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Research report

Excitotoxic action of NMDA agonists on nigrostriatal dopaminergic neurons: modulation by inhibition of nitric oxide synthesis

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Abstract

Focal infusions of *N*-methyl-D-aspartate (NMDA) or an endogenous NMDA agonist, quinolinic acid (QUIN), into the substantia nigra pars compacta (SNc) of adult Sprague–Dawley rats resulted in a dose-dependent depletion of ipsilateral striatal tyrosine hydroxylase (TH) activity, a biochemical marker for dopaminergic neurons. To assess the intermediary role of nitric oxide in the neurotoxicity elicited by these toxins, their action was tested in animals treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME). Systemic injections (2 injections; 8 h apart) of L-NAME (100, 150 and 250 mg/kg) produced a dose-related inhibition of cerebellar nitric oxide synthase (NOS) activity. The time–course of cerebellar NOS inhibition following L-NAME (250 mg/kg) was rapid in onset and lasted for at least 24 h following the second injection. An L-NAME treatment regimen of 250 mg/kg, with the second injection given 24 h prior to assessment of NOS activity, produced an 87 and 91% inhibition of cerebellar and nigral NOS activity, respectively. Intranigral infusion of 40 and 60 nmol QUIN reduced ipsilateral striatal TH activity by 62 and 75%, respectively. However, 40 and 60 nmol QUIN infusions into animals pretreated with L-NAME (250 mg/kg) reduced striatal TH activity by 83 and 96%, respectively. Intranigral infusion of 15 and 30 nmol NMDA produced a 48 and 77% decrease in striatal TH activity, respectively, whereas the same doses of NMDA given to animals pretreated with L-NAME (250 mg/kg) resulted in a 59 and 88% decrease in TH activity. Thus, both QUIN and NMDA toxicity was enhanced following L-NAME pretreatment. The destruction of the nigrostriatal pathway was verified using TH immunocytochemistry of the SNc. It was also observed that a low dose of L-NAME (1.5 mg/kg), previously shown to be neuroprotective in cerebral ischemic damage, did not influence NMDA (15 nmol) neurotoxicity. The results of this study show that extensive inhibition of NOS activity enhances NMDA receptor-mediated excitotoxicity.

Keywords: Quinolinic acid; Tyrosine hydroxylase; Excitotoxicity; L-NAME; Nitric oxide synthase

1. Introduction

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopaminergic neurons of the nigrostriatal pathway. The causes of dopaminergic neuronal cell degeneration are not known but it has been postulated that excitotoxicity due to overactivation of excitatory amino acid receptors may be a factor in this cell loss [26,48]. Glutamate receptors have been localized on the dopaminergic neurons of the substantia nigra pars compacta (SNc) [10], and experiments on cultured mesencephalic dopaminergic neurons have shown that activation of NMDA receptors on these neurons can cause toxicity [24]. Additionally, excitotoxicity has been implicated in the selective cell loss produced by 1-methyl-4-phenylpyridinium (MPP^+), a toxic metabolite formed by oxidation of MPTP via monoamine oxidase B. In some studies MPP^+ toxicity has been reported to be attenuated by the *N*-methyl-D-aspartate (NMDA) antagonist MK-801 [39,42], although others have failed to show the protective action of NMDA antagonists in this toxicity [38].

It is widely acknowledged that nitric oxide (NO) is produced in response to activation of NMDA receptors [15]. Recent studies employing neuronal culture, brain slices and animal models of focal ischemia have implicated NO as a mediator of NMDA receptor toxicity [4,5,8,23,25,36]. However, other studies using similar protocols have provided contradictory evidence with

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respect to the role of NO in neurotoxicity [7,9,17,18,33,35,44,45]. It has been suggested that these discrepancies may be partly due to variations in the amount of nitric oxide synthase (NOS) inhibited by different doses, routes of administration and potencies of NOS inhibitors used in these studies [21]. A recent report has suggested that NO destroys dopaminergic neurons by competing with iron for ferritin binding sites thereby leaving more iron free to react with H_2O_2 in the Haber-Weiss reaction [47]. However, the role of NO in dopaminergic neuronal toxicity in vivo has not received much attention.

The goal of the present study was to investigate the sensitivity of the nigrostriatal dopaminergic pathway to two excitatory amino acid agonists, quinolinic acid and NMDA, and to study the role of NO in NMDA receptor-mediated toxicity using the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME). On the basis of previous studies implicating NO in neurotoxicity, it was hypothesized that L-NAME would protect against the neurotoxic action of NMDA receptor agonists on dopaminergic neurons. This hypothesis was evaluated by examining whether an extensive reduction in brain NOS activity prevents the neurotoxic action of quinolinic acid and NMDA following intranigral injection of these agents.

2. Materials and methods

2.1. Inhibition of nitric oxide synthase

Prior to determining the role of NOS inhibition on NMDA and quinolinic acid (QUIN) toxicity, the ability of systemic administration of L-NAME to inhibit NOS activity was investigated. To determine the relationship between the dose of L-NAME and brain NOS activity, male Sprague–Dawley rats (250–275 g) were given two intraperitoneal (i.p.) injections (8 h apart) of the vehicle (distilled water) or different doses of L-NAME (100, 150 and 250 mg/kg). 14 h after the last injection, the animals were sacrificed and the cerebella removed and placed in ice-cold saline for assay of NOS activity. To determine the time–course of inhibition of NOS activity, the dose of L-NAME which produced the largest inhibition in the dose–response experiments was examined with sacrifice occurring at 4, 14, 24, 48 and 96 h following the last injection. The animals were sacrificed and cerebella removed and placed in ice-cold saline for assay of NOS activity. To determine the effect of L-NAME on NOS activity in the SNc, nigral tissues from both hemispheres were pooled for the enzyme assay. The substantia nigra from each hemisphere was dissected from a 3-mm thick coronal section which was removed 4.5 mm posterior to the optic chiasm.

The respective tissue samples were homogenized with a Teflon pestle in a solution containing 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 mM EDTA and 50 mM HEPES buffer at pH 7.4. Each nigral or cerebellar tissue sample was homogenized in a volume of 175 μl and 1 ml, respectively. The homogenates were centrifuged at 20,000 $\times g$ for 30 min at 4° C and the supernatant retained for the NOS assay.

2.2. Nitric oxide synthase assay

NOS activity was assayed using a modification of a previously described method [3] which measures the amount of [^{14}C]citrulline formed from [^{14}C]arginine. Tissue supernatant samples (25 μl) were incubated for 1 h at 37° C with 25 μl of 100 μM [^{14}C]arginine and 100 μl of reaction buffer: 1 mM DTT, 1.25 mM $CaCl_2$, 1 mM valine, 2 mM NADPH, 1 mM EDTA and 50 mM HEPES buffer, pH 7.4. The reaction was terminated by the addition of 2 ml ice-cold stop buffer which contained 2 mM EDTA and 20 mM HEPES buffer at pH 5.5. Each sample was passed through a 1 ml AG50W-X8 cationic-exchange column (Na^+ form) which was washed with 2 ml of distilled water, and the eluate collected and counted using a Beckman scintillation counter. Protein was determined by the method of Bradford [2] and the enzyme activity was expressed as nmol citrulline/mg protein/h.

2.3. Intranigral infusions of excitotoxins

Male Sprague–Dawley rats (250–275 g) were anesthetized with sodium pentobarbital (50 mg/kg) and placed in a Narashige stereotaxic apparatus. NMDA (15 and 30 nmol), QUIN (20, 40, 60 and 120 nmol) or 0.9% saline was infused unilaterally through a cannula into the SNc according to the following stereotaxic coordinates [34]: 5.3 mm posterior to bregma, 2.2 mm lateral to the midline, and 7.7 mm ventral to bregma. NMDA and QUIN were dissolved in 0.9% saline and titrated to pH 7.4 with 1 N NaOH. Each agent was infused for 75 s through the cannula (30 gauge) connected to a Hamilton syringe to deliver a total volume of 0.5 μl. The cannula was left in place for an additional 2 min and then withdrawn, bone wax applied and the scalp incision sutured. Four days after infusion, the animals were decapitated and each brain was rapidly removed and placed in ice-cold saline. The ipsilateral and contralateral striata were dissected and homogenized separately with a glass hand-held homogenizer in 150 μl of buffer consisting of 0.3 M sucrose in 0.01 M Tris-HCl at pH 7.3. The homogenate samples were subsequently centrifuged at 12,000 $\times g$ for 6 min at 4° C and the supernatant retained for the assay of tyrosine hydroxylase (TH) activity.

In the L-NAME pretreatment experiments, animals were injected twice (8 h apart) with 1.5 or 250 mg/kg L-NAME, 24 h prior to stereotaxic injection of QUIN, NMDA or 0.9% saline into the SNc. Animals were sacrificed 4 days post-injection for assay of striatal TH activity.

2.4. Tyrosine hydroxylase assay

Tyrosine hydroxylase (TH) activity in the dissected striatum was assayed using a modification of the method described by Yamauchi and Fujisawa [46]. The modification to the method included a 1.5 h incubation period at 37° C as well as centrifugation at $2,000 \times g$ for 10 min following each vortex of the alumina. The amount of L-dopa formed from L-tyrosine was determined by the trihydroxyindole fluorometric method [46]. Protein was determined by the method of Bradford [2] and TH enzyme activity was expressed as nmol L-dopa/mg protein/h. For each animal, the results were expressed as the % decrease from the enzyme activity measured in the contralateral (uninjected) side.

2.5. TH immunocytochemistry

Four days following stereotaxic infusions of either QUIN or saline (described above), animals were anesthetized and transcardially perfused with 200 ml of 0.9% saline followed by 300 ml of 4% paraformaldehyde. The brain was removed, post-fixed in 4% paraformaldehyde for 12 h, and then immersed in 30% sucrose for 48 h. Sections (50 μm) were cut on a cryostat and stored in phosphate buffered saline at 4° C. The tissue sections were stained according to the Vectastain ABC technique [19] for TH immunoreactivity using a monoclonal TH antibody.

2.6. Drugs and chemicals

Radiolabelled [^{14}C]arginine (0.319 Ci/mmol, 50 μCi/ml) was obtained from Amersham, Ont., Canada. The monoclonal TH antibody was provided by Dr. A.C. Cuello, M^{c}Gill University, Canada. All other drugs and chemicals were obtained from the Sigma Chemical Co., MO, USA.

2.7. Statistical analyses

Data were compared using analyses of variance (ANOVA) with a Newman-Keuls post-hoc test for statistical significance at $P < 0.05$. The number of independent experiments ranged between 4 and 8 for all points and values were expressed as mean ± S.E.M.

3. Results

3.1. Inhibition of brain NOS

The cerebellum was chosen as a measure of NOS activity since this region has the highest specific NOS activity in the rat brain [13], and thus pooling of tissue samples was not required. Cerebellar NOS activity was characterized in order to determine an appropriate L-NAME dosing regimen that would provide extensive inhibition of brain NOS activity following i.p. administration of the enzyme inhibitor. Fig. 1 shows the dose-related effect of L-NAME on cerebellar NOS activity. Basal cerebellar NOS activity was found to be 5.1 ± 0.6 nmol/mg/h. A dose of 250 mg/kg L-NAME (2 injections, 8 h apart) produced an 86% decrease in cerebellar NOS activity. In separate experiments, the time-course of NOS inhibition by this dose was evaluated. As shown in Fig. 2, L-NAME produced maximal inhibition of enzyme activity by 4 h following the second injection, an effect which was sustained for at least 24 h. Thereafter, NOS activity showed a progressive recovery returning to pre-injection levels 96 h after the second injection. From these results, a dose of 250 mg/kg L-NAME administered twice (8 h apart), with the second injection occurring 24 h prior to excitotoxin infusions was selected as the pretreatment regimen for the excitotoxin experiments.

The action of L-NAME on nigral NOS activity was also evaluated. Basal nigral NOS activity was found to be 3.1 nmol/mg/h. A dose of 1.5 mg/kg L-NAME

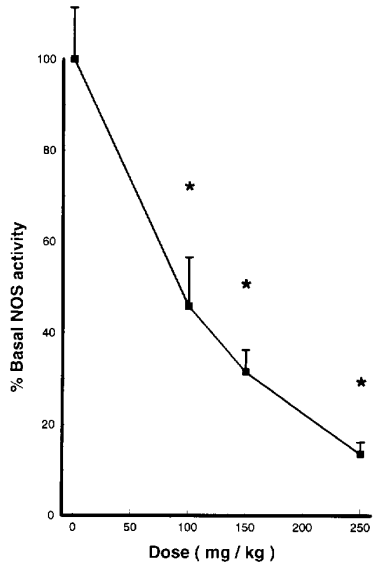


Fig. 1. Cerebellar NOS activity. Dose–response curve following two i.p. injections of varying doses of vehicle or L-NAME (8 h apart) with dissection of the cerebellum 14 h after the last injection. Values are expressed as mean ± S.E.M. with $n = 4$–6 for each point. * $P < 0.05$.

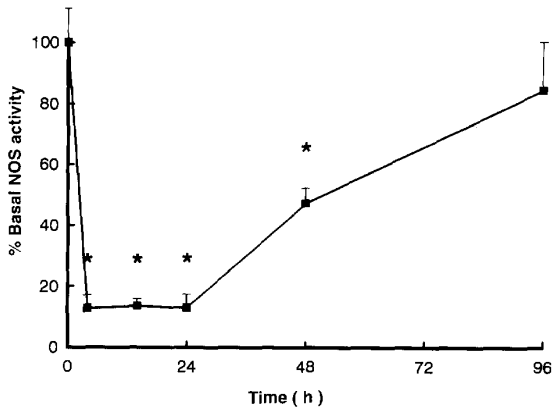


Fig. 2. Cerebellar NOS activity following two i.p. injections of vehicle or 250 mg/kg L-NAME (8 h apart) with dissection of the cerebellum at varying intervals after the last injection. Values are expressed as mean ± S.E.M. with $n = 4–6$ for each point. * $P < 0.05$.

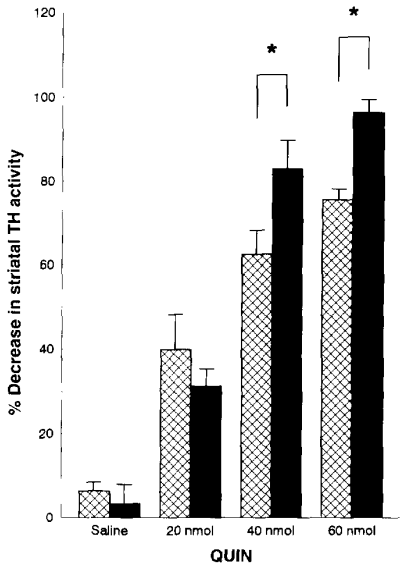


Fig. 3. Striatal TH activity following intranigral infusions of varying doses of QUIN into non-pretreated (hatched) and 250 mg/kg L-NAME pretreated animals (solid). Values are expressed as % decrease in striatal TH activity from the contralateral (unlesioned) side. Data are mean ± S.E.M. with $n = 5–8$ for each point. * $P < 0.05$.

inhibited nigral NOS activity by 2.6 ± 7.8%, a value which was not significantly different from vehicle treated animals. The nigral NOS activity following a dose of 250 mg/kg L-NAME was inhibited by 91.0 ± 1.6%, a value comparable to that seen in cerebellar NOS activity following the same L-NAME pretreatment (Fig. 2).

3.2. Effects of NOS inhibition on QUIN toxicity

The TH activity in the contralateral striatum of naive animals was found to be 570 ± 40 pmol/mg/h which is in agreement with values reported by Vrana et al. [43] who used an HPLC assay to measure TH activity. The TH activity obtained from the striata of vehicle pretreated (water, i.p.) animals was not significantly different from that of naive animals (data not shown). No difference in the striatal TH activity between the contralateral (uninjected) sides of saline, QUIN (20, 40, and 60 nmol) or NMDA (15 and 30 nmol) infused animals was observed.

The effects of L-NAME-induced NOS inhibition on the neurotoxic action of QUIN on nigrostriatal dopaminergic neurons are shown in Fig. 3. The doses of QUIN shown are submaximal since it was found that an infusion of 120 nmol QUIN into the SNc produced an 87.9 ± 4.2% decrease in striatal TH activity. The slight depletion in striatal TH activity observed following a saline infusion into the SNc was not altered by L-NAME (250 mg/kg) pretreatment. Infusion of QUIN (20, 40, 60 nmol) produced a dose-related decrease in ipsilateral striatal TH activity. The action of 20 nmol QUIN was not influenced by L-NAME pretreatment. In animals receiving 40 and 60 nmol of intranigral QUIN, the striatal TH activity was reduced by 62 and 75%, respectively, indicating a significant action of the excitotoxin on dopaminergic cells. However, in the L-NAME (250 mg/kg) pretreated animals 40 and 60 nmol QUIN reduced striatal TH activity by 83 and 96%, respectively. These values were significantly higher than corresponding values observed in animals not pretreated with L-NAME. Thus, L-NAME pre-

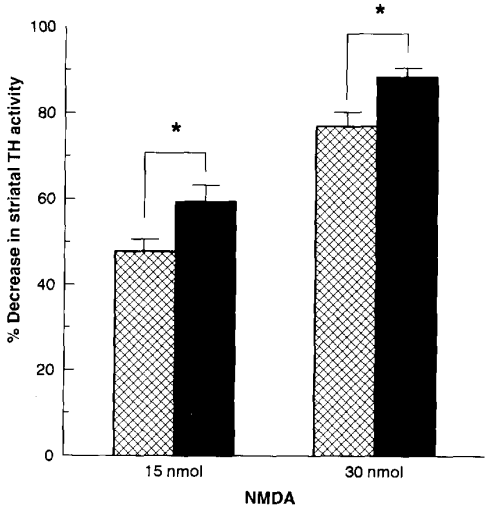
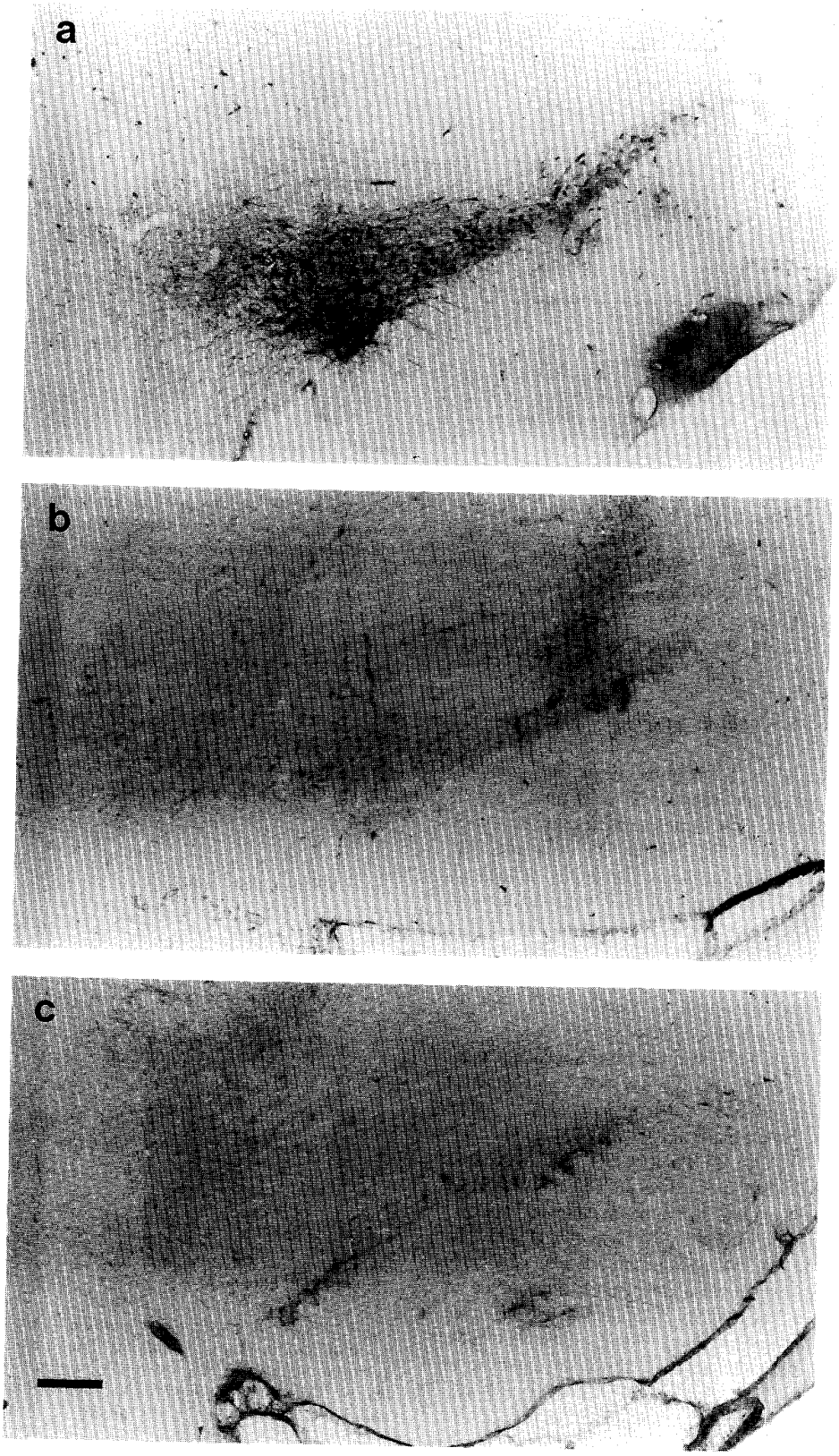


Fig. 4. Striatal TH activity following intranigral infusions of varying doses of NMDA into vehicle (hatched) and 250 mg/kg L-NAME pretreated animals (solid). Values are expressed as % decrease in striatal TH activity from the contralateral (unlesioned) side. Data are mean ± S.E.M. with $n = 5–8$ for each point. * $P < 0.05$.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treatment enhanced the effect of two submaximal doses of QUIN.

3.3. Effects of NOS inhibition on NMDA toxicity

The effects of L-NAME-induced NOS inhibition on the neurotoxic action of NMDA on nigrostriatal dopaminergic neurons are shown in Fig. 4. Infusion of NMDA (15, 30 nmol) also produced a dose-related decrease in ipsilateral striatal TH activity. In vehicle pretreated animals receiving 15 and 30 nmol of intranigral NMDA, the striatal TH activity was reduced by 48 and 77%, respectively, indicating a significant action of the excitotoxin on the nigrostriatal dopaminergic cells. However, in the L-NAME (250 mg/kg) pretreated animals 15 and 30 nmol NMDA reduced striatal TH activity by 59 and 88%, respectively. These values were significantly higher than corresponding values observed in animals not pretreated with L-NAME. Thus, as was seen in QUIN experiments on dopaminergic neurons, L-NAME pretreatment enhanced the effect of NMDA.

In a separate group of animals, the effect of a low dose (1.5 mg/kg) of L-NAME on NMDA toxicity was also investigated since Buisson et al. [5] showed that a single injection of 3 mg/kg L-NAME provided neuroprotection against an NMDA (300 nmol) infusion into the striatum. A 15 nmol infusion of NMDA into vehicle pretreated animals produced a $47.8 \pm 2.6\%$ decrease in striatal TH activity, and the same dose of NMDA produced a $45.5 \pm 7.2\%$ decrease in the L-NAME (1.5 mg/kg) pretreated animals. The difference between these values was not significant, thus, pretreatment of animals with a low dose of L-NAME did not alter the nigral toxicity of NMDA (15 nmol).

3.4. TH immunocytochemistry

In order to verify that the depletion of TH activity in the striatum following a nigral infusion of QUIN was indeed due to the destruction of nigral dopaminergic neurons, the nigral tissue was examined for the presence of dopaminergic cell bodies using TH immunocytochemistry. The TH immunoreactivity following an infusion of 0.9% saline into the SNc of an animal pretreated with 250 mg/kg L-NAME is shown in Fig. 5a. An infusion of 40 nmol QUIN into a vehicle pretreated animal resulted in a very substantial loss of TH immunoreactivity in the SNc (Fig. 5b). A similar loss was seen in animals pretreated with 250 mg/kg L-NAME following an infusion of 40 nmol QUIN (Fig. 5c).

4. Discussion

In the present study, nigrostriatal dopaminergic neurons were found to be sensitive to focal infusions of both QUIN and NMDA. Infusions of both agonists into the SNc significantly depleted striatal TH activity which serves as a biochemical marker for these neurons. The toxicity of both NMDA receptor agonists was potentiated by pretreatment with a dose of L-NAME which inhibited nigral and cerebellar NOS by approximately 90%. The effect of systemic L-NAME on cerebellar NOS activity in the present study is comparable to that reported by Iadecola et al. [22]. Interestingly, this effect of systemic L-NAME on NOS activity has been found to be less potent than that of systemic N^{ω}-nitro-L-arginine (L-NA) [11]. This difference has been suggested to be due to a decreased ability of L-NAME to access the CNS [22].

The results demonstrate that the nigrostriatal neurons are sensitive to the neurotoxic effects of QUIN (an endogenous NMDA receptor agonist) and NMDA. In contrast to some other studies showing that inhibition of NOS is neuroprotective, the present study shows that inhibition of NOS activity by L-NAME does not protect these neurons from an excitotoxic insult. L-NAME, in fact, augmented NMDA receptor-mediated toxicity.

Although some studies have provided support for the intermediary role of NO in NMDA receptor-mediated excitotoxicity and cortical ischemic damage, other studies have provided evidence against this notion. It has been suggested that discrepancies between the results of ischemia studies may arise from varying levels of NOS inhibition produced by the various inhibitors examined [21]. Indeed, very few studies have provided measures of NOS activity while utilizing an inhibitor of this activity as a potential neuroprotectant. Thus, the present study sought to evaluate QUIN and NMDA toxicity under experimental conditions which provided near maximal inhibition of NOS activity in the cerebellum and importantly in the SNc, the site of excitotoxin infusions. When NOS activity in these areas was reduced by about 90%, neither QUIN nor NMDA action was attenuated. At doses of QUIN and NMDA which produced submaximal toxicity, the action of these two agonists was significantly enhanced by L-NAME. A low dose of L-NAME has been reported to attenuate NMDA-induced excitotoxicity and ischemic damage [5]. Similarly, Carreau et al. have shown that low but not high doses of L-NA protect against ischemic damage [6]. However, in the present study a low dose

Fig. 5. TH immunocytochemistry of the substantia nigra pars compacta following 0.5 μl infusion of saline or 40 nmol QUIN. Saline infusion with 250 mg/kg L-NAME pretreatment (a), 40 nmol QUIN infusion with vehicle pretreatment (b) and 40 nmol QUIN infusion with 250 mg/kg L-NAME pretreatment (c). Bar = 200 μm.

of L-NAME, reported to be neuroprotective by Buisson et al. [4] failed to inhibit nigral NOS activity or alter the toxic action of NMDA on the SNc.

The lack of a protective effect observed in both low and high dose L-NAME pretreated groups implies that NO is not directly involved in NMDA receptor-mediated toxicity of the nigral dopaminergic neurons in vivo. The results of this study showing that extensive inhibition of NOS enhances NMDA receptor-mediated excitotoxicity imply that endogenous NO may have a protective role in this experimental paradigm. The neuroprotective potential of NO in vivo is suggested by several studies reported in the literature.

First, NO may inhibit activation of the NMDA receptor by a feedback mechanism [14,30,31]. Manzoni et al. [31] demonstrated blockade of NMDA receptor function by 3-morpholino-sydnonimine (SIN-1), which acts as an NO donor. In that study, hemoglobin, an agent which binds NO, enhanced NMDA responses by removing basal levels of NO. Recently, it has been suggested that the oxidized form of NO, the nitrosonium ion (NO^+), inhibits currents associated with NMDA receptor activation by causing S-nitrosylation of the NMDA redox modulatory site [28]. Removal of basal NO^+ production by an NOS inhibitor can be anticipated to remove this negative feedback and consequently enhance NMDA toxicity. The enhancement of QUIN and NMDA toxicity in the L-NAME pretreated animals in the present study may result from inhibition of NO^+ formation. However, NO^+ formation can not explain the basal level of toxicity associated with an infusion of excitotoxins into the SNc of naive animals.

Second, inhibition of neuronal or endothelial NOS may interfere with regulation of local cerebral blood flow (LCBF) [20,21]. In the brain, glutamate receptor agonists can produce activity-dependent vasodilation which can be blocked by inhibition of NOS activity [12,37]. Thus, a high dose of L-NAME resulting in almost complete inhibition of nigral NOS, may have potentiated excitotoxicity by attenuating the activity-dependent vasodilation and thus metabolically compromising nigral neurons activated by QUIN or NMDA. The possibility that metabolic compromise may potentiate excitotoxicity is supported by recent findings that inhibition of oxidative phosphorylation by various mitochondrial inhibitors can lead to 'excitotoxic' lesions [1,16,39,40]. This might also explain why a critical dose of QUIN was required before an enhancement of toxicity was observed. It has been previously shown that systemic administration of the NOS inhibitor N^{ω}-monomethyl-L-arginine (30 mg/kg, i.v.) decreased LCBF in the SNc by 25% [41], and that L-NAME (30 mg/kg, i.v.) produced a 40% decrease in LCBF throughout the entire brain [29]. In addition, the study by Faraci and Breese [12] demonstrated that the local vasodilation induced by NMDA could be blocked by another NOS inhibitor, L-NA. However, the hypothesis that the loss of activity-dependent vasodilation is able to impair metabolic function to the point where excitotoxicity is potentiated remains to be evaluated in future studies.

Recently, we have also shown that the selective neuronal NOS inhibitor 7-nitro indazole (7-NI) can also potentiate NMDA-mediated toxicity on the nigrostriatal pathway [7] suggesting that the results of the present study are not due to a non-specific effect of L-NAME. While 7-NI appears to be selective for neuronal NOS in vivo [32], L-NAME inhibits all isozymes of NOS throughout the body. The ability of both inhibitors to influence NMDA receptor-mediated toxicity suggests that selectivity for neuronal NOS is not a crucial factor in regard to enhancement of excitotoxicity. In addition to the nitro-arginine analogs, 7-NI has also been found to decrease LCBF [27]. Thus, reduction of LCBF is one of the common factors which may influence excitotoxic action at NMDA receptors.

In conclusion, we have demonstrated that the nigrostriatal dopaminergic neurons are sensitive to NMDA receptor-mediated neurotoxicity, and that under conditions of very extensive nigral NOS inhibition this toxicity is significantly enhanced. Consequently, the present study does not provide support for an intermediary role of NO in excitotoxic damage to dopaminergic neurons in vivo. It remains to be seen if the observations made on the dopaminergic neurons extend to other neurons in the CNS.

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