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A glycine antagonist reduces ischemia-induced CA1 cell loss in vivo

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Excessive activation of the *N*-methyl-D-aspartate (NMDA) receptor–channel complex has been implicated as one of the mechanisms by which ischemia-induced neuronal damage is mediated. Elevated glycine levels during ischemia may contribute to damage mediated by the NMDA receptor as glycine binding potentiates NMDA responses, and may be necessary for channel opening. We investigated the protective effects of 7-chlorokynurenic acid – a competitive antagonist at the glycine binding site associated with the NMDA receptor – against hippocampal CA1 cell loss induced by transient forebrain ischemia in rats. Intraventricular administration of the drug immediately before the onset of ischemia significantly attenuated neuronal loss compared to vehicle-treated animals.

Transient cerebral ischemia can lead to permanent damage of selectively vulnerable neurons [25]. A prominent theory concerning the mechanism of ischemia-induced cell loss is that it is mediated in part by the excitotoxic actions of excitatory amino acids [26]. In particular, excessive activation of the *N*-methyl-D-aspartate (NMDA) receptor by glutamate (or aspartate) during ischemia is thought to cause pathological levels of calcium influx and subsequent cell death by various calcium-mediated processes [3].

In support of the excitotoxic theory of ischemic damage, several studies have demonstrated protective effects of NMDA receptor antagonists both in vivo [9, 29] and in vitro [4, 13]. The excitotoxic theory is also supported by in vivo neurochemical evidence showing that extracellular glutamate levels increase dramatically during ischemia [2, 7, 10]. The extracellular levels of other amino acid neurotransmitters also increase during ischemia [11]. A recent study utilizing the in vivo microdialysis technique shows that there is a close correlation between the areas of the brain that suffer cell loss and those in which extracellular glycine is elevated during ischemia and remains elevated following reperfusion. In contrast, changes in extracellular glutamate levels during ischemia and reperfusion are equivalent across both vulnerable and non-vulnerable brain areas [11]. In addition, glycine has been shown to potentiate NMDA-mediated toxicity in vitro [24]. These findings suggest that glycine may play a role in the pathological consequences of transient cerebral ischemia in vivo.

One mechanism by which glycine may contribute to ischemia-induced damage is by its action at the strychnine-insensitive glycine binding site associated with the NMDA receptor [15]. Glycine has been shown to potentiate NMDA channel activation [12] and it has been suggested that glycine may be a co-agonist at the NMDA receptor–channel complex. Specifically, simultaneous binding of glycine and glutamate to their respective binding sites on the NMDA receptor–channel complex is thought to be required, with concomitant cell depolarization, for ion conductance through the channel [6, 16–18]. It is possible that elevated extracellular levels of glycine during and following ischemia may contribute to or potentiate the damage mediated by calcium influx via the NMDA channel. Prevention of glycine binding might therefore be expected to attenuate the cell loss associated with ischemia. To investigate this possibility we have assessed the protective effects of 7-chlorokynurenic acid (7-ClK) – a potent competitive antagonist at the strychnine-insensitive glycine binding site [14] – against CA1 pyramidal cell loss in a rat model of transient forebrain ischemia.

29 male Wistar rats (300–350 g) received transient forebrain ischemia, and a further 20 rats received sham ischemia, using the procedures described by Mudrick et al. [22] which were modified from the model developed

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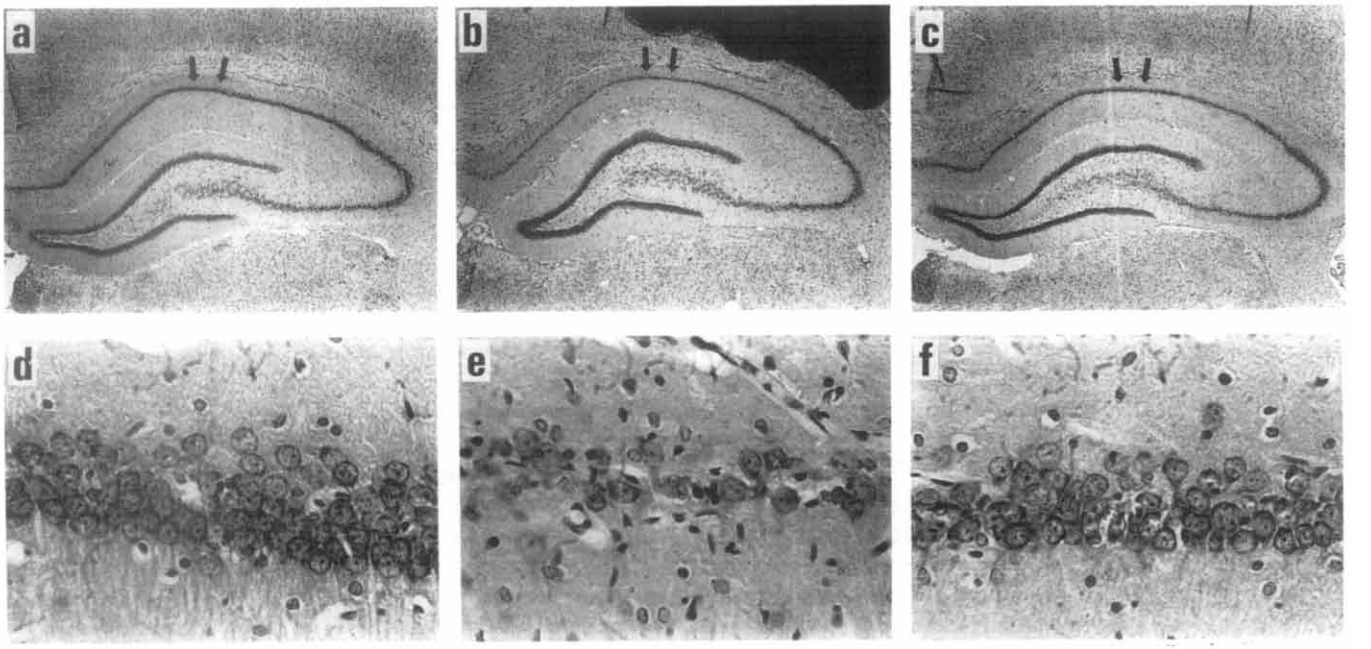

Fig. 1. Representative photomicrographs of the hippocampal formation (a–c) and of part of the CA1 cell field (d–f) at level 2, from representative rats in the SS group (a,d), the IS group (b,e) and the JD group (c,f). Arrows on the low power photomicrographs indicate the region of CA1 from which the high power photomicrographs were taken. Ischemic damage in the IS condition can be seen in b as a thinner cell layer, and in e as a substantial decrease in the number of viable pyramidal cell bodies.

by Smith et al. [32]. Rats were anesthetized with sodium pentobarbital (65 mg/kg i.p.), and both carotid arteries isolated. One femoral artery was cannulated with PE50 tubing connected to a saline-primed reservoir via a pressure transducer (Electromedics Inc.). Arterial pressure was recorded on a Y-T chart recorder. Ischemia was induced by hemorrhaging via the femoral artery until the mean arterial pressure dropped to 30 mmHg, followed by clamping of both carotid arteries. After 20 min bilateral carotid occlusion, during which the arterial pressure was maintained at 30 mmHg by withdrawing or reinfusing blood as necessary, the carotids were released and the shed blood reinfused. Rats receiving sham ischemia were subjected to the same procedures with the exception of hemorrhaging and carotid occlusion. Rats' core temperature was maintained at 36°C throughout the course of surgery, and for 30 min after reinfusion of the shed blood.

Half of the rats in each group received bilateral intraventricular injections of 2 μl 500 μM 7-ClK (Tocris Neuramin) at an infusion rate of 1 μl/min immediately before carotid occlusion. The remaining rats received bilateral intraventricular injections of vehicle (2 μl phosphate-buffered saline over 2 min). There were therefore 4 groups of animals: ischemia + drug (ID), sham ischemia + drug (SD), ischemia + saline (IS), sham ischemia + saline (SS).

Several of the rats subjected to ischemia exhibited motor seizures within 3 days of surgery, and of these, 5 in the ID group and 4 in the IS group subsequently died. The incidence of motor seizures in the surviving rats was comparable in both ischemia groups. The surviving rats (n=10 in each of the 4 groups) were maintained in their home cages for either 6 or 12 weeks at which time they were re-anaesthetized with sodium pentobarbital (65 mg/kg i.p.) and perfused transcardially with 10% formalin in 0.05% phosphate buffer. The brains were removed immediately and stored overnight in phosphate-buffered formalin. The brains were then processed in graded ethanols and xylene and embedded in paraffin. 10-μm coronal sections were cut and every 10th section mounted and stained for Nissl substance with 1% Cresyl violet. Quantification of CA1 pyramidal cells was performed by direct counting of viable neurons using a light microscope at 40× power. Neurons were counted from one section at each of 6 levels distributed evenly along the septo-temporal axis of the hippocampus and cell counts were expressed as the number of viable cells present per unit length of the cell field (cells/125 μm). These cell counts were averaged across the right and left hippocampi to generate a mean cell count expressed as no. cells/125 μm at each of the 6 levels for each rat. During histological evaluation the experimenter was blind with respect to the experimental treatment of the animals.

7-ClK significantly attenuated ischemia-induced CA1 pyramidal cell loss (Figs. 1 and 2). Relative to the SS

group, the ischemic rats treated with 7-ClK (ID) had only 22.7% CA1 cell loss averaged across all levels of the hippocampus, whereas the ischemic animals not treated with the drug (IS) suffered 53.8% cell loss. One-way analysis of variance of the cell counts averaged across the 6 levels revealed a significant difference among the 4 groups (F=15.046, df=3, P<0.01). Post hoc analyses (Tukey tests) showed that the IS group had significantly fewer viable CA1 neurons than each of the control groups (P<0.01) and the ID group (P<0.05). The ID group also had significantly fewer cells than both control groups (P<0.05), but significantly more than IS (P<0.05). There were no significant differences between SS and SD groups. To investigate whether the pattern of CA1 cell loss varied across the different levels, a repeated measures two-way analysis of variance was conducted with groups and levels as independent variables. There was a significant difference among the 4 groups (F=12.18, P<0.01), and a significant group × level interaction (F=5.65, P<0.01), but there was no significant main effect for level. Post hoc analyses (Tukey) showed that IS rats had significantly fewer CA1 cells than SS and SD rats at all 6 levels (P<0.01). ID rats had fewer cells than SS and SD at levels 1 and 2 (P<0.01), and 3 and 4 (P<0.05), but not at levels 5 and 6. Ischemic rats treated with 7-ClK (ID) had more cells than untreated ischemic rats in levels 2, 4, 5 and 6 (P<0.05), but not in levels 1 and 3. These data show that the degree of protection offered by 7-ClK is greater at more temporal levels of the hippocampus, while the drug is less effective in preventing CA1 cell loss at the septal pole. Previous studies have shown that the septal hippocampus is more susceptible to ischemia than the temporal hippocampus [31], suggesting that 7-ClK was more effective in an area that is generally more resistant to ischemia. However, this cannot be confirmed, as the degree of cell loss in untreated ischemic rats (IS) was not significantly different across the 6 levels in this experiment.

The finding that 7-ClK reduces the CA1 pyramidal cell loss produced by transient forebrain ischemia is consistent with the excitotoxic hypothesis of ischemia-induced neuronal damage, as antagonism of the glycine binding site on the NMDA receptor would be expected to decrease NMDA channel opening [6], thus attenuating calcium entry via the NMDA receptor during ischemia. The fact that 7-ClK imparts only partial protection is not surprising, given that it competes with glycine for binding and, as discussed above, levels of glycine during ischemia are relatively high. Furthermore, only one dose of the drug was given in the present study. These data confirm a preliminary report of protective effects of glycine site antagonists against ischemia in gerbils [23], and are consistent with previous reports of protective

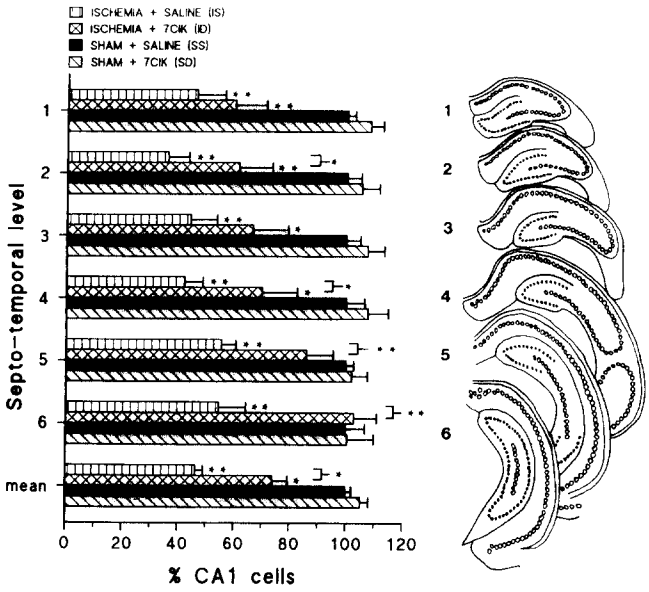

Fig. 2. Histograms showing the mean (± S.E.M.) CA1 cell counts expressed as a % of the mean SS cell count for each group (IS, ID, SS and SD) at each of the 6 hippocampal levels.

effects of 7-ClK against NMDA-mediated neurotoxicity both in neocortical cultures [20, 24] and in vivo [8, 33].

These data do not address the question on the contribution of *elevated* glycine levels to NMDA receptor mediated damage during ischemia. One issue yet to be resolved is whether resting levels of glycine in the synapse are sufficient to fulfill the binding requirement for NMDA channel activation, or whether this requirement is met only when glycine levels are increased transiently, for example as a result of stimulated release from neurons or glia. Most in vitro evidence supports the theory that the glycine site is saturated tonically [1, 14]. However, addition of glycine or D-serine (a potent glycine site agonist) has been shown to potentiate NMDA responses in vivo [5, 27, 30, 34], suggesting that resting levels of glycine in the synapse may not be high enough to saturate the site completely. If this is the case, it is likely that elevated glycine during ischemia can potentiate glutamate's actions at the NMDA receptor. In addition to its role as a co-agonist, glycine may reduce desensitization of the NMDA receptor [18, 19]. Under conditions of prolonged elevation of extracellular glutamate concentrations, as is the situation during ischemia, one might predict desensitization of the NMDA receptor site, which in turn may be attenuated by high extracellular glycine levels. Administration of a competitive glycine-binding site antagonist may therefore maintain or enhance desensitization of the NMDA receptor, thereby decreasing the influx of calcium.

While 7-ClK is one of the most selective antagonists for the glycine site on the NMDA receptor [14], and does not seem to affect the affinity of the NMDA binding site

for either agonists or antagonists, it is only 40-fold more selective for the glycine site on the NMDA receptor than for the kainate binding site [6]. Given that the density of kainate receptor expression on CA1 pyramidal cells is extremely low, it is unlikely that the protective effects of 7-ClK against cell loss in CA1 can be explained by prevention of kainate receptor-mediated excitotoxicity. Another possible mechanism by which 7-ClK may be effective in attenuating neuronal damage during ischemia is by virtue of its free-radical scavenging properties [21], as free radical formation during ischemia is thought to contribute to neuronal damage [28]. Regardless of the mechanism of action, the experimental results provide strong evidence for the prophylactic effects of 7-ClK and other antagonists acting at the glycine binding site on the NMDA receptor, against ischemia-induced cell loss.

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