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A glycine antagonist 7-chlorokynurenic acid attenuates ischemia-induced learning deficits

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TRANSIENT global ischemia can result in permanent neuronal damage and impairments in learning and memory. We investigated the therapeutic potential of 7-Chlorokynurenic acid, a potent antagonist at the glycine-modulatory site on the NMDA receptor, in terms of both neuroprotection and behavioral outcome in rats following transient forebrain ischemia. Intraventricular administration of the drug immediately before ischemia significantly attenuated ischemia-induced CA1 pyramidal cell loss. Morevoer, ischemic rats treated with 7-Chlorokynurenic acid showed unimpaired acquisition of a delayed nonmatching to sample task 8 weeks following surgery, whereas saline-treated ischemic rats were significantly impaired. These data provide preliminary evidence that the glycine site may be an appropriate target for therapeutic agents in ischemia.

Key words: Excitotoxicity; Ischemia, Glycine; Glutamate; Hippocampus; Learning; Memory; NMDA receptor

Introduction

Ischemia-induced neurodegeneration can result in marked learning and memory impairments both in humans[1] and in other animals.[2–4] Substantial evidence has accumulated suggesting a role for endogenous excitatory amino acids (EAAs) in the mediation of ischemia-induced neurodegeneration in the CNS.[5] Consequently, drugs that block the excitatory effects of glutamate and other EAAs have attracted much attention as possible neuroprotective agents.[6] Recently, we have shown that administration of 7-Chlorokynurenic acid, an antagonist acting at the glycine-sensitive modulatory site associated with the NMDA subtype of glutamate receptor, significantly decreases ischemia-induced loss of hippocampal CA1 pyramidal neurons in rats.[7] Independently, we have found that following ischemia, rats show impaired acquisition and performance of a non-spatial recognition memory task, delayed nonmatching to sample (DNMS).[8] This task[9] is analogous to a DNMS task on which monkeys[2,4] and humans[10] are impaired following ischemia. In the present study we have investigated whether the neuroprotection afforded by 7-Chlorokynurenic acid is sufficient to ameliorate the DNMS acquisition deficits produced by transient forebrain ischemia in rats.

Materials and Methods

Subjects: The subjects were 29 Male Wistar rats weighing 300–325 g at the time of surgery. Before surgery and for 21 days following surgery, rats were housed in groups of 4–5 under a 12/12 h light/dark cycle with continuous access to food and water. After 21 days of post surgery recovery the rats were housed individually with continuous access to water, and placed on a restricted feeding schedule to lower their body weights to 85%.

Surgery: The surgical procedure used to induce transient forebrain ischemia has been described in detail previously.[8] Briefly, rats were anesthetized with sodium pentobarbital (65 mg kg^{-1} i.p.) and treated with atropine sulphate (1.0 mg kg^{-1} i.p.). The common carotid arteries were isolated and encircled with silk ligatures. The femoral artery was cannulated with PE50 tubing connected to a saline-primed reservoir via a pressure transducer. The rats received a 0.1 ml bolus of heparinized saline (100 units) via the arterial cannula and were treated periodically with small volumes (0.1 ml) of more dilute heparinized saline (20 units ml^{-1}) to prevent the cannula from becoming occluded. To induce ischemia, the mean arterial blood pressure was first reduced to 30 mmHg by allowing the animals to hemorrhage via the arterial cannula over approximately 10 min. As soon as this level of hypotension was reached, atraumatic arterial clamps were placed on the isolated common carotid arteries. After 20 min of carotid occlusion, during which mean arterial pressure was maintained at 30 mmHg by withdrawing or reinfusing blood as necessary, the arterial clamps were removed and the collected blood reinfused under 200 mmHg pressure (over approximately 10 min). During the course of surgery, body temperature was monitored and maintained at 36°C using a YSI regulator. The sham-ischemia control rats were subjected to the same surgical procedures as the ischemic rats except hemorrhaging and carotid artery occlusion. Half of the rats in each group received bilateral intraventricular injections of 2 µl 500 µM 7-Chlorokynurenic acid at an infusion rate of 1 µl min^{-1} two min before occlusion. The remaining rats received bilateral intraventricular injections of vehicle (2 µl phosphate-buffered saline over 2 min). This yielded 4 groups of animals: ischemia + drug (ID, $n = 8$), sham ischemia + drug (SD, $n = 6$), ischemia + saline (IS, $n = 9$), sham ischemia + saline (SS, $n = 6$).

Apparatus: The testing apparatus has been described in detail elsewhere.[9] It consisted of a straight runway with identical goal areas at each end that were separated from a central area by two guillotine doors. Each goal area contained two recessed food wells, into which food pellets could be delivered through silicone tubing. 350 different "junk" objects, divided into seven sets of 50 objects, served as test stimuli.

Behavior: Behavioral testing began 35 days after surgery. The experimenter was blind with respect to the experimental treatment of the animals throughout behavioral testing. Each rat was first habituated to the testing apparatus as described previously.[8,9] Following habituation, each rat progressed through two phases of testing: acquisition of an object-discrimination task and acquisition of DNMS. During object discrimination training only two test objects were used; one was randomly designated as S+ (reward) and the other as S− (no reward). At the beginning of each test session, one of the doors was closed, and the S+ and S− were placed over the two food wells behind the door. The rat was placed in the center of the apparatus, and the door was opened, allowing access to the test objects. If the rat displaced the S+ from over a food well, a food pellet was delivered to that well. If it displaced S−, no reward was given. After the rat had displaced one of the objects, the far door was closed, and the same test objects were positioned (left *vs* right for a given object varied randomly) over the two food wells behind that door in preparation for the next trial. This procedure was repeated for 25 trials, after which the rat was returned to its home cage. For DNMS training, one set of 50 test objects was used for each daily session, and different sets were used on successive sessions. A different pair of objects was used for each of the 20 trials within each session. Rats started each session in the center of the box with both doors closed. Two objects were chosen from the pool and placed over randomly designated food wells, one at each end of the apparatus. One was designated at random as the sample object, and the other as the novel object. One of the doors was opened to allow access to the sample object, which the rat displaced to obtain a food pellet. The sample object was then removed, and placed over the vacant food well at the other end of the apparatus. After a 4 s retention delay, the second door was opened, and the rat was allowed to displace one of the two objects. The rat obtained a food pellet only if it displaced the novel object. After the rat had displaced one object, both objects were removed, and both doors were closed once the rat had returned to the central area. A different pair of objects were then positioned for the next trial. Daily DNMS and object discrimination training continued until rats reached a learning criterion of at least 85% correct on two consecutive sessions.

Neuropathology: After behavioral testing, rats were anesthetized with sodium pentobarbital (65 mg kg^{-1} i.p.) and perfused transcardially with 10% formalin in 0.05% phosphate buffer. The brains were removed immediately, stored overnight in phosphate buffered formalin, processed in graded ethanols and xylene, and then embedded in paraffin. 10 μm thick coronal sections were cut, mounted, and stained with 1% cresyl violet. CA1 pyramidal neurons were quantified directly by counting viable neurons in each of six coronal sections distributed evenly along the septo-temporal axis of the hippocampal formation. The cell counts at each level were averaged across the right and left hippocampi, to generate a mean cell count for each level expressed as the number of viable neurons present per unit length of the CA1 cell field (cells 125 μm^{-1}). The experimenter was blind with respect to the experimental treatment of the animals during histological evaluation.

Results

Neuropathology: Three rats in the IS group and two rats in the ID group died the day after surgery: this left six rats in each of the four groups. Upon investigation of the brains, it was observed that one rat in the ID group had sustained a large amount of damage in the rostral half of the brain; the ventricles were grossly enlarged, and the striatum, thalamus and hippocampus appeared misshapen. We surmised that this rat had suffered mechanical brain damage, perhaps related to the intraventricular drug injection. As this pattern of dam-

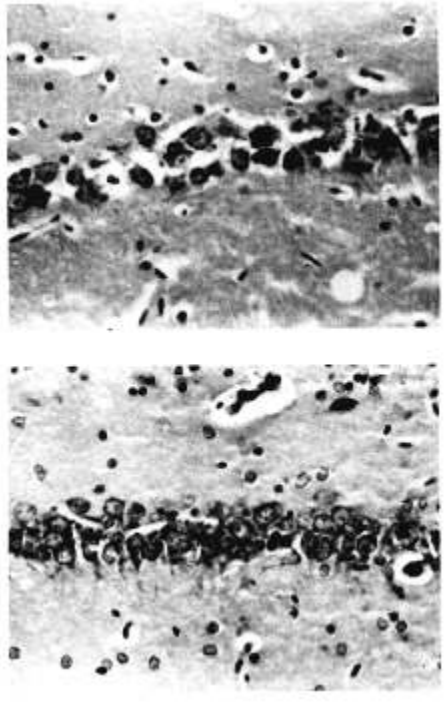

FIG. 1. Representative high power (×400) photomicrographs of a coronal section of the CA1 cell field in the hippocampus of rats subjected to ischemia plus intraventricular injections of saline (upper panel) or 7-chlorokynurenic acid (lower panel). Ischemic damage in the saline condition is indicated by fewer viable pyramidal cells.

age was not representative of that produced in either of the two ischemia groups, and was unlike anything we have observed previously, the histological and behavioral data from this rat were excluded from the subsequent analyses. As illustrated in Figures 1 and 2, 7-Chlorokynurenic acid significantly decreased ischemia-induced CA1 pyramidal cell loss. The ischemic rats treated with 7-Chlorokynurenic acid (ID) suffered only 21.9 ± 7.2% cell loss compared with sham ischemia saline control rats (SS), whereas the ischemic rats that received saline (IS) suffered 57.0 ± 6.5% cell loss. One-way analysis of variance on the CA1 pyramidal cell counts averaged across the six levels revealed a significant difference among the four groups ($F = 23.24$, df = 3, $p < 0.01$). *Post hoc* analyses (Tukey tests) showed that the IS group had significantly fewer neurons than each of the control groups (SS and SD), and the ID group ($ps < 0.01$). The ID group had significantly fewer CA1 cells than the SS group ($p < 0.05$), but not significantly fewer than the SD group. The two control groups (SS and SD) did not differ significantly. To determine whether the pattern of cell loss varied across the septo-temporal extent of the hippocampus, a two-way repeated measures ANOVA was conducted, with groups as the between subjects variable, and hippocampal level as the repeated measures factor. There was a significant difference among the four groups ($F = 23.24$, $p < 0.01$), a significant effect of hippocampal level ($F = 5.61$, $p < 0.01$), and a significant group × level interaction ($F = 4.37$, $p < 0.01$). *Post hoc* analyses showed that IS rats had significantly fewer CA1 cells than SS and SD rats at each of the six levels ($ps < 0.01$). ID rats had fewer cells than SD rats at level 1, and less than SS rats at levels 2 and 3 ($ps < 0.05$). At levels 4, 5, and 6 the ID rats were not significantly different from either of the control groups (SS and SD). Comparing the two ischemic groups, ID rats had more CA1 cells than IS rats at all six levels (levels 1, 2, 3 and 4, $p < 0.05$; levels 5 and 6, $p < 0.01$). These data are illustrated in Figure 2.

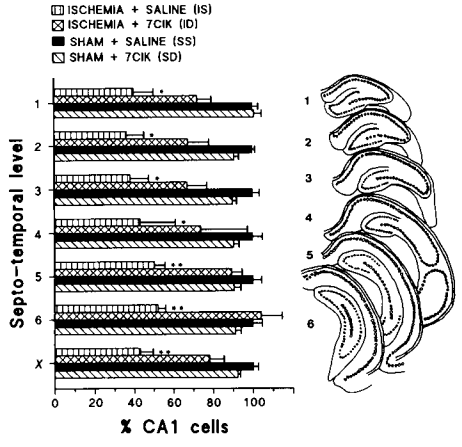

FIG. 2. Histograms showing the mean (± s.e.m.) CA1 pyramidal cell counts for each group, expressed as a % of the mean cell count for sham ischemia + saline (SS), at each of 6 hippocampal levels along the septo-temporal axis.

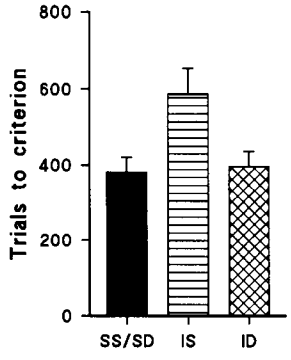

FIG. 3. Histograms showing the mean (± s.e.m.) trials to criterion for acquisition of a delayed non-matching to sample task. SS/SD = combined data from sham ischemia + saline (SS) and sham ischemia + drug (SD) groups; IS = ischemia + saline, ID = ischemia + drug groups.

Behavior: All rats reached criterion on the object discrimination task within five sessions of 25 trials each. For each group, the trials to criterion were IS = 95.8 ± 7.0, ID = 90.0 ± 8.9, SS ± 87.5 ± 7.8, SD = 87.5 ± 5.1. A one-way ANOVA on the number of trials required to reach criterion on the object-discrimination showed that there were no significant differences among the four groups ($F = 0.26$, df = 3, $p > 0.05$). The IS rats required a mean of 586.7 ± 67.9 trials to reach criterion on DNMS, while ID rats required a mean of only 396 ± 41.2 trials. The SS and SD groups required 416.7 ± 40.14 and 346.7 ± 67.5 respectively. A Student's *t*-test on the trials to criterion for the two control groups (SS and SD) showed that 7-Chlorokynurenic acid did not significantly affect the ability of sham-ischemia control rats to learn the DNMS task (pooled $t = 0.89$, df = 10, $p > 0.05$). In addition, the cell counts for these two groups were not significantly different (pooled $t = 1.73$, df = 10, $p > 0.05$). Accordingly, the behavioral data from the two control groups (SS and SD) were combined for subsequent analyses. A one-way ANOVA on the trials to criterion for the three groups (combined controls, IS and ID) revealed a significant difference among the groups ($F = 5.15$, df = 2, $p < 0.05$). *Post hoc* tests revealed that the IS group required significantly more trials to reach criterion than both the combined control group and the ID group ($ps < 0.05$). The difference between the ID group and the combined control group was not significant (see Fig. 3). There was also a significant negative correlation between the number of intact CA1 pyramidal neurons averaged across the six levels of the hippocampus, and the trials to criterion ($r = -0.4337$, df = 22, $p < 0.05$). Rats with the fewest CA1 cells tended to require more trials to learn the DNMS task.

Discussion

7-Chlorokynurenic acid administered immediately prior to bilateral carotid occlusion significantly attenuated ischemia-induced CA1 pyramidal cells loss in rats. These data replicate our previous report of significant neuroprotection against transient forebrain ischemia by 7-Chlorokynurenic acid.[7] The partial neuroprotective effect of 7-Chlorokynurenic acid was associated with a significant improvement in behavioral outcome, ischemic rats that received this drug (ID) learned the DNMS task in significantly fewer trials than ischemic rats administered saline (IS), and their performance was not significantly different from control rats (SS and SD). We have shown previously that trials to criterion on DNMS is a sensitive index of ischemia-induced disruption of learning in this model of ischemia.[8] Moreover, slow acquisition of DNMS by ischemic rats is typically associated with poor DNMS performance when rats are tested at longer retention delays.[8] This poor performance may reflect a memory deficit. From this, it may be inferred that 7-Chlorokynurenic acid could also be used to prevent ischemia-induced memory deficits. However, further behavioral assessment will be necessary to confirm this conjecture. The possibility that delay testing might reveal a memory deficit in ID rats must also be acknowledged.

7-Chlorokynurenic acid did not appear to have non-specific performance enhancing effects, as there were no significant differences between the drug-treated and saline-treated sham-ischemia rats in acquisition of the DNMS task. Therefore, the absence of DNMS deficits in ischemic rats treated with 7-Chlorokynurenic acid, is consistent with the hypothesis that a reduction in ischemic brain damage is responsible for the improved behavioral outcome observed in this study. It must be noted that the present data do not specify whether the improvement in DNMS performance by ischemic rats treated with the drug is due to neuroprotection within the hippocampus and/or in extrahippocampal brain structures. Furthermore, the present findings of normal acquisition of DNMS by the ID rats, do not preclude other memory deficits that may have been revealed by different tests.

The neuroprotection afforded by 7-Chlorokynurenic acid is probably a consequence of its antagonistic actions at the glycine site associated with the NMDA receptor.[11] Agonist binding to this site is required, in combination with agonist binding to the NMDA binding site, and cell depolarization, for NMDA channel opening.[12–15] Calcium influx via the NMDA channel, and the subsequent activation of calcium-mediated processes is thought to be the primary mechanism of excitotoxic damage in ischemia.[5] Indeed, both competitive and noncompetitive NMDA antagonists are effective neuroprotectants in transient forebrain ischemia.[16,17] However, the clinical use of drugs acting directly on the NMDA binding site or at the level of the NMDA channel may be precluded by their undesirable side effects, which include behavioral activation, elevated blood pressure, and increased glucose metabolism and, in some cases, neurotoxicity.[6,18] The results of the present experiment indicate that antagonists acting at the glycine-sensitive modulatory site may provide an alternative approach to neuroprotection, since glycine site antagonists appear to lack the negative behavioral and autonomic side effects of traditional NMDA antagonists.[6]

Conclusions

The results of this study suggest that 7-Chlorokynurenic acid provides significant protection against ischemia-induced cell loss, and that this is sufficient to improve neurobehavioral outcome after transient forebrain ischemia in rats. Therefore, drugs acting at the glycine-sensitive modulatory site associated with the NMDA receptor may prove useful as therapeutic agents in ischemia.

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