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Modulation of quinolinic acid-induced depletion of striatal NADPH diaphorase and enkephalinergic neurons by inhibition of nitric oxide synthase

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Abstract

The present study was designed to examine the role of nitric oxide (NO) in quinolinic acid (QUIN)-induced depletion of rat striatal nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase and enkephalinergic neurons. Intrastriatal injection of QUIN produced a dose-dependent decrease in NADPH diaphorase and enkephalin positive cells, with cell loss being evident following the injection of 6 and 18 nmol QUIN, respectively. To evaluate the role of NO in QUIN-induced toxicity, animals were pretreated with the non-specific nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine (L-NAME) or the selective neuronal NOS inhibitor, 7-nitro indazole (7-NI). L-NAME (2×250 mg/kg, i.p. 8 h apart) maximally inhibited striatal NOS activity by 85%, while 7-NI (50 mg/kg, i.p.) maximally inhibited striatal NOS activity by 60%. Pretreatment with L-NAME or 7-NI potentiated the loss of NADPH diaphorase neurons resulting from intrastriatal injection of low doses of QUIN (18 nmol). Neither NOS inhibitor had any effect on the loss of striatal NADPH diaphorase neurons induced by a higher dose of QUIN (24 nmol). In contrast, 7-NI partially prevented the QUIN (18 and 24 nmol)-induced loss of enkephalinergic neurons, while L-NAME had no effect. These results indicate that NO formation may play a role in QUIN-induced loss of enkephalinergic neurons, but not in the loss of NADPH diaphorase neurons. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Neurotoxicity; Nitric oxide; Quinolinic acid; Enkephalinergic

1. Introduction

Huntington's disease, an inherited neurodegenerative disorder, is characterized by a profound and early degeneration of striatal γ-aminobutyric acid (GABA)-containing neurons [18,22,42] and relative sparing of interneurons containing nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase [18,22]. The projection neurons constitute the majority of striatal cells and the neuropeptides substance P, dynorphin and enkephalin are co-localized with GABA [1,9,21,50]. The enkephalin/GABA-containing neurons, which project to the globus pallidus, have been reported to be depleted in the early stages of Huntington's disease [54]. Although the cause of neurodegeneration in Huntington's disease is not known, the phenomenon of excitotoxicity has been implicated in the selective neuron loss seen in this neurodegenerative disease.

Striatal NADPH diaphorase neurons are consistently spared in Huntington's disease, however, there is controversy regarding the sparing of this neuron population following exposure to the endogenous tryptophan metabolite quinolinic acid (QUIN) which acts on N-methyl-D-aspartate (NMDA) receptors. Beal et al. [4–6] reported that rat striatal NADPH diaphorase neurons are relatively resistant to QUIN, while others have reported that these neurons are destroyed following intrastriatal injection of QUIN [7,8,16,17,38,39]. At present, the basis for these discrepant results remains unresolved. Despite the fact that enkephalinergic neurons are depleted in Huntington's disease, little information is available regarding the action of QUIN on this specific neuron population. Roberts et al. [55] examined the action of QUIN on enkephalinergic neurons and found that following intrastriatal QUIN administration,

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both NADPH diaphorase and enkephalin immunoreactive neurons were depleted to the same extent in the lesion core but that there was selective sparing of the NADPH diaphorase but not enkephalin-positive neurons in lateral regions of the transition zone (area immediately adjacent to the lesion core). Most studies investigating the effects of QUIN on striatal neurons have used relatively high doses of QUIN (120 nmol). However, we have found that injection of QUIN at a dose as low as 7.5 nmol produces extensive depletion of rat striatal NADPH diaphorase neurons in the lesion core (C.M. Wray, unpublished observations). The response of enkephalinergic neurons to low-dose QUIN has not been investigated. Thus, one goal of this study was to examine the sensitivity of the striatal NADPH diaphorase and enkephalinergic neurons to low doses of focal QUIN injections.

Excitotoxicity induced by QUIN results from overactivation of the NMDA receptor [52,57]. Following NMDA receptor activation, there is a sustained increase in intracellular Ca^{+2} [23,58], which activates a cascade of biochemical events resulting in neurotoxicity. One consequence of NMDA receptor-mediated Ca^{+2} influx is the activation of Ca^{+2} -calmodulin-dependent neuronal nitric oxide synthase (NOS) [11,26,34] and subsequent formation of nitric oxide (NO) [43]. Dawson et al. [19] reported that NMDA receptor-mediated neurotoxicity in cultured cortical neurons was prevented by inhibition of NOS activity, implicating NO as a mediator of NMDA receptor toxicity. NO was also found to be neurotoxic in models of focal cerebral ischemia [12,13,49], in hippocampal slice studies [31] and in excitotoxicity experiments involving intrastriatal infusion of NMDA [56]. Recently, Inagaki et al. [30] reported that mice lacking neuronal NOS were resistant to neuronal damage following permanent middle cerebral artery occlusion. In contrast, a large number of studies have provided opposite results with regard to the role of NO in NMDA receptor-mediated toxicity. NOS inhibition has been found to either augment or have no effect on excitotoxicity in cell culture [25,29], hypoxic/ischemic injury [20,61,63] and following the infusion of excitotoxins [14,15,38,39].

Since QUIN has been reported by a number of laboratories to be highly neurotoxic to both NADPH diaphorase and enkephalinergic neurons, it is of interest to examine whether NO plays a role in this toxicity. The role of NO in NADPH diaphorase neuron toxicity is of special interest as these neurons themselves generate this messenger molecule. In the present study, N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor [24], and 7-nitro, indazole a selective neuronal NOS inhibitor [3,46,47] were selected to inhibit striatal NOS activity. The ability of these agents to influence the neurotoxic action of QUIN on two populations of striatal neurons under treatment conditions resulting in significant NOS inhibition was examined. Part of this work has been presented in abstract form [33].

2. Materials and methods

2.1. Source of compounds

7-NI was obtained from Lancaster Synthesis (Windham, NH). L-NAME, QUIN, NADPH, nitro blue tetrazolium (NBT), dithiothreitol, *N*-[2-hydroxyethyl]-piperazine-*N*′-12-ethanesulfonic acid (HEPES), leupeptin, dowex were purchased from Sigma (St. Louis, MO). Radiolabelled [^{14}C]arginine (0.321 Ci/mmol, 50 μCi/ml) was obtained from New England Nuclear (Lachine, Que.). Anti-leucine enkephalin antibody was purchased from Incstar (Stillwater, MN), reconstituted in 10 μl of double distilled water, aliquotted and frozen at −20°C until use. Normal goat serum (NGS), biotinylated goat anti-rabbit IgG, and avidin–biotin complex were purchased from Dimension Labs (Mississauga, Ont.). All other chemicals used were at least reagent grade and were obtained from Fisher Scientific (Ottawa, Ont.) or BDH (Toronto, Ont.).

7-NI was prepared by sonicating and heating in peanut oil. QUIN was dissolved initially in 1 N NaOH, the pH adjusted to 7.4 with 1 N HCl and the desired concentration then achieved by the addition of 0.9% saline.

2.2. Animals

Adult male, Sprague–Dawley rats (Charles River, St. Constant, Que.) weighing 250 to 350 g were used for all experiments. Animals were housed in wire cages, maintained on a 12 h light/12 h dark cycle at 21°C and received food and water ad libitum.

2.3. Inhibition of nitric oxide synthase

To determine the extent of striatal NOS inhibition following L-NAME treatment, animals were given systemic injections of L-NAME using the procedure previously described by Connop et al. [14]. Rats were given two i.p. injections (8 h apart) of L-NAME (100 or 250 mg/kg) or vehicle and sacrificed 12 h after the second injection. To determine the duration of rat striatal NOS inhibition, animals were given two injections of L-NAME (250 mg/kg; 8 h apart) and sacrificed 4, 12, 15, 48 and 72 h following the second injection. In the L-NAME pretreatment experiments, animals were injected twice (8 h apart) with 250 mg/kg L-NAME or vehicle with the second injection occurring 4 h prior to stereotaxic injection of QUIN. In 7-NI pretreatment experiments, rats were given three injections of 50 mg/kg 7-NI at 4 h intervals, with the first injection occurring 0.5 h prior to stereotaxic injection of QUIN. The extent and duration of striatal NOS inhibition following 7-NI treatment previously have been determined [32].

2.4. Nitric oxide synthase assay

Treated animals were sacrificed at a designated time point and the brain was removed rapidly and rinsed with ice-cold saline. A micropunch (2.5 mm, i.d.) was used to remove the right and left striatum from a brain slice 3 mm in thickness, using the anterior commissure, lateral ventricles and corpus callosum as borders. The striatal tissue was homogenized in 100 μl of 50 mM HEPES buffer containing 1 mM dithiothreitol, 10 μg/ml leupeptin and 1 mM EDTA, at pH 7.4. The homogenates were centrifuged at $20\,000 \times g$ for 30 min at 4°C and the supernatants stored up to 7 days at −20°C for the NOS assay.

Each supernatant sample (25 μl) was incubated with 25 μl of 100 μM [^{14}C]L-arginine and 100 μl of reaction buffer for 1 h at 37°C. The reaction buffer consisted of 1 mM dithiothreitol, 1.25 mM $CaCl_2$, 1 mM valine, 2 mM NADPH and 1 mM EDTA in 50 mM HEPES buffer at pH 7.4. The reaction was stopped by the addition of 2 ml ice-cold 20 mM HEPES buffer containing 2 mM EDTA at pH 5.5. Each sample was passed through a 0.75 ml Dowex 50WX8-400 (Na^+) column to separate [^{14}C]L-arginine from [^{14}C]citrulline. The columns were subsequently washed with 2 ml of distilled water, 10 ml of scintillation cocktail added and the [^{14}C]citrulline in the resulting eluate was counted. The amount of protein present in striatal supernatant was determined by the method of Bradford [10].

2.5. Intrastriatal injection

Rats were anesthetized with halothane (4% halothane/96% O_2 via inhalation) and positioned in a Narashige small animal stereotaxic apparatus with the incisor bar set at −3.3 mm. Anaesthesia was maintained by the inhalation of 2% halothane/98% O_2 and an injection cannula was placed into the striatum at the following coordinates: 1.0 mm anterior, 2.5 mm lateral and 5 mm ventral to Bregma [51]. A volume of 1 μl of saline or QUIN was infused unilaterally into the striatum over a period of 2 min and 33 s. After the injection, the cannula was withdrawn, the skin sutured and the animal was allowed to recover for 4 days prior to sacrifice.

2.6. Histology

Animals used for histological assessment of NADPH diaphorase and enkephalinergic neurons were anaesthetized with pentobarbital (50 mg/kg) and perfused through the ascending aorta with 50 ml of ice-cold saline (0.9% at pH 7.4) followed by 400 ml of ice-cold paraformaldehyde (4% in 0.1 M phosphate buffer at pH 7.4). Each brain was dissected out and post-fixed in paraformaldehyde for at least 24 h. Transverse 50 μm sections were serially cut with a vibratome and collected in 24-well culture plates containing phosphate-buffered saline (PBS).

2.7. NADPH diaphorase histochemistry

Histochemical assessment of striatal NADPH diaphorase neurons was carried out by staining for NADPH diaphorase activity using the procedure described by Vincent and Kimura [60]. Alternate sections (five in total) containing the cannula tract were stained. Free-floating sections were incubated in PBS containing 1.2 mM NADPH, 0.12 mM NBT and 0.3% Triton X-100 for 30 to 120 min and subsequently rinsed with PBS. Sections were mounted on gelatinized glass slides and air-dried. Slides were then rinsed with water and dehydrated in a series of ethanol solutions (50 to 100%), coverslipped and viewed under a light microscope. Cells that stained positive for NADPH diaphorase were counted in the plane of the cannula. The lesion core was designated as a rectangular area totalling 1.6 mm^2. For each animal, five tissue sections, which demonstrated the presence of the cannula tract, were counted. All histological slides were coded

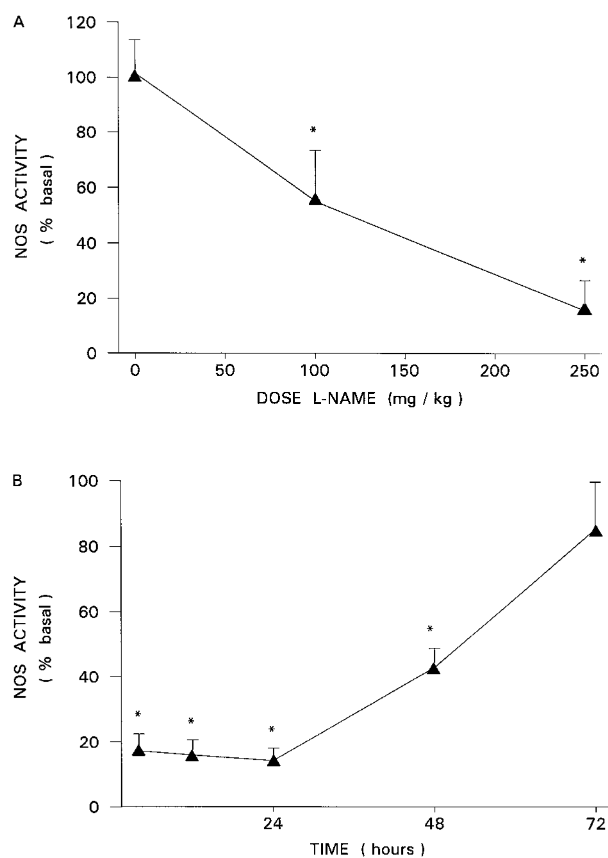

Fig. 1. (A) Striatal NOS activity following two i.p. injections (8 h apart) of vehicle or L-NAME (100 and 250 mg/kg) with dissection occurring 12 h after the second injection. (B) Time course of striatal NOS activity following two i.p. injections of 250 mg/kg L-NAME (8 h apart) with dissection of the striatum occurring at varying intervals after the second injection. NOS activity is expressed as a percentage of untreated animals. Each point represents the mean ± S.D. of four experiments. A significant difference from vehicle-injected animals is represented by $* P < 0.05$.

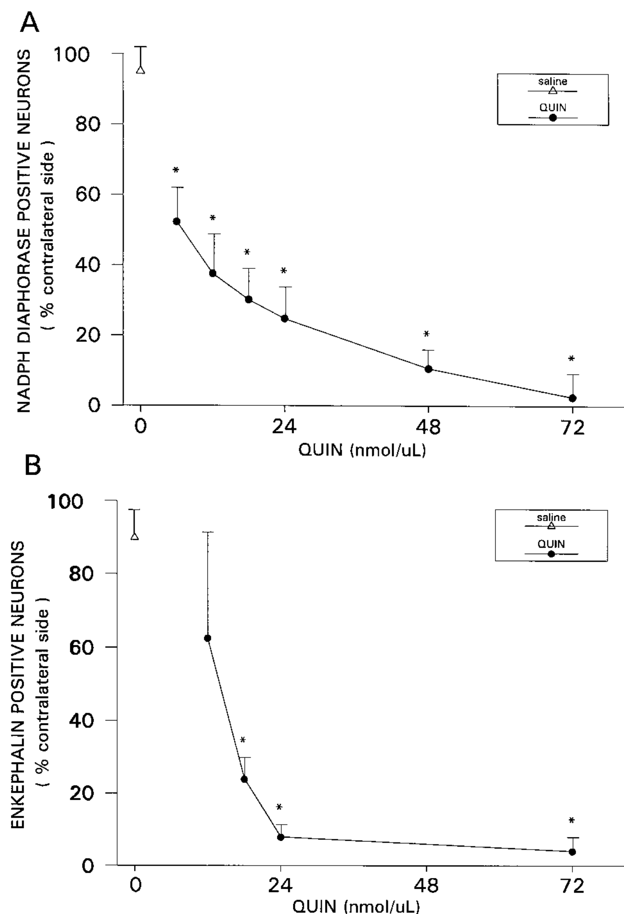

Fig. 2. Cell counts of NADPH diaphorase- (A) or enkephalin- (B) positive neurons in the lesion core of the rat striatum following injection of 0.9% saline or QUIN (6 to 72 nmol) delivered in 1 ml. Cell counts are expressed as a percentage of the contralateral side. Each point represents the mean ± S.D. of four to seven experiments. A significant difference from saline is represented by $* P < 0.05$.

such that the treatment condition was not known at the time of histological assessment.

2.8. Enkephalin immunohistochemistry

Alternate sections (five in total) of those showing the cannula tract, were stained for enkephalin immunoreactivity using a modified procedure described by Roberts et al. [55]. Free-floating sections were incubated in PBS containing 3% NGS and anti-leucine enkephalin at a dilution of 1/1000 for 72 h at 4°C. Sections were then rinsed twice for 10 min in PBS and transferred to PBS containing 3% NGS and biotinylated goat anti-rabbit IgG diluted 1/500. Following incubation for 2 h, sections were again rinsed twice for 10 min and incubated with the avidin–biotin complex (0.9% avidin and biotin) with 3% NGS in PBS for 1 h. The avidin–biotin complex was mixed 1/2 h prior to the addition of tissue slices. Sections were then rinsed twice for 10 min in 0.1 M Tris-buffered saline (TBS). Subsequently, sections were incubated in 0.5 mg/ml DAB in TBS containing 0.01% hydrogen peroxide and 0.4% nickel chloride for 5 to 10 min. Slices were rinsed in TBS, mounted on gelatinized slides and air-dried. They were then rinsed with water and dehydrated in a series of alcohol solutions, coverslipped and viewed under a light microscope. Enkephalin-immunoreactive cells were counted in the lesion core and transition zone as described above. All cells exhibiting enkephalin immunoreactivity were considered positive as no cell staining was evident in sections stained in the absence of primary antibody.

2.9. Data analysis

Nitric oxide synthase activity was expressed as nmol [^{14}C]citrulline formed per milligram protein per hour. Inhibition of NOS activity was expressed as a percentage of the NOS activity in control (vehicle-treated) animals. The sum of cell counts for the injected striatum of each animal

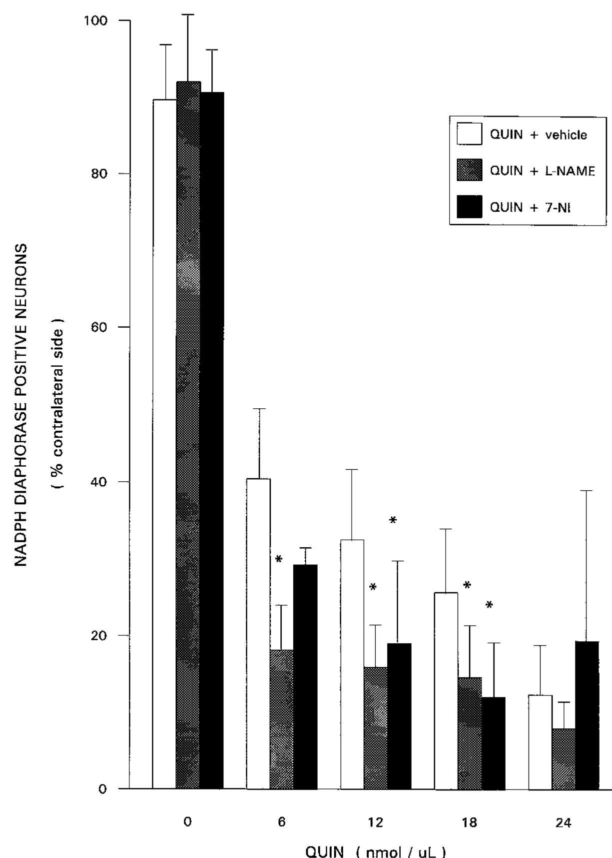

Fig. 3. Cell counts of NADPH diaphorase neurons of the rat striatum following injection of 0.9% saline or QUIN (6 to 24 nmol) delivered in 1 μl into vehicle-, 250 mg/kg L-NAME- or 50 mg/kg 7-NI-pretreated animals. Cell counts are expressed as a percentage of the contralateral side. Each bar represents the mean ± S.D. of four to six experiments. A significant difference from vehicle pretreatment is represented by $* P < 0.05$.

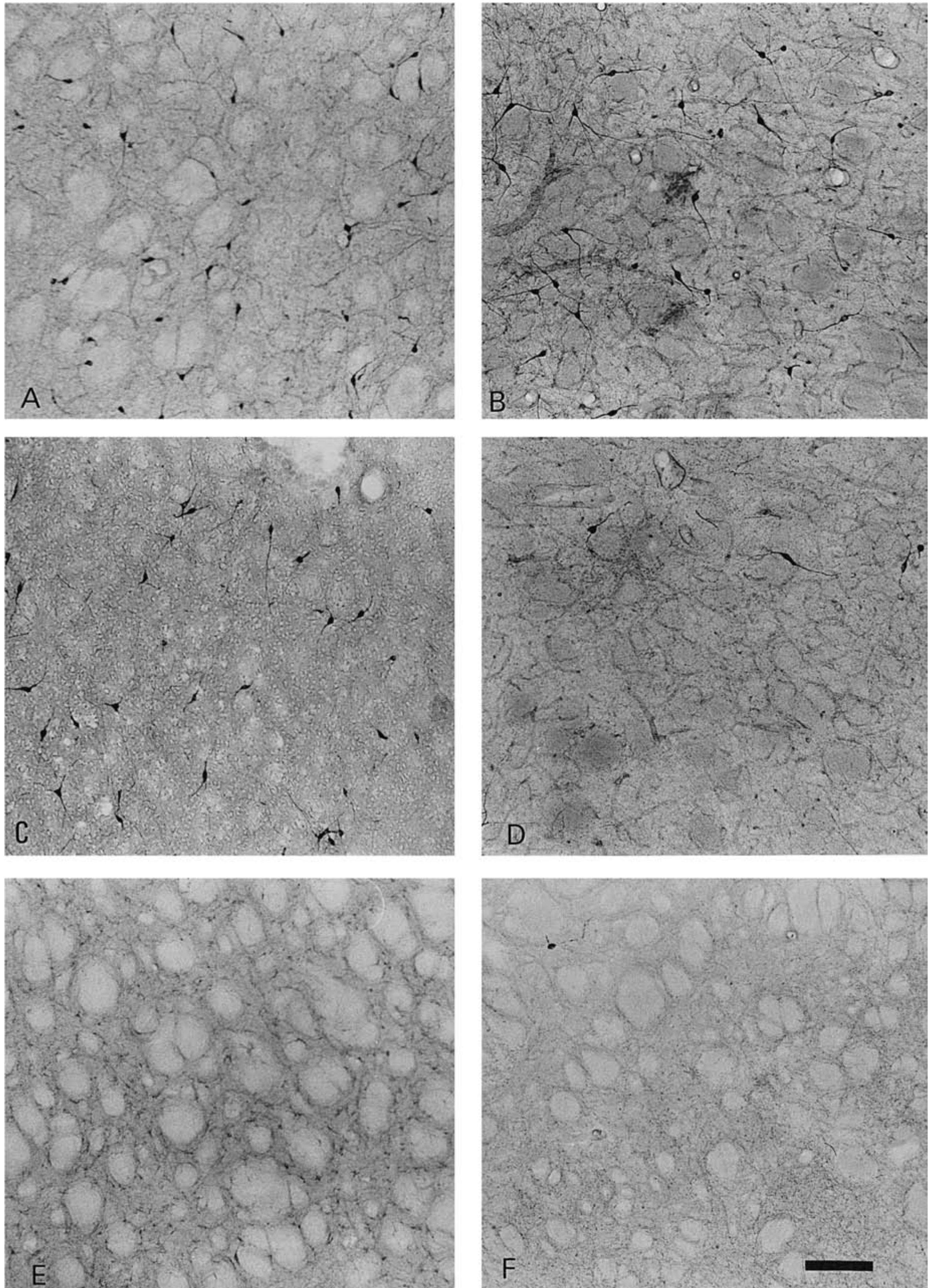

Fig. 4. Photomicrographs of 50 mm coronal sections of rat striatum stained for NADPH diaphorase following a 1-μl injection of 0.9% saline in vehicle- (A), L-NAME- (B) or 7-NI- (C) pretreated animals and 12 nmol QUIN in vehicle (D), L-NAME- (E) or 7-NI- (F) pretreated animals. Bar = 100 μm.

is expressed as a percentage of the sum of cell counts at a similar site on the contralateral side of each section. For histological assessment, four to seven animals were used for each treatment group.

Results are expressed as the mean ± S.D. All data have been assessed for homogeneity of variance using Cochran's test and subsequent parametric statistical analysis performed using a randomized One-way ANOVA followed by Newman–Keul's test to determine which groups were significantly different. Differences in mean values were considered significant if $P < 0.05$.

3. Results

3.1. Effect of L-NAME and 7-NI on rat striatal NOS activity

The action of L-NAME and 7-NI on striatal NOS activity was first examined to determine a dosing regimen that would provide extensive and sustained inhibition of NOS activity following systemic administration of the drug. Basal striatal NOS activity in vehicle (water or peanut oil)-treated animals was 3.2 ± 1.1 nmol $[^{14}C]$citrulline per milligram protein per hour ($n = 11$). Fig. 1A depicts the effects of 100 and 250 mg/kg L-NAME i.p. (two injections, 8 h apart). A dose of 250 mg/kg L-NAME produced an 85% decrease in striatal NOS activity. The enzyme activity was maximally decreased 4 h following the second injection of L-NAME and this level of inhibition persisted for at least 24 h (Fig. 1B). The NOS activity recovered to approximately 50% at 48 h and returned to control levels by 72 h. To ensure prolonged and sustained inhibition of NOS activity, in subsequent experiments, L-NAME (250 mg/kg) was administered 12 and 4 h prior to intrastriatal injection of saline or QUIN.

Previously, we found that a dose of 50 mg/kg 7-NI produced a maximal inhibition of striatal NOS activity (40.3 ± 11.6% of control NOS activity [32]). The recovery of this activity begins approximately 4 h following injection with 7-NI but this is not complete [32,40]. Thus, animals were injected with 50 mg/kg 7-NI, every 4 h for a 12-h period. Animals received intrastriatal injection of saline or QUIN 0.5 h following the first injection of 7-NI.

3.2. Effect of QUIN on NADPH diaphorase and enkephalinergic neurons

Following injection of saline, NADPH diaphorase neurons were observed throughout the striatum, including the area surrounding the cannula tract. In animals exposed to QUIN, the lesion core was originally defined as the area of the striatum with extensive cell loss, as determined from Cresyl violet sections (not shown). At all doses of QUIN used, the cerebral cortex was not affected and, additionally, there appeared to be no visible change in appearance of NADPH diaphorase-positive cells in the contralateral striatum. In sections prepared from the injected side and stained for NADPH diaphorase, the size of lesion core increased with increasing concentrations of QUIN. At lower doses (6 and 12 nmol QUIN), the lesion core occupied approximately one-third of the striatum and the remaining neurons appeared normal. However, injection of higher doses (24 to 72 nmol) produced a lesion core occupying one-half to two-thirds of the striatum. The surviving neurons in the lesion core appeared rounded, lacked processes and were found only along the outer edges of the lesion core area. At these doses of QUIN, occasional rounded cells were visible in the transition zone as well.

The dose-related effects of QUIN on striatal NADPH diaphorase neurons are shown in Fig. 2A. Saline injection resulted in cell survival of 94.7 ± 5.7% relative to the contralateral (uninjected) side. A reduction in cell counts

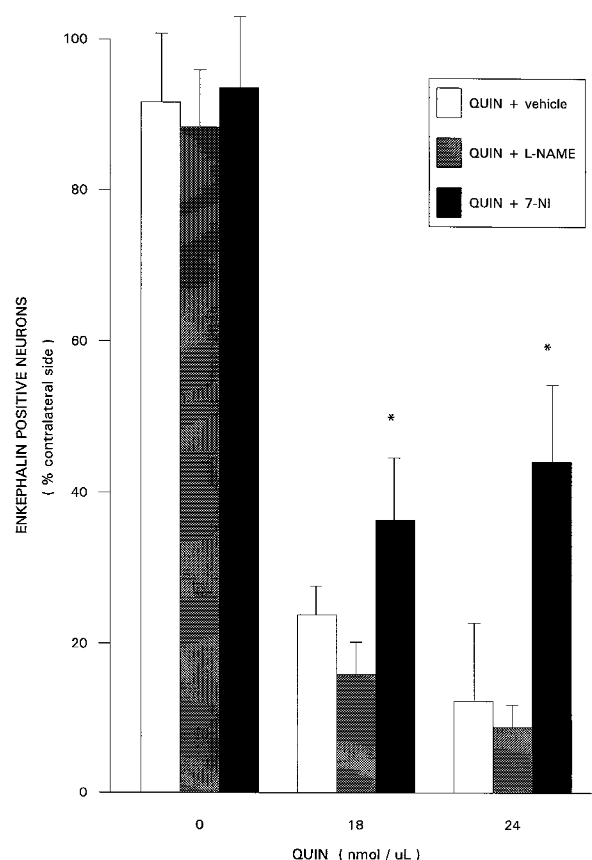

Fig. 5. Cell counts of enkephalin-immunoreactive neurons of the rat striatum following injection of 0.9% saline or QUIN (18 or 24 nmol) delivered in 1 μl into vehicle-, 250 mg/kg L-NAME- or 50 mg/kg 7-NI-pretreated animals. Cell counts are expressed as a percentage of the contralateral side. Each bar represents the mean ± S.D. of four to six experiments. A significant difference from vehicle pretreatment is represented by * $P < 0.05$.

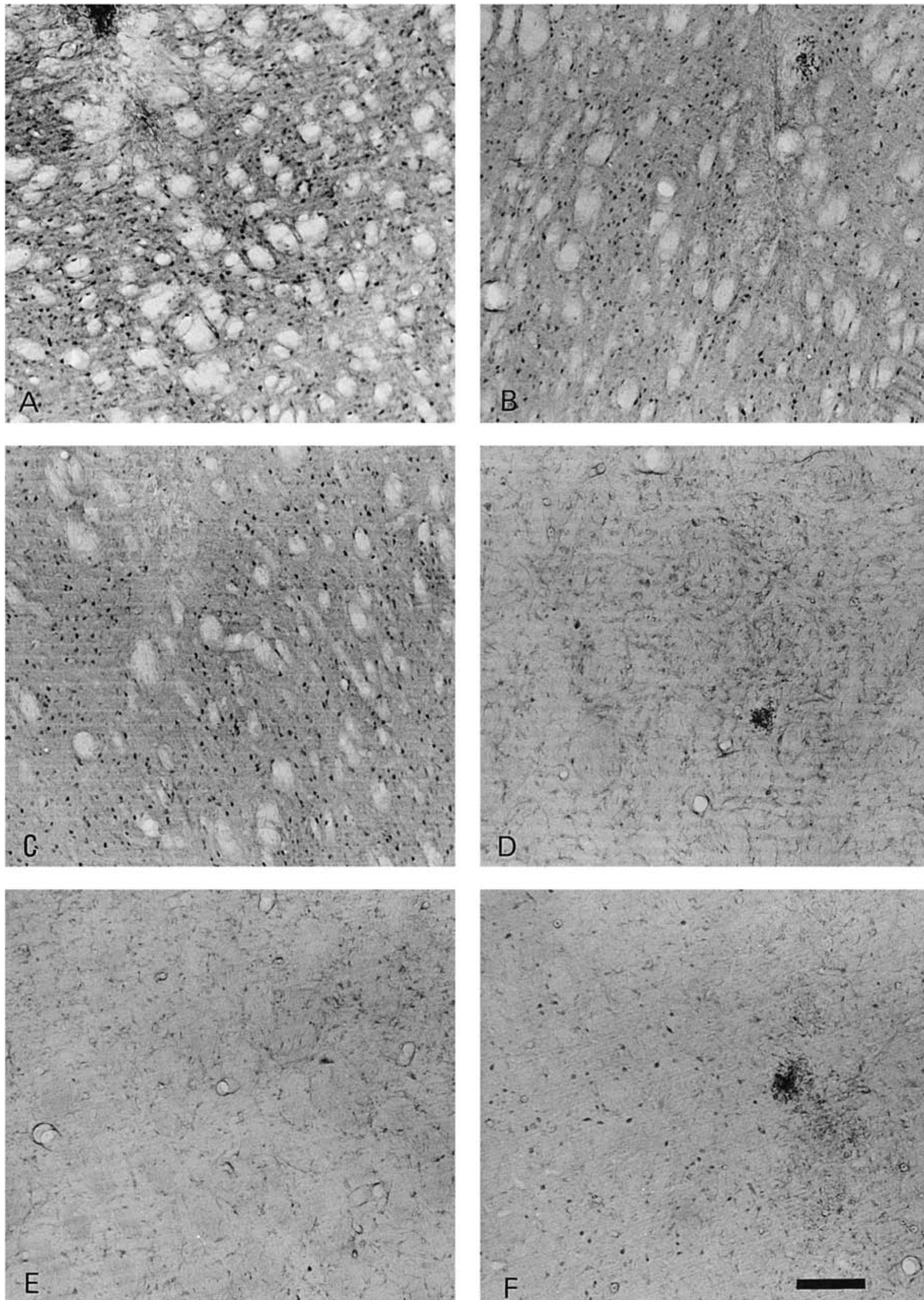

Fig. 6. Photomicrographs of 50 mm coronal sections of rat striatum stained for enkephalin immunoreactivity following a 1-μl injection of 0.9% saline in vehicle- (A), L-NAME- (B) or 7-NI- (C) pretreated animals and 24 nmol QUIN in vehicle (D), L-NAME- (E) or 7-NI- (F) pretreated animals. Bar = 100 μm.

within the lesion core was evident at the lowest dose of QUIN (6 nmol; 54.7 ± 7.1% of contralateral). Following the injection of 12 to 72 nmol QUIN, there was a steep decrease in cell counts, from 38.6 to 3.9% of cells, respectively, remaining in the injected side relative to the contralateral side. In subsequent experiments, doses of 6 to 24 nmol QUIN were selected to study the action of NOS inhibition on QUIN-induced toxicity.

Fig. 2B shows the effects of acute injections of saline and QUIN on enkephalin positive cells in the rat striatum. In saline-injected rats, enkephalin-positive neurons were present throughout the striatum and around the cannula tract with 92.1 ± 7.9% of cells remaining in the injected side relative to the contralateral side. In enkephalin-stained sections obtained from animals injected with 12 nmol QUIN, a cell loss was evident only some of the time, with cell survival in the lesion core ranging from 36.9 to 88.7% relative to the contralateral side. Due to the variability of the action of this dose of QUIN, cell loss following intrastriatal injection of 12 nmol QUIN was not statistically different from that observed in tissue obtained from saline-injected animals. However, at higher doses of QUIN (18, 24 and 72 nmol), there was a steep decline in cell counts within the lesion core. The injection of 18 nmol revealed consistent depletion of neurons with 20.6 ± 9.9% of enkephalin cells remaining in the lesion core region relative to the contralateral side. Intrastriatal injection of 24 or 72 nmol QUIN produced almost complete depletion of enkephalinergic neurons in the lesion core, with 7.9 ± 5.1 and 4.1 ± 4.3% of cells remaining, respectively. At all doses used, there was no alteration in the appearance of enkephalin-positive cells in the cortex or contralateral striatum. In subsequent experiments, doses of 18 and 24 nmol QUIN were selected to study the action of NOS inhibition on QUIN-induced loss of enkephalin immunoreactive neurons.

3.3. *Effect of L-NAME and 7-NI on QUIN-induced toxicity in the striatum*

The effects of L-NAME or 7-NI pretreatment followed by intrastriatal injection of saline or QUIN on NADPH diaphorase-positive cell counts are shown in Fig. 3. Representative photomicrographs of NADPH diaphorase neurons following the injection of saline and QUIN (6 or 12 nmol) in the absence or presence of L-NAME or 7-NI pretreatment are presented in Fig. 4. L-NAME or 7-NI pretreatment in the intrastriatal saline-injected rats did not influence cell counts in the injected or uninjected striatum. The delivery of 6, 12 or 18 nmol QUIN into the striatum reduced cell counts in the lesion core to 40.5 ± 12.6, 32.6 ± 10.4 and 25.8 ± 9.0% of the contralateral side, respectively. The injection of 6, 12 or 18 nmol QUIN into L-NAME pretreated rats reduced cell counts to 18.2 ± 8.0, 16.0 ± 5.9 and 14.7 ± 8.8% of the contralateral side. The values in L-NAME-treated animals were significantly different from those obtained from animals pretreated with vehicle, indicating a greater degree of cell loss due to intrastriatal QUIN toxicity after NOS inhibition. In contrast, injection of a higher dose of QUIN (24 nmol) into the striatum decreased NADPH diaphorase cell counts to 12.5 ± 6.8% in vehicle-treated rats and 8.0 ± 3.9% in L-NAME-pretreated animals. These values were not significantly different. Thus, L-NAME did not influence the toxic effect of a higher dose of QUIN.

Injection of 6 or 24 nmol QUIN, representing lowest and highest QUIN doses, into the 7-NI-pretreated rats decreased NADPH diaphorase cell counts to 29.3 ± 3.0% and 19.5 ± 16.9%, respectively. These values were not significantly different from values obtained from intrastriatal injection of QUIN into vehicle-treated animals. In contrast, injection of intermediate QUIN doses (12 or 18 nmol) into the striatum of 7-NI-pretreated rats decreased cell counts to 19.1 ± 11.3% and 12.1 ± 7.7% of the contralateral side, respectively. These values were significantly different from vehicle-treated animals, indicating that NOS inhibition enhanced QUIN toxicity at these doses. Thus, both NOS inhibitors significantly augmented the neurotoxic response to QUIN.

The effects of L-NAME or 7-NI pretreatment followed by intrastriatal injection of saline or QUIN on enkephalinergic neurons are shown in Fig. 5. Representative photomicrographs of enkephalinergic neurons following the injection of saline or QUIN, in the absence or presence of L-NAME or 7-NI pretreatment, are presented in Fig. 6. L-NAME or 7-NI pretreatment did not influence cell counts in the saline-injected striatum. The intrastriatal injection of 18 or 24 nmol of QUIN reduced enkephalin cell counts to 23.9 ± 4.6% and 12.3 ± 14.2% of the contralateral side, respectively. In animals pretreated with L-NAME, injection of 18 or 24 nmol QUIN reduced cell counts to 15.8 ± 4.6% and 8.8 ± 3.9%, respectively. These values were not different from those obtained from vehicle-pretreated rats, indicating that L-NAME pretreatment did not significantly modify the toxic action of QUIN on enkephalinergic neurons. In contrast, injection of the same doses of QUIN into the striatum of the 7-NI-pretreated animals resulted in cell counts of 36.4 ± 11.9% and 44.2 ± 12.6%, respectively. These values were significantly higher than those obtained from the vehicle-pretreated rats. Thus, the enkephalinergic cell loss was partially attenuated by pretreatment of animals with 7-NI.

To ensure that the observed differences in cell counts were strictly due to intrastriatal injection of QUIN, NADPH diaphorase or enkephalin-immunoreactive cell counts of the contralateral (uninjected) side were compared for all the treatment groups. Total cell counts (for five sections) in an area of the uninjected side corresponding to the lesion core of the injected side ranged from 162 ± 34 to 209 ± 22 and 1215 ± 207 to 1490 ± 56 for NADPH diaphorase and enkephalin-immunoreactive cells, respec-

tively. The values obtained in different treatment groups were not significantly different from each other.

4. Discussion

In the present study, NADPH diaphorase and enkephalin-containing neurons of the rat striatum were found to be highly sensitive to the acute toxic action of QUIN, the NMDA receptor-based excitotoxin that is endogenous to the brain. The toxic action of QUIN on the NADPH diaphorase neuron population was enhanced by pretreatment of animals with a dose of L-NAME or 7-NI that produced a high level of inhibition of striatal NOS activity. In contrast, under these treatment conditions, the loss of striatal enkephalinergic neurons was not influenced by L-NAME but was partially attenuated by pretreatment of animals with 7-NI. Thus, inhibition of NOS exerted a differential action on the two neuron populations.

The observed high sensitivity of striatal NADPH diaphorase neurons to low dose QUIN is in agreement with the findings of previous investigators who reported that the number of these neurons is markedly reduced following local exposure to QUIN [8,16,17], but is in conflict with the findings of Beal et al. [4–6] who reported a relative sparing of NADPH diaphorase neurons. The present study also showed that at relatively low QUIN doses, that are toxic to the NADPH diaphorase neurons, the enkephalinergic neurons are also affected. This is in agreement with the findings of Roberts et al. [55], who reported that both neuron types were depleted to the same extent in the lesion core. However, these investigators also reported that the enkephalinergic neurons were depleted to a greater extent than the NADPH diaphorase neurons in an area adjacent to the lesion core—the transition zone. It was suggested that a larger transition zone was present with enkephalinergic neurons and that methods used to quantitate cells in the transition zone may explain the discrepancy regarding the relative sparing of NADPH diaphorase neurons of the striatum [55]. A major difference between the present study and previous studies of Beal et al. [4] and Roberts et al. [55] is the dose of QUIN injected into the striatum. In earlier work, a dose of 120 nmol QUIN was employed, while in the present study, doses ranging between 6 and 24 nmol were used. Indeed, at the 24 nmol dose, QUIN produced a nearly complete depletion of both neuron populations within the lesion core.

To investigate the role of NO in QUIN-induced loss of striatal NADPH diaphorase and enkephalinergic neurons, striatal NOS activity was inhibited by pretreatment of animals with L-NAME or 7-NI. Systemic administration of L-NAME inhibited striatal NOS activity by approximately 85%, an effect which was sustained for at least 24 h. The dose-response and time course of the effects of systemic L-NAME are in agreement with previous studies of Connop et al. [14] and Iadecola et al. [29]. In contrast, 7-NI maximally inhibited striatal NOS activity by only 60% [32]. The difference in action of these NOS inhibitors may be due to the selective action of 7-NI on neuronal NOS in vivo.

Pretreatment with either L-NAME or 7-NI-enhanced NADPH diaphorase cell loss in the lesion core produced by intrastriatal injection of low doses (≤ 18 nmol) of QUIN but not by a higher dose (24 nmol). The enhanced toxicity at the low dose of QUIN is in contrast to that of MacKenzie et al. [38,39], who reported that both L-NAME and 7-NI had no significant effect on the depletion of striatal NADPH diaphorase neurons induced by 15, 30 and 60 nmol of QUIN. This difference may be due to counting the entire striatal section by MacKenzie et al. [38], while in the present study, cell counting was restricted to the lesion core. We observed a consistent loss of NADPH-diaphorase neurons following 12 nmol QUIN and both L-NAME and 7-NI clearly increased the cell loss. The results obtained with a higher dose of QUIN (24 nmol) are in agreement with those of MacKenzie et al. [38,39]. However, at this dose, injection of QUIN alone produced a near maximal cell loss in the lesion core and potentiation of toxicity would be difficult to observe.

The finding that NOS inhibitors enhanced the loss of striatal NADPH diaphorase neurons in QUIN injected rats points to a protective role for NO in NMDA receptor-mediated toxicity and is in agreement with earlier studies involving different neuronal populations [14,15,61,63]. Several hypotheses have been put forward to explain the mechanisms involved in the protective role of NO. One involves a direct NO-mediated inhibition of NMDA receptor function [37,41]. These authors suggest that NO produced following NMDA receptor activation feeds back to inhibit the NMDA receptor as an autoregulatory response. It has also been reported that NO may be neuroprotective by scavenging free radicals [62]. An increase in intracellular Ca^{+2} resulting from NMDA receptor activation can lead to the activation of proteases and lipases and ultimately free radicals that can contribute to irreversible cell damage [36,45]. The lack of NO synthesis following NOS inhibitIon and resultant loss of scavenging may result in enhanced toxicity. Alternatively by acting in association with divalent cations in particular Zn, NO has been shown to decrease both ionic conductance and the gating process of NMDA channels in cerebellar granule cells [20]. This effect which is independent of the redox, pH or glycine modulatory sites [20] could be responsible for the increased neurotoxicity we observed in the presence of NOS inhibitors. In other studies, NO has been suggested to protect neurons indirectly by producing regional activity-dependent vasodilation in response to NMDA-induced excitation. Under ischemic conditions, the initial production of NO was found to be neuroprotective [63,64] possibly due to its ability to increase collateral blood flow in regions at risk of infarct [64]. The vasodilatory response

would satisfy the increased energy requirements of excited neurons [29]. Loss of activity-dependent vasodilation by inhibition of NOS would result in compromised metabolism of neurons undergoing excitation and this phenomenon may underlie the potentiation of toxicity observed following NMDA receptor activation [14,15,27]. In the present study, L-NAME and 7-NI augmented the loss of NADPH diaphorase neurons following injections of low dose QUIN, while cell loss resulting from exposure to higher doses of QUIN was unaffected.

The fact that both L-NAME and 7-NI potentiated QUIN-induced loss of NADPH diaphorase neurons but not of enkephalinergic neurons is of interest as, in both cases, the neurotoxic response was NMDA receptor-mediated. This finding suggests that these two types of striatal neurons respond differently to NO. Lipton et al. [37] has proposed that the redox state may play a role in the neurotoxic or neuroprotective action of NO. A potential difference in the redox milieu within NADPH diaphorase and enkephalinergic neurons could underlie the different action of NOS inhibitors on NMDA-mediated toxicity in these neuron populations. Since NADPH diaphorase neurons are NOS-containing cells and thus produce NO following excitation, they may preferentially form NO^+, which could feed back to the NMDA receptor on these neurons. In addition, superoxide dismutase (SOD) is protective against excitotoxicity and levels of SOD have been reported to be higher in striatal NOS containing neurons [30]. However, Medina et al. [44] reported that striatal cholinergic and parvalbumin-containing neurons, but not somatostatin positive (co-localized with NADPH diaphorase) neurons have high levels of SOD. Since SOD is neuroprotective, its differential distribution may explain the differential sensitivity of neurons to the toxic properties of NO.

In addition to different cellular constituents, different NMDA receptor subtypes on the NADPH diaphorase and enkephalinergic neurons may explain their vulnerability to NO. Messenger RNA (mRNA) for the NMDAR1 receptor subunit was reported to be high in NOS-containing cells [53] although others have reported low levels of this subunit in these neurons [2,35]. Landwehrmeyer et al. [35] reported that levels of NMDAR2B mRNA were lower in somatostatin- than enkephalin-positive neurons and that the NMDAR2D mRNA was expressed in somatostatin but not in enkephalinergic neurons. In addition, NO may modulate different splice variants of the NMDA receptor indirectly, via inhibition of protein kinase C. Landwehrmeyer et al. [35] reported that NADPH diaphorase and cholinergic neurons lack one or both of the alternatively spliced carboxy-terminal regions of the NMDAR1 receptor subunit. Since alternative splicing of the NMDAR1 mRNA regulates phosphorylation of the NMDA receptor by protein kinase C [59], NO could modulate NMDA receptor function through inhibition of protein kinase C [28,48]. Thus, NO may alter NMDA receptor function differently depending on which receptor subunit or splice variant is expressed by a particular group of neurons.

The present study shows that under similar conditions of NOS inhibition, neurons show qualitative differences in sensitivity to NMDA receptor activation. The basis for this difference remains to be explored in future work

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