Neuroprotective Effects of SKF 10,047 in Cultured Rat Cerebellar Neurons and in Gerbil Global Brain Ischemia

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Background and Purpose: Excitatory amino acids and their receptors are involved in mediating ischemic neuronal damage. The σ-agonists are believed to interact with the N-methyl-D-aspartate receptor. Therefore, we studied the neuroprotective, hypothermic, and motor deficit effects of the σ-agonist SKF 10,047 and the N-methyl-D-aspartate antagonist MK-801.

Methods: Neuroprotective effects were compared using an in vitro ischemia model of cultured rat cerebellar granule cells and the gerbil model of global brain ischemia induced by 5 minutes of bilateral carotid artery occlusion followed by 7 days of reperfusion.

Results: In vitro, (+)MK-801 protected against 100 μM glutamate with a 50% protective concentration of 30 nM, followed by (-)MK-801 (150 nM), cyclazocine (0.5 μM), (+)SKF 10,047 (3.3 μM), pentazocine (5 μM), and (-)SKF 10,047 (10 μM). In vivo, (+)SKF 10,047 pretreatment (60 mg/kg) or multiple postischemic treatments provided neuroprotection comparable with MK-801 pretreatment (10 mg/kg). When ischemic animals were administered the multiple dosing regimen of (+)SKF 10,047, no hypothermic effect was noted in the temporalis muscle over 4 hours postischemia. Motor deficits monitored by a swing grid test showed that 50% recovery from (+)SKF 10,047 was 5.5 times faster than recovery from MK-801.

Conclusions: These results are the first to report a hypothermia-free, in vivo neuroprotective effect of (+)SKF 10,047, a prototypical drug of the σ-agonist class.

KEY WORDS • cerebral ischemia • neuroprotection • gerbils • rats

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f the many noncompetitive antagonists of the N-methyl-D-aspartate (NMDA) receptor, MK-801 has been found to be the most efficacious compound interacting at this site. In binding studies, MK-801 has been found to have the highest affinity for ligands binding to the NMDA channel both in brain membranes and in vivo. In neuroprotection studies, MK-801 has been found to protect striatal and hippocampal neurons against damage induced by ischemic insult or exogenous glutamate or NMDA, and at lower doses than other comparable compounds. This holds for in vitro cell culture models as well, with regard to inhibition of NMDA-modulated second messenger systems and protection of neurons and astrocytes. However, use of MK-801 is not without side effects, the most noticeable being the psychotomimetic effect, probably due to binding to the phencyclidine (PCP) binding site. In addition, there are reports of neurotoxicity, stimulation of phosphoinositide hydrolysis, and oncogene induction, suggesting a mitogenic action. Because drug discrimination studies have shown that MK-801 causes generalization to most other non-competitive NMDA antagonists, they, too, have been tested for efficacy in the various models cited above, with the hope of achieving neuroprotection without untoward side effects. We have recently reported that the prototypical σ-agonist SKF 10,047 (N-allylnormetazocine) was neuroprotective against the excitotoxic action of glutamate in an in vitro ischemia model system of cultured rat cerebellar granule cells. This type of neuroprotection has been seen in other in vitro model systems as well. The compounds (+)SKF 10,047 and MK-801 are thought to be neuroprotective by acting as noncompetitive antagonists at the NMDA receptor channel, blocking cation influx initiated by the excitatory amino acids glutamate and aspartate. However, (+)SKF 10,047 also binds with higher affinity to a haloperidol-sensitive σ-site, which is better defined by the more specific σ-ligands (+)-3-PPP [3-(3-hydroxyphenyl)-N-(1-propyl)piperidine] and DTG (1,3-di-ortho-tolyl-guanidine). This σ-site has little affinity for MK-801, yet animals trained to recognize MK-801 also recognize (+)SKF 10,047 in drug discrimination studies (cross-generalizing) through interactions at the PCP site. The higher affinity σ-site is thought not to play a role in (+)SKF 10,047 discrimination. Because of the complex pharmacology of these drug interactions, recently dubbed “the sigma enigma,” we decided to compare the neuroprotective effects of SKF 10,047 and MK-801 in our in vitro cell culture system and in the gerbil model of global brain ischemia.
Primary cultures of rat cerebellar neurons were prepared from 8-day-old Sprague-Dawley rat pups (Taconic Farms, Germantown, N.Y.) and used after 8 or 9 days in culture for neuroprotection studies as previously described.12-14 This in vitro ischemia model differs from other models of glucose deprivation or hypoxia13,15 by demonstrating rapid (30-minute) excitotoxicity by exogenous glutamate through the NMDA receptor16 and emphasizes an enhanced response to low concentrations of agonist once the voltage-dependent Mg2+ block is removed as a result of energy depletion.30,31

The Mongolian gerbil model of global forebrain ischemic stroke has been extensively studied and characterized.4,6,7,20-35 In brief, male Mongolian gerbils weighing 60-70 g (Tumblebrook Farms, West Brookfield, Mass.) were anesthetized with 2.5% isoflurane in a mixture with 100% O2, placed onto a heating pad to maintain body temperature at 37°C, exposed to a 5-minute bilateral carotid occlusion, and allowed to recover on the heating pad for 10 minutes. At appropriate times, bilateral neuronal counts over a 750-μm section of the hippocampal CA1 regions stained in 0.1% Thionin were determined by microscopic examination of coded slides.

Untreated, conscious gerbils were injected with either 60 mg/kg s.c. (+)SKF 10,047 or 3 mg/kg i.p. MK-801 and compared with saline-injected animals for motor function over time as monitored by a swing grid test, which is an adaptation of the inclined plane test.36 In brief, gerbils were placed onto a 5 x 7-inch metal mesh grid held horizontally. The grid was rotated 180° about a fixed axis, starting in a downward direction, over a 2-second interval. The number of degrees at which the animal lost its grip was recorded. Results were expressed as the number of degrees needed to complete 180°; this value was 0° for untreated animals able to hold on to the grid while upside down.

The rectal temperature of gerbils was monitored with a RET-3 rectal probe for mice. Temporalis muscle temperature was measured with a TYPE MT-29/2 hypodermic needle microphone attached to a Sensortek TH-8 Temperature Monitor (Physitemp Instruments, Inc., Clifton, N.J.).

The enantiomers of SKF 10,047 were either obtained through the National Institute on Drug Abuse, Rockville, Md., or purchased from Research Biochemicals, Inc., Natick, Mass., which was also the source of the enantiomers of MK-801. Cyclazocine and pentazocine were obtained as racemic mixtures from in-house supplies.

Data in text and figures are mean±SEM values of the indicated number of experiments. Comparisons between treatment groups and vehicle were analyzed with the most rigorous tests applicable to each pool of data and are specified in the figure legends. Significant difference was accepted at p<0.05, and probability values for each data set were calculated.

Results

Figure 1 shows the protective effects of the enantiomers of MK-801 and SKF 10,047 along with two related benzomorphans that act at the NMDA receptor.37 The data from our previous study38 was included for comparison. The most potent compound tested was (+)MK-801, with a 50% protective concentration (PC50) of 30 nM (Figure 1). (-)MK-801 was fivefold less protective, with a PC50 of 150 nM. Among the benzomorphans, (+)-cyclazocine was the most potent (PC50=0.5 μM), followed by (+)SKF 10,047 (PC50=3.3 μM), (±)-pentazocine (PC50=5 μM), and (-)SKF 10,047 (PC50=10 μM). This relative order of potency is the same as defined by binding studies performed on rat brain membranes.4,24

Three or 7 days after 5-minute ischemia, there was 86% or 71% respective protection of CA1 neurons when (+)SKF 10,047 was given 15 minutes before occlusion at 60 mg/kg but not at 30 mg/kg (Figures 2 and 3). A variation of the treatment regimen is also shown in Figure 3, where administering (+)SKF 10,047 as a bolus of 15 mg/kg at 15 minutes and 1 hour after reperfusion followed by 30 mg/kg at 2, 4, and 6 hours after ischemia was nearly as effective (61% protection, p=0.03) as a single 60-mg/kg pretreatment dose. However, there was no protective effect when (+)SKF 10,047 was given as a single 60-mg/kg dose 30 minutes after reperfusion.

Previously, MK-801 had been found to be optimally neuroprotective at a dose of 3 mg/kg when administered by intraperitoneal injection.6,9 Our results using this treatment regimen show that pretreatment with MK-801 at 3 mg/kg given 1 hour before occlusion gave 82% or 64% protection of CA1 hippocampal neurons 3 or 7 days after ischemia (Figure 4); MK-801 at 10 mg/kg (85%) was statistically no more protective than at 3 mg/kg 7 days after reperfusion (Figure 4). We tested the hypothermic effects of 3 mg/kg MK-801 on conscious gerbils to avoid anesthetic or surgical effects on temperature36-38 and monitored both rectal and temporalis muscle temperatures, the latter as a reliable indirect estimate of brain temperature39 (Figure 5). At both sites temperatures decreased significantly by approximately one degree, 1 hour after intraperitoneal administration of MK-801. The hypothermic response

**Figure 1.** In vitro protective effects of various noncompetitive antagonists against glutamate-mediated excitotoxicity in rat cerebellar granule cells. Cells were incubated in the absence of glucose for 40 minutes before addition of 100 μM glutamate, and toxicity was assessed 30 minutes later by staining with fluorescein diacetate. Antagonists were added 10 minutes before addition of glutamate. Protection of neurons was determined by counting cells and expressing the percentage viability. Results represent single titrations repeated three times for each compound.
FIGURE 2. In vivo protective effects of (+)SKF 10,047 3 days after 5-minute ischemia. (+)SKF 10,047 was administered as a bolus of either 30 or 60 mg/kg s.c. in saline 15 minutes before 5-minute bilateral carotid occlusion. Results represent average CA1 hippocampal neuronal counts over a 750-μm length of both left and right hemispheres. Number of gerbils used is indicated over each bar. Comparisons within group receiving 30 mg/kg were performed separately using Student's t test and found to be nonsignificant. Data obtained for group receiving 60 mg/kg was analyzed by two-factor analysis of variance and Student's t test. Protection by 60 mg/kg was highly significant compared with vehicle-treated gerbils (p=0.0003).

was maintained at a maximal level for at least 24 hours (Figure 5). To evaluate the hypothermic effect of (+)SKF 10,047, we monitored the temporalis muscle temperature of ischemic gerbils receiving (+)SKF 10,047 and found no significant decrease in temperature within the assumed 45-minute therapeutic window of opportunity35 (Figure 6). Significant changes from baseline temperatures after 4 hours of treatment were resolved by 30 minutes.

As seen in Figure 7, normal, conscious gerbils receiving an equally neuroprotective dose of each drug were ataxic within as early as 15 minutes and were unable to hold on to the grid. (+)SKF 10,047-treated gerbils had 50% return of function by 2 hours and showed no further differences. In contrast, animals given MK-801 were severely disabled for 8 hours (Figure 7). Return of motor function for the MK-801 test group was apparent by 13 hours but was still significantly different from saline-treated animals (p=0.0002). By analyzing the time required for 50% recovery (T½), we calculated a T½ of 2 hours for (+)SKF 10,047 and a T½ of 11 hours for MK-801, establishing a 5.5 ratio of motor impairment.

Discussion

The relative order of potency of the drugs tested in our cerebellar granule cell model of in vitro ischemia was the same as that defined by inhibition of [3H]MK-801 binding to rat brain membranes.424 Our in vitro neuroprotection results are most likely a reflection of drug interaction at the NMDA receptor to prevent glutamate-induced excitotoxicity.12,14,25,30-31 The same antagonist order of potency has also been seen for neuroprotection of cortical neurons exposed to NMDA or hypoxia,13 for blocking NMDA excitotoxicity in ex vivo chick embryo retina,22,39 for inhibition of NMDA modulation of muscarinic-stimulated phosphoinositide
Lysko et al  Neuroprotection by α-Agonist SKF 10,047  417

hydrolysis in rat cortical slices,11 and for ability to reduce NMDA-evoked and spontaneous epileptiform activity of neocortical slices.40 The 10-fold protective difference that we saw between ±cyclazocine and ±pentazocine mirrors their differential potency as antagonists of N-methylaspartate-evoked excitations of spinal neurons.37 It is likely that the in vitro data are predictive of activity in vivo, in which only twenty times more (+)SKF 10,047 was needed to achieve comparable neuroprotection in the gerbil model of global brain ischemia. This is an underestimation of comparable efficacy because subcutaneous administration of (+)SKF 10,047 is likely to result in slower absorption than intraperitoneal administration of MK-801.

The protective actions of MK-801 in reducing neuronal damage in gerbils exposed to 5 minutes of cerebral ischemia have been recently attributed solely to its hypothermic effect.33,34 In those studies, MK-801 failed to protect against hippocampal cell loss or spatial memory impairment when treated animals were maintained as normothermic during and after ischemia. Busto et al38 showed in the rat four-vessel occlusion model that simply lowering brain temperature by only two degrees prevented ischemic damage to neurons. Even preventing the postischemic rise in temperature

FIGURE 5. Changes in temporalis muscle and rectal temperatures in conscious gerbils treated with MK-801. Temperature was monitored at selected time intervals in conscious gerbils receiving either vehicle (n=10) or 3 mg/kg i.p. MK-801 (n=14). Data were analyzed in a split-plot analysis of variance and comparisons between vehicle and MK-801 groups made with Student’s t tests. Temperature changes were highly significant compared with vehicle-treated gerbils, and probability values are listed for each time point.

FIGURE 6. Changes in temporalis muscle and rectal temperatures in ischemic gerbils treated with (+)SKF 10,047. Temperature was monitored at selected time intervals before, during, and after anesthesia, ischemia, and reperfusion in gerbils receiving either vehicle (n=10) or (+)SKF 10,047 (n=6) administered as the multiple dosing regimen in Figure 3 (arrows). The baseline temperature average (37.7 rectal, 36.8 temporalis) was taken just before anesthesia was administered, and temperature was continually recorded before and during ischemia and at selected times thereafter. Zero time indicates start of reperfusion. Data were analyzed overall by three-factor analysis of variance (ANOVA) followed by two-factor ANOVA for each treatment, with repeated measures over time for each gerbil. Temperature changes that were highly significant compared with vehicle-treated gerbils are noted with a probability value for each time point.
reiterate that their dysphoric and psychotomimetic effects in humans reside in the levorotatory enantiomer (as reviewed in reference 44). Despite the animal discrimination studies that link MK-801 and (+)SKF 10,047 behaviorally,18,24,28 (+)SKF 10,047 has been shown to produce unique changes in encephalographic spectral parameters that are distinctly different from those produced by PCP or MK-801.45 Such differences may be related to the distinctly faster recovery from motor deficits induced by (+)SKF 10,047 versus MK-801, as shown in the present study.

Thus, the mode of action of drugs that cross-react at both PCP and α-sites may never be well defined by either behavioral or binding studies, as we have suggested previously.22 By studying neuroprotection, we hope to bypass some of the confusion and contradictions encountered when investigating these interesting classes of compounds, which may be neuroprotective by simply inducing hypothermia.33,34 We recognize that separate PCP/MK-801, dextromethorphan, and α-sites may coexist within the NMDA channel and that there may be utility in using a ligand as cross-reactive as (+)SKF 10,047.22,25,46 The neuroprotection studies reported here are an indication that this class of compound may prove useful in the treatment of cerebral ischemia and stroke.

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References


FIGURE 7. Comparison of (+)SKF 10,047 and MK-801 in swing grid test. Motor function of gerbils given equivalently neuroprotective doses of either (+)SKF 10,047 (n=5–10) or MK-801 (n=10) was assessed over time with a swing grid test as described in "Materials and Methods." Values of 140° were obtained for completely atoxic animals because gravity overcame friction at 40°. Data were analyzed by Kruskal-Wallis non-parametric analysis of variance and Bonferroni correction. (+)SKF 10,047-treated gerbils were significantly different from saline-treated gerbils for up to 2 hours (p=0.0001), whereas MK-801-treated gerbils were different for up to 13 hours (p≤0.0002). *Significantly less motor impairment in (+)SKF 10,047 group compared with MK-801 group (p=0.0001).

has proven beneficial to different brain regions in rat41 and gerbil models of global brain ischemia, in which a critical period for reversing CA1 injury was identified as being between 15 and 45 minutes after reperfusion.35

To attempt to separate the MK-801-induced hypothermic effect from the decrease in body temperature resulting from anesthesia, surgery, and the resulting inactivity, we looked at direct temperature effects in conscious animals and still saw an immediate 1- to 2-degree hypothermic effect, which has been shown to enhance the MK-801 protective effect.42 Because brain hypothermia induced by (+)SKF 10,047 was significant only after 4 hours in animals given a neuroprotective regimen, the therapeutic effect seems independent of hypothermia, considering that the window of vulnerability is within the first hour of reperfusion.35 Some investigators have agreed that the MK-801–induced hypothermia is responsible for only 30% of its neuroprotective action, which corroborates earlier findings.42 A sustained dosage may be more important: 60 mg/kg (+)SKF 10,047 did not protect when given 30 minutes after reperfusion, yet produces 2° hypothermia in 15 minutes.

A case has been made for the clinical use of NMDA antagonists to treat ischemic brain injury, with appropriate cautions and safety issues considered.43 The potential usefulness of NMDA antagonists and α-compounds for specific, acute disorders such as stroke, cardiac arrest, or neurotrauma may dispel the concerns over dysphoria or psychotomimetic side effects, especially for acute, limited treatment. Regarding the side effects of benzomorphan α-ligands, it is imperative to...
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