Neuroprotective Mechanism of (+)SKF 10,047
In Vitro and in Gerbil Global Brain Ischemia

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Background and Purpose: The N-methyl-D-aspartate receptor is believed to mediate part of the ischemic neuronal damage caused by the excitatory amino acid glutamate. (+)SKF 10,047, the prototypic \( \alpha \)-agonist, interacts with the N-methyl-D-aspartate receptor. Therefore, we studied the neuroprotective effect of (+)SKF 10,047 on cultured rat cerebellar neurons and on CA1 hippocampal neurons of gerbils exposed to brain ischemia.

Methods: Mechanisms of neuroprotection were studied in vitro by measuring calcium influx into cultured rat cerebellar granule cells loaded with fura 2-AM. In vivo neuroprotection of gerbil CA1 hippocampal neurons was studied in a posttreatment regimen following 5 minutes of bilateral carotid artery occlusion and 7 days of reperfusion.

Results: In primary cultured rat cerebellar granule cell neurons, (+)SKF 10,047 in a dose-dependent manner diminished intracellular calcium levels of N-methyl-D-aspartate-stimulated neurons by a maximum of 87% (TI=8), with a 50% inhibitory concentration of 0.8 \( \mu \)M. (+)SKF 10,047 did not prevent subsequent calcium influx stimulated by kainic acid or KCl, nor did it interfere with modulation of the kainate response by quisqualic acid. Neuroprotection of 64% (p=0.006, n=15) of gerbil CA1 hippocampal neurons was achieved by posttreatment injection followed by minipump infusion.

Conclusions: Neuroprotection by (+)SKF 10,047 most likely involves interaction at the N-methyl-D-aspartate receptor. These results suggest that the benzomorphan class of \( \alpha \)-agonists may provide neuroprotection in cerebral ischemia and stroke. (Stroke 1992;23:1319-1324)

KEY WORDS • amino acids • cerebral ischemia • neuroprotection • gerbils
slotted, Plexiglas holder designed by J. Paul Hieble of this department to fit tightly inside the cuvette. F$_{\text{max}}$ was determined by adding ionomycin to 35 μM and F$_{\text{min}}$ by adding 400 mM ethylene glycol-bis(β-aminoethyl ether) N$_{2}$N$'_2$N$''_2$N$'''_2$-tetraacetic acid (EGTA) in 4M Tris (pH 10) to a final concentration of 10 mM EGTA, essentially the same as has been recently reported$^{13,14}$; our results gave very similar values. A prior report of a procedure$^{15}$ for determining these values in granule cell neurons caused (in our hands) a falsely high F$_{\text{max}}$, leading to an underestimation of basal [Ca$^{2+}$]$_j$ levels.

The Mongolian gerbil model of global forebrain ischemic stroke has been extensively studied and characterized.$^{16-20}$ 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% O$_2$, placed on a 37°C heating pad to keep body temperature changes to <1°C during ischemia,$^2$ exposed to bilateral carotid artery occlusion for 5 minutes, and allowed to recover on the heating pad for 10 minutes. After 7 days, 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 to ensure unbiased observation. For protection studies, (+)SKF 10,047 was dissolved in saline and administered subcutaneously as a bolus and was infused with the use of minipumps. Minipumps (Alzet model 1003D, Alza Corp., Palo Alto, Calif.) were loaded with 100 μl (+)SKF 10,047, activated by immersion in normal saline for 4 hours at 37°C, and implanted subcutaneously (two per gerbil to deliver a total of 270 mg/kg (+)SKF 10,047 over 3 days).

The enantiomer (+)SKF 10,047 was obtained through the National Institute on Drug Abuse, Rockville, Md.

Data in text and figures are mean±SEM values of the indicated number of animals. Statistical analysis was performed as indicated in the figure legends; a significant difference was accepted at p<0.05.

Results

The effect of 5 minutes' ischemia is seen in Figure 1 for vehicle-treated gerbils, in which the CA1 neuronal count was decreased to an average of 27% (44/163, n=15) of the mean count in sham-operated animals (163) 7 days after ischemia. Administering (+)SKF 10,047 as a bolus of 15 mg/kg at 1 and 2 hours after reperfusion and as a 90 mg/kg/day infusion for 3 days was an effective posttreatment regimen (64% protection, 104/163, n=15, p=0.006).

In our previous investigation we never saw any evidence for (+)SKF 10,047-induced neurotoxicity,$^2$ and here we examined the effects of (+)SKF 10,047 on conscious, nonischemic gerbils. There was no difference in CA1 neuronal counts when animals treated with 60 mg/kg s.c. (+)SKF 10,047 were compared with saline-treated control gerbils 7 days after injection (Figure 2).

Measurements of [Ca$^{2+}$], in primary cultured rat cerebellar granule cells gave resting values of 145±19 nM (n=8), similar to recently published values$^{13,14}$ that were higher than the low levels previously reported,$^{15}$ most likely due to differences in the determination of F$_{\text{max}}$ and F$_{\text{min}}$ (see "Materials and Methods"). Stimulation with 5 μM glycine and 100 μM NMDA increased [Ca$^{2+}$], almost threefold to 419±73 nM (n=8) over basal levels, providing sustained or gradually increasing levels of [Ca$^{2+}$], (Figure 3). (+)SKF 10,047 diminished the stimulated [Ca$^{2+}$], by a maximum of 87±2% (n=8), as
Figure 3. Tracings showing inhibition by (+)SKF 10,047 of sustained intracellular calcium concentration ([Ca^{2+}]_i) increases in rat cerebellar granule cells stimulated with N-methyl-D-aspartate (NMDA). A: After additions of 5 μM glycine (△) and 100 μM NMDA (△), (+)SKF 10,047 was sequentially added to trace 1 (downward arrows) at increasing micromolar concentrations shown. Subsequent additions included 10 μM MK-801, 100 μM kainic acid (KA), 100 μM quisqualic acid (QA), and 30 mM KCl. B: Neurons received 5 μM each of glycine and nimodipine before addition of 100 μM NMDA (△). (+)SKF 10,047 was added as in A (upward arrows, trace 2) at increasing micromolar concentrations shown, followed by same additions as in A.
granule cells. Percent inhibition of NMDA/glycine-induced excitotoxicity was calculated for each cumulative concentration of (+)SKF 10,047, after the addition of [Ca^{2+}]_i. Results are expressed as % inhibition at each cumulative concentration. IC_{50} (0.8 \mu M) was determined by linear regression analysis.

Discussion

The interruption of cell calcium homeostasis by excitatory amino acids is considered to be an important initiator of excitotoxic damage, especially in delayed neuronal death. In cerebellar granule cells, NMDA causes sustained increases in [Ca^{2+}]_i as clearly shown in the present study. The primary influx is through the receptor-associated channel, with secondary influx through voltage-sensitive calcium channels that are inhibited by nifedipine. The agonist-operated calcium channel influx can be blocked rapidly by MK-801, as we have shown here for (+)SKF 10,047. The IC_{50} of 0.8 \mu M and the cumulative levels of (+)SKF 10,047 needed to totally block the channel (16.65 \mu M, Figure 4) reflect the levels we reported for protection against glutamate-induced excitotoxicity. The (+)SKF 10,047 effect was selective for only the NMDA subtype of glutamate receptor because, as reported for MK-801, subsequent stimulation of neurons with kainate caused no change in calcium influx. The NMDA influx was not altered by MK-801, which has been assessed in a variety of ways in these cells, persisted after the addition of (+)SKF 10,047, again supporting the selectivity of (+)SKF 10,047's action at the NMDA receptor. These results strengthen our previous suggestion that benzomorphans afford neuroprotection with diminished side effects. Although the protective mechanism of (+)SKF 10,047 in vitro indicates efficacy at, and selectivity for, the NMDA receptor, the neuronal activation in vivo is not the only mechanism involved in selective neuronal vulnerability. Other factors, such as non-NMDA receptors, must be involved in neuronal death in vivo because noncompetitive inhibitors such as (+)SKF 10,047 do not provide total neuroprotection. The results of the minipump protection study, in which treatment was delayed until 1 hour after reperfusion, reinforce our previous findings that (+)SKF 10,047 is an effective neuroprotectant even when treatment is initiated after ischemia. The neuroprotective effect of the minipump treatment was the same as that of our previous posttreatment regimen of 15 mg/kg at 15 minutes and 1 hour and 30 mg/kg at 2, 4, and 6 hours, which produced no significant decrease in temporal muscle temperature until after 4 hours of treatment. The minipump was implanted 20 minutes after the 100-minute therapeutic window of opportunity for reversal of delayed neuronal injury with halothane, an inhibitor of postsynaptic hyperthermia. Other studies of the temperature effects of SKF 10,047 in mice and rats have shown mostly no effect, with occasional hyperthermia. However, doses of (+)SKF 10,047 higher than used here (80 mg/kg) have been reported to produce acute hypothermia in rats as we have reported for 60 mg/kg in gerbils. Because acute administration of (+)SKF 10,047 at 5–40 mg/kg s.c. produced no temperature effects in rats and we saw either no hypothermia or no temperature change (15–30 minutes) of any hypothermia in gerbil brain, neuroprotection by (+)SKF 10,047 is likely to be independent of hypothermia. We also do not envision a neuroprotective effect in vivo by increasing cerebral blood flow because (+)SKF 10,047 causes only small increases in heart rate and mean arterial blood pressure in conscious dogs and has no contractile effect at up to 100 \mu M in rat tail artery. In fact, (+)SKF 10,047 potentiated norepinephrine-induced contractions in rat tail artery and, in general, benzomorphans have been shown to constrict isolated canine cerebral vessels, but only at concentrations around 0.1–1 mM. Therefore, if (+)SKF 10,047

![Figure 4](https://stroke.ahajournals.org/)

**Figure 4.** Plot showing blockade by (+)SKF 10,047 of N-methyl-D-aspartate (NMDA)/glycine-induced increases in intracellular calcium concentration ([Ca^{2+}]_i) in rat cerebellar granule cells. Percent inhibition of NMDA/glycine-induced [Ca^{2+}]_i increase was determined by calculating [Ca^{2+}]_i after each cumulative addition of (+)SKF 10,047. Results are expressed as % inhibition at each cumulative concentration. IC_{50} (0.8 \mu M) was determined by linear regression analysis.
has an effect on cerebral vessels, one could expect only vasoconstriction and reduced blood flow.

Although noncompetitive inhibitors of the NMDA receptor have been reported to cause neuropathologic changes such as vacuole formation and stimulation of endothelial cells, one could expect only protective effects of (+)SKF 10,047 are manifested at the NMDA receptor.

Therefore, this class of noncompetitive NMDA receptor inhibitors may be better tolerated. These results offer further evidence that the neuroprotective effects of (+)SKF 10,047 are manifested at the NMDA receptor and that this class of compound may prove useful in the treatment of cerebral ischemia and stroke.

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References

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