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European Journal of Pharmacology 390 (2000) 267–274

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Systemic administration of 4-chlorokynurenine prevents quinolinate neurotoxicity in the rat hippocampus

Hui-Qiu Wu *, Song-Chu Lee, Robert Schwarcz

Maryland Psychiatric Research Center, University of Maryland School of Medicine, P.O. Box 21247 Baltimore, MD 21228, USA

Received 30 September 1999; received in revised form 31 December 1999; accepted 4 January 2000

Abstract

The synthetic compound 4-chlorokynurenine has been shown to be enzymatically transaminated to the selective glycine$_B$ receptor antagonist 7-chlorokynurenate. Since 4-chlorokynurenine, in contrast to 7-chlorokynurenate, readily penetrates the blood–brain barrier, the present study evaluated its neuroprotective properties after systemic administration in rats. Intrahippocampal injection of the NMDA receptor agonist quinolinate (15 nmol/1 μl) was used as the neurotoxic paradigm. Serum and hippocampal tissue measurements confirmed that 4-chlorokynurenine serves as an effective pro-drug of 7-chlorokynurenate both in the periphery and in the brain. These studies and complementary hippocampal microdialysis experiments compared the effects of single and repeated injections of 4-chlorokynurenine (50 or 200 mg/kg, intraperitoneal (i.p.), 10 min prior to an intrahippocampal quinolinate injection; or 50 mg/kg, i.p., 10 min before and 30, 120 and 360 min after quinolinate). With the multiple-dosing regimen, extracellular 7-chlorokynurenate levels in the hippocampus reached a maximum of approximately 750 nM 7 h after quinolinate and gradually decreased with a half-life of about 3 h. In contrast, a single injection of 200 mg/kg 4-chlorokynurenine resulted in a considerably shorter rise in extracellular 7-chlorokynurenate without yielding higher peak levels. In separate animals, repeated treatment with 50 mg/kg 4-chlorokynurenine, but not a single injection of 200 mg/kg of the pro-drug, provided total protection against quinolinate-induced excitotoxicity. These data suggest that a prolonged and functionally relevant blockade of hippocampal glycine$_B$ receptors can be achieved after the systemic administration of 4-chlorokynurenine.

Keywords: 7-Chlorokynurenate; Excitotoxicity; Glycine$_B$ receptor; Neuroprotection

1. Introduction

NMDA receptors play a critical role in neurotransmission and are also involved in the occurrence of excitotoxic neuronal death (Schwarcz and Meldrum, 1985; Dingledine et al., 1999). In fact, a large number of NMDA receptor antagonists have shown anticonvulsive and neuroprotective properties in vivo and in vitro (Parsons et al., 1998). Among these, antagonists of the glycine co-agonist site of the NMDA receptor (the ''glycine$_B$'' receptor) are of particular interest for drug development (Leeson and Iversen, 1994; Danysz and Parsons, 1998). 7-Chlorokynurenate, a synthetic derivative of the tryptophan metabolite kynurenic acid, is a potent and specific glycine$_B$ receptor blocker (Kemp et al., 1988), but penetrates poorly through the blood–brain barrier (Rao et al., 1993; Ginski and Witkin, 1994). However, 7-chlorokynurenate can be produced in vivo by enzymatic transamination of 4-chlorokynurenine, a synthetic compound with easy access to brain (Hokari et al., 1996) and negligible affinity to the glycine$_B$ receptor (Salituro et al., 1994). We have shown previously that an intracerebral infusion of 4-chlorokynurenine, after enzymatic conversion to 7-chlorokynurenate, dose-dependently reduces excitotoxic neurodegeneration in the rat hippocampus (Wu et al., 1997).

The present study was designed to examine if anti-excitotoxic effects of 4-chlorokynurenine could also be observed following an intraperitoneal (i.p.) administration of the pro-drug. Using intrahippocampal injections of the excitotoxin quinolinate as the experimental test system (Schwarcz et al., 1983), we indeed observed pronounced 4-chlorokynurenine-induced neuroprotection. These data and accompanying biochemical analyses are described in the present report.

* Corresponding author. Tel.: +1-410-402-7491; fax: +1-410-747-2434.

E-mail address: hwu@umaryland.edu (H.-Q. Wu).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00024-8

2. Materials and methods

2.1. Animals

Fourteen day-old male Sprague–Dawley rats were used in all experiments. The animals were housed with their mother under standard laboratory conditions, i.e., a 12 h: 12 h light:dark cycle and free access to food and water. Experimental protocols were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland.

2.2. Surgical procedures

In preparation for an intrahippocampal microinjection, the animals were anesthetized with an i.p. injection of chloral hydrate (360 mg/kg) and placed in a small animal stereotaxic apparatus (David Kopf, Tujunga, CA) using a wooden platform. A 30 gauge stainless steel cannula was then lowered into the dorsal hippocampus (coordinates in mm: A, 2.0 posterior to bregma; L, 1.9 from the midline; V, 2.9 below the skull) for the microinjection of the excitotoxin quinolinate (Sigma, St. Louis, MO). Quinolinate was dissolved in phosphate-buffered saline (PBS), and the solution was adjusted to pH 7.4 with 1 N NaOH. Fifteen nanomoles of quinolinate in 1 μl were injected over 10 min using a microinfusion pump (Carnegie Medicin, Stockholm, Sweden). The needle was left in position for an additional 1 min before being slowly withdrawn. After surgery, the scalp was closed with Crazyglue®, and the animals were returned to their mother.

2.3. Microdialysis

Microdialysis was performed as described previously (Wu et al., 1992). Briefly, the animals were anesthetized with chloral hydrate (360 mg/kg, i.p.) and mounted in a David Kopf stereotaxic frame. A guide cannula (outer diameter: 0.65 mm) was positioned on top of the dorsal hippocampus (AP, 2.0 mm posterior to bregma; L, 1.9 mm from the midline; V, 1.5 mm below the skull) and secured to the skull with anchor screws and acrylic dental cement. Two hours after surgery, a microdialysis probe (CMA/10, membrane length: 1 mm, Carnegie Medicin) was inserted through the guide cannula, extending vertically throughout the dorsal hippocampus. The probe was then connected to a microperfusion pump (CMA/100, Carnegie Medicin) set to a speed of 1 μl/min and perfused with Ringer solution containing (in mM): NaCl, 144; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 1.7; pH 6.8. Dialysate was collected every 60 min. The first fraction was routinely discarded. During microdialysis, the probe was temporarily removed, and quinolinate was infused into the hippocampus as described above, using an injection needle coupled to an infusion pump. The microdialysis probe was reinserted immediately after the quinolinate infusion, and perfusion was continued as needed.

2.4. Administration of 4-chlorokynurenine

4-Chlorokynurenine (L-enantiomer; kindly provided by Dr. R. Tomlinson, Marion-Merrell-Dow, Cincinnati, OH) was dissolved in 3% hydroxypropyl-β-cyclodextrin (Aldrich, Milwaukee, WI), and the solution was adjusted to pH 7.0 using 1 N NaOH. 4-Chlorokynurenine was injected i.p. using either a single dose of 50 or 200 mg/kg (10 min before an intrahippocampal quinolinate injection) or four doses of 50 mg/kg (administered 10 min before, 30, 120 and 360 min after quinolinate). Control animals received equivalent treatments with vehicle.

2.5. Histological analysis

Four days after the intrahippocampal quinolinate injection, animals were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with buffered 4% paraformaldehyde. The brain was removed and post-fixed in the same fixative overnight at 4°C. Following immersion in 0.1 M phosphate-buffered saline (pH 7.4) containing 20% sucrose for 48 h (4°C), the brain was rapidly frozen and stored at −80°C. Cryostat sections (30 μm) were then cut coronally through the dorsal hippocampus. Every second section was collected on gelatin-coated slides and stained with cresyl violet.

Lesions were assessed by volume measurement in area CA1 of the dorsal hippocampus and by cell counting in the polymorphic area of the hilus. The lesion volume in CA1 was determined in a 420-μm range around the center of the injection (one section at the needle track and three sections each anterior and posterior to the needle track) using an image analysis system (Inquiry software version 3.01). Nonspecific damage due to the injection needle was approximately 1% of the quinolinate-induced cell loss and was therefore ignored in the analysis. Neuron loss in the hilus was determined quantitatively by counting all polymorphic neurons within the hilar region using an ocular grid. This was done at 200× magnification at four levels, two anterior and two posterior to the track of the injection needle. Only neuronal profiles with a visible nucleus and/or a complete cell contour and with a diameter above 20 μm were counted.

2.6. 4-Chlorokynurenine and 7-chlorokynurenate measurements

Animals were deeply anesthetized with chloral hydrate (360 mg/kg, i.p.), and cardiac blood samples were obtained with a syringe. The animals were subsequently perfused transcardially with 20 ml of ice-cold 0.9% saline solution. Blood samples were immediately centrifuged, and the resulting supernatant plasma was frozen on dry ice.

Table 1
Production of 7-chlorokynurenate from its pro-drug 4-chlorokynurenine
A single injection of 4-chlorokynurenine (4-Cl-KYN) was made 10 min before the intrahippocampal infusion of quinolinate (15 nmol/1 μl). Repeated injections of 4-chlorokynurenine were made 10 min before and 30, 120 and 360 min after the quinolinate infusion. Animals were killed 2 h after the last 4-Cl-KYN administration. Data are the mean ± S.E.M. of six animals per group. 7-Cl-KYNA: 7-chlorokynurenate.

Treatment	Serum		Hippocampus	
	4-Cl-KYN (μM)	7-Cl-KYNA (μM)	4-Cl-KYN (μM)	7-Cl-KYNA (nM)
4-Cl-KYN (50 mg/kg, i.p.)	179.8 ± 9.5	6.2 ± 0.7	10.7 ± 1.6	30.8 ± 5.3
4-Cl-KYN (50 mg/kg, i.p., ×4)	316.7 ± 17.0	36.8 ± 3.9*	29.8 ± 3.7*	206.9 ± 35.4*

*$P < 0.01$ compared to single treatment (Student's *t*-test).

After removal of the brain, the hippocampi were rapidly dissected out and immediately frozen on dry ice. Plasma and tissue samples were stored at −80°C until the day of the assay.

Thawed plasma was diluted (1:10, v/v) with ultrapure water, deproteinated with 6% perchloric acid and centrifuged (5 min, 12 000 × *g*). An aliquot of the supernatant was diluted (1:100, v/v) with ultrapure water. Tissue was thawed and sonicated (1:10, w/v) in ultrapure water. The resulting homogenate was further diluted (1:4 or 1:10, v/v, for single or multiple dosing, respectively) with ultrapure water, deproteinated using 6% perchloric acid and centrifuged (5 min, 12 000 × *g*).

For 4-chlorokynurenine determinations, appropriate aliquots of plasma and tissue extracts were diluted (1:1, v/v) with high performance liquid chromatography (HPLC) mobile phase (0.1 M ammonium acetate buffer containing 18% acetonitrile, pH 4.65). The samples were then subjected to HPLC analysis using a 5 μm reverse phase C_{18} column (Adsorbosil, Alltech, Deerfield, IL) and a flow rate of 1.0 ml/min. 4-Chlorokynurenine was detected by UV absorption at 365 nm using a 160 Absorbance Detector (Beckman, Fullerton, CA). The retention time of 4-chlorokynurenine was approximately 5 min.

For 7-chlorokynurenate measurements, appropriate aliquots of diluted plasma or original tissue homogenates were deproteinated as described above. Aliquots of the extracts were diluted (1:1, v/v) with HPLC mobile phase (50 mM sodium acetate buffer containing 0.25 M zinc acetate and 10% acetonitrile, pH 6.2), and samples were applied to a 3 μm reverse phase HR-80 C_{18} column (ESA, Chelmsford, MA). After elution at a flow rate of 1.0 ml/min, 7-chlorokynurenate was detected by fluorescence using an excitation wavelength of 344 nm and an emission wavelength of 398 nm (Swartz et al., 1990). 7-Chlorokynurenate eluted with a retention time of 5–6 min.

The 7-chlorokynurenate content of microdialysis samples was determined as described above by directly injecting 100 μl of diluted dialysate (1:3, v/v, with HPLC mobile phase) onto the HPLC column. The data presented were not corrected for recovery from the dialysis probe (15.4 ± 0.9%, $n = 6$; determined in vitro).

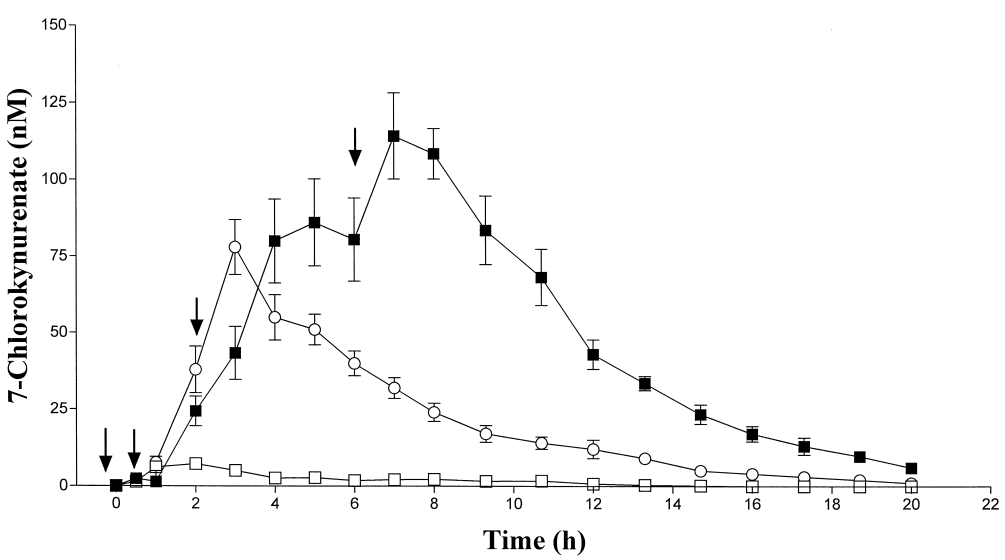

Fig. 1. Extracellular levels of 7-chlorokynurenate in the dorsal hippocampus after a single i.p. injection (50 mg/kg, open squares; 200 mg/kg, open circles) or multiple i.p. administration of 50 mg/kg (solid squares) of 4-chlorokynurenine. Arrows indicate the times of pro-drug application in the repeated treatment regimen. Quinolinate (15 nmol/1 μl) was infused intrahippocampally at time 0 as described in the text. Data (not corrected for recovery from the dialysis probe) are the mean ± S.E.M. of six to seven animals per group.

2.7. Data analysis

After completion of the experiments, the position of the injection needle or microdialysis probe was examined histologically in all animals. Only animals showing correct intrahippocampal track positions were used for data analysis. Neurochemical and histological data were statistically analyzed with repeated measurement of variance and with Bonferroni corrections for specific group/time comparisons.

3. Results

3.1. 4-Chlorokynurenine and 7-chlorokynurenate levels in serum and hippocampus

In a first set of experiments, we determined the content of 4-chlorokynurenine and 7-chlorokynurenate in serum and hippocampus 2 h after the systemic administration of

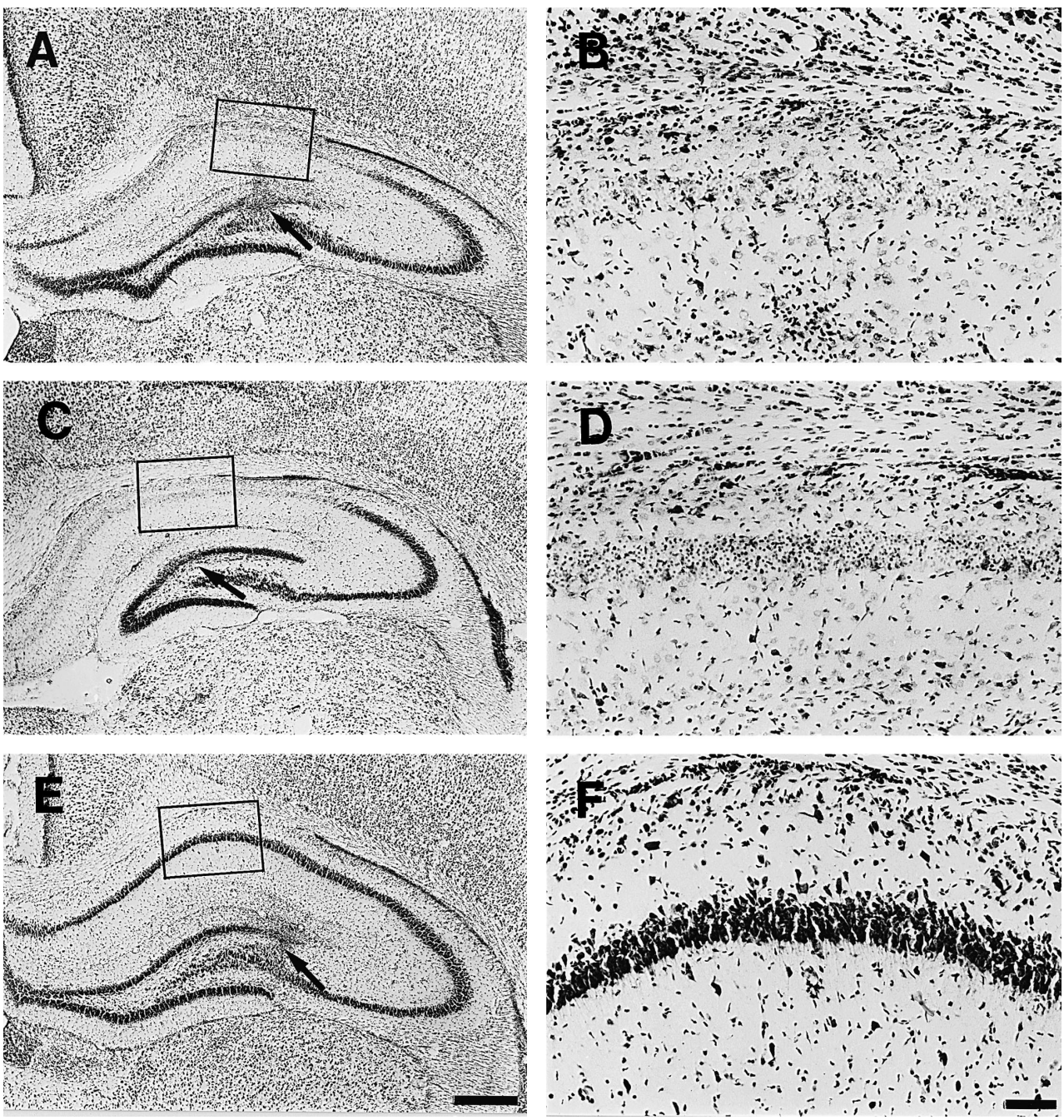

Fig. 2. Micrographs of Nissl-stained coronal sections showing low (A, C, E) and high (B, D, F) magnifications (corresponding to the boxes in A, C and E) of quinolinate (15 nmol/1 μl)-injected hippocampi. (A, B) Controls, (C, D) treatment with a single dose of 200 mg/kg 4-chlorokynurenine, (E, F) treatment with four successive injections of 50 mg/kg 4-chlorokynurenine. Arrows indicate the track of the injection needle. See text for experimental details. Scale bars: 500 μm (A, C, E); 200 μm (B, D, F).

the pro-drug, comparing single and multiple injections of 50 mg/kg 4-chlorokynurenine. After a single injection, serum 4-chlorokynurenine and 7-chlorokynurenate levels were in the μM range, with the pro-drug attaining an approximately 30 times higher concentration than 7-chlorokynurenate. Multiple dosing of 4-chlorokynurenine (10 min before and 30, 120 and 360 min after the quinolinate infusion) resulted in a modest further elevation in serum 4-chlorokynurenine levels (from 180 to 317 μM), and a sixfold increase in serum 7-chlorokynurenate (from 6 to 37 μM; Table 1). Essentially the same 4-chlorokynurenine/7-chlorokynurenate ratios were found in the hippocampus, with the yield of 7-chlorokynurenate improving with repeated systemic pro-drug application. 207 fmol 7-chlorokynurenate/mg tissue were thus recovered from the hippocampus after intermittent treatment with 4-chlorokynurenine (Table 1).

3.2. 7-Chlorokynurenate levels in microdialysate

The extracellular levels of 7-chlorokynurenate following the systemic application of 4-chlorokynurenine were monitored by hippocampal microdialysis in vivo. Analyses were made after single injections of 50 or 200 mg/kg 4-chlorokynurenine, or using the repeated injection paradigm described above. In all experiments, microdialysis was performed continuously for 20 h following the quinolinate infusion.

As illustrated in Fig. 1, 50 mg/kg 4-chlorokynurenine, applied 10 min before quinolinate, resulted in 7.2 ± 1.0 nM 7-chlorokynurenate/100 μl dialysate 2 h after the quinolinate infusion, and 7-chlorokynurenate levels dropped beneath the level of assay sensitivity (~ 200 pM) by 15 h. A single injection of 200 mg/kg 4-chlorokynurenine yielded a peak 7-chlorokynurenate concentration of 78 ± 9 nM 3 h following quinolinate application. These levels decreased gradually with a half life of 2.5–3 h. After repeated administration of 4-chlorokynurenine, 7-chlorokynurenate in the microdialysate continued rising to reach maximal levels approximately 7 h after the quinolinate infusion (114 ± 14 nM).

3.3. Neuroprotection by systemic 4-chlorokynurenine administration

Histological examination revealed that multiple administration of 4-chlorokynurenine produced significant protection against quinolinate-induced hippocampal neurotoxicity, as judged by the preservation of neurons in both area CA1 and the hilus of the dorsal hippocampus. Thus, the lesion volume in area CA1 was reduced by 87% (from 0.072 ± 0.005 to 0.009 ± 0.003 mm^3; $P < 0.05$), and hilar cell count increased from 10.4 ± 0.8 cells (quinolinate alone) to 88.3 ± 7.0 cells (quinolinate + 4 × 4-chlorokynurenine; $P < 0.05$). In contrast, a bolus treatment of 200 mg/kg 4-chlorokynurenine, i.e., the same total amount of the pro-drug, did not convey neuroprotection against a quinolinate-induced lesion in the dorsal hippocampus (Figs. 2 and 3).

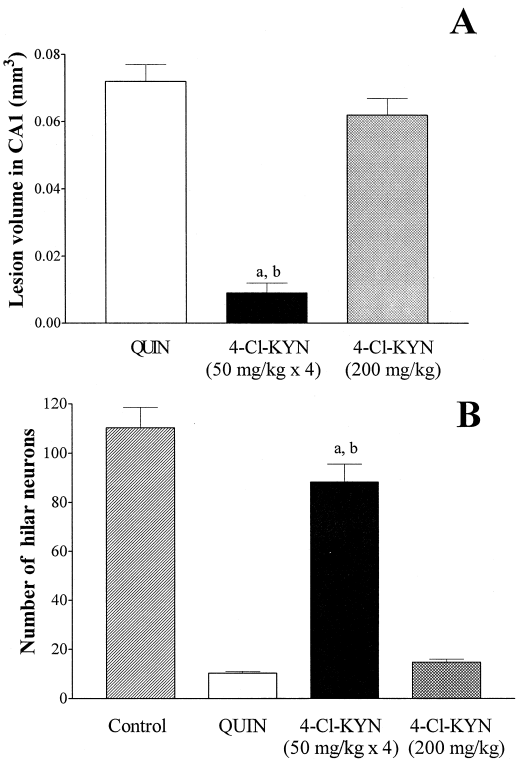

Fig. 3. Quantitative assessment of neurodegeneration and neuroprotection in the dorsal hippocampus following a focal infusion of quinolinate (15 nmol/1 μl). (A) Bar graph showing the lesion volume in area CA1 of the dorsal hippocampus. (B) Bar graph illustrating hilar neuronal loss. See text for experimental details. Data are the mean ± S.E.M. of 6–13 animals per group. a: $P < 0.05$ as compared to quinolinate-lesioned controls; b: $P < 0.05$ as compared to animals receiving a single injection of 200 mg/kg 4-chlorokynurenine (Bonferroni's test).

4. Discussion

The present study provides evidence that a systemic pro-drug approach can be successfully used to deliver neuroprotective quantities of a selective glycine$_B$ receptor antagonist to the brain. The results are in line with the demonstration that 4-chlorokynurenine readily penetrates the blood–brain barrier using the large neutral amino acid transporter (Hokari et al., 1996) and with experiments using intracerebral application of 4-chlorokynurenine (Salituro et al., 1994; Wu et al., 1997). The latter studies documented the efficient enzymatic transamination of 4-chlorokynurenine to 7-chlorokynurenate in the brain and illustrated the neuroprotective potential of the newly formed glycine$_B$ receptor antagonist.

Various drug administration paradigms were examined to optimize the formation of 7-chlorokynurenate from 4-chlorokynurenine for neuroprotection. The first set of experiments was designed to find a treatment schedule that would yield brain concentrations of 7-chlorokynurenate close to its IC_{50} at the glycine$_B$ receptor (0.56 μM; Parsons et al., 1997). Since in vivo neuroprotection against acute excitotoxic injury can be expected to be more effective when receptor blockade is extended beyond the initial insult, we also attempted to maintain the presumed neuroprotective levels of 7-chlorokynurenate for a prolonged period of time. Using animals that received a unilateral intrahippocampal injection of 15 nmol of quinolinate, i.e., the test dose of the excitotoxin used in subsequent neurotoxicity/neuroprotection experiments, the effects of a single injection of 50 mg/kg 4-chlorokynurenine were therefore compared with those of repeated injections of 50 mg/kg (totalling 200 mg/kg of the pro-drug). In both cases, serum and hippocampal content of 4-chlorokynurenine and 7-chlorokynurenate were determined 2 h after the last application of 4-chlorokynurenine. In separate animals, hippocampal microdialysis was performed to monitor the levels of extracellular 7-chlorokynurenate over time.

The results of the initial study revealed several notable features. In both serum and brain, repeated injection of 4-chlorokynurenine improved the yield of 7-chlorokynurenate, indicating a relatively slow removal or degradation of newly synthesized 7-chlorokynurenate compared to a more rapid metabolism or disposal of the pro-drug. Thus, although not investigated in the present study, it can be assumed that 4-chlorokynurenine is not only transaminated to produce 7-chlorokynurenate, but is also degraded via the competing quinolinate branch of the kynurenine pathway, eventually forming the 3-hydroxyanthranilate oxygenase inhibitor 4-Cl-3-hydroxyanthranilate (Parli et al., 1980; Naritsin et al., 1995; Guidetti et al., in press). In contrast, and in analogy to the endogenous excitatory amino acid receptor antagonist kynurenate (Turski and Schwarcz, 1988), newly formed 7-chlorokynurenate is probably not metabolized enzymatically. In practical terms, this produced disproportionately high levels of 7-chlorokynurenate upon repeated pro-drug application, causing an approximately seven-fold increase in hippocampal 7-chlorokynurenate content (from 31 to 207 fmol/mg tissue) while 4-chlorokynurenine levels in the same animals rose less than three-fold (cf. Table 1). The relative persistence of newly produced 7-chlorokynurenate bodes well for future chronic applications of 4-chlorokynurenine, since it may be possible to achieve desirable brain levels of the antagonist with reduced doses of the pro-drug.

Hippocampal microdialysis, using identical treatment regimens and including a group of animals receiving a single injection of 200 mg/kg 4-chlorokynurenine, confirmed the superior efficacy of intermittent pro-drug application. Thus, as compared to a bolus administration of 200 mg/kg, de novo synthesized 7-chlorokynurenate reached slightly higher peak levels (approximately 750 nM, calculated for recovery from the dialysis probe) following the injection of 4×50 mg/kg 4-chlorokynurenine. More importantly, repeated pro-drug injection maintained the extracellular presence of the antagonist for a considerably longer period of time. Thus, by 12 h after the intracerebral quinolinate infusion, multiple application of 50 mg/kg 4-chlorokynurenine still yielded approximately 250 nM 7-chlorokynurenate, i.e., a concentration that may convey significant inhibition of the glycine$_B$ receptor (Kemp et al., 1988; Parsons et al., 1997). Pharmacological efficacy is even more likely in view of the fact that microdialysate measurements probably underestimated the true synaptic concentration of 7-chlorokynurenate (cf. Scharfman et al., 1999).

In accordance with these considerations and the well-established prevention of quinolinate neurotoxicity by glycine$_B$ receptor blockade (Foster et al., 1990; Hartley et al., 1990; Wu et al., 1997), intermittent systemic pro-drug administration (4×50 mg/kg 4-chlorokynurenine) protected hippocampal neurons against quinolinate-induced injury. In these animals, both CA1 pyramidal cells and polymorphic hilar neurons were found to survive exposure to a normally toxic dose of quinolinate (15 nmol). Notably, no neuroprotection was observed after a bolus injection of 200 mg/kg 4-chlorokynurenine. Taken together with the results of the chemical analyses described above, these data therefore clearly demonstrate that both the degree and the duration of receptor blockade constitute critical elements of an effective defense against an acute excitotoxic insult in vivo.

During the past decade, the glycine$_B$ receptor has been identified as a premier target of drug development to combat neurological and psychiatric diseases which are thought to be directly or indirectly linked to abnormal glutamatergic neurotransmission (Leeson and Iversen, 1994; Danysz and Parsons, 1998). In particular, this site has attracted attention because its pharmacological manipulation does not appear to be associated with the untoward side effects of several of the drugs acting at other sites of the NMDA receptor complex (Fix et al., 1994; Javitt, 1996; Malhotra et al., 1996; Herrling et al., 1997; Tamminga, 1998). For example, glycine$_B$ receptor antagonists do not share a discriminative cue with NMDA channel blockers and are therefore unlikely to have the abuse potential of phencyclidine (PCP) and related agents (Balster et al., 1995). Moreover, they have a comparatively low risk of producing memory impairment (Baron and Moerschbacher, 1996; Bordi et al., 1999) and do not seem to cause neuronal vacuolization in limbic brain areas. These side effects have substantially impeded the development of both competitive and noncompetitive NMDA receptor antagonists for clinical use (Hargreaves et al., 1993; Auer, 1997; Hawkinson et al., 1997), though it is now clear that relatively wide therapeutic margins can be achieved with

newer NMDA receptor channel blockers (Parsons et al., 1998).

Poor brain penetration, rapid brain efflux, or nephrotoxicity have so far constituted the main impediments in the progress of glycine$_B$ receptor-based therapies. To varying degrees, these problems have foiled or delayed the development of compounds such as MDL 104,653, L-701,324, L-705,022, ACEA-1021 and MRZ/576 (Danysz and Parsons, 1998). Limited brain access after systemic administration is also a significant deficiency of 7-chlorokynurenate, the prototypical and probably most widely used full glycine$_B$ receptor antagonist (Rao et al., 1993; Ginski and Witkin, 1994). The results of the present study demonstrate that a pro-drug approach using 4-chlorokynurenine may provide the means to reevaluate the clinical efficacy of 7-chlorokynurenate, especially for the treatment of chronic pain, drug abuse, and acute and chronic neurodegenerative diseases (Danysz and Parsons, 1998).

Acknowledgements

We gratefully acknowledge the excellent secretarial assistance of Mrs. Joyce Burgess. This work was supported by USPHS grants NS 16102 and NS 28236.

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