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Modulation of striatal quinolinate neurotoxicity by elevation of endogenous brain kynurenic acid

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1 Nicotinylalanine, an inhibitor of kynurenine metabolism, has been shown to elevate brain levels of endogenous kynurenic acid, an excitatory amino acid receptor antagonist. This study examined the potential of nicotinylalanine to influence excitotoxic damage to striatal NADPH diaphorase (NADPH-d) and γ-aminobutyric acid (GABA)ergic neurones that are selectively lost in Huntington's disease.

2 A unilateral injection of the N-methyl-D-aspartate (NMDA) receptor agonist, quinolinic acid, into the rat striatum produced an 88% depletion of NADPH-d neurones. Intrastriatal infusion of quinolinic acid also produced a dose-dependent reduction in striatal GABA content.

3 Nicotinylalanine (2.3, 3.2, 4.6, 6.4 nmol 5 μl^{-1}, i.c.v.) administered with L-kynurenine (450 mg kg^{-1}), a precursor of kynurenic acid, and probenecid (200 mg kg^{-1}), an inhibitor of organic acid transport, 3 h before the injection of quinolinic acid (15 nmol) produced a dose-related attenuation of the quinolinic acid-induced loss of NADPH-d neurones. Nicotinylalanine (5.6 nmol 5 μl^{-1}) in combination with L-kynurenine and probenecid also attenuated quinolinic acid-induced reductions in striatal GABA content.

4 Nicotinylalanine (4.6 nmol, i.c.v.), L-kynurenine alone or L-kynurenine administered with probenecid did not attenuate quinolinic acid-induced depletion of striatal NADPH-d neurones. However, combined administration of kynurenine and probenecid did prevent quinolinic acid-induced reductions in ipsilateral striatal GABA content.

5 Injection of nicotinylalanine, at doses (4.6 nmol and 5.6 nmol i.c.v.) which attenuated quinolinic acid-induced striatal neurotoxicity, when combined with L-kynurenine and probenecid produced increases in both whole brain and striatal kynurenic acid levels. Administration of L-kynurenine and probenecid without nicotinylalanine also elevated kynurenic acid, but to a lesser extent.

6 The results of this study demonstrate that nicotinylalanine has the potential to attenuate quinolinic acid-induced striatal neurotoxicity. It is suggested that nicotinylalanine exerts its effect by increasing levels of endogenous kynurenic acid in the brain. The results of this study suggest that agents which influence levels of endogenous excitatory amino acid antagonists such as kynurenic acid may be useful in preventing excitotoxic damage to neurones in the CNS.

Keywords: Nicotinylalanine; endogenous kynurenic acid; quinolinic acid; neurotoxicity

Introduction

The enzymatic metabolism of tryptophan in the central nervous system (CNS) via the kynurenine pathway yields several intermediates that have the ability to influence excitatory amino acid (EAA) neurotransmission (Schwarcz *et al*., 1984). One such product, quinolinic acid, a pyridine dicarboxylic acid, behaves as an agonist at N-methyl-D-aspartate (NMDA) receptor sites; another, kynurenic acid, acts as an EAA receptor antagonist. Neuronal application of quinolinic acid produces excitatory responses (Stone & Perkins, 1981) and its focal injection in brain areas causes excitotoxic damage to neurons bearing NMDA receptors and receiving glutamatergic innervation (Schwarcz, 1983; Schwarcz *et al*., 1984; El-Defrawy *et al*., 1985). In contrast, kynurenic acid blocks excitatory responses produced by NMDA and non-NMDA receptor agonists (Perkins & Stone, 1982), and prevents the excitotoxic effects of quinolinic acid (Foster *et al*., 1984; Boegman *et al*., 1985; Winni *et al*., 1991; Cockhill *et al*., 1992).

Both quinolinic acid and kynurenic acid are derived from the catabolism of kynurenine, the first metabolic product of tryptophan degradation via the kynurenine pathway. The synthesis of kynurenic acid is catalyzed by kynurenine aminotransferase, while quinolinic acid is produced from kynurenine via the activity of three enzymes: kynureninase, kynurenine hydroxylase and 3-hydroxyanthranilic acid oxygenase. Recent studies have shown that in the CNS production of kynurenines, including kynurenic acid and quinolinic acid, occurs primarily in glial cells (Roberts *et al*., 1992; 1995; Schwarcz, 1993).

Since quinolinic acid originates in the CNS and can cause profound neuronal damage, it has been designated as an endogenous excitotoxin (Schwarcz *et al*., 1984). The phenomenon of excitotoxicity, involving neuronal damage due to overstimulation of NMDA and non-NMDA ionotropic receptors has been implicated in acute neuronal disorders such as stroke, and chronic disorders such as Huntington's disease and Parkinson's disease (Rothman & Olney, 1986; Choi, 1988). In contrast to quinolinic acid, an EAA receptor agonist, kynurenic acid can be designated as an endogenous EAA receptor antagonist. As both are derived from a common precursor (kynurenine) and exert opposite actions on neuronal viability, a balance between these metabolites in the CNS may influence neuronal survival. Indeed, it has been shown that kynurenic levels are depleted in the striatum and cortex of patients with Huntington's disease (HD) (Beal *et al*., 1990; 1992) and the neuronal pathology produced by the intrastriatal infusion of quinolinic acid closely mimics that found in HD (Beal *et al*., 1986). Thus, a pharmacological strategy that increases kynurenic acid levels in the CNS could potentially

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inhibit the neuronal loss that results from excessive stimulation of NMDA receptors. The recent discovery that certain agents can elevate levels of endogenous kynurenic acid in the brain makes it possible to evaluate this strategy in experimental models of excitotoxic damage.

One of the agents that elevates brain kynurenic acid level is nicotinylalanine, a structural analogue of kynurenine and inhibitor of the enzymes kynureninase and kynurenine hydroxylase (Decker *et al*., 1963). Administration of nicotinylalanine to rats, in combination with L-kynurenine and probenecid, compounds which act as a precursor of kynurenic acid and block its egress from the brain, respectively (Miller *et al*., 1992), produces a significant increase in brain kynurenic acid content (Moroni *et al*., 1991; Russi *et al*., 1992; Miranda *et al*., 1997). Nicotinylalanine was also shown to reduce the incidence of chemical seizures, an effect which has been attributed to its ability to augment brain kynurenic acid (Connick *et al*., 1992). Nicotinylalanine, through its ability to increase endogenous kynurenic acid levels in the CNS, might be expected to inhibit excitotoxic damage. Indeed, it has been demonstrated that nicotinylalanine in combination with L-kynurenine and probenecid can attenuate quinolinic acid-induced excitotoxicity in the substantia nigra pars compacta (Miranda *et al*., 1997). However, the potential of nicotinylalanine to influence excitotoxic damage in the striatum, has not been explored. The goal of this study was to investigate if nicotinylalanine, administered under conditions which lead to increased brain kynurenic acid, influences excitotoxic damage to specific populations of striatal neurones. This goal was addressed by evaluating the effects of centrally administered nicotinylalanine on the survival of striatal NADPH-diaphorase (NADPH-d) neurones and striatal GABA content, a marker of GABAergic neuronal viability, following a toxic injection of quinolinic acid into the rat striatum. Previous work in this and other laboratories has demonstrated that these neuronal populations are readily destroyed by intrastriatal quinolinic acid injections (Beal *et al*., 1986; Boegman & Parent, 1988; Davies & Roberts, 1987; Roberts *et al*., 1993; Kalisch *et al*., 1994). To optimize the action of nicotinylalanine, this agent was co-administered with L-kynurenine, the precursor of kynurenic acid and probenecid, an inhibitor of organic acid transport, which reduces the efflux of kynurenic acid from the brain (Moroni *et al*., 1991; Miller *et al*., 1992; Vescei *et al*., 1992).

Methods

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec), weighing between 125–150 g, were given free access to food (Purina rat chow) and drinking water. Experiments described here were performed in accordance with the Guidelines of the Canadian Council on Animal Care using protocols approved by Queen's University Animal Care Committee.

Surgery and stereotaxic injections

Animals were anaesthetized with a 4% halothane-96% oxygen mixture and positioned in a Narashighe small animal stereotaxic apparatus. The skull was exposed and a small hole drilled for placement of an intraventricular cannula (0.18 min i.d.; 0.36 mm, o.d., 2 cm long). Following insertion into the lateral ventricle, the cannula was connected to a Hamilton syringe filled with normal saline or a nicotinylalanine solution and mounted in an infusion pump. The saline or nicotinylalanine solution (2.3–6.4 nmol) was infused (5 μl volume) into the lateral ventricle over a 100 second period. In order to prevent back flow of nicotinylalanine, the cannula was left in place for 2 min to allow nicotinylalanine to diffuse away and then be removed. Injections of L-kynurenine (450 mg kg^{-1}) and probenecid (200 mg kg^{-1}) were then administered by the intraperitoneal route. Control animals received injections of saline over the same time period. Both L-kynurenine and probenecid were dissolved in 1 N NaOH and titrated to pH = 10 with 1 N HCl.

Three hours following drug treatment a single unilateral injection of quinolinic acid (15 nmol in 0.5 μl saline or 30 nmol in 1 μl) was delivered into the right striatum. Briefly, the animal was placed in a stereotaxic frame, the skull exposed and a small hole drilled by use of the coordinates: 1.0 mm anterior to bregma, 3.2 mm lateral to midline and 5.0 mm ventral to the skull surface with incisor bar set at −3.3 mm. The intrastriatal injection was delivered over a 75 s period and the injection cannula was left in place for an additional 2 min to prevent the back flow before its removal. Control animals received normal saline instead of quinolinic acid.

Two days following the intrastriatal injection, the animals were anaesthetized and prepared for fixation of brain tissue as described in a previous study (Kalisch *et al*., 1994). The fixed brain was sectioned in a coronal plane using a cryostat and tissue slices (40 μm thickness) were collected in culture plates containing 0.9% saline. The sections incorporating the striatum were subsequently stained to visualize NADPH-diaphorase neurones by use of a modification of the method described by Sims *et al*. (1974). Briefly, tissue sections in which the injection cannula tract was clearly visible, and those anterior and posterior to the cannula, were incubated in phosphate buffered saline containing 1.4 mM NADH, 1.1 mM nitroblue tetrazolium and 12% dimethylsulphoxide for 15–30 min and subsequently rinsed twice with saline. Sections were mounted on glass slides and coverslips applied. Cells staining positive for NADPH-d in the striatum within a defined area (1.6 mm × 1.0 mm), which included the tip of the infusion cannula track, were counted under bright field illumination. For each animal, three tissue sections containing the cannula tract were counted. All histological slides used in cell counts were coded to mask the treatment condition of the animal from which the tissue section was obtained. The pooled total of cell counts for the injected side in each animal was expressed as a percentage of the total of cell counts at the corresponding site on the contralateral (uninjected) striatum.

Kynurenic acid measurements

In a separate group of saline- or drug-treated animals, whole brain kynurenic acid levels were measured by use of a modification of a method described by Russi *et al*. (1992). Three hours following central injection the whole brain from each animal was homogenized in an ethanol-0.1 M NaOH mixture (3 : 1). The homogenate was centrifuged (10 min, 5000 × *g*) and the pellet resuspended in 5 ml of 90% ethanol. The supernatants were pooled and placed overnight at −80°C to precipitate fatty materials which were discarded after centrifugation (2000 × *g* for 5 min). Following this, 200–300 mg of an ion exchange resin (Dowex AG1 WX8, acetate form, 100–200 mesh) were added to the supernatant and the mixture centrifuged for 5 min. The supernatant was discarded, the resin suspended in 2 ml of water and placed in glass columns (pasteur pipettes) plugged with glass wool. The column was washed with 5 ml of water and with 10 ml of 1 N formic acid. Kynurenic acid was eluted with 5 ml 1 N formic

acid. This eluate was mixed with 250–300 mg of Dowex AG-5 WX8 resin, in the H^+ form centrifuged at 20 000 × *g* for 5 min and the pellet resuspended in 2 ml of water and placed in the pasteur pipette. Kynurenic acid was eluted with 5 ml 3 N NH_4OH. The eluate was concentrated to dryness and resuspended in 200 ml of sodium acetate buffer (pH 6.2), and 50 μl injected into the high performance liquid chromatographic (h.p.l.c.) system. The h.p.l.c. utilized a mobile phase consisting of 4.5% acetonitrile and 50 mM sodium acetate (pH = 6.2). The mobile phase was pumped through a reverse phase column (CSC-spherisorb-ODS2, 10 cm × 0.46 cm, 3 μm particle size) at a flow rate of 1.0 ml min^{-1}. A solution of zinc acetate (0.5 M) was delivered post-column to enhance the detection of kynurenic acid by a fluorescence detector (Shimadzu RF-551) set at excitation and emission wavelengths of 344 and 398 nm, respectively. The peak areas of the chromatographic signal were integrated. Kynurenic acid standards and a sodium acetate blank were analysed with each set of experimental samples and a standard curve was generated. The detection limit of this assay was 35 fmol.

In another group of animals that had been pretreated with L-kynurenine (450 mg kg^{-1}, i.p.) and probenecid (200 mg kg^{-1}, i.p.) with or without intraventricular nicotinylalanine (5.6 nmol), the whole striatum from each animal was dissected out at 1.5, 3, 6 and 9 h post treatment. The content of kynurenic acid in the striatal tissue was measured with the procedure described above.

Striatal GABA content

In a separate group of animals, the ability of nicotinylalanine to influence quinolinic acid-induced reduction in striatal GABA content was evaluated. The animals were decapitated and the brain rapidly removed washed with ice cold saline and placed in a brain mould. A 2 mm slice of striatum from each animal was dissected out and a 2 mm diameter punch at the tip of the cannula track was homogenized in 500 μl of nanopure water and centrifuged at 5000 × *g* for 30 min. Striatal GABA content was assayed by h.p.l.c. Briefly, 200 μl of the supernatant was derivitized by mixing with 200 μl of *o*-phthaldialdehyde reagent solution before application to a reverse phase column (LC-18-Supelcosil, 15 cm × 4.6 mm, 5 μm particle size). A portion (100 μl) of the supernatant was retained for protein determination (Bradford, 1976). The mobile phase consisted of 0.04 M sodium acetate buffer with 30% methanol (pH = 7.5). GABA was detected with a Shimadzu RF-590 fluorescence detector at an excitation wavelength of 345 and a detection wavelength of 470. In order to determine GABA concentrations a standard curve was constructed for each assay. The detection limit of this assay was 1 pmol. GABA content in the ipsilateral striatum was expressed as a percentage of that in the contralateral side.

Statistical analyses

The data were compared by a one way analysis of variance (ANOVA), followed by Newman-Keul's test to determine differences between various drug treatments. Differences in mean values were considered significant at $P < 0.05$.

Results

Figure 1 shows the effect of saline and quinolinic acid (15 nmol) on the survival of the NADPH-d neurones 2 days after a single injection into the striatum. The data, expressed as % cell survival, represent the number of NADPH-d neurones on the injected side as a % of cells in corresponding area of the contralateral uninjected striatum. The total cell counts in designated area of the uninjected side were 99.3 ± 5.4 (saline group) and 105.3 ± 8.2 (quinolinic acid group). Following intrastriatal saline (0.5 μl) there was over 90% survival of NADPH-d neurones. In contrast, injection of quinolinic acid (15 nmol) produced a severe neuronal depletion with only 12% of NADPH-d neurones surviving in the injected side. This effect of quinolinic acid was very reproducible (see below).

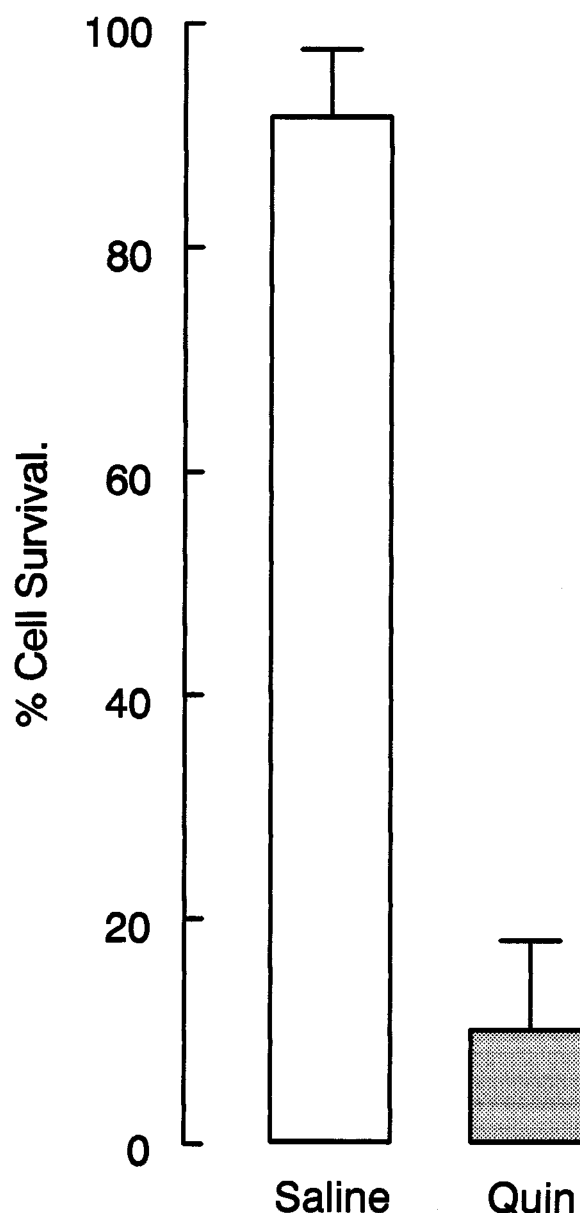

Figure 1 The effect of a unilateral intrastriatal injection of saline (0.5 μl) or quinolinic acid (Quin, 15 nmol, 0.5 μl) on the survival of neurones staining for NADPH-d diaphorase in the rat striatum. Values shown (mean ± s.e.mean) represent cell counts in the Quin-injected side expressed as percentage of cells in a corresponding area of the striatum on the uninjected side in the same animal; $n = 4$ animals. *$P < 0.001$, value significantly different from saline-injected animals.

To investigate the action of nicotinylalanine on quinolinic acid-induced toxicity, this agent was injected into the lateral ventricle 3 h before an intrastriatal quinolinic acid (15 nmol) injection. L-Kynurenine (450 mg kg^{-1}, i.p.) and probenecid

(200 mg kg^{-1}, i.p.) were co-administered by the i.p. route immediately after the nicotinylalanine injection. As shown in Figure 2, central injection of nicotinylalanine (2.3–6.4 nmol) and co-treatment produced a dose-dependent increase in the survival of NADPH-d neurones in the quinolinic acid-injected side. In animals receiving the highest dose (6.4 nmol) of the drug, approximately 72% of neurones in the injected striatum survived quinolinic acid toxicity. Statistical analysis confirmed a significant treatment effect. Thus, centrally injected nicotinylalanine with the co-treatment afforded protection against quinolinic acid-induced neurotoxicity.

To determine if nicotinylalanine or co-treatment alone had the ability to influence quinolinic acid toxicity, drugs were injected separately and their action on toxicity examined in similar experiments. The treatments evaluated were: saline (i.c.v.), nicotinylalanine (4.6 nmol i.c.v.), L-kynurenine (450 mg kg^{-1}, i.p.), nicotinylalanine in combination with L-kynurenine (450 mg kg^{-1}, i.p.) or with probenecid (200 mg kg^{-1}, i.p.), and L-kynurenine in combination with probenecid. Each evaluation included a control group of animals which received the treatment under study but was administered intrastriatal saline (0.5 μl) instead of quinolinic acid (15 nmol). As shown in Figure 3, after an injection of intrastriatal saline, the survival of NADPH-d neurones ranged between 89 and 94%. This effect was similar to that observed in the preceding experiments (Figure 1). Intrastriatal quinolinic acid, nicotinylalanine alone or the L-kynurenine-probenecid combination did not influence quinolinic acid toxicity. In the quinolinic injected acid groups, the NADPH-d neurone survival ranged between 8–17%, values which were similar to that obtained with quinolinic acid in untreated animals (Figure 1). Thus, none of the treatments evaluated in this part of the study exerted an anti-quinolinic acid action comparable to that observed with nicotinylalanine, in combination with L-kynurenine and probenecid (Figure 2).

In order to validate further the neuroprotective actions of nicotinylalanine, its ability to influence quinolinic acid-induced reductions in striatal GABA content was assessed. Striatal GABA content in the injection side, expressed as a percentage of that from the intact side, was used as a biochemical marker of the viability of the GABAergic projection neurones (Figure 4). Focal infusion of saline into the striatum did not significantly alter GABA content in the ipsilateral striatum. However, intrastriatal infusion of quinolinic acid resulted in dose-dependent reductions in ipsilateral GABA content ($P<0.05$). In rats infused with the lowest dose of quinolinic acid, ipsilateral striatal GABA content was 64 ± 13.5% of the contralateral side. At the highest concentration of quinolinic acid employed in this study (240 nmol), ipsilateral striatal GABA content was 27.8 ± 5.3% of the contralateral side. In subsequent experiments the dose of quinolinic acid (30 nmol) that produced approximately 50% reduction in striatal GABA content was selected to examine the effect of nicotinylalanine on quinolinic acid-induced excitotoxic response.

As illustrated in Figure 5, injection of quinolinic acid (30 nmol) in the striatum of animals pretreated with probenecid (200 mg kg^{-1}, i.p.) or with L-kynurenine (450 mg kg^{-1}, i.p.) also produced approximately 50% depletion of striatal GABA, indicating failure of these agents to prevent quinolinic acid toxicity. A combination of L-

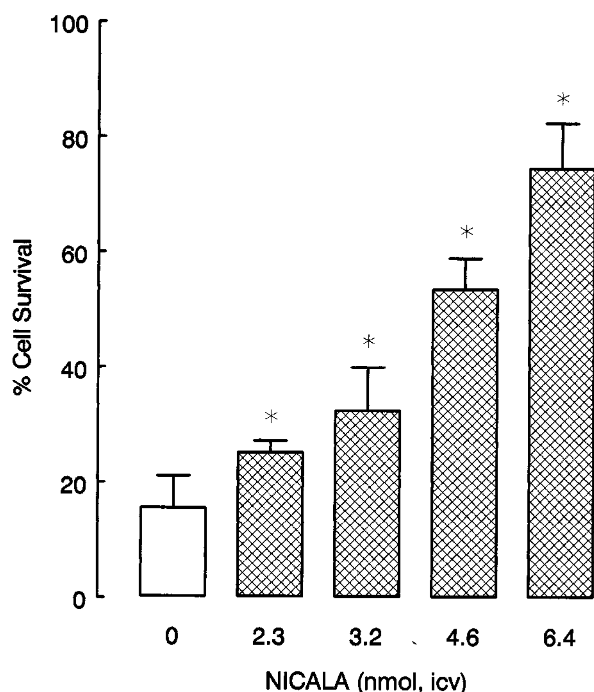

Figure 2 Dose-response relationship for the action of nicotinylalanine (NICALA) on the survival of NADPH-d neurones of the rat striatum following injection of quinolinic acid (QA). NICALA or saline was injected, together with L-kynurenine (450 mg kg^{-1}, i.p.) and probenecid (200 mg kg^{-1}, i.p.), 3 h before a unilateral injection of QA (15 μmol, 0.5 μl) into the right striatum. Values (mean$\pm$s.e.mean) shown represent counts of NADPH-d neurones in the QA-injected side expressed as a percentage of similar counts in a corresponding area of the uninjected striatum; $n=4$ animals. *$P<0.05$, values significantly different from those in saline-injected animals.

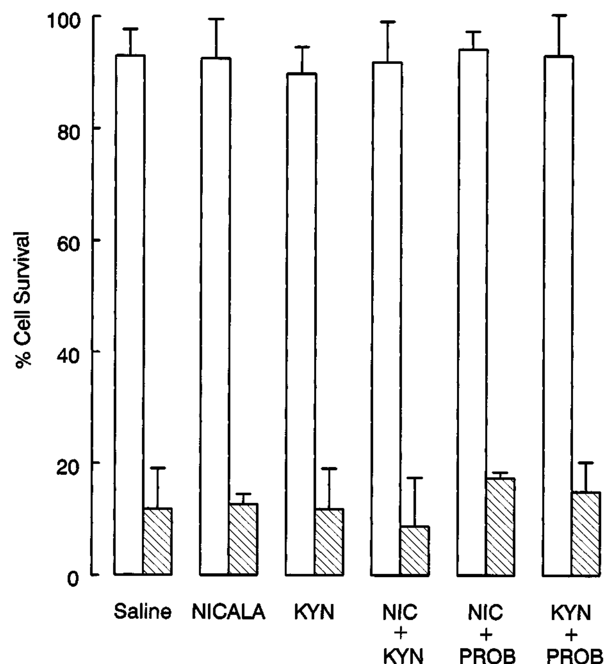

Figure 3 Effect of nicotinylalanine (NICALA; 4.6 nmol, i.c.v.), L-kynurenine (KYN; 450 mg kg^{-1}, i.p.) and their combination with each other or with probenecid (PROB; 200 μg kg^{-1}, i.p.), on the survival of NADPH-d diaphorase neurones in the rat striatum after an intrastriatal injection of quinolinic acid (QA) or saline. All treatments shown were delivered 3 h before a unilateral injection of QA (15 nmol, 0.5 μl) or saline (5 μl) into the striatum. Values (mean$\pm$s.e.mean) represent counts of NADPH-d diaphorase neurones in the QA-injected side expressed as a percentage of similar counts in a corresponding area of the uninjected striatum; $n=4-5$ animals.

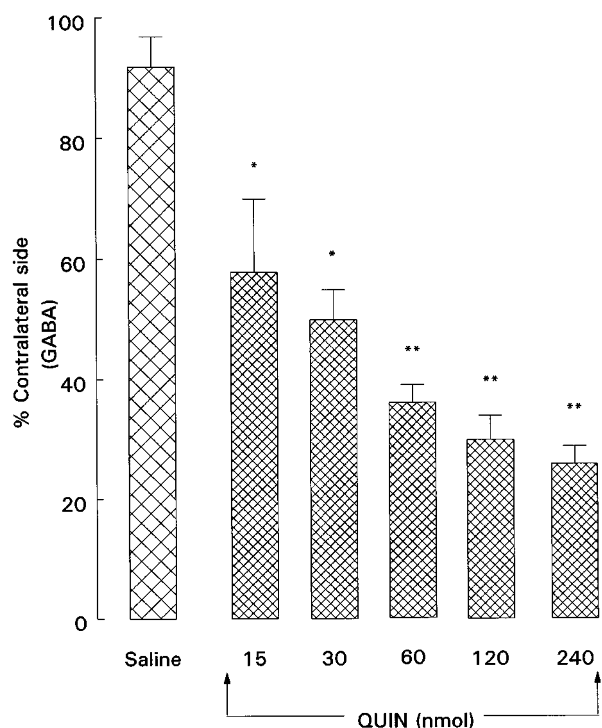

Figure 4 Effect of quinolinic acid (QUIN) on striatal GABA content. Rats received intrastriatal infusions of saline or increasing doses of quinolinic acid. Striatal GABA content on the injection side is expressed as a percentage of GABA content on the uninjected side. Differences between groups were determined by use of ANOVA followed by Newman-Keuls *post-hoc* testing ($P<0.05$). *Represents a significant difference from saline treated animals. Each column represents the mean ± s.e.mean ($n=5-8$). **($P<0.01$).

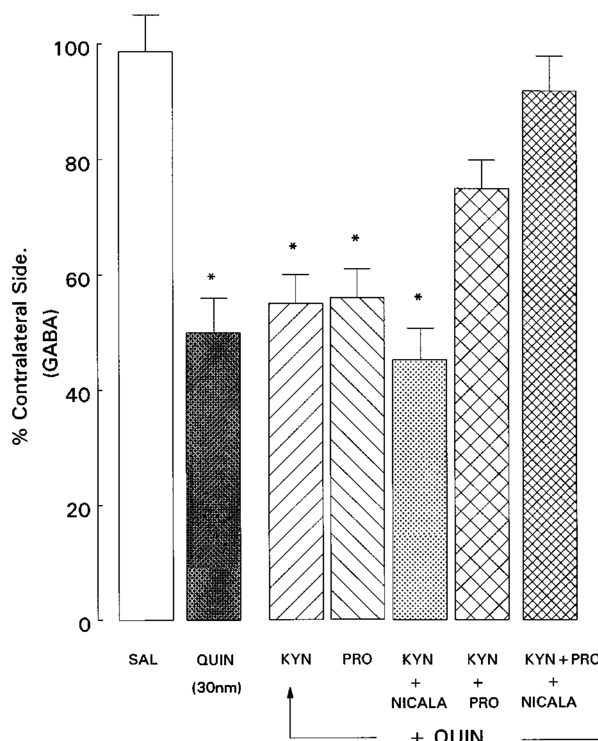

Figure 5 Effect of nicotinylalanine (NICALA) on quinolinic acid (QUIN)-induced reductions of striatal GABA. Striatal GABA content in the injection side is expressed as a percentage of GABA content in the uninjected side. Rats received intrastriatal injection of saline or quinolinic acid (30 nmol). Rats were pretreated with probenecid (PRO; 200 mg kg^{-1}, i.p.), L-kynurenine (KYN; 450 mg kg^{-1}, i.p.), nicotinylalanine (5.6 nmol 5 μl^{-1}) and L-kynurenine, saline with L-kynurenine and probenecid, or nicotinylalanine, L-kynurenine and probenecid 3 h before the infusion of quinolinic acid. Each column represents the mean ± s.e.mean ($n=5-8$). Differences between groups were determined by ANOVA followed by Dunnett's *post-hoc* test to determine differences from saline treated rats ($P<0.05$). *Represents a statistically significant difference from saline-treated animals.

kynurenine with nicotinylalanine (5.6 nmol 5 μl^{-1}, i.c.v.) also did not significantly alter the magnitude of quinolinic acid-induced striatal GABA depletion. However, when animals were pretreated with the L-kynurenine/probenecid combination in the absence of nicotinylalanine, intrastriatal injection of quinolinic acid produced a 19% decrease in GABA; addition of nicotinylalanine to this combination resulted in 8% decrease in GABA content. A statistical comparison revealed a significant effect of these two sets of treatment on the quinolinic acid-induced depletion of striatal GABA observed in untreated animals (Figure 5. Thus, combined administration of L-kynurenine and probenecid with or without nicotinylalanine significantly inhibited the quinolinic acid-induced excitotoxic response.

The effects of nicotinylalanine (4.6 nmol 5 μl^{-1}) and co-treatment with L-kynurenine and probenecid on the content of brain kynurenic acid is represented in Figure 6. In untreated animals, the levels of kynurenic acid was 42.1 ± 20 pmol g^{-1}. Administration of L-kynurenine plus probenecid raised the level to 3778 ± 519 pmol g^{-1}. However, when this combination was delivered with nicotinylalanine (4.6 nmol, i.c.v.) the level of kynurenic acid rose to 5638 ± 1203 pmol g^{-1}. This value was significantly greater than that obtained after all other treatments used in this part of the study. The effects of nicotinylalanine (5.6 nmol) on striatal levels of kynurenic acid are presented in Figure 7. The level of kynurenic acid in the striatum of untreated animals was 964 ± 240 pmol g^{-1} tissue ($n=5$). Following L-kynurenine and probenecid treatment produced a 2 fold increase, while addition of nicotinylalanine produced an almost 4 fold increase in kynurenic acid levels. This increase in the nicotinylalanine group was significantly different from that in the untreated group and L-kynurenine/probenecid-treated group at the three hour post-injection period ($P<0.05$) but not at subsequent time periods.

Discussion

The results of this study demonstrate that a unilateral injection of quinolinic acid into the rat striatum leads to a severe depletion of NADPH-d neurones and a reduction in striatal GABA content. Nicotinylalanine, an inhibitor of kynurenine metabolism, elevated the brain level of endogenous kynurenic acid, a broad spectrum EAA receptor antagonist, and significantly attenuated the neurotoxic action of quinolinic acid on NADPH-d neurones and GABA containing neurones.

In this study, conditions for the expression of the action of nicotinylalanine were optimized by selection of a highly sensitive model of toxicity, and by administration of co-treatments which would favour accumulation of endogenous kynurenic acid in the brain. The NADPH-d neurones of the rat striatum have been found in previous work to be highly sensitive to the action of quinolinic acid (Boegman & Parent, 1988; Kalisch *et al.*, 1994). Indeed, an almost complete loss of striatal NADPH-d neurones in the injected area could be produced with a relatively low dose of quinolinic acid. GABA containing neurones in the striatum have also been found to be

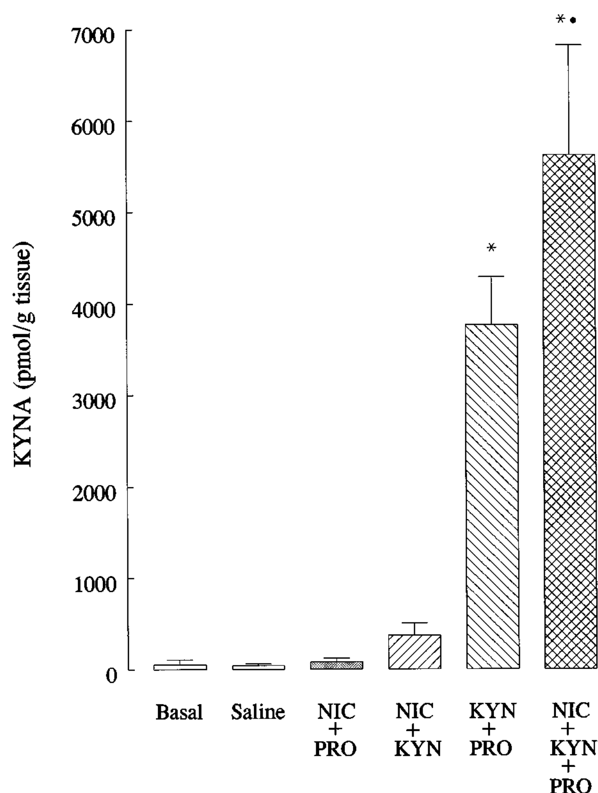

Figure 6 Effect of nicotinylalanine (NICALA) injection, administered alone (4.6 nmol i.c.v.) or in combination with L-kynurenine (KYN, 450 mg kg^{-1}, i.p.) and/or probenecid (PRO; 200 mg kg^{-1}, i.p.), on the content of kynurenic acid (KYNA) in the whole rat brain. The injections were delivered 3 h before the estimation of kynurenic acid. The basal values were obtained from uninjected animals and the saline value represents that obtained from animals injected with 0.9% saline (5 μl, i.c.v.). Values shown represent mean ± s.e.mean. *$P<0.01$, value significantly different from saline-injected (shown) animals; •$P<0.05$, value significantly different from all other values.

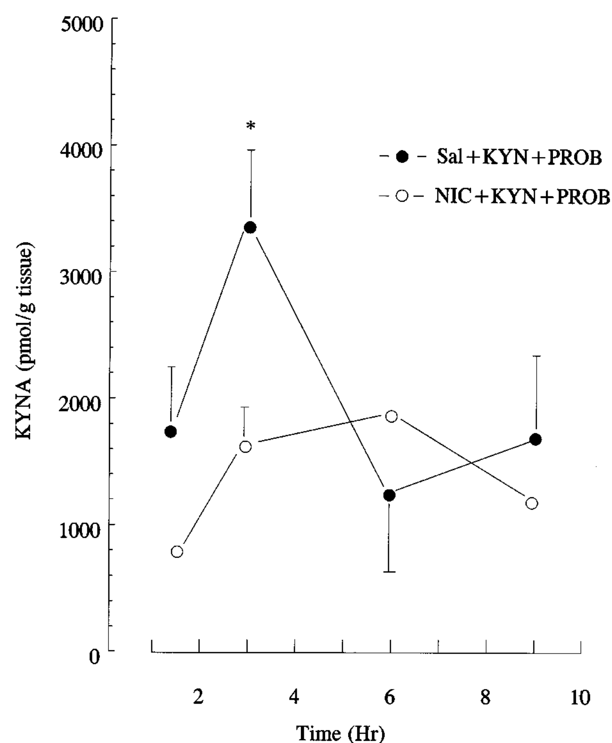

Figure 7 Effect of nicotinylalanine (NIC) on striatal kynurenic acid (KYNA). Rats received nicotinylalanine or saline in combination with L-kynurenine (KYN; 450 mg kg^{-1}, i.p.) and probenecid (PROB; 200 mg kg^{-1}, i.p.). Differences between groups were determined by use of ANOVA followed by Dunnett's *post-hoc* test to determine significant differences from saline treated animals. Each point represents the mean of 3–9 determinations; vertical lines show s.e.mean. *Represents a significant difference from control ($P<0.05$).

sensitive to quinolinic acid-induced toxicity (Santamaria *et al.*, 1996). Thus, the fact that striatal cholinergic and non-striatal cholinergic and dopaminergic neurones, although vulnerable to quinolinic acid, are less sensitive to its toxic action (Schwarcz *et al.*, 1983; El Defrawy *et al.*, 1985; Connop *et al.*, 1995) and the sensitivity of the NADPH-d neurones and GABA containing neurones to quinolinic acid, coupled with the reproducibility of the effects of quinolinic acid action, influenced the choice of this model. The use of this model was based on the previous findings showing that kynurenine which acts as the source of kynurenic acid, and probenecid, which blocks organic acid transport and thus reduces egress from the brain, can elevate levels of endogenous kynurenic acid in the brain (Moroni *et al.*, 1991; Miller *et al.*, 1992).

The anti-neurotoxic action of nicotinylalanine observed most likely results from elevation of endogenous brain kynurenic acid (KA) and subsequent blockade of quinolinic acid (QA)-sensitive NMDA receptors. This explanation is favoured by previous observations that exogenous KA readily blocks the biochemical and behavioural manifestations of QA neurotoxicity (Foster *et al.*, 1984; Boegman *et al.*, 1985; Wirsching *et al.*, 1989; Winn *et al.*, 1991; Cockhill *et al.*, 1992), and that the administration of nicotinylalanine elevates endogenous KA in the brain (Moroni *et al.*, 1991). In the present study administration of a dose of nicotinylalanine (delivered in combination with L-kynurenine and probenecid) which blocked QA-induced toxicity in two neuronal populations also produced a very large increase in whole brain KA and an almost 2 fold increase in striatal KA. The protection against QA-induced toxicity afforded by the administration of nicotinylalanine in combination with L-kynurenine and probenecid, in spite of only a modest increase in striatal KA when compared to that in whole brain is surprising. However, KAT, the enzyme catalyzing KA synthesis, is found in astrocytes surrounding excitatory synapses in the striatum (Roberts *et al.*, 1995) and thus, KA levels may be greatly increased in the synapse when compared to the surrounding tissue. Previous microdialysis experiments have shown that treatments which elevate endogenous brain KA can also increase its efflux (Swartz *et al.*, 1990; Wu *et al.*, 1992; Carpenedo *et al.*, 1994). However, the relationship between the tissue content of endogenous KA and its availability at the synapse is not well understood. A study of this relationship including the measurement of synaptic concentrations of KA may reveal the extent to which tissue KA must be augmented to produce a neuroprotective effect. Rats administered nicotinylalanine, L-kynurenine and probenecid or the combination of kynurenine and probenecid alone appeared to be sedated. These effects were more pronounced in the nicotinylalanine, kynurenine and probenecid treatment group (experimental observations) and may reflect the higher synaptic concentrations of KA achieved in this treatment group.

Interestingly, combined administration of L-kynurenine and probenecid also provided a significant increase in whole brain KA. Although unable to prevent QA-induced depletions in NADPH-d neurones, this combination attenuated QA-induced reductions in striatal GABA content. The inability of

L-kynurenine and probenecid in the absence of nicotinylalanine to protect NADPH-d neurones may indicate that these neurones are less sensitive to the actions of KA than are GABA containing neurones. Alternatively, the inability of the combination of L-kynurenine and probenecid to protect against QA-induced destruction of NADPH-diaphorase neurones may reflect relatively poor synthesis of KA in synapses surrounding these neurones. Indeed, as KAT is found in astrocytes surrounding excitatory synapses in the striatum (Roberts *et al*., 1995) and GABA-containing neurones are the target for the majority of glutamatergic afferents from the cortex, it is possible that KA levels at synapses surrounding GABA neurones exceed that at NADPH-d neurones. It is also likely that the differential ability of endogenous KA against QA-induced excitotoxicity in the striatum reflects differences in the expression of NMDA receptor subtypes on GABA-containing neurones relative to NADHP-diaphorase neurones. However, at present no evidence exists for a differential effect of KA on QA-induced toxicity in the striatum.

In contrast to the findings of Santamaria *et al*. (1996), administration of L-kynurenine or probenecid alone in this study did not protect against QA-induced reductions in striatal GABA content. These results are surprising, given that the concentration of QA employed in this study (30 nmol) is quite low compared to that used by Santamaria *et al*. (1996) (240 nmol). The reason for the discrepancy between the results of this study and that of Santamaria *et al*. (1996) are unknown. However, in support of the present data a recent study found that probenecid alone was unable to suppress NMDA-evoked responses in striatal neurones *in vivo* (Urenjak *et al*., 1997). The authors of this study suggest that inhibition of organic anion transport alone is not sufficient to protect against toxicity associated with excessive NMDA-receptor activation (Urenjak *et al*., 1997). The absence of protection following the administration of L-kynurenine can be explained by the fact that L-kynurenine (450 mg kg^{-1}, i.p.) while significantly increasing the concentration of KA in the striatum (Vescei *et al*., 1992), also increases the concentration of QA in the brain (Luthman *et al*., 1996). It has been shown that brain tissue has the capacity to retain QA while KA can be removed from the brain through an organic acid transporter (Vescei *et al*., 1992; Speciale *et al*., 1993). Thus, administration of L-kynurenine in the absence of nicotinylalanine or probenecid most likely increases the concentration of QA in the brain and thus counteracts the neuroprotective effects of elevated endogenous KA. The administration of nicotinylalanine and L-kynurenine was unable to prevent QA-induced depletions of NADPH-d and GABA containing neurones. It has been suggested that enzymatic degradation of KA does not play a significant role in the removal of KA from the brain and the majority of KA in the brain is eliminated via the probenecid sensitive organic acid transporter (Stone, 1993). The absence of protection in this treatment group appears to confirm this hypothesis, as in the absence of probenecid it is likely that KA leaves the brain and the concentration of KA in the synapse is insufficient to antagonize QA-induced toxicity.

It is possible that the effects on GABA content in the various treatment groups are due to non-specific effects on GABA synthesis and degradation. Thus, the neuroprotection in the present study may have been the result of an increase in GABA synthesis or a decrease in GABA degradation. However, histological studies have confirmed that QA is toxic to GABA-containing neurones in the striatum (Figueredo-Cardenas *et al*., 1994), suggesting that QA-induced depletions in striatal GABA content are a result of the destruction of GABA-containing neurones and not a result of effects on GABA synthesis or degradation. In addition, probenecid and L-kynurenine alone or L-kynurenine and nicotinylalanine did not have any effect on QA-induced reductions in striatal GABA content. Thus, it is unlikely that the preservation of GABA levels in the groups pretreated with kynuerenine and probenecid with or without nicotinylalanine before the infusion of QA are due to non-specific effects.

The possibility of a direct action of nicotinylalanine on NMDA receptors was excluded by its failure to influence QA-induced depletion of NADPH-d neurones when administered in the absence of co-treatment. Nicotinylalanine itself also failed to elevate KA level. This lack of effect of nicotinylalanine on its own may arise from a lack of precursor availability for KA synthesis and/or a rapid removal of KA from the brain.

Although administration of nicotinylalanine in combination with L-kynurenine and probenecid produced a large increase in brain KA, blockade of QA-induced destruction of NADPH-d neurones was incomplete. Exogenous KA, when co-injected locally with QA, has been found to produce a complete blockade of QA toxicity (Boegman *et al*., 1985; Jhamandas *et al*., 1990). A local injection has the potential to produce high levels of extracellular KA at the target sites. However, such levels may not be achieved with agents that produce a global *in vivo* increase in endogenous KA. Indeed as stated previously, the production of kynurenine metabolites in the brain occurs mainly in synapses surrounding excitatory synapses (Roberts *et al*., 1995). Thus, to be pharmacologically effective the endogenous KA produced in astrocytes must diffuse to neuronal cell bodies and block the EAA receptors located on their surface. It is likely that despite the use of probenecid a significant portion of synthesized KA is dissipated by diffusion or non-specific binding and is less available to such receptors. Thus, endogenously produced KA may be less effective than exogenous KA injected in the vicinity of receptors.

The metabolism of kynurenine is also known to yield several other products (quinaldic, hydroxy quinaldic and xanthurenic acid) which are structurally rated to KA and some of which act as EAA antagonists. However, in comparative studies on the pharmacological effects of these products, KA has been shown to be the most potent (Perkins & Stone, 1982; Robinson *et al*., 1985; Jhamandas *et al*., 1990). Also, the extent to which these agents are produced in the brain is presently unclear. Thus, endogenous KA is likely to be a major contributor to the observed anti-QA action of nicotinylalanine, although potential contributions from other less active antagonist metabolites to this action cannot be overlooked. The relationship between endogenous KA and cell survival in the CNS remains to be explored further.

The present findings, indicating a potential role of endogenous KA as an anti-neurotoxic agent, suggest a novel strategy of neuronal protection from an excitotoxic insult. Excitotoxicity has been implicated in diverse conditions such as brain hypoxia/ischaemia, hypoglycaemia, status epilepticus and chronic neurodegenerative disease (Olney, 1990). Thus, agents that produce optimal elevation of endogenous EAA receptor antagonists in the CNS may prove useful as novel neuroprotectants.

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