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PROTECTION AGAINST QUINOLINIC ACID-MEDIATED EXCITOTOXICITY IN NIGROSTRIATAL DOPAMINERGIC NEURONS BY ENDOGENOUS KYNURENIC ACID

A. F. MIRANDA,* R. J. BOEGMAN,* R. J. BENINGER† and K. JHAMANDAS*‡

*Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario, K7L 3N6, Canada

†Department of Psychology and Psychiatry, Queen's University, Kingston, Ontario, K7L 3N6, Canada

Abstract—Endogenous excitotoxins have been implicated in the degeneration of dopaminergic neurons in the substantia nigra compacta of patients with Parkinson's disease. One such agent quinolinic acid is an endogenous excitatory amino acid receptor agonist. This study examined whether an increased level of endogenous kynurenic acid, an excitatory amino acid receptor antagonist, can protect nigrostriatal dopamine neurons against quinolinic acid-induced excitotoxic damage.

Nigral infusion of quinolinic acid (60 nmoles) or N-methyl-D- aspartate (15 nmoles) produced a significant depletion in striatal tyrosine hydroxylase activity, a biochemical marker for dopaminergic neurons. Three hours following the intraventricular infusion of nicotinylalanine (5.6 nmoles), an agent that inhibits kynureninase and kynurenine hydroxylase activity, when combined with kynurenine (450 mg/kg i.p.), the precursor of kynurenic acid, and probenecid (200 mg/kg i.p.), an inhibitor of organic acid transport, the kynurenic acid in the whole brain and substantia nigra was increased 3.3-fold and 1.5-fold respectively when compared to rats that received saline, probenecid and kynurenine. This elevation in endogenous kynurenic acid prevented the quinolinic acid-induced reduction in striatal tyrosine hydroxylase. However, 9 h following the administration of nicotinylalanine with kynurenine and probenecid, a time when whole brain kynurenic acid levels had decreased 12-fold, quinolinic acid injections produced a significant depletion in striatal tyrosine hydroxylase. Intranigral infusion of quinolinic acid in rats that received saline with kynurenine and probenecid resulted in a significant depletion of ipsilateral striatal tyrosine hydroxylase. Administration of nicotinylalanine in combination with kynurenine and probenecid also blocked N-methyl-D-aspartate-induced depletion of tyrosine hydroxylase. Tyrosine hydroxylase immunohistochemical assessment of the substantia nigra confirmed quinolinic acid-induced neuronal cell loss and the ability of nicotinylalanine in combination with kynurenine and probenecid to protect neurons from quinolinic acid-induced toxicity.

The present study demonstrates that increases in endogenous kynurenic acid can prevent the loss of nigrostriatal dopaminergic neurons resulting from a focal infusion of quinolinic acid or N-methyl-D-aspartate. The strategy of neuronal protection by increasing the brain kynurenic acid may be useful in retarding cell loss in Parkinson's disease and other neurodegenerative diseases where excitotoxic mechanisms have been implicated. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: nicotinylalanine, Parkinson's Disease, NMDA, tyrosine hydroxylase, dopamine, neuroprotection.

Parkinson's disease, involves a relatively selective degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNc). Excitatory amino acids (EAAs) have been implicated in the degeneration of the nigrostriatal dopaminergic pathway.[24,41] EAAs are known to play a critical role in normal functions of the SNc including the control of dopamine release[1,17,28] and the excitation of dopaminergic neurons projecting to the striatum.[26] These phenomena are mediated, at least in part by EAA receptor subtypes: N-methyl-D-aspartate

(NMDA), kainate and 2-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA) receptors.[11,12] It has been suggested that excessive activation of EAA receptors localized on cell bodies of nigral dopaminergic neurons[12] may play a role in the loss of these neurons.[24,26] Thus, activation of NMDA receptors located on dopaminergic neurons has been found to produce toxicity both *in vitro*[23] and *in vivo*.[9]

Certain endogenous excitotoxins have been implicated in the cell loss seen in Parkinson's Disease.[29] In the CNS tryptophan metabolism via the kynurenine pathway yields several metabolites which have the potential to activate or to block EAA receptors. One such metabolite, quinolinic acid, excites neurons by activation of NMDA receptors.[30] Microinjection of quinolinic acid into specific brain regions produces

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‡To whom correspondence should be addressed.
Abbreviations: AMPA, 2-amino-3-hydroxy-5-methylisoxazole propionic acid; EAA, excitatory amino acid; NICALA, nicotinylalanine; NMDA, N-methyl-D-aspartate; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

axon sparing lesions, an effect which can be attenuated by NMDA receptor antagonists.[14,15,16,34] Expression of the toxic action of quinolinic acid, unlike that of NMDA,[25] requires the presence of an intact presynaptic glutamatergic input to target neurons.[13,14,35,37] Another tryptophan metabolite, kynurenic acid acts as an antagonist at ionotropic EAA receptor subtypes but has greater affinity for NMDA receptors.[22] Kynurenic acid acts by blocking the glycine site associated with the NMDA receptor complex.[22] Although kynurenic acid can attenuate the neurotoxic effects of both quinolinic acid and NMDA in the striatum it was reported to be more effective in blocking the neurotoxic actions of quinolinic acid.[14]

Both kynurenic acid and quinolinic acid are produced in the vicinity of the SNc and the local concentration of these compounds may be sufficiently high to influence EAA receptor function in vivo.[31,36] Kynurenine aminotransferase and 3-hydroxyanthranlic acid oxygenase, the enzymes responsible for the production of kynurenic acid and quinolinic acid, respectively, have been found in astrocytes surrounding glutamatergic afferents and dopaminergic neurons in the SNc.[31] Thus, it is likely that a balance exists between the production of quinolinic acid and kynurenic acid in the SNc. Specifically, an increase in the production of quinolinic acid or a decrease in the production of kynurenic acid may result in excessive activation of EAA receptors on nigrostriatal neurons. Recent work in our laboratory has shown that intra-nigral infusions of quinolinic acid can destroy the nigrostriatal dopaminergic neurons, as reflected in the depletion of striatal tyrosine hydroxylase (TH) and loss of TH immunoreactivity.[4,9] The role of kynurenic acid in protecting against quinolinic acid-mediated toxicity in the SNc has not been determined.

The level of endogenous kynurenic acid in the brain can be influenced by nicotinylalanine (NICALA), an inhibitor of kynureninase and kynurenine hydroxylase[10,27] enzymes that are critical to the biosynthesis of quinolinic acid. Inhibition of this pathway elevates the concentration of kynurenine, a substrate for the enzyme kynurenine aminotransferase which catalyses the production of kynurenic acid. The increase in brain kynurenic acid produced by NICALA has been linked to sedative and anticonvulsant effects in mice.[33] We have recently demonstrated that systemic or central administration of NICALA at a dose which significantly elevates brain kynurenic acid, when coupled with systemic injections of kynurenine and probenecid, prevents quinolinic acid-induced damage to the NADPH-diaphorase neurons in the rat striatum.[19]

Since quinolinic acid infusion into the SNc destroys dopaminergic neurons, pharmacological strategies that elevate the level of endogenous EAA receptor antagonists such as kynurenic acid, might be useful in preventing cell death. In the present study

we examined this possibility by determining whether elevations in endogenous kynurenic acid produced by intraventricular injections of NICALA (coupled with administration of kynurenine and probenecid) can inhibit quinolinic acid-induced damage to nigrostriatal neurons.

EXPERIMENTAL PROCEDURES

Experimental animals

Male Sprague–Dawley rats (Charles River, St Constant, Que.) weighing 100–125 g were given free access to food (Purina rat chow) and water and were maintained in an alternating light–dark environment (12 h light: 12h dark) at a constant temperature of 20°C. Treatment of all rats used in the present study was in accordance with the Animals for Research Act, the Guidelines of the Canadian Council on Animal Care and relevant University policy, and was approved by the Queen's University Animal Care Committee.

Stereotaxic injections and drug treatments

Rats were anaesthetized using a 2% halothane, 98% O_2 mixture and positioned in a Narashige small animal stereotaxic apparatus for intranigral injection. Due to limited quantities, NICALA was administered intraventricularly to juvenile rats. The treatment regimen employed in this study has previously been shown to produce the greatest increase in whole brain kynurenic acid concentration.[19] NICALA (5.6 nmoles) (Colour Your Enzyme, Kingston Ont.) was dissolved in 0.9% saline (pH 7.0)and administered in a volume of 5 μl into the lateral ventricle over a period of 2 min, 47 s using the following coordinates: 0.8 mm posterior to bregma, 1.45 mm lateral to the midline and 3.6 mm ventral to the surface of the skull with the incisor bar set at −3.3 mm. The injection apparatus consisted of a stainless steel cannula (0.18 mm, i.d.; 0.36 mm, o.d.; approximately 2 cm in length), connected to a Hamilton syringe via PE-20 polyethylene tubing (0.38 mm, i.d.). The syringe was mounted in a Sage Instruments electric pump to ensure a constant pressure and flow of NICALA into the brain. Following injection, the cannula was left in place for an additional 2 min period, to allow for the diffusion of NICALA from the infusion site, and subsequently removed. Probenecid (200 mg/kg) and kynurenine (450 mg/kg) (Sigma, St Louis, MO) were administered by intraperitoneal injection during NICALA infusion. Control animals received saline (5 μl i.c.v.) with kynurenine and probenecid. Kynurenine and probenecid were dissolved in 1 N NaOH and titrated to pH 10.0 with 1 N HCl.

Quinolinic acid or NMDA (Sigma, St Louis, MO) were infused into the SNc using the following stereotaxic coordinates: 5.3 mm posterior to bregma, 2.2 mm lateral to the midline and 7.7 mm ventral to the surface of the skull.[9] Both quinolinic acid and NMDA were dissolved in 0.9% saline and titrated to pH 7.4 with 1 N NaOH. Each drug was infused for 75 s in a volume of 0.5 μl through the cannula using the procedure described above.

Kynurenic acid measurement

Brain kynurenic acid was measured according to the method of Russi et al.[32] with some modifications. Briefly, the animal was killed by decapitation and the brain rapidly removed. The whole brain, or substantia nigra pooled from two animals, was homogenized in 4 ml of a mixture (3:1) of ethanol and 1 N NaOH. After centrifugation (10 min, 5000 g) the pellet was resuspended in 5 ml of 90% ethanol and centrifuged again (10 min, 5000 g). The supernatants were pooled and placed at −80°C overnight to precipitate fatty materials which were discarded. Dowex AG1 Wx8 (acetate form 100–200 mesh, 250–300 mg), was added to the

supernatant. The suspension was mixed for 5 min and centrifuged (10 min, 5000 g). The supernatant was discarded, and the resin resuspended in 2 ml of distilled water and placed in a pasteur pipette in which a pellet of glass wool had been inserted previously. The column was washed with 5 ml of distilled water followed by 10 ml of 1 N formic acid. Kynurenic acid was eluted with 5 ml of 10 N formic acid and this was directly passed through a similarly prepared pasteur pipette containing 250–300 mg of Dowex resin (AG50 W × 8 H⁺ form). The column was washed with 2 ml of water and kynurenic acid eluted with 5 ml of 3 N ammonium hydroxide. In order to determine the % recovery, a control sample containing a known amount of kynurenic acid was taken through the same purification procedure. Recovery using this procedure was 50%. The eluate containing kynurenic acid was lyophilized, resuspended in 200 µl of 50 mM Na acetate (pH 6.2), and 50 µl applied to the high performance liquid chromotography column. A mobile phase consisting of 4.5% acetonitrile and 50 mM sodium acetate (pH 6.2) was pumped through a CSC-Spherisorb-ODS2, 3 mm reverse-phase column at a flow rate of 1.0 ml/min. A solution of 0.5 M zinc acetate was delivered postcolumn at a flow rate of 1.0 ml/min. This procedure was carried out to stabilize the unionized form of the kynurenic acid in the sample and to enhance the fluorescence signal. Kynurenic acid was detected by a Shimadzu model RF-551 fluorescence detector operating at excitation and emission wavelengths of 344 nm and 398 nm, respectively. The peak area under the curve was integrated and used for data analysis.

Tyrosine hydroxylase assay

Four days following the infusion of quinolinic acid or NMDA into the SNc, the rats were decapitated and the brain rapidly removed and placed in ice-cold saline. The striatum from each hemisphere was dissected and homogenized separately with a glass hand-homogenizer in 175 µl of buffer consisting of 0.3 M sucrose in 0.01 M Tris–HCL at pH 7.3. The samples were centrifuged at 12000 g for 6 min at 4°C and the supernatant retained for the assay of TH. The TH activity in the striatum from each hemisphere was assayed using a modification of a method previously described.[40] The modification included a 1.5 h incubation period at 37°C as well as centrifugation at 2000 g for 10 min following each reaction with alumina. The amount of L-DOPA formed from L-tyrosine was determined by the trihydroxyindole fluorometric method.[40] Protein was determined by the method of Bradford[5] and TH activity expressed as pmol L-DOPA formed/mg protein/h. For each animal, the results were expressed as a percentage of the enzyme activity measured in the contralateral (uninjected) side.

Tyrosine hydroxylase immunohistochemistry

Four days following stereotaxic infusions, animals were anaesthetized and transcardially perfused with 100 ml of 0.9% saline followed by 200 ml of 4% paraformaldehyde. The brain was removed, post-fixed in paraformaldehyde for 12 h, and then immersed in 30% sucrose for 48 h. Transverse tissue sections (50 µm) incorporating the nigral region were cut on a cryostat and stored in phosphate-buffered saline at 4°C. The sections were mounted and stained according to the Vectastain avidin–biotin-complex technique[21] for TH immunoreactivity using a monoclonal TH antibody (Eugenetech, New Jersey, U.S.A.).

Statistical analysis

Data were compared using analyses of variance (ANOVA) followed by Newman–Keuls *post hoc* tests to determine differences between the various treatment groups. The level of significance chosen was $P<0.05$. Values were expressed as mean ± S.D.

RESULTS

Kynurenic acid level

The concentration of kynurenic acid in the brain of naive animals was 49.1 pmol/g tissue. Brain kynurenic acid following the administration of NI-CALA (5.6 nmoles i.c.v.), with kynurenine (450 mg/kg i.p.) and probenecid (200 mg/kg i.p.), is shown in Fig. 1. Kynurenic acid was significantly increased over that in naive animals 1.5 h following the administration of NICALA. These levels reached a peak value 3 h post-NICALA and declined at 6 and 9 h. Control rats receiving saline (5 µl i.c.v.) kynurenine (450 mg/kg i.p.) and probenecid (200 mg/kg i.p.) also showed a significant increase in brain kynurenic acid levels relative to naive animals.

Nigral kynurenic acid 3 h following the administration of saline with kynurenine and probenecid was 786 pmol/g tissue. Three hours following the administration of NICALA with probenecid and kynurenine nigral kynurenic acid levels were 1.5-fold higher than those in control rats (Table 1).

Nigral dopaminergic neurotoxicity

Tyrosine hydroxylase in the striatum on the injection side, expressed as a percentage of that from the intact side, was used as a biochemical marker of dopaminergic neurotoxicity in the SNc (Fig. 2). TH activity in the contralateral striatum of untreated animals was 570 ± 40 pmol/mg protein/h which is in agreement with a previous report.[39] Focal infusion of saline into the SNc was not toxic, as TH activity in the ipsilateral striatum showed little change. In contrast, TH activity following a quinolinic acid infusion was reduced significantly ($P<0.01$), indicating the neurotoxic action of this NMDA receptor agonist. Infusion of exogenous kynurenic acid (60 nmoles) alone into the SNc was not toxic to nigrostriatal dopaminergic neurons (results not shown). To determine the effect of exogenous kynurenic acid on quinolinic acid-induced toxicity, quinolinic acid (60 nmoles) and kynurenic acid (60 or 30 nmoles) were co-infused into the SNc. Following the co-treatment, striatal TH in the injection side was not significantly different from that seen in the striatum of saline-treated animals indicating that exogenous kynurenic acid prevented quinolinic acid-induced depletion of nigrostriatal dopaminergic neurons.

To determine the effect of an increase in endogenous kynurenic acid on quinolinic-induced toxicity, a group of rats was pretreated with NICALA (5.6 nmoles, i.c.v.), kynurenine (450 mg/kg i.p.) and probenecid (200 mg/kg i.p.) prior to an infusion of quinolinic acid (60 nmoles) into the SNc. Quinolinic
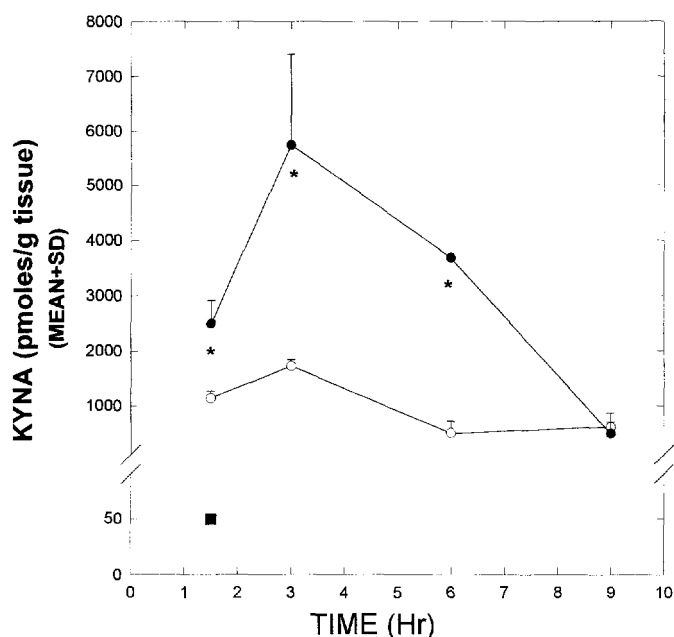
Fig. 1. Concentration of whole brain kynurenic acid following the administration of nicotinylalanine (NICALA) (5.6 nmoles i.c.v.) with kynurenine (450 mg/kg i.p.) and probenecid (200 mg/kg i.p.) (solid circles). Control animals (open circles) received only kynurenine (i.p.) and probenecid (i.p.) and saline (i.c.v.). Kynurenic acid in naive rats is indicated by the solid square. Each point represents the mean ± S.D. ($n=3$). Data was compared using ANOVA followed by Neuman–Keuls *post hoc* test to determine differences between groups. *Represents a significant difference from control values for a given time-point ($P<0.05$).

Table 1. Concentration of kynurenic acid in the substantia nigra compacta 3 h following the administration of nicotinylalanine (5.6 nmoles i.c.v.) with kynurenine (450 mg/kg i.p.) and probenecid (200 mg/kg i.p.); or saline (5 μl i.c.v.) with kynurenine (450 mg/kg i.p.) and probenecid (200 mg/kg)

	Treatment groups	
Brain region	saline+kynurenine+probenecid	NICALA+kynurenine+probenecid
Substantia nigra pars compacta	786 ± 104 ($n=3$)	1154 ± 138* ($n=3$)

Each value represents the mean ± S.D. Groups were compared using the Student's *t*-test.
*Represents a significant difference from rats that received saline instead of NICALA ($P<0.05$).

acid was infused 3 h following NICALA administration, a time-period at which brain kynurenic acid levels were maximally elevated (see Fig. 1). In this group the value of ipsilateral striatal TH was significantly greater than that measured in rats that received quinolinic acid alone (Fig. 2). Thus, treatment with NICALA in combination with probenecid and kynurenine significantly attenuated the depletion of TH induced by an intranigral quinolinic acid injection, reflecting the protective action of this treatment regimen on nigral dopaminergic neurons.

To determine if the protection afforded by NICALA was indeed dependent on elevated endogenous kynurenic acid levels, striatal TH was measured in rats that had received an intranigral injection of quinolinic acid 9 h after NICALA, a time-period at which the level of brain kynurenic acid had declined (Fig. 1). Quinolinic acid infusion reduced ipsilateral striatal TH to a value that was not significantly different ($P<0.05$) from that measured in rats that

had received intranigral quinolinic acid alone (Fig. 2). Thus, infusion of quinolinic acid at a time when kynurenic acid had declined, produced a significant depletion of dopaminergic neurons. In control rats that had received kynurenine and probenecid and saline rather than NICALA prior to the quinolinic acid injection, TH in the ipsilateral striatum was not significantly different ($P<0.05$) from that measured in rats in which quinolinic acid alone was infused. Thus, in the absence of NICALA, these two agents did not afford protection against quinolinic acid-induced toxicity.

Intranigral injection of NMDA (15 nmoles) like quinolinic acid, was toxic to dopaminergic neurons as evidenced by the reduction of ipsilateral striatal TH (Table 2). When NMDA was co-injected with exogenous kynurenic acid (60 nmoles), TH was not significantly different from that measured in saline-treated rats, indicating that exogenous kynurenic acid prevented NMDA-induced depletion
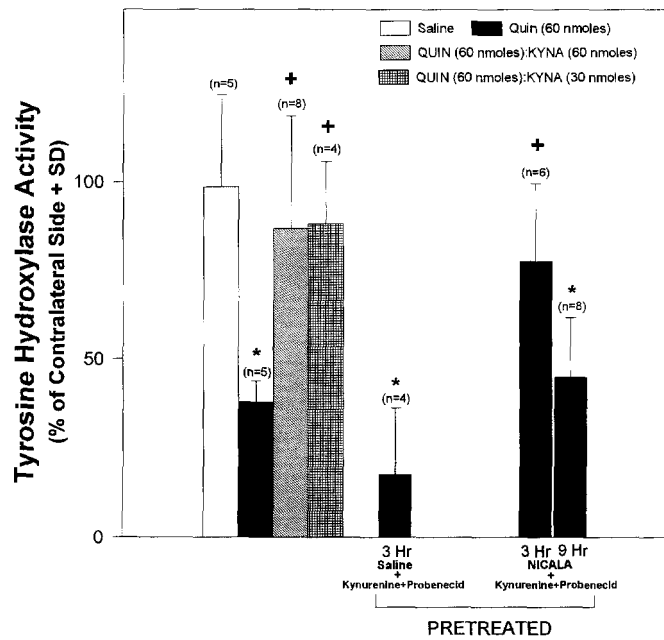
Fig. 2. Effect of nicotinylalanine (NICALA) on the depletion of striatal tyrosine hydroxylase (TH) induced by unilateral intranigral infusion of quinolinic acid. Tyrosine hydroxylase in the injected side is expressed as a percentage of the activity in the uninjected side in the same animal (570 ± 40 pmol/mg protein/h). The bars on the left represent injections of saline, quinolinic acid (60 nmoles, QUIN) alone and quinolinic acid in combination with exogenous kynurenic acid (60 or 30 nmoles). The bar in the middle represents the action of quinolinic acid in animals injected with saline (i.c.v.), kynurenine (450 mg/kg i.p.) and probenecid (200 mg/kg) 3 h before quinolinic acid. The bars on the right represent the action of quinolinic acid in animals receiving NICALA (5.6 nmoles i.c.v.) with kynurenine and probenecid. The intranigral injection of quinolinic acid was delivered 3 h and 9 h after treatment with NICALA in combination with kynurenine and probenecid. Each bar represents the mean $\pm$ S.D. *Values are significantly different from the corresponding value in saline-injected animals ($P<0.05$); + values are significantly different from animals injected with QUIN alone ($P<0.05$).

Table 2. Effect of nicotinylalanine on the depletion of striatal tyrosine hydroxylase activity induced by unilateral intranigral infusion of N-methyl-D-aspartate

Treatment Group	Pretreatment	Tyrosine hydroxylase activity
Saline	–	98.6 ± 26 ($n=5$)
NMDA (15 nmoles)	–	$49.5 \pm 10^*$ ($n=4$)
NMDA (15 nmoles): kynurenic acid (60 nmoles)	–	88.4 ± 17 ($n=8$)
NMDA (15 nmoles)	NICALA+kynurenine+probenecid	$91 \pm 22^+$ ($n=7$)

Tyrosine hydroxylase in the injected side is expressed as a percentage of the activity in the uninjected side in the same animal (570 ± 40 pmol/mg protein/h). The intranigral injection of NMDA was delivered 3 h after the injection of NICALA (5.6 nmoles i.c.v.), kynurenine (450 mg/kg i.p.) and probenecid (200 mg/kg i.p.). Each value represents the mean $\pm$ S.D.
*Values are significantly different from the corresponding value in saline-injected animals ($P<0.05$).
+Value is significantly different from animals injected with NMDA alone ($P<0.05$).

of nigrostriatal dopaminergic neurons (Table 2). To determine the effect of elevations in endogenous kynurenic acid levels on NMDA-induced toxicity, rats were pretreated with NICALA (5.6 nmoles), kynurenine (450 mg/kg) and probenecid (200 mg/kg) prior to the intranigral infusion of NMDA. Ipsilateral TH in this group was not different from that in the saline-injected animals (Table 2). Thus, NICALA in combination with kynurenine and probenecid was able to block the NMDA-induced neurotoxicity.

In order to further examine the action of NICALA in combination with probenecid and kynure-

nine on the damage produced by quinolinic acid to the SNc, histological assessment of neurons in the SNc was undertaken using an antibody specific for TH. The results are shown in Fig. 3. Intranigral injection of quinolinic acid produced a significant depletion of neurons in the SNc on the injected side (Fig. 3a) relative to the contralateral uninjected side (Fig. 3c). Focal infusion of quinolinic acid, 3 h following the administration of NICALA in combination with probenecid and kynurenine, resulted in a sparing of neurons in the ipsilateral SNc (Fig. 3b).

A

B

C
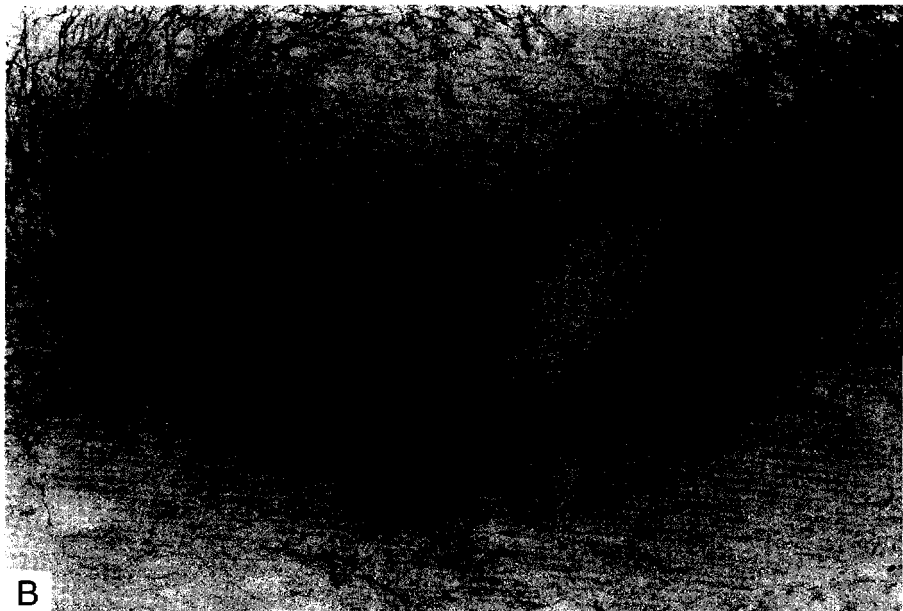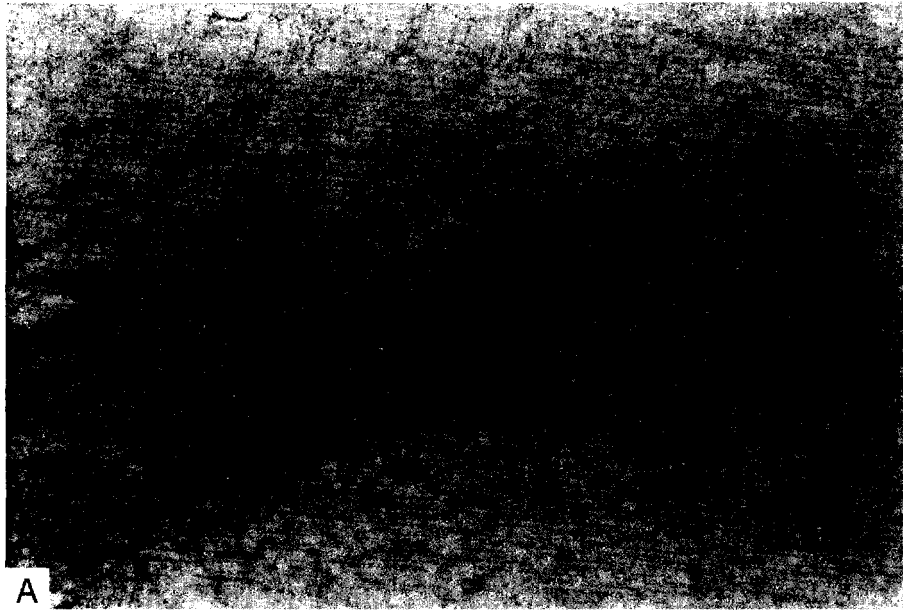
DISCUSSION

The present study examined the possibility that an elevation of endogenous brain kynurenic acid, which acts as an EAA receptor antagonist, affords protection against quinolinic acid-induced excitotoxicity in the nigrostriatal pathway. Previous studies in this laboratory have demonstrated that an infusion of quinolinic acid into the SNc destroys dopaminergic neurons projecting to the striatum,[4,9] an effect reflected in the depletion of striatal TH, a biochemical marker for dopaminergic neurons projecting to the striatum. Using this model of neurotoxicity, the present study demonstrated that intraventricular injection of NICALA, an inhibitor of kynureninase and kynurenine hydroxylase,[10] in combination with kynurenine, the precursor to kynurenic acid and probenecid, an inhibitor of organic acid transport from the brain, elevated endogenous kynurenic acid and afforded protection against the neurotoxicity produced by an intranigral infusion of quinolinic acid or NMDA.

Previous studies have shown that treatment with NICALA alone, or probenecid combined with kynurenine, elevates brain kynurenic acid and has the ability to inhibit convulsions, an effect which has been attributed to an increase in the level of brain kynurenic acid.[8,20,27,33,38] However, it has been demonstrated that a significantly greater increase in kynurenic acid is produced when NICALA is combined with kynurenine and probenecid.[19] Thus, it was this paradigm that was used in the present study. The neuroprotection afforded by treatment with NICALA in combination with kynurenine and probenecid most likely resulted from an elevation of brain kynurenic acid, since neurotoxicity was attenuated at a time period (3 h post-treatment) which co-incided with the highest level of brain kynurenic acid. When kynurenic acid had declined to a lower level (9 h post-treatment) NICALA in combination with kynurenine and probenecid did not afford significant protection against quinolinic acid-induced toxicity. The combination of saline with kynurenine and probenecid while producing almost a 35-fold increase in whole brain kynurenic acid and increasing nigral kynurenic acid did not protect against quinolinic acid or NMDA-mediated excitotoxicity. This suggests that a threshold level of kynurenic acid is necessary in order to protect dopaminergic nigrostriatal neurons from excitotoxic damage. The absence of protection following the combination of saline with kynurenine and probenecid may also indicate that NICALA is particularly effective because of its ability to inhibit the synthesis of quinolinic acid. In control animals

that received saline instead of NICALA, administration of kynurenine and probenecid would be expected to increase not only the level of kynurenic acid in the SNc but also that of quinolinic acid.[18] Thus, in animals receiving saline instead of NICALA the increased level of quinolinic acid may antagonize the neuroprotection afforded by increased kynurenic acid.

While co-infusion of exogenous kynurenic acid (60 or 30 nmoles) also attenuated quinolinic acid excitotoxicity, the concentration at the injection site following co-administration of kynurenic acid is difficult to determine. This makes it impossible to compare the absolute amount of exogenous kynurenic acid infused with the concentration of endogenous kynurenic acid necessary to protect nigrostriatal dopaminergic neurons.

Interestingly, elevations of nigral kynurenic acid (1154.6 ± 137.9 pmoles/g tissues) 3 h following treatment with NICALA, kynurenine and probenecid were not as high as those in whole brain (5745 ± 1654.4 pmoles/g tissue). However, recent studies have shown that kynurenine aminotransferase, the enzyme responsible for the biosynthesis of kynurenic acid, exist in astrocytes abutting excitatory synapses in the SNc.[31,36] Thus, the 1.5-fold increase in total nigral kynurenic acid produced by NICALA, probenecid and kynurenine relative to control rats treated with saline, kynurenine and probenecid may not be a true reflection of the local kynurenic acid concentraion at synapses in the SNc. Alternatively, the protection against quinolinic acid-mediated destruction of nigrostriatal dopaminergic neurons afforded by only a 1.5-fold increase in nigral kynurenic acid suggests that a steep dose–response relationship may exist between the concentration of kynurenic acid in the SNc and its ability to antagonize quinolinic acid toxicity. It is also possible that the elevation of kynurenic acid in the SNc may follow a different time-course from that in whole brain.

Since quinolinic acid exerts its neurotoxic action via activation of NMDA receptors, it is highly likely that the elevation of endogenous kynurenic acid by NICALA produced its neuroprotective effect by blocking these receptors. The neurotoxicity of quinolinic acid, unlike that of other NMDA receptor agonists such as NMDA and ibotenate is critically dependent on intact glutamatergic input to the target.[13,14,25,35] Some studies have suggested that in the striatum exogenous kynurenic acid is more effective against quinolinic acid-induced damage than against damage produced by other NMDA receptor agonists such as ibotenic acid.[14] However, other studies have shown effective blockade of quinolinic acid and

Fig. 3. TH immunohistochemistry of the SNc following the infusion of (a) quinolinic acid (60 nmoles) alone or (b) following the administration of NICALA (5.6 nmoles i.c.v.) with kynurenine (450 mg/kg i.p.) and probenecid (200 mg/kg i.p.). Arrow indicates cannula tip; (c)contralateral (non-injection) side. Scale bar=100 μm.

ibotenate toxicity by exogenous kynurenic acid in the basal forebrain.[7] In accord with results of a previous study,[9] infusions of 15 nmoles NMDA into the SNc produced a depletion of striatal TH comparable to that produced by 60 nmoles of quinolinic acid, reflecting its higher potency as a neurotoxin. Co-injection of exogenous kynurenic acid abolished NMDA-induced neurotoxicity. Administration of NICALA (in combination with kynurenine and probenecid) also blocked the neurotoxic effect of NMDA. Thus, elevations in endogenous kynurenic acid afforded protection against the toxic action of both quinolinic acid and NMDA. This action of endogenous kynurenic acid is consistent with its ability to act as an antagonist at NMDA receptors.[30]

The findings in this study may have relevance to Parkinson's disease or other neurodegenerative disorders such as Huntington's disease, in which excitotoxic mechanism have also been implicated in neuronal damage.[2,3,34] Specifically, there may exist a balance between the formation of quinolinic acid, an agonist that is neurotoxic, and of kynurenic acid, an agent which is neuroprotective. Thus, increased formation of quinolinic acid or decreased production of kynurenic acid, may result in excessive activation of NDMA receptors resulting in excitotoxic injury to specific neurons.

CONCLUSIONS

This study demonstrates that elevation of endogenous kynurenic acid protects dopaminergic neurons in the SNc from excitotoxic damage mediated by quinolinic acid or NMDA. The novel strategy employed in this study may be able to protect neuronal populations in diseases that involve excitotoxic damage. It remains to be seen whether pharmacologically relevant elevations in brain kynurenic acid can be achieved by enzyme inhibitors in the absence of adjunct treatments. Recently, more potent analogues of NICALA have been developed and found to have anticonvulsant properties.[6] It will be interesting to determine the potential of these agents as neuroprotectants. The present study provides a rationale for examining their effects.

REFERENCES

1. Areneda R. and Bustos G. (1989) Modulation of dendritic release of dopamine by N-methyl-D-aspartate receptors in rat substantia nigra. *J. Neurochem.* **52,** 962–970.
2. Beal M. F., Kowall N. W., Ellison D. W., Mazurek M. F., Swartz K. J. and Martin J. B. (1986) Replication of the neurochemical characteristics of Huntinton's disease by quinolinic acid. *Nature* **321,** 168–171.
3. Beal M. F., Matson W. R., Swartz K. J., Gamache P. H. and Bird E. D. (1990) Kynurenine pathway measurements in Huntington's disease striatum:evidence for reduced formation of kynurenic acid. *J. Neurochem.* **55,** 1327–1339.
4. Beninger R. J., Colten A. M., Ingles J. L., Jhamandas K. and Boegman R. J. (1994) Picolinic acid blocks the neurotoxic but not the neuroxexcitant properties of quinolinic acid in the rat brain: evidence from turning behaviour and tyrosine hydroxylase immunocytochemistry. *Neuroscience* **61,** 603–612.
5. Bradford M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72,** 248.
6. Carpenedo R., Chiarugi A., Russi P., Lombardi G., Carla V., Pellicciari R., Mattoli L. and Moroni F. (1994) Inhibitors of kynurenine hydroxylase and kynureninase increase cerebral formation of kynurenate and have sedative and anticonvulsant activities. *Neuroscience* **612,** 237–243.
7. Cockhill J., Jhamandas K., Boegman R. J. and Beninger R. J. (1992) Action of picolinic acid and structurally related pyridine carboxylic acids on quinolinic acid-induced cotrical cholinergic damage. *Brain Res.* **599,** 57–63.
8. Connick J. H., Heywood G. C., Sills G. J., Thompson G. G., Brodie M. J. and Stone T. W. (1992) Nicotinylalanine increases cerebral kynurenic acid content and has anticonvulsant activity. *Gen. Pharmac.* **23,** 235–239.
9. Connop B. P., Boegman R. J., Jhamandas K. and Beninger R. J. (1995) Excitotoxic action of NMDA agonists on nigrostriatal dopaminergic neurons: Modulaton by inhibition of nitric oxide synthesis. *Brain Res.* **676,** 124–132.
10. Decker R. H., Brown R. R. and Price J. M. (1963) Studies on the biological activity of nicotinylalanine, an analogue of kynurenine. *J. biol. Chem.* **238,** 1049–1053.
11. Di Loreto S., Florio T. and Scarnate E. (1992) Evidence that non-NMDA receptors are involved in the excitatory pathway from the pedunculopontine region to the nigrostriatal dopaminergic neurons. *Expl Brain. Res.* **89,** 79–86.
12. Difazio M. C., Hollingsworth Z., Young A. B. and Penney J. B. (1992) Glutamate receptors in the substantia nigra of Parkinson's disease brains. *Neurology* **42,** 402–406.
13. Foster A. C., Collins J. F. and Schwarcz R. (1983) On the excitotoxic properties of quinolinic acid, 2,3-piperidine dicarboxylic acids and structurally related compounds. *Neuropharmacology* **22,** 1331–1342.
14. Foster A. C., Vezzani A., French E. D. and Schwarcz R. (1984) Kynurenic acid blocks neurotoxicity and seizures induced in rats by the related brain metabolite quinolinic acid. *Neurosci. Lett.* **48,** 273–278.
15. Foster A. C., Gill R. and Woodruff G. N. (1988) Neuroprotective effects of MK-801 *in vivo*: selectivity and evidence for delayed degeneration mediated by NMDA receptor activation. *J. Neurosci.* **8,** 4745–4754.
16. Foster A. C., Willis C. L. and Tridgett R. (1990) Protection against N-methyl-D-aspartate receptor mediated neuronal degeneration in rat brain by 7-chlorokynurenic acid and 3-amino-1hydroxypyrrolid-2-one, antagonists at the allosteric site for glycine. *Eur. J. Neurosci.* **2,** 270–277.

17. Galli T., Godeheu G., Artaud F., Desce J. M., Pittaluga A., Barbeito L., Glowinski J. and Cheramy A. (1991) Specific role of N-acetyl-glutamate in the in vivo regulation of dopamine release from dendrites and nerve terminals of nigrostriatal dopaminergic neurons in the rat. Neuroscience 42, 19–28.

18. Guidetti P., Eastman C. L. and Schwarcz R. (1995) Metabolism of [5-³ H] kynurenine in the rat brain in vivo: Evidence for the existence of a functional kynurenine pathway. J. Neurochem. 65, 2621–2632.

19. Harris C. A., Boegman R. J., Jhamandas K. and Beninger R. J. (1993) Antagonism of quinolinic acid-induced toxicity on striatal diaphorase neurons following administration of kynurenine, probenecid and nicotinylalanine. Soc. Neurosci. Abstr. 19, 1345.

20. Hiller J. M., MacGarvey V. and Beal M. F. (1992) The effect of peripheral loading with kynurenine and probenecid on extracellular striatal kynurenic acid concentrations. Neurosci. Lett. 1461, 115–118.

21. Hsu S. M., Raine L. and Fager H. (1981) Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques. Am. J. clin. Path. 75, 734.

22. Kessler M., Terramani T., Lynch G. and Baudry M. (1989) A glycine site associated with N-methyl-D-aspartic acid receptors: Characterization and identification of a new class of antagonists. J. Neurochem. 52, 1319–1328.

23. Kikuchi S. and Kim S. U. (1993) Glutamate neurotoxicity on mesencephalic dopaminergic neurons in culture. J. Neurosci. Res. 36, 558–569.

24. Kopin I. J. (1993) The pharmacology of Parkinson's disease therapy: an update. A. Rev. Pharmac. Toxicol. 33, 467–495.

25. McDonald J. W., Silverstein F. S. and Johnston M. V. (1988) Neurotoxicity of N-methyl-D-aspartate is markedly enhanced in developing rat central nervous system. Brain Res. 200, 200–203.

26. Merue G., Costa E., Armstrong D. M. and Vicini S. (1991) Glutamate receptor subtypes mediate excitatory synaptic currents of dopamine neurons in midbrain slices. J. Neurosci. 11, 1359–1366.

27. Moroni F., Russi P., Gallo-Mezo M. A., Moneti G. and Pellicciari R. (1991) Modulation of quinolinic and kynurenic acid content in the rat brain: effects of endotoxin and nicotinylalanine. J. Neurochem. 57, 1630–1635.

28. Mount H., Quirion R., Kohn-Alexander J. and Boksa P. (1990) Subtypes of excitatory amino acid receptors involved in the stimulation of dopamine release from cell cultures of rat ventral mesecephalon. Synapse 5, 271–280.

29. Ogawa T., Matson W. R., Beal M. F., Myers R. H., Bird E. D., Milbury P. and Saso S. (1992) Kynurenine pathway abnormalities in Parkinson's disease. Neurology 42, 1702–1706.

30. Perkins M. N. and Stone T. W. (1982) An iontophoretic investigation of the actions of the cunvulsant kynurenines and their interaction with the endogenous excitant quinolinic acid. Brain Res. 247, 184–187.

31. Roberts R. C., McCarthy K. E., Du F., Okuno E. and Schwarcz R. (1994) Immunocytochemical localization of the quinolinic acid synthesizing enzyme, 3-hydroxyanthranilic acid oxygenase, in the rat substantia nigra. Brain Res. 650, 229–238.

32. Russi P., Carla V. and Moroni F. (1989) Indolpyruvic acid administration increases the brain content of kynurenic acid. Biochem. Pharmac. 38, 2405–2409.

33. Russi P., Alesiani M., Lombardi G., Davolio P., Pellicciari R. and Moroni F. (1992) Nicotinylalanine increases the formation of kynurenic acid in the brain and antagonizes convulsions. J. Neurochem. 59, 2076–2080.

34. Schwarcz R., Whetsell W. O. Jr. and Mangano R. M. (1983) Quinolinic acid: an endogenous metabolite that produces axon-sparing lesions in rat brain. Science 219, 316–318.

35. Schwarcz R., Foster A. C., French E. D., Whetsell W. O. Jr. and Kohler C. (1984) Excitotoxic models for neurodegenerative disorders. Life Sci. 35, 19–32.

36. Schwarcz R., Du F., Schmidt W., Turski W. A., Gramsbergen J. B. P., Okuno E. and Roberts R. C. (1992) Kynurenic acid: A potential pathogen in brain disorders. Ann. N. Y. Acad. Sci. 648, 140–153.

37. Stone T. W. and Connick J. H. (1985) Quniolinic acid and other kynurenines in the central nervous system. Neuroscience 15, 597–617.

38. Vescei L., Miller J., MacGarvey V. and Beal M. F. (1992) Kynurenine and probenecid inhibit pentylenetetrazol- and NMDA-induced seizures and increase kynurenic acid concentrations in the brain. Brain Res. Bull. 282, 233–238.

39. Vrana S. L., Azzaro A. J. and Vrana K. E. (1992) Selegiline administration transiently decreases tyrosine hydroxylase activity and mRNA in the rat nigrostriatal pathway. Molec. Pharmac. 41, 839–844.

40. Yamauchi T. and Fujisawa H. (1978) A simple and sensitive fluorometric assay for tyrosine hydroxylase. Analyt. Biochem. 89, 143–150.

41. Zeevalk G. D., Nicklas W. J. and Sonsalla P. K. (1994) NMDA receptor involvement in two animal models of Parkinson's disease. Neurobiol. Agng 15, 269–270.