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Effect of glycine and glycine receptor antagonists on NMDA-induced brain injury

John E. Uckele¹, John W. McDonald³, Michael V. Johnston⁴ and Faye S. Silverstein^{1,2}

Departments of ¹Pediatrics, ²Neurology, ³Neuroscience and Medical Scientist Training Program, University of Michigan, Ann Arbor, MI (U.S.A.) and ⁴Johns Hopkins University School of Medicine and the Kennedy Institute, Baltimore, MD (U.S.A.)

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In postnatal day 7 rats, a unilateral intrastriatal injection of 12.5 nmol of *N*-methyl-D-aspartate (NMDA) reproducibly injures the ipsilateral striatum, adjacent hippocampus and overlying cortex. The severity of injury can be quantified by comparing cerebral hemisphere weights in animals sacrificed 5 days after the injection. Co-injection of NMDA and the glycine receptor antagonists kynurenic acid (KYN) or 7-chlorokynurenic acid (7-CKA) reduced the severity of NMDA-induced damage in a dose-dependent fashion. One hundred nmol of KYN with 12.5 nmol of NMDA reduced average % damage from $19.3 \pm 0.9\%$ ($n=9$) to $2.3 \pm 0.5\%$ ($n=6$), $P < 0.001$, ANOVA. Co-injection of 40 nmol of 7-CKA with 12.5 nmol of NMDA ($n=6$) reduced average % damage from $17.1 \pm 1.6\%$ ($n=15$) to $3.0 \pm 0.6\%$, $P < 0.001$, ANOVA. Concurrent injection of 1000 nmol glycine with 5 nmol NMDA did not increase the extent of NMDA-induced damage. Our results demonstrate that glycine receptor antagonists attenuate NMDA-induced brain injury in vivo.

The *N*-methyl-D-aspartate (NMDA) receptor/channel complex includes the NMDA-selective glutamate binding site, a cationic channel, and a strychnine-insensitive glycine receptor which is closely associated with the receptor/channel complex [1]. In vitro studies have demonstrated that glycine is required for NMDA receptor activation [12] and potentiates NMDA responses [3]. Kynurenic acid and 7-chlorokynurenic acid (7-CKA) are competitive antagonists of strychnine-insensitive [³H]glycine binding [4, 5].

Intrastriatal injection of NMDA in postnatal day (PND) 7 rats produces reproducible damage to the ipsilateral striatum, adjacent hippocampus, and the overlying cortex [8]. The severity of brain injury can be quantified by comparing the weights of

Correspondence: F.S. Silverstein, Neuroscience, Laboratory Building, 1103 E. Huron Street, Ann Arbor, MI 48104-1687, U.S.A.

the injected and contralateral cerebral hemispheres in animals sacrificed 5 days later, on PND 12 [9, 10]. In this study, we examined the ability of glycine receptor agonists and antagonists to potentiate or attenuate NMDA-induced brain injury in the immature brain. To ensure consistent drug delivery, all drugs were administered by direct intra-cerebral injection.

Seven-day-old Sprague–Dawley albino rats were anesthetized with diethyl ether. Stereotaxic injections of NMDA in 0.5 μ l phosphate-buffered saline (PBS) were made in the right striatum of each rat pup (co-ordinates referenced to bregma: AP 2 mm, ML 2.5 mm, V 4 mm). In the first experiment, pups received 5 nmol NMDA, with or without co-injection of 1000 nmol glycine. The next group of experiments included equal numbers of pups that received 12.5 nmol NMDA with or without co-injection of kynurenic acid (4 doses, 12.5–100 nmol) or 7-CKA (4 doses, 2.5–40 nmol). After recovery from anesthesia, the pups were returned to the dam. Animals were sacrificed by decapitation on postnatal day 12, 5 days after lesioning.

The severity of brain injury was quantified by deriving a % damage value, calculated as the difference between the contralateral hemisphere (*C*) weight and the injected (*I*) hemisphere weight, divided by the contralateral hemisphere weight (% damage = $C - I / C \times 100$). Previous studies indicate that in this experimental model, the % damage value is a consistent measure of the severity of neuronal injury and the efficacy of neuroprotective agents [9]. At doses of NMDA ranging from 5 to 25 nmol, there is a direct linear correlation between % damage and progressive reductions in both choline acetyltransferase activity and striatal cross-sectional area [10]. Data was expressed as average % damage \pm S.E.M. in all groups. At 12.5 nmol NMDA ($n = 24$) produced an average % damage of $18.1 \pm 1.2\%$. Brain injury was also evaluated by histologic examination of Nissl-stained coronal brain sections.

The doses of drug that reduced damage by 50% (protective dose 50, PD₅₀) were determined by the method of Litchfield and Wilcoxon [6]. Statistical comparisons were made by analysis of variance (ANOVA).

To examine the issue of potentiation of NMDA-induced injury by glycine, we used the lowest dose of NMDA, 5 nmol, that elicited a reproducible lesion. Injection of 1000 nmol of glycine with 5 nmol NMDA did not increase NMDA-induced brain injury when compared to controls which received 5 nmol NMDA alone. Average % damage for experimentals ($n = 6$) was $9.3 \pm 1.2\%$, compared with $10.5 \pm 1.2\%$ for controls ($n = 6$), $P = \text{N.S.}$, by ANOVA.

In contrast, co-injection of increasing doses of the glycine receptor antagonist kynurenic acid (KYN) with 12.5 nmol NMDA progressively reduced the extent of NMDA-induced brain injury. Four doses of KYN were evaluated: 12.5 nmol ($n = 6$), 25 nmol ($n = 6$), 50 nmol ($n = 8$) and 100 nmol ($n = 5$). At each dose, the extent of NMDA-induced brain injury was reduced, $P < 0.001$, ANOVA (Fig. 1A); % damage in brains injected with 100 nmol KYN fell to $2.3 \pm 0.5\%$. The PD₅₀ for KYN was 34.1 nmol. In addition, histologic sections confirmed a marked reduction in injury to the ipsilateral striatum, hippocampus and cortex in animals injected with 100 nmol KYN and 12.5 nmol NMDA (Fig. 2B) when compared to controls injected with 12.5 nmol NMDA only (Fig. 2A).

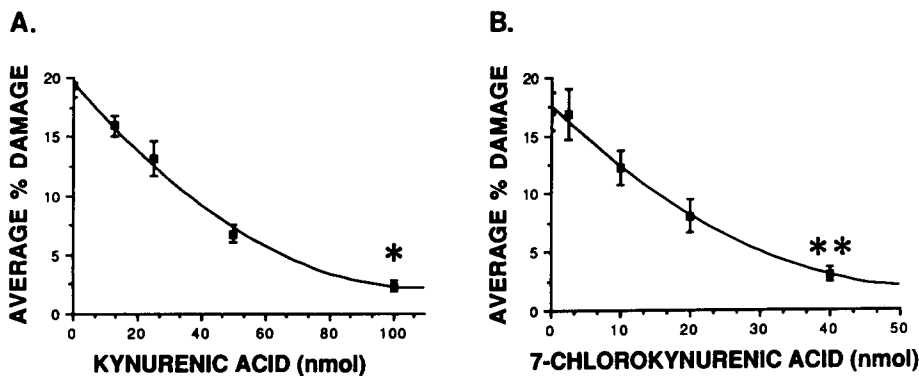


Fig. 1. Dose-response curves showing average % damage vs dose of kynurenic (KYN) acid (A) and 7-chlorokynurenic acid (7-CKA) (B) with concurrent administration of 12.5 nmol *N*-methyl-D-aspartate (NMDA). Postnatal day (PND) 7 rats received right intrastratial injection of 12.5 nmol NMDA with KYN (dose range: 0–100 nmol) or 7-CKA (dose range: 0.40 nmol). Animals were sacrificed on PND 12. % damage was calculated by comparison of the injected (*I*) and contralateral (*C*) hemisphere weights ($C - I / C \times 100$) for each group and expressed as mean \pm S.E.M. * $P < 0.001$, compared with 12.5 nmol NMDA-injected controls, ANOVA, post-hoc *t*-test indicated significant differences at each dose: ** $P < 0.001$, compared with 12.5 nmol NMDA-injected controls, ANOVA, post-hoc *t*-tests indicated significant differences at doses 10, 20 and 40 nmol 7-CKA.

The neuroprotective effects of 7-CKA on NMDA-induced brain injury were similar. Doses of 7-CKA including 2.5 nmol ($n = 9$), 10 nmol ($n = 6$), 20 nmol ($n = 6$) and 40 nmol ($n = 6$) were co-injected with 12.5 nmol NMDA. The lowest dose tested, 2.5 nmol 7-CKA, did not reduce injury. Doses of 10, 20, and 40 nmol 7-CKA progressively reduced the severity of NMDA-induced brain injury, $P < 0.001$, ANOVA (Fig. 1B). The PD_{50} of 7-CKA calculated from these data was 17.6 nmol. Higher doses of 7-CKA could not be tested because of limitations in solubility of the drug. Coronal sections of brains injected with 40 nmol 7-CKA and 12.5 nmol NMDA (Fig. 2C) demonstrated relative preservation of histologic integrity of the striatum, hippocampus and cortex as compared to controls injected with 12.5 nmol NMDA only (Fig. 2A).

KYN has mixed glutamate antagonist properties, inhibiting [3 H]glycine binding in a competitive manner and also inhibiting [3 H]glutamate binding to NMDA receptors [2]. However, 7-CKA has 70–80 times the specificity of kynurenic acid for the strychnine-insensitive glycine site [4]. The increased efficacy (lower PD_{50}) observed of 7-CKA over KYN may be attributable to increased specificity of 7-CKA at the glycine modulatory site. These results are consistent with a recent report [11] which demonstrated that 7-CKA blocked glutamate neurotoxicity in cell culture and that glycine could reverse its neuroprotective effects.

These data demonstrate that glycine receptor antagonists can attenuate NMDA-induced neurotoxicity in vivo. The inability of exogenous glycine to potentiate NMDA-induced brain injury may be due to maximal receptor occupation by physiologic concentrations of glycine in vivo. Drugs which act as glycine receptor antago-

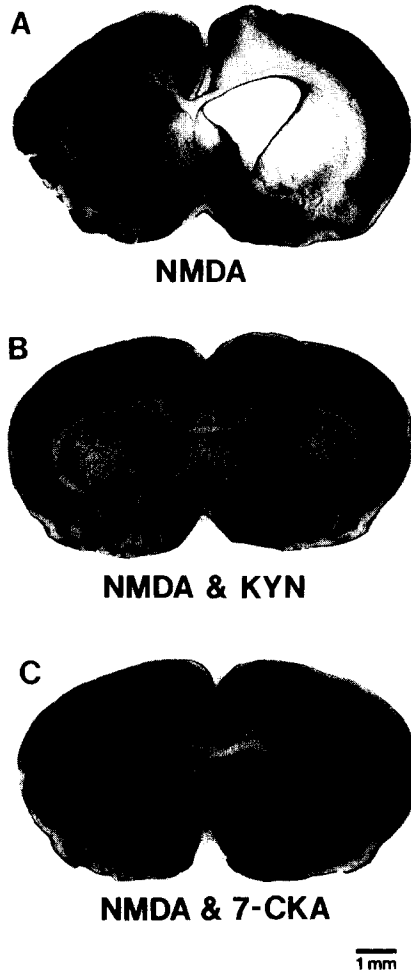


Fig. 2. Representative Nissl-stained coronal brain section ($75 \mu\text{m}$) from rat pups sacrificed at postnatal (PND) day 12. On PND 7, ether anesthetized rats received right intrastriatal injections of 12.5 nmol NMDA (A), 12.5 nmol NMDA and 100 nmol KYN (B), and 12.5 nmol NMDA and 40 nmol 7-CKA (C). The severity of injury is markedly reduced by both KYN acid and 7-CKA.

nists may have neuroprotective properties in neurologic disorders associated with over-activation of NMDA receptors.

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