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HA-966 (1-hydroxy-3-aminopyrrolidone-2) selectively reduces *N*-methyl-D-aspartate (NMDA)-mediated brain damage

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The neuroprotective effects of the strychnine-insensitive glycine receptor antagonist, HA-966, against *N*-methyl-D-aspartate (NMDA)- and quisqualate (QA)-mediated brain injury were determined in perinatal rats. Postnatal day (PND) 7 rats received intrastriatal injections of NMDA (25 nmol) or QA (100 nmol) and then were administered intraperitoneal (i.p.) injections of varying doses of HA-966 or vehicle 15 min later. Animals were sacrificed 5 days later and the degree of brain injury was calculated by comparison of the weights of injected and contralateral cerebral hemispheres. HA-966 selectively reduced the degree of NMDA-mediated brain injury in a dose-dependent manner. However, HA-966 did not attenuate QA-mediated brain injury.

The *N*-methyl-D-aspartate (NMDA)-responsive glutamate receptor is part of a receptor/ion channel complex with multiple regulatory sites including recognition sites for NMDA, phencyclidine-like compounds, cations (magnesium and zinc), and a strychnine-insensitive glycine modulatory site [12]. Glycine potentiates NMDA-mediated responses and enhances binding of phencyclidine-like ligands [6, 11, 13]. Intracerebral administration of nanomolar quantities of NMDA produces focal brain injury in postnatal day (PND) 7 rats [8, 10]. Systemically administered competitive (CPP, 3-((±)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid) and non-competitive (phencyclidine and MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) NMDA receptor antagonists prevent this damage [9, 10]. As well, multiple doses of magnesium attenuate NMDA-mediated brain injury [9]. NMDA receptor antagonists also reduce NMDA- and quinolinate-

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induced neuronal injury in adult rats [1, 4] and glutamate and NMDA neurotoxicity in neocortical cell cultures [2, 5].

In initial studies, we found that co-injection of glycine or structurally related analogues (25–1000 nmol) with NMDA did not enhance NMDA-induced brain injury in PND 7 rats (data not shown); this observation suggested that endogenous levels of glycine in the extracellular fluid of the brain are sufficient to maximally potentiate NMDA toxicity. Yet, it is possible that antagonists of the strychnine-insensitive glycine modulatory site could be effective blockers of NMDA-mediated brain injury. We tested the neuroprotective effects of a putative antagonist of the glycine modulatory site, HA-966 [3], against NMDA-induced brain injury in PND 7 rats. To test the selectivity of any observed neuroprotective properties of HA-966, in a second group of animals the glutamate agonist QA was used to induce brain injury.

NMDA (25 nmol in 0.5 μl) was stereotaxically injected into the right posterior striatum of ether anesthetized PND 7 Sprague–Dawley rats using a 26 gauge Hamilton syringe as described previously [9, 10]. Coordinates were AP 2.0 mm, ML 2.5 mm, at a depth of 4.0 mm from the dura using bregma as a landmark. The needle was left in place for 2 min following injection to limit leakage. Fifteen minutes later, a single dose of HA-966 (5 mg/kg, $n=6$; 25 mg/kg, $n=7$; 50 mg/kg, $n=6$) was administered i.p. in 0.05 ml phosphate-buffered saline (PBS), pH 7.4. NMDA-injected littermate controls received an equivalent volume of PBS ($n=19$). Additional animals received intrastriatal injections of QA (100 nmol in 0.5 μl) and were treated (i.p.) either with 50 mg/kg HA-966 ($n=6$) or PBS ($n=6$). All animals were sacrificed on

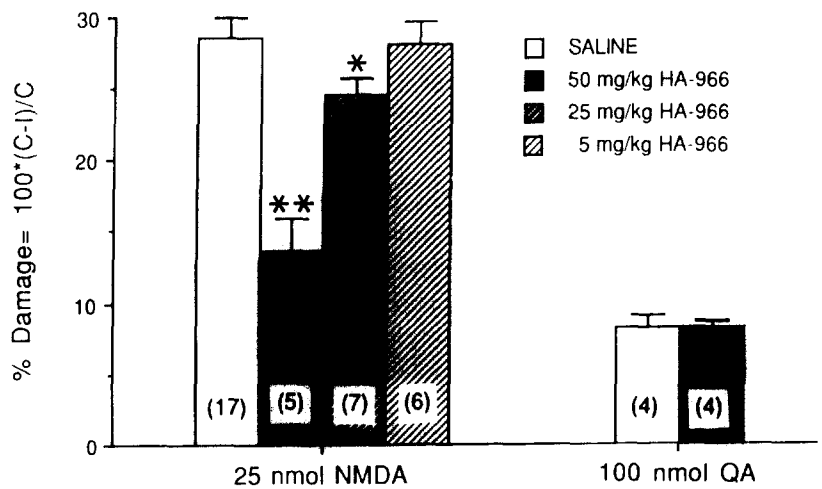

Fig. 1. Quantitative comparison of the neuroprotective effects of HA-966 against NMDA- and QA-mediated brain injury. Unilateral intrastriatal stereotaxic injections of either NMDA (25 nmol) or QA (100 nmol) were performed in ether anesthetized postnatal day (PND) 7 rats. Fifteen min later, HA-966 was administered i.p. in 0.05 ml saline. NMDA-injected littermate controls received equivalent volumes of saline. Animals were sacrificed 5 days later on PND 12. The degree of resulting brain injury was assessed by comparison of the weights of the injected (*I*) and contralateral (*C*) cerebral hemispheres and expressed as %Damage (mean ± S.E.M.) using the formula indicated on the vertical axis. The number of surviving animals in each group is indicated in parenthesis. $*P<0.05$, $**P<0.001$, ANOVA, HA-966-treated vs saline-treated.

PND 12, 5 days after intrastriatal injection. The degree of resulting brain injury was quantitated by comparison of injected (*I*) and contralateral (*C*) cerebral hemisphere weights using the formula, %Damage = $100\,(C-I)/C$, expressed as mean $\pm$ S.E.M. This is a sensitive and reliable method for quantitating unilateral brain injury in this experimental model [9, 10]. Values for percent damage are highly correlated with ipsilateral reductions in both choline acetyl transferase activity and hemisphere cross-sectional area measurements (submitted for publication).

In PND 7 rats that receive an intrastriatal injection of 25 nmol NMDA and are sacrificed 5 days later there is unilateral gross hemisphere deformity and confluent brain necrosis in the corpus striatum which extends into adjacent areas of neocortex, dorsal hippocampus and thalamus [8, 10]. In this study, intrastriatal injection of 25 nmol NMDA produced $28.6 \pm 1.3\%$ reduction in the weight of the injected cerebral hemisphere relative to the contralateral side and resulted in 10% mortality ($n=19$). Administration of 50 mg/kg HA-966 produced a $52 \pm 7.5\%$ reduction in NMDA-mediated brain injury, as assessed by comparison of cerebral hemisphere weights on PND 12 ($P<0.001$, ANOVA, HA-966 treated, $n=5$ vs PBS treated, $n=17$). Twenty-five mg/kg HA-966 reduced damage by $29.7 \pm 0.7\%$ ($P<0.05$, ANOVA, HA-966 treated, $n=7$ vs PBS treated, $n=17$) while a 5 mg/kg dose had no effect on NMDA-mediated brain injury (Fig. 1). Animals that received 50 mg/kg of HA-966 exhibited a behavioral state of deep sedation with hypotonia and also showed short periods of apnea (less than 30 s). This dose resulted in a 17% mortality rate ($n=6$) while lower doses were associated with 100% survival. All animals that received 100 mg/kg HA-966 died about 1 h later.

The neuroprotective effects of HA-966 were selective for NMDA-mediated injury. HA-966 (50 mg/kg) did not attenuate QA-induced brain injury when assessed either in histologic sections or by comparison of cerebral hemisphere weights (Fig. 1). The mortality rate in both QA injected groups was identical (33% mortality, $n=6$/group).

Preliminary studies indicate that co-injection of the selective glycine antagonist 7-chlorokynurenate or kynurenate [7] with NMDA also reduces the degree of resulting brain injury [14].

Our laboratory as well as others have shown that competitive NMDA antagonists and phencyclidine-like compounds attenuate NMDA-mediated brain injury [4, 9, 10]. We have now demonstrated that HA-966, an antagonist of the glycine modulatory site, attenuates NMDA-mediated brain injury. HA-966 was less potent than other non-competitive (MK-801, PCP) and competitive NMDA antagonists in reducing brain injury [9, 10]. The maximal level of neuroprotection achieved by HA-966 without increasing mortality was 52% protection relative to NMDA injected controls. Low doses of HA-966 appeared to reduce the mortality rate associated with intrastriatal injection of NMDA.

The dose-dependent neuroprotective effects of HA-966 against NMDA induced brain injury are consistent with electrophysiological [3, 4] and biochemical studies [11, 13] and lend support to the hypothesis that endogenous glycine facilitates NMDA-mediated neurotoxicity in vivo. These observations also suggest that antagonists of the glycine modulatory site may effectively limit neuronal injury in a variety of acute and chronic neurologic diseases.

Since the glycine modulatory site functions to facilitate NMDA receptor channel

activation, partial antagonism of the glycine modulatory site may prevent excessive activation of the NMDA receptor channel complex but still allow limited channel activation. By titration of the dosage of glycine antagonists it may be possible to attain neuroprotection with fewer adverse behavioral effects than are observed in animals treated with competitive NMDA antagonists and phencyclidine-like compounds. In HA-966-treated rats, we did not observe the sympathomimetic effects and turning behavior typical of animals treated with phencyclidine-like compounds and competitive NMDA antagonists. Finally, competitive antagonism of both the glycine modulatory and NMDA recognition sites may be expected to have synergistic neuroprotective effects against NMDA-mediated neuronal injury.

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