following all national guidelines for the care of experimental animals.

Our surgical procedure, the bioassay, and the behavioral methods have been published in detail and will only be summarized here.27-30 To avoid any confounding interaction between anesthesia and ischemia, we implanted catheters in the left internal carotid artery of male Sprague-Dawley rats (250 to 300 g) and waited 3 hours for recovery from inhaled halothane anesthesia before embolization. We injected a bolus of 125I-labeled, 25-μm-diameter plastic microspheres (3M Corporation, Minneapolis, Minn) through the implanted catheter directly into the middle cerebral artery. We randomly divided 92 animals into four treatment groups and reserved 26 as unoperated controls. Within 5 minutes of embolization we administered by tail vein a bolus of saline (n=27), 1.0 mg/kg MK-801 (a gift from Merck, New York, NY, n=23), 1.0 mg/kg muscimol (Sigma Chemical Co, St Louis, Mo, n=17), or a combination of 0.5 mg/kg MK-801 plus 0.5 mg/kg muscimol (n=25). All solutions were prepared so that each animal received an infusion of 1.0 mL/kg over 0.5 minutes. Forty-eight hours later an examiner blinded to each group assignments rated each subject as follows: normal, abnormal (diminished level of consciousness, reduced exploratory behavior, circling, limping, or inability to maintain upright posture), or dead. These ratings are valid and highly reproducible and are used for the bioassay, which is described completely elsewhere.27-29 At death or euthanasia each brain was placed into a gamma counter and from the measured radioactivity and the specific activity an estimate of the quantity of trapped microspheres was calculated. The radioactivity measurements were corrected for decay over the time interval between injection and euthanasia. For each group of animals we estimated the quantity of trapped microspheres that caused 50% of the animals to be abnormal or dead, a parameter termed the ED50. The ED50 and its standard error were calculated using the probit analysis method available in the SPS package (SPSS Inc, Chicago, Ill) by comparing the global neurological rating to the amount of microspheres recovered in the brain of each subject.28 In several previous investigations, the ED50 has been shown to be a highly reproducible and efficient measure of the efficacy of cerebral protective agents.27-30 A protective drug causes the ED50 to be larger in a treated group of subjects.

Two to 4 weeks after embolization, all surviving rats were tested for visual-spatial learning using a Morris water maze.28,34 For this study, we tested all animals daily using a visible escape platform that is 1.5 cm above water level. On the fourth day of preliminary testing with the visible platform, all animals could swim directly to the platform, averaging 5 to 8 seconds per swim. Animals with severe motor, sensory, or visual deficits that would confound testing of visual-spatial memory cannot complete this task and are eliminated from further testing, but no animals were so excluded in this study. Next, learning of the visual-spatial information necessary to navigate to the escape platform was tested by using a submerged platform. The swimming rat cannot see the platform and the unlesioned control subjects (n=26) constructed learning curves from the escape latency data. To do this, we transformed the latencies from all 10 days by taking the inverse and calculated a bivariate regression equation between (latency)-1 and trial day. A straight line can be fit to the data with a reasonable goodness-of-fit.37 The slope of the line fit to the data is a direct measure of the rate of learning for each group of subjects.

Three months after ischemia we performed detailed morphometry on all animals that survived the embolization and water maze testing. After anesthesia with halothane, the descending aorta was clamped, and 60 mL of normal saline was perfused via a transcardiac catheter at room temperature and physiological pressure using a pulsatile flow infusion pump (Harvard Apparatus, Cambridge, Mass). After clearing, another 60 to 100 mL of 4% buffered paraformaldehyde was perfused. Fixation occurred in situ for 30 to 60 minutes at 4°C, after which each brain was removed and immersion fixed for an additional 24 hours. The cerebellum and brain stem were removed after 24 hours' fixation by cutting through the midbrain. We sectioned each brain entirely using a freezing microtome, selecting a 30-μm-thick section every 400 μm throughout. The sections were mounted on gelatin-substrate slides and stained with cresyl violet and Luxol Fast Blue. Each microscopic section was examined using a video-enhanced image analysis system (Optimas Image Analyzer, Bioscan Co, Seattle, Wash). The area of each hemisphere was determined by threshold analysis and automated planimetry.28 Following the method of Cavaleri, the hemisphere volume was calculated by summing the areas of the individual sections and multiplying by the mean distance between adjacent sections.39 To obtain compartment volumes rapidly, we adapted the stereological method of point counting, and compartment planimetry to be inaccurate and time consuming. We divided the total number of points falling on each compartment by the total number of points falling on the entire cerebrum to determine the proportion of the cerebrum made up by each compartment. This ratio is called the compartment volume density because it expresses the volume of a substructure as a fraction of the larger containing volume.39

Results

No attempt was made to control brain temperature or blood pressure in these unanesthetized subjects. Ischemia after embolization with microspheres caused a range of abnormal clinical findings, consistent with previous experience using this model.31-34 All three treatments caused sedation in the subjects, which resolved within 24 hours. The bioassay results are depicted in Fig 1 and show that muscimol and the combination treatment were effective neuroprotectants, as indicated by the increase in the ED50, compared with the saline-treated group. In this study MK-801 did not show a statistically significant benefit, although it has proven effective in previous studies using the same model.44 The ED50 for the combination therapy was greater than that for either treatment used alone, but this increase was not statistically increased over the muscimol group. The potency ratio, defined as the ratio of the ED50 in a treatment group versus that for the saline group, was approximately 2.0 for MK-801 (P=NS), 4.1 for muscimol (P<.05), and 4.9 (P<.05) for the combination group.

The water maze test results are summarized in Fig 2 and generally confirm the bioassay results. The latency data are illustrated in Fig 2, top panel, and show that during the testing period all animals learned to find the escape platform. The unlesioned control subjects (n=26)
Ischemia significantly reduced the volume of the hemisphere ipsilateral to the side of microsphere injection. In the unlesioned control group, hemisphere volume on either side was 0.42±0.05 mL (mean±SD) (n=15). In the saline-treated group the hemisphere ipsilateral to the side of the microsphere injection was significantly reduced by approximately 20%, to 0.34±0.05 mL (mean±SD, P<.0001). The contralateral hemisphere was reduced by approximately 9%, to 0.38±0.03 mL (P<.001), suggesting that some of the injected microspheres crossed to the contralateral hemisphere. To account for this and other potential confounding effects, we compared the volume of the hemisphere ipsilateral and contralateral to the embolization and expressed the result as the hemisphere volume ratio
By guest on August 14, 2017

This mean ratio for the muscimol group appears in the top panel. For clarity we present only the most

multivariate ANOVA revealed a significant effect of treatment (P<0.001); to examine the effect of each treatment, we applied the least significant difference test (significance level of P<0.05) to account for multiple comparisons and reduce the chance of finding a difference due to chance alone. Cortex was approximately 22% of total cerebral volume in the unlesioned control hemisphere. Cortex and hippocampus were significantly reduced in volume by ischemia in the saline-treated group, but other large compartments were unaffected. MK-801 and combination treatment ameliorated this effect. The volume density of white matter in muscimol-treated animals was significantly reduced compared with controls and combination treatment. Infarction volume was 9% of the hemisphere, and whereas MK-801 and combination reduced this significantly, muscimol was not protective. The volume of the ipsilateral ventricle was significantly reduced in the muscimol and MK-801 groups but not in the combination group. In the hemisphere contralateral to the side of the embolization (bottom panel), ischemia significantly reduced the volume density of cortex and hippocampus in the saline-treated group. In cortex both muscimol and MK-801 eliminated the damage, and in hippocampus all three treatments were effective. In the thalamus only muscimol was protective, and no significant reduction of the basal ganglia occurred. The ventricle was significantly enlarged in the contralateral hemisphere, an effect not present in the muscimol or MK-801 specimens. *Significantly different from controls and + significantly different from saline treatment (both P<.05 after correction for multiple comparisons).

Treatment also protected cerebral compartment volume densities, as illustrated in Fig 3, middle panel. For each structure there are 10 nonredundant post hoc procedures that correct for multiple comparisons. As shown in the middle panel of Fig 3, ipsilateral to the side of the embolization, cerebral cortex was reduced from 22.4±1.2% of cerebrum (mean±SD) in 15 unlesioned control subjects to 17.5±3.6% in 13 saline-treated subjects (P<.0001). Hippocampus was reduced by approximately 31%, and MK-801 (n=8) and the combination therapy (n=13) blocked this reduction, whereas muscimol (n=13) did not (P<.05). In the muscimol group, the white matter and thalamus compartments were significantly reduced, but this may represent an artifact or chance finding since there is no known toxicity of muscimol to myelin. As shown in the bottom panel of Fig 3, the contralateral
cortex was 22.3 ± 1.2% (mean ± SD) of total cerebrum, and ischemia reduced this value in the saline-treated group to 20.8 ± 1.0%, a statistically significant result (group effect F_4,57 = 9.16, P < .001; post hoc comparisons, P < .05). Muscimol and MK-801 blocked this reduction, but the combination treatment did not. Similarly, the volume density of hippocampus contralateral to the side of the embolization was reduced from 4.2% to 3.6% of total cerebral volume (F_3,57 = 10.49, P < .0001), and all three treatments blocked this damage.

The volume of visible infarction was 8.7 ± 10.8% of cerebrum in the saline-treated group, compared with 9.9 ± 11.8%, 1.8 ± 3.9%, and 1.4 ± 4.1% for muscimol, MK-801, and combination treatment, respectively (Fig 3, middle panel). The overall ANOVA for an effect of treatment group on lesion size is significant (F_3,57 = 4.6, P = .003), and post hoc comparisons revealed that the MK-801 and combination results are significantly different from the saline and muscimol treatments (P < .05).

The morphometric and behavior testing results are confounded by the fact that muscimol treatment enabled animals with larger insults to survive. Mortality was 52% (14/27) in the saline group, 65% (15/23) in the MK-801 group, 24% (4/17) in the muscimol group, and 48% (12/25) in the combination group. Therefore, we found significantly more microspheres in the brains of the surviving muscimol-treated animals (Fig 4). In the other groups, animals with larger amounts of microspheres did not survive to be tested in the water maze and undergo morphometric analysis, and hence the results are biased away from showing a beneficial effect in the muscimol-treated animals.

**Discussion**

The results show that combination treatment with muscimol and MK-801 is protective following cerebral ischemia as measured with a bioassay, behavioral testing, and the hemisphere volume ratio. We confirmed our earlier report that a GABA-A agonist is at least as potent as MK-801 as a neuroprotectant, but the data do not clearly support a synergistic effect of the combination treatment at the doses we used. This report is of particular significance because there are several GABA-A agonists either currently available or nearing clinical use, while no glutamate antagonist has progressed beyond early phase I clinical testing. In previous studies, including our own, MK-801 has shown protective effects in the bioassay, and the current findings likely reflect chance variation.

\(\gamma\)-Aminobutyric acid blocks the voltage-gated calcium influx associated with glutamate, and this is the likely mechanism of the protective effect of muscimol. Muscimol is derived from the mushroom Amanita muscaria, readily crosses the blood-brain barrier, and is the most avid agonist of the GABA-A receptor known. It has variable effects on cerebral blood flow and appears to cause metabolic inhibition in the brain. In addition to membrane voltage stabilization, agonists of the GABA-A receptor increase cerebral blood flow and decrease cerebral metabolism in humans. Muscimol given before the onset of ischemia was protective in a previous animal study and in our prior study when given 5 minutes after ischemia. The GABA-B agonist baclofen was not protective in a previous study of radial arm maze learning after bilateral forebrain ischemia, further suggesting that the protection of GABA is a postsynaptic effect.

This report also contains a new, quantitative method for measuring the effect of an insult, such as ischemia, on brain compartment volumes. We found that unilateral cerebral embolization significantly reduced the ipsilateral cortex volume from 22% to 17% of whole cerebrum (see Fig 3) and had a similar although lesser effect in the contralateral hemisphere. Other structures known to be vulnerable during ischemia tend to contain a much higher density of glutamate receptors, including hippocampus and, to a lesser extent, the striatum. Structures known to be more resistant to ischemia, such as the cerebral white matter, were not affected by ischemia.

The reduction in cortex and hippocampus volume contralateral to the side of the embolization may reflect ischemia due to the migration of some microspheres via the circle of Willis. Conversely, several authors have noted depression in cerebral metabolism contralateral to ligation of the middle cerebral artery. The morphometric method we developed is ideally suited to quantitative study of the effect of such "diaschisis" associated with unilateral middle cerebral artery occlusion, if it exists, on cerebral compartment volumes. Further studies are also needed to determine the effect of other insults, such as trauma or cerebral hematoma, on compartment volumes. After these lesions there may be a differential effect on other compartments, such as the white matter degeneration associated with mild, concussive head trauma. Our morphometric method is applicable to such studies without modification and was efficient and reproducible in pilot studies.
The combination and muscimol treatments were highly effective as measured by the bioassay (Fig 1). All three treatments ameliorated the learning deficit typically caused by ischemia in rodents, and the combination was also superior to muscimol alone (Fig 2, bottom panel). The hemisphere volume ratio clearly shows that MK-801 and the combination are superior to muscimol alone (Fig 3, top panel). Despite showing protection in the bioassay, however, muscimol was not as protective as MK-801 on the volumetric outcomes. This apparent contradiction may be addressed in several ways. First, the bioassay takes into account all subjects, including those that died, while morphometry can only be performed on survivors. It is clear that muscimol and the combination treatment had a greater positive effect on survival than did MK-801 used alone (see Fig 1). Second, because of the enhanced survivability with muscimol treatment, the groups are not well matched with respect to the quantity of microspheres lodged in the brains of the survivors (Fig 4). This imbalance would bias the muscimol-treated group away from benefit, since the surviving subjects might do less well in the water maze task and suffer greater volumes of infarction. Third, we used 1.0 mg/kg of MK-801 alone but only 0.5 mg/kg of MK-801 in the combination group to avoid the excessive sedation we noted in pilot dose-finding studies. It is possible that the combination should include a higher dose of MK-801, and this should be the subject of future investigations. Also, we treated the subjects very soon after onset of ischemia to simplify the treatment protocol. Clearly it will be necessary to test the combination treatment at later time points that are more relevant to treating human stroke.

The treatment effects are not consistent across the several cerebral compartments. For example, the middle panel of Fig 3 shows that MK-801 and the combination were protective as measured by the volume densities of cortex and visible infarction, but only MK-801 was effective in hippocampus. The bottom panel of Fig 3 shows that while muscimol and MK-801 were individually protective of the cortex, the combination was not. The most likely explanation for these discrepancies is that we found some small changes that spuriously achieved statistical significance due to the large number of comparisons, despite our use of post hoc testing procedures to prevent this. Therefore, these results will require confirmation using an alternative model before they can be fully accepted. Alternatively, some of the seemingly discrepant results may reflect the inhomogeneous distribution of NMDA and GABA-A receptors in the cerebrum. For example, MK-801 was most protective in areas that are relatively higher in NMDA receptors, cortex, and hippocampus (Fig 3). On the other hand, combination treatment was more effective in basal ganglia, a region known to have a relatively higher density of GABA-A receptors. Some evidence suggests that ischemic damage in the thalamus is not mediated by the NMDA receptor, and therefore treatment results in this region may be unpredictable.

The inhomogeneity of receptor distribution may also help explain the discrepancy between the behavioral and morphologic outcomes. Cerebral hemisphere volume represents the sum of all the compartment volumes, and the infarction volume is the sum of the visible damage that occurs in all brain structures. It is not proven that learning and total brain volume are tightly linked, but it is quite clear that some structures, notably hippocampus and thalamus, are critical areas subserving learning and memory. It is possible, although speculative, that a putative neuroprotectant may have a greater positive effect on brain elements required for learning and memory than on the grossly visible volume of infarction. While Lashley and others have tried to associate a "mass effect" of brain volume on behavior, it is now fairly well accepted that strategically placed lesions may impair learning disproportionately to the total volume of brain removed. In support of this speculation is the observation that glutamate receptors are found with highest concentration in some areas critical to memory. Thus, although it would be gratifying to find a simple correlation between the neuroprotective effects of these drugs on learning and brain compartment volumes, the complexity of brain/behavior interactions argues strongly against such a result.

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