Blockade of Neuronal Nitric Oxide Synthase Protects against Excitotoxicity *in vivo*

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Nitric oxide may be a key mediator of excitotoxic neuronal injury in the central nervous system. We examined the effects of the neuronal nitric oxide synthase inhibitor 7-nitroindazole (7-NI) on excitotoxic striatal lesions. 7-NI significantly attenuated lesions produced by intrastriatal injections of NMDA, but not kainic acid or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). 7-NI attenuated secondary striatal excitotoxic lesions produced by the succinate dehydrogenase inhibitor malonate, and the protection was reversed by L-arginine but not by D-arginine. 7-NI produced nearly complete protection against striatal lesions produced by systemic administration of 3-nitropropionic acid (3-NP), another succinate dehydrogenase inhibitor. 7-NI protected against malonate induced decreases in ATP, and increases in lactate, as assessed by ^{1}H magnetic resonance spectroscopy. 7-NI had no effects on spontaneous electrophysiologic activity in the striatum *in vivo*, suggesting that its effects were not mediated by an interaction with excitatory amino acid receptors. 7-NI attenuated increases in hydroxyl radical, 8-hydroxy-2-deoxyguanosine and 3-nitrotyrosine generation *in vivo*, which may be a consequence of peroxynitrite formation. The present results implicate neuronal nitric oxide generation in the pathogenesis of both direct and secondary excitotoxic neuronal injury *in vivo*. As such they suggest that neuronal nitric oxide synthase inhibitors may be useful in the treatment of neurologic diseases in which excitotoxic mechanisms play a role.

[*Key words: nitric oxide synthase, excitotoxicity, 7-nitroindazole, nitrotyrosine, free radicals, Huntington's disease*]

The role of nitric oxide (NO˙) in neuronal injury is an area of intense investigation. The initial studies of Dawson and colleagues implicated NO˙ in excitotoxic cell death following the activation of NMDA receptors (Dawson et al., 1991). Subsequently inhibition of NOS was reported to show protection in a model of cerebral ischemia *in vivo* (Nowicki et al., 1991). These findings; however, have been controversial since some authors found no protection. The absence of consensus may be due to the prior lack of NO˙ synthase inhibitors with specificity for various isoforms of the enzyme. Strong evidence for a role of neuronal nitric oxide synthase (NOS) in focal ischemia has come from studies in mice with a knockout of the neuronal isoform of NOS. These mice show a significant attenuation in the size of focal ischemic lesions (Huang et al., 1994).

Improved inhibitors of NOS have recently been described. One of these is 7-nitroindazole (7-NI) which is a relatively specific inhibitor of the neuronal isoform of NOS *in vivo*. Although *in vitro* studies suggest that it inhibits both endothelial and neuronal NOS, *in vivo* studies showed no effects on blood pressure, on endothelium-dependent blood vessel relaxation and on ACh induced blood vessel relaxation (Babbedge et al., 1993; Moore et al., 1993; Wolff and Gribin, 1994; Yoshida et al., 1994). 7-NI is efficacious against focal ischemic lesions *in vivo* (Yoshida et al., 1994), and we found that it blocks MPTP neurotoxicity *in vivo* (Schulz et al., 1995b).

In the present study we examined the hypothesis that neuronal NOS plays a role in excitotoxicity *in vivo*. We examined whether 7-NI can block striatal lesions produced by NMDA, kainic acid or AMPA. We also investigated its effects on secondary excitotoxic lesions produced by the reversible succinate dehydrogenase inhibitor malonate, and the irreversible succinate dehydrogenase inhibitor 3-nitropropionic acid. To evaluate the mechanism of neuroprotection we investigated the effects of 7-NI on spontaneous striatal electrophysiologic activity, and ATP depletions produced by malonate. We also investigated its effects on NMDA and 3-NP induced hydroxyl (˙OH) radical generation, increases in 8-hydroxy-2-deoxyguanosine a marker of oxidative damage to DNA, and increases in 3-nitrotyrosine, which may be a consequence of peroxynitrite formation (Beckman et al., 1990, 1992; Crow et al., 1994).

Materials and Methods

Chemicals. Malonate, NMDA, kainic acid (KA), L-arginine, D-arginine, nitroblue tetrazolium, and peanut oil were obtained from Sigma (St. Louis, MO), α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) from Research Biochemicals (Natick, MA), 3-nitropropionic acid (3-NP) from Aldrich (Milwaukee, WI), and 7-NI from Cookson Chemical Ltd. (Southampton, UK).

Stereotaxic lesion technique. Male Sprague–Dawley rats (Charles River, Cambridge, MA) weighing 300–325 gm were anesthetized with

pentobarbital (50 mg/kg i.p.) and positioned in a David Kopf stereotaxic instrument with the incisor bar set at 3.3 mm below the interaural line. Malonate, NMDA, KA, and AMPA were dissolved in 0.1 M phosphate-buffered saline (pH 7.4). Intrastriatal injections were made with a 10 μl Hamilton syringe fitted with a 26 gauge blunt-tipped needle into the left striatum at the coordinates 0.5 mm anterior bregma, 2.6 mm lateral to the midline, and 5 mm ventral to the dura. Injection volumes were 1 μl (5 nmol KA, 200 nmol NMDA, and 30 nmol AMPA), and 1.5 μl (3 μmol malonate). All injections were made over 1 min and the needle was left in place for an additional 1 min before being slowly withdrawn. 7-NI was dissolved and sonicated in peanut oil at a concentration of 10 mg/ml. Animals were treated with peanut oil (vehicle) or 50 mg/kg of 7-NI were administered i.p. 0.5 hr before striatal injections of malonate, NMDA, KA, and AMPA. The ability of 300 mg/kg i.p. of L-arginine and D-arginine coinjected with 7-NI, 0.5 hr before striatal injections to block the neuroprotective effects of 7-NI on striatal malonate lesions was also examined. Since this paradigm did not show any protective effects for NMDA, KA, and AMPA injections as compared to controls, in a second experiment two doses of 50 mg/kg of 7-NI were administered i.p. at 4 hr and 0.5 hr before striatal injections of NMDA, KA, and AMPA. An identical protocol was used to assess the effects of 7-NI on neurochemical measurements following intrastriatal injections of saline, NMDA or KA. Initial results showed that 7-NI significantly decreased body temperature in rats under pentobarbital anesthesia measured 1 hr after administration of 50 mg/kg 7-NI i.p. (35.9 ± 0.2°C vs 36.5 ± 0.1°C, $n = 10$, $p < 0.02$). Since hypothermia may produce neuroprotection, the body temperature was maintained at 37.5°C in an incubator as long as the animals were anesthetized. Temperature was monitored using a rectal thermister. Nine to 10 animals were used per group in each experiment.

Quantification of lesion volume. One week after the striatal injections, animals were sacrificed by decapitation and the brains were rapidly removed, placed in cold saline for 10 min, and sectioned coronally into slices at 2 mm intervals. Slices were stained in 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC, Sigma, St. Louis, MO) solution at room temperature in the dark for 30 min followed by fixation in phosphate-buffered 4% paraformaldehyde (Bederson et al., 1986). The lesioned area (noted by pale staining) was measured on the posterior surface of each section using an Apple Macintosh based image analysis system [Sony color video camera; software: COLORSNAP (Computer Friends Inc., Portland, OR) and IPLAB SPECTRUM (Signal Analytics, Vienna, VA)]. The lesions were evaluated by an experienced histologist blinded to the experimental conditions. We previously verified the reliability of the TTC measurements in animals injected with malonate on adjacent sections stained with either TTC or Nissl stain (Schulz et al., 1995a).

Systemic 3-NP treatment. 3-NP was diluted in water and adjusted to pH 7.4 with NaOH and administered at a dose of 10 mg/kg i.p. every 12 hr. With this dosing regimen the animals become acutely ill after 4–5 d and show large striatal lesions (Beal et al., 1993b). At the same time points 25 mg/kg of 7-NI dissolved in peanut oil at a concentration of 10 mg/ml or vehicle alone (peanut oil) was administered s.c. Since there was variability in the times at which animals became ill, they were clinically examined 3 hr after the injections and one animal of each group was sacrificed when an animal was acutely ill, regardless of whether it was a vehicle treated control or a 7-NI treated animal. In an initial experiment 12 animals per group were studied. In this experiment following sacrifice striatal lesion size was assessed by TTC staining.

In a second experiment five animals per group were studied with the same treatment paradigm for histology, and five animals received saline every 12 hr for 4–5 d. Three animals were treated with 7-NI alone every 12 hr for 5 d as an additional control. A third group of five animals was treated with more prolonged administration of 3-NP and 7-NI: these animals were treated with 3-NP and 7-NI identically to animals of the other 7-NI treated group, but were not sacrificed when vehicle treated animals became ill but only later, when they showed symptoms. In this experiment animals were deeply anesthetized and transcardially perfused with ice cold saline, followed by phosphate buffered 4% paraformaldehyde (Beal et al., 1991). Brains were sectioned at 50 μm intervals on a freezing microtome.

Enzyme histochemistry. Free floating sections were stained, using a modification of the direct method of Vincent and Johansson (1983) for demonstrating nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH-d). Tissue sections were incubated at 37°C and inter-

mittently monitored for intensity for 1–3 hr in a solution of 10 ml 0.1 M Tris HCl buffer (pH 7.4) containing 4 mg NADPH and 10 mg nitroblue tetrazolium salt (NBT). Increased intensity of reaction product was achieved by the addition of 0.8% Triton. Sections were double-labeled, using a combined technique for NADPH-d enzyme histochemistry and Nissl staining. Heat treated tissue sections at 60°C for 2 hr or incubating sections with NBT alone served as controls for specificity of enzyme activity.

Immunocytochemistry. Immunocytochemistry was performed on 50 μm thick tissue sections, using a conjugated second antibody method. The antisera used include those against 3-nitrotyrosine (monoclonal clone 1A6), courtesy of Dr. Joseph Beckman, used at a dilution of 1:100 for free floating sections (Beckman et al., 1994); and 8-hydroxy-2-deoxyguanosine, courtesy of Dr. Bruce Ames, used at a dilution of 1:750 (Park et al., 1992). Preabsorption of antibodies with 3-nitrotyrosine and 8-hydroxy-2-deoxyguanosine respectively abolished staining. The procedure was as follows: the tissue sections were preincubated in absolute methanol 0.3% hydrogen peroxide solution for 30 min, washed (3×) in phosphate-buffered saline (PBS) (pH 7.4) 10 min each, placed in 10% normal goat serum (GIBCO Labs) for 1 hr, incubated free floating in primary antiserum at room temperature for 12–18 hr (all dilutions of primary antisera above included 0.3% Triton X-100 and 10% normal goat serum), washed (3×) in PBS for 10 min each, placed in periodate-conjugated goat anti-rabbit IgG (1:300 in PBS) (Boehringer-Mannheim), washed (3X) in PBS 10 min each, and reacted with 3,3′ diaminobenzidine HCl (1 mg/ml) in Tris HCl buffer with 0.005% hydrogen peroxide.

Blood pressure and heart rate measurements. Male Sprague–Dawley rats weighing 300–325 gm were anesthetized with pentobarbital and 22 gauge angiocatheters (Becton Dickinson, Sandy, UT) were placed in the femoral artery for arterial blood pressure measurements (ETH-400 transducer-amplifier, AD Instruments, Milford, MA). After recording baseline measurments 50 mg/kg of 7-NI in peanut oil was injected i.p. at time 0 and blood pressure and heart rate were recorded for 180 min.

Salicylate assay, 3-nitrotyrosine, and 8-hydroxy-2-deoxyguanosine measurements. The salicylate hydroxyl trapping method (Floyd et al., 1984; Hall et al., 1993) was used for measuring ·OH radicals in striatal tissue following intrastriatal injection of NMDA and KA and i.p. administration of 3-NP. Male Sprague–Dawley rats weighing 300 gm ($n = 8$ per group) received intrastriatal injections of saline, NMDA, or KA as described above. Animals were treated with 7-NI (50 mg/kg) i.p. at 0.5 hr before and at the time of striatal injections. Salicylate (150 mg/kg i.p.) was administered at the time of the striatal injections.

In the 3-NP experiments rats weighing 125–150 gm ($n = 10$ per group) were injected i.p. every 12 hr for three doses with either (1) saline, (2) 20 mg/kg of 3-NP and peanut oil vehicle, or (3) 20 mg/kg of 3-NP and 25 mg/kg of 7-NI dissolved in peanut oil. At 1.5 hr after the last injection of 3-NP or saline, salicylate (150 mg/kg) was administered intraperitoneally. Sixty minutes later the animals were sacrificed and the right and left striata were rapidly dissected from a 2 mm thick slice on a chilled glass plate, and placed in 0.5 ml chilled 0.1 M perchloric acid. The samples were sonicated, rapidly frozen, thawed and centrifuged twice. Aliquots of the supernatant were stored at −70°C until the time of assay.

Salicylate and its metabolites 2,3 and 2,5 dihydroxybenzoic acid (DHBA) were quantified by HPLC with 16-electrode electrochemical detection (Beal et al., 1990). Salicylate, 2,3 and 2,5 DHBA, tyrosine and 3-nitrotyrosine were measured electrochemically by oxidation at 840, 240, 120, 600, and 840 mV, respectively, with retention times of 20.5, 9.4, 6.3, 10.5, and 18.2 min, respectively. 3-Nitrotyrosine measurements were validated by changing chromatographic conditions, overspiking samples with authentic standards, demonstrating the correct electrochemical signature across 2 electrodes and by treating both samples and standards with 1 M sodium hydrosulfite which converts 3-nitrotyrosine to aminotyrosine eliminating the peak at 840 mV. The data were expressed as the ratio of 2,3 and 2,5 DHBA to salicylate and 3-nitrotyrosine to tyrosine to normalize the DHBA and 3-nitrotyrosine concentrations for differing brain concentrations of salicylate and tyrosine, which could be a consequence of impairment of the blood-brain barrier (salicylate) or neuronal loss during treatment (tyrosine). Eight saline treated and eight 3-NP (20 mg/kg) treated 250 gm male Sprague–Dawley rats were sacrificed at 3 hr and whole brain DNA was extracted and 8-hydroxy-2-deoxyguanosine was measured as previously described (Mecocci et al., 1993).

ATP measurements. The effects of 7-NI on malonate induced de-

creases in striatal ATP concentrations were examined. 50 mg/kg of 7-NI dissolved in peanut oil or peanut oil vehicle were administered i.p. 0.5 hr before intrastriatal injection of 3 μmol malonate. At 3 hr rats were deeply anesthetized and the skull surface was exposed. After decapitation the heads were rapidly frozen in liquid nitrogen and subsequently stored at −70°C. The striata were dissected from a 2 mm slice frontal to and adjacent to the anterior commissure on a freezing cold plate. The corpus callosum, the septal complex, and the anterior commissure were used as dorsal and lateral, medial, and ventral boundaries. ATP was measured by the luciferin-luciferase assay (Lust et al., 1981). Proteins were measured on the pellets using a fluorimetric assay. Eight animals per group were examined.

Localized lactate measurements by ^1H *magnetic resonance spectroscopy.* The effects of intrastriatal injections of 3 μmol malonate on lactate production and the influence of pretreatment with 7-NI on lactate production were assessed *in vivo* using magnetic resonance imaging (Brouillet et al., 1993b). Male Sprague–Dawley rats weighing 275–325 gm received either i.p. injections of peanut oil (vehicle) or 50 mg/kg 7-NI (*n* = 5–6 per group). One h later striatal injections of malonate were made as described above using halothane anesthesia. Rats were imaged under halothane/N_2O/O_2 anesthesia on a 4.7 Tesla GE Omega CSI Imager using a 30 mm bird cage coil at 1.0–2.5 hr after striatal malonate injections. Animals were maintained at normal body temperature by use of a temperature regulated circulating water blanket placed on the body of the animal. Lesion volumes were measured using a T_2-weighted sequence (TR/TE 3200/80 msec) with slice thickness of 2.5 mm, and a field of view of 40 mm. Lesion volumes were measured using a criterion for abnormal tissue of being greater than 2 SDs above the mean signal intensity in the contralateral unaffected striatum. Lactate was measured using single phase encoding two-dimensional (y,ω) water supressed chemical shift imaging sequence with an inversion pulse for lipid supression described earlier (Jenkins et al., 1991; Henshaw et al., 1994). Parameters were TR/TE/TI = 2200/272/208 msec, with a field of view of 35 mm, 16 phase encode steps and a slice thickness of 8 mm. Lactate concentrations were measured in the lesioned striatum by averaging spectra through the striatum. The lactate signal was integrated using the NMR 2 package (New Method Research Inc., Syracuse, NY) and then normalized to the N-acetylaspartate (NAA) signal intensity in the contralateral striatum, which was assumed to be 7 mM in concentration (Birken and Olendorf, 1989; and references therein).

Electrophysiology. Four male Sprague–Dawley rats weighing 300–400 gm were used for electrophysiological studies. Animals were anesthetized with urethane 1.5 gm/kg i.p. and placed in a stereotaxic apparatus with their body temperatures maintained at 37°C by a feedback heating pad. A 0.5 × 0.5 cm craniotomy was made and recording electrodes were placed in the anterior striatum using stereotaxic coordinates and aural monitoring. Following electrode placement the craniotomy was covered with 4 % agar in saline and 30 min were allowed for stabilization before recordings were made. The spontaneous activity of multiple units were recorded extracellularly in the striatum, 1 mm anterior to the bregma and 3 mm lateral to the midline at a depth of 3.5 mm using commercially available tungsten microelectrodes (4 MΩ at 1 kHz, F. Haer, Inc.). The neuronal activity was amplified, broadcast with an audio monitor, displayed on an oscilloscope, and recorded with a desktop computer. Data acquisition and analysis of mean firing rate was performed using DISCOVERY, EXPERIMENTER'S WORKBENCH, and PERSONAL SCIENTIFIC WORKSTATION software (DataWave Technologies, Longmont, CO). We investigated the electrophysiological effects of the NMDA antagonist MK-801 and the NOS inhibitor 7-NI. Initially, baseline spontaneous activity was collected over a 20 min period for 1 sec every 19 sec (60 samples). The animal was then given an i.p. injection of 50 mg/kg 7-NI. After 60 minutes, an additional 20 min of spontaneous neuronal activity was collected from the same recording site in the same manner. Finally, spontaneous activity was recorded after the NMDA channel mediated activity was blocked by injection of MK-801 (4 mg/kg).

Statistical methods. Data are expressed as means ± SEM values. Side-to-side comparisons were made by two-tailed paired *t* test. The statistical significance of differences in lesion volume, DHBA and nitrotyrosine values, lactate production and ATP depletion between groups were determined by unpaired Student's *t* test (two tailed) or one-way analysis of variances (ANOVA) followed by Fisher's PLSD (protected least significant difference) post-hoc test to compare group
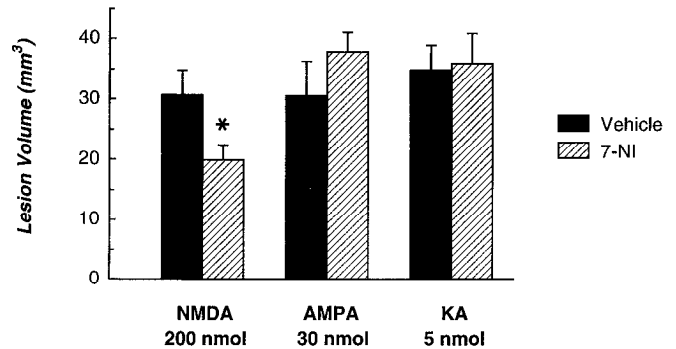

Figure 1. Effects of pretreatment with 7-NI (2 × 50 mg/kg i.p.) on striatal lesions produced by NMDA, AMPA and KA. *p<0.05, (Student's *t* test). Nine to 10 animals were used in each group.

means. The number of animals showing striatal lesions after systemic 3-NP treatment were compared by χ^2 test.

Animal guidelines. All animal use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the local Animal Care Committee.

Results

Stereotaxic lesions. We examined whether pretreatment with the neuronal NOS inhibitor 7-nitroindazole (7-NI) can attenuate striatal excitotoxic lesions produced by the excitatory amino acid receptor agonists NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA), and kainic acid (KA). Pilot experiments showed that a single dose of 7-NI (50 mg/kg i.p.) administered i.p. 0.5 hr before striatal injections produced no protection (200 nmol NMDA 25.0 ± 2.4 mm³ vs 21.3 ± 3.4 mm³ (vehicle vs 7-NI treatment); 30 nmol AMPA 33.6 ± 4.6 mm³ vs 30.4 ± 5.2 mm³; 5 nmol KA 39.5 ± 2.9 mm³ vs 36.3 ± 4.4 mm³; *n* = 9–10). Treatment with two doses of 50 mg/kg 7-NI 4 hr and 0.5 h before the striatal injection of the excitotoxins showed significant protection against lesions produced by 200 nmol NMDA (35%), but no protection against lesions produced by 30 nmol AMPA or 5 nmol KA (Fig. 1).

We and others recently reported that malonate, a reversible inhibitor of succinate dehydrogenase, produces secondary excitotoxic lesions in the striatum (Beal et al., 1993a; Greene et al., 1993; Henshaw et al., 1994). Malonate produces ATP depletions and striatal lesions which are blocked by both competitive and noncompetitive NMDA antagonists (Beal et al., 1993a; Greene et al., 1993; Henshaw et al., 1994). We examined the effects of both 25 mg/kg and 50 mg/kg of 7-NI administered i.p. 0.5 hr before striatal injections of 3 μmol malonate. 7-NI significantly and dose-dependently attenuated the striatal lesions produced by 3 μmol of malonate (Fig. 2, top). Furthermore the administration of L-arginine (300 mg/kg i.p. 0.5 hr before the striatal injection) completely blocked the neuroprotective effects of 7-NI whereas D-arginine had no significant effect (Fig. 2, bottom).

Blood pressure and heart rate. To verify that 7-NI was not having effects on endothelial NOS when administered at 50 mg/kg i.p. blood pressure and heart rate were measured at 30 min intervals for 180 min as shown in Table 1. There were no significant effects on heart rate or blood pressure.

ATP and lactate measurements. A possible mechanism for neuroprotective effects of 7-NI would be to prevent the effects of peroxynitrite which can inhibit mitochondrial function (Bolanos et al., 1994), or which can deplete ATP by activation of poly (ADP-ribose) synthetase (Zhang et al., 1994). As compared

A

B
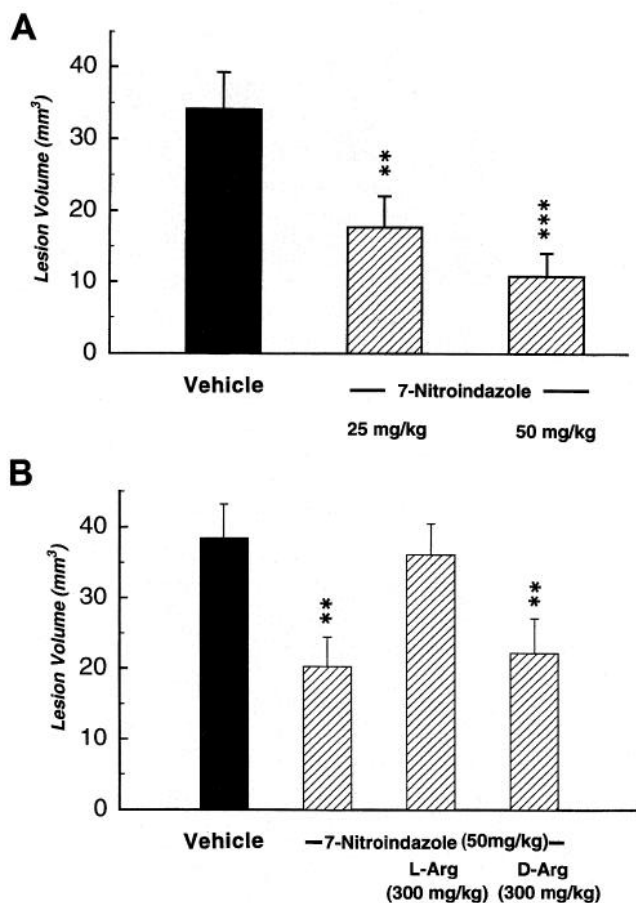
Figure 2. Effects of pretreatment with 7-NI (25 or 50 mg/kg i.p.) on striatal lesions produced by 3 μmol of malonate (top). The effects of L-arginine (*L-Arg*) and D-arginine (*D-Arg*) on the protection produced by 7-NI (50 mg/kg i.p.) are shown at the bottom. **, $p < 0.01$; ***, $p < 0.001$ (ANOVA). Ten animals were used in each group.

Table 1. Blood pressure and heart rate after 7-NI administration

Time (min)	Mean arterial blood pressure (MAP) (mm Hg)	Heart rate (HR) (beats/min)
0	111 ± 3	342 ± 11
30	111 ± 3	330 ± 9
60	111 ± 2	354 ± 9
90	110 ± 3	348 ± 6
120	112 ± 3	358 ± 15
180	108 ± 2	349 ± 10

Data are expressed as means ± SEM ($n = 6$). The 0 min time point is just prior to i.p. injection of 50 mg/kg 7-NI dissolved in peanut oil. ANOVA for repeated measurements did not show a significant change in mean arterial blood pressure or heart rate over time. MAP: $F(5, 5, 25) = 0.285$, $p = 0.917$; HR: $F (5, 5, 25) = 1.364$, $p = 0.2714$.

12 hr completely prevented striatal lesions produced by 3-NP (Fig. 5).

We carried out a similar experiment for histologic evaluation and immunocytochemistry for 8-hydroxy-2-deoxyguanosine and 3-nitrotyrosine. 8-hydroxy-2-deoxyguanosine is a well accepted marker of oxidative damage to DNA (Park et al., 1992; Mecocci et al., 1993), while 3-nitrotyrosine is produced by peroxynitrite-mediated tyrosine nitration (Ischiropoulos et al., 1992). 3-NP (10 mg/kg) was administered every 12 hr. Vehicle and 7-NI treated animals were sacrificed in pairs ($n = 5$). Since the first experiment showed complete protection a second group of 7-NI treated animals was included. These animals were not sacrificed in pairs with the controls, but only later when they became symptomatic. Controls were sacrificed after a median cumulative 3-NP dose of 80 mg/kg (range 70–90 mg/kg), whereas the 7-NI treated animals first showed systemic toxicity after a median cumulative dose of 130 mg/kg (range 90–130 mg/kg, $p < 0.02$, Mann-Whitney U test). The animals treated with 3-NP and vehicle showed extensive bilateral lesions in the striatum. Nissl stains

to vehicle treated animals pretreatment with 50 mg/kg of 7-NI significantly attenuated decreases of striatal ATP concentrations measured 1.5 hr after injections of 3 μmol of malonate (Fig. 3). Localized lactate concentrations were measured *in vivo* using ^{1}H magnetic resonance spectroscopy. There was a profound increase in lactate production 1.5 hr after striatal injection of malonate compared to the contralateral unlesioned striatum (Fig. 3). The increased concentrations of lactate after striatal malonate lesions confirm our earlier reports (Beal et al., 1993a; Henshaw et al., 1994), in which we reported similar values. Pretreatment with 50 mg/kg of 7-NI i.p. 0.5 hr before the striatal lesion significantly attenuated this increase in striatal lactate concentrations (Figs. 3, 4). At 2 hr after striatal injections of malonate in animals treated with 7-NI lesion volumes were significantly attenuated (34.9 ± 11.3 mm³, $n = 6$) as compared with vehicle treated controls (84.0 ± 7.2 mm³, $n = 5$, $p < 0.01$) on T$_2$-weighted MR images (Fig. 4).

Toxicity by systemic 3-NP treatment. Following subacute administration of 3-NP by i.p. injection of 10 mg/kg every 12 hr, rats developed severe dystonic posturing and rigidity by the fourth to fifth day. At these time points basal ganglia lesions were detectable by TTC staining. Vehicle and 7-NI treated animals were sacrificed in pairs at the fourth or fifth day when symptoms developed. Treatment with 25 mg/kg of 7-NI every
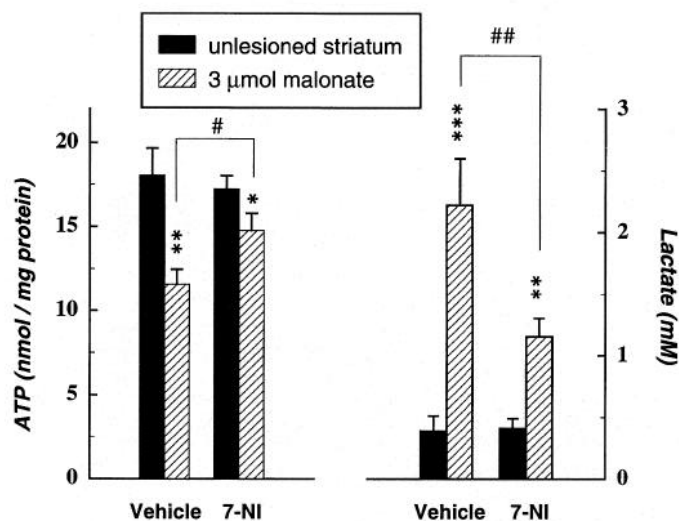

Figure 3. Effects of treatment with 50 mg/kg of 7-NI on ATP depletions at 3 hr ($n = 8$) and lactate increases detected by ^{1}H magnetic resonance spectroscopy at 1.5 hr ($n = 5–6$) induced by malonate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as compared with the unlesioned striata (paired Student's t test). #, $p < 0.05$; ##, $p < 0.01$ (unpaired Student's t test).
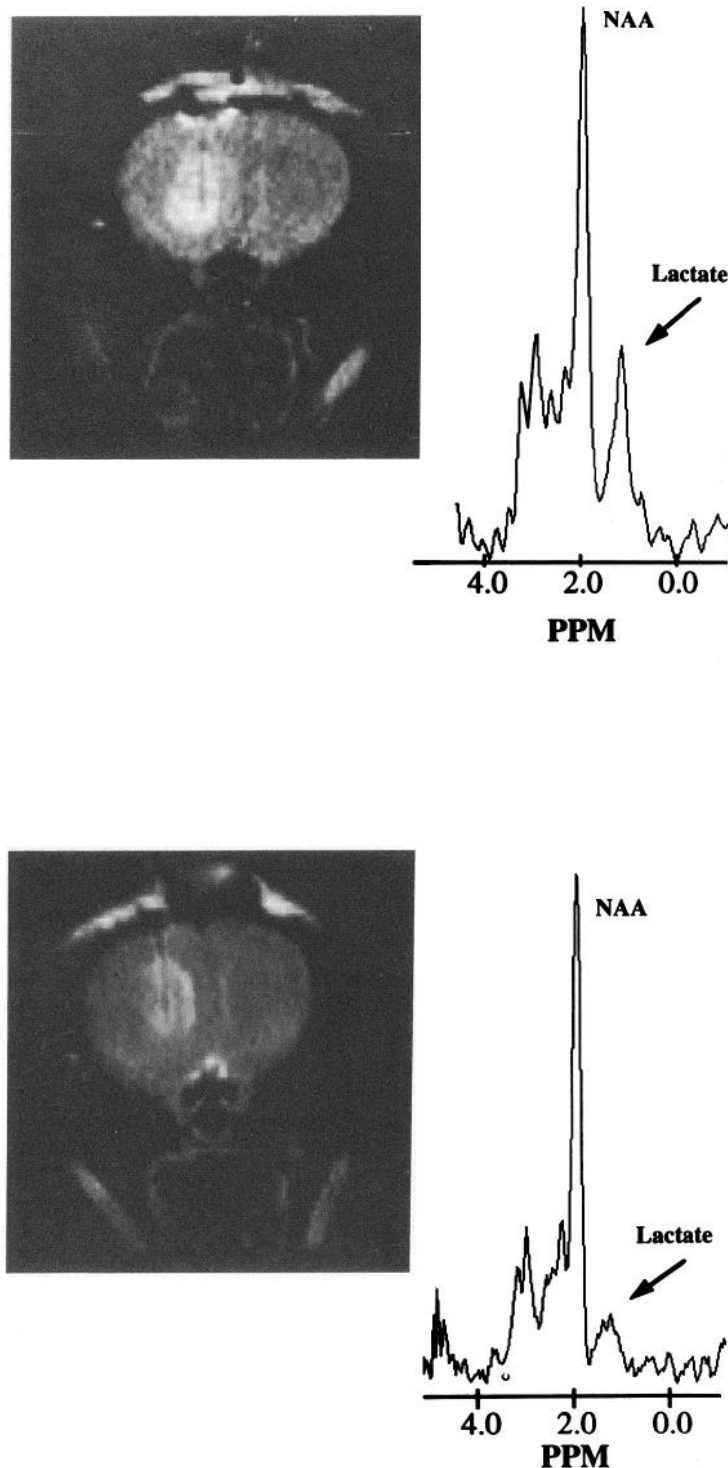
Figure 4. T$_2$-weighted images (TR 3200/TE 80) and striatal proton spectra from two lines of a one dimensional chemical shift imaging sequence (magnitude spectrum TR 2200/TE 272) in a rat 1.5 hr after striatal injection of 3 µmol malonate without (*upper panel*) and with pretreatment of 50 mg/kg of 7-NI (*lower panel*). Rats were imaged on a 4.7-T General Electric Omega CSI Imager with a 30 mm bird-cage coil. *NAA*, N-acetylaspartate; *PPM*, parts per million.

showed loss of neurons with relative sparing of NADPH-diaphorase positive neurons (Fig. 6). In animals concomitantly treated with 3-NP and 7-NI with either dosing regimen, 7-NI completely protected the striatum from lesions and there was no immunoreactivity for either 8-hydroxy-2-deoxyguanosine or 3-nitrotyrosine. Compared to saline treated controls and 7-NI treated 3-NP animals there was a marked increase of 8-hydroxy-2-deoxyguanosine and 3-nitrotyrosine immunoreactivity in vehicle treated 3-NP animals (Fig. 7). Treatment with 7-NI alone had no effect on immunocytochemistry in animals not receiving

3-NP. Biochemical measurements of 8-hydroxy-2-deoxyguanosine confirmed that it was increased from 9.1 ± 3.1 to 44.1 ± 8.0 fmol/µg DNA ($p < 0.001$) following 3-NP treatment.

Measurements of DHBA and 3-nitrotyrosine in 3-NP toxicity. The concentrations of 2,3 and 2,5 DHBA, which are formed by salicylate reacting with ·OH radicals, were significantly increased following intrastriatal injection of NMDA and 2,5 DHBA was increased by KA injections (Fig. 8). 7-NI treatment significantly ($p < 0.05$) attenuated the increase in 2,5 DHBA produced by NMDA. The concentrations of 2,3 and 2,5 DHBA,
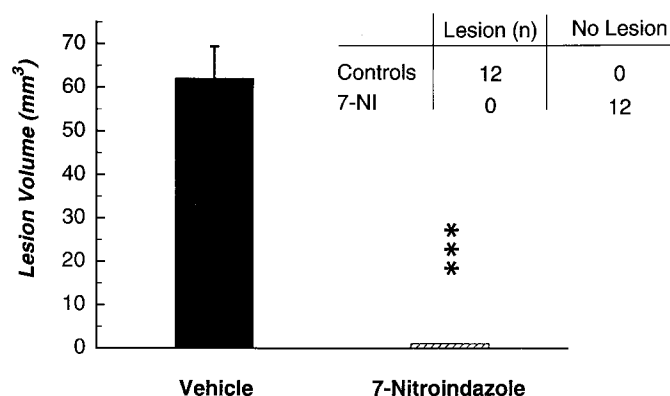
Figure 5. Effects of 7-NI on striatal lesions produced by i.p. injection of 10 mg/kg of 3-NP every 12 hr. Animals were treated with either 25 mg/kg 7-NI or vehicle at the same time points. Animals were sacrificed at the 4th and 5th day. The table gives the number of animals showing a striatal lesion per group ($n = 12, p < 0.001$ by χ^2 test). For calculation of lesion volume the lesions in both hemispheres were combined. ***, $p < 0.001$ (Student's t test). Twelve animals per group were used.

were significantly increased after 3-NP treatment as compared to saline treated controls (Fig. 9). Treatment of 3-NP animals with 7-NI significantly attenuated the increase of 2,3 DHBA. Peroxynitrite, a product of NO• reaction with O_2^-, has recently been defined as a potent oxidant (Beckman et al., 1990) and mediates the nitration of tyrosine (Beckman et al., 1992; Ischiropoulos et al., 1992). Both intrastriatal injection of NMDA and KA siginficantly increased the ratio of 3-nitrotyrosine to tyrosine in the striatum and the increases produced by NMDA but not KA were blocked by 7-NI pretreatment (Fig. 8). 3-NP treatment increased the ratio of 3-nitrotyrosine to tyrosine in the striatum compared to saline treated controls. Treatment with 7-NI significantly attenuated the increase of 3-nitrotyrosine as compared to vehicle treated animals in 3-NP animals (Fig. 9).

Electrophysiology. The effects of 7-NI on spontaneous striatal electrophysiologic activity were compared with those of the NMDA antagonist MK-801 in urethane anesthetized rats, to determine whether 7-NI could antagonize NMDA excitatory amino acid receptors. Spontaneous electrophysiologic activity of neurons returned to normal within 30 min after insertion of an electrode into the striatum. After stabilization the mean spontaneous firing rate in the striatum was recorded (Fig. 10). Sixty minutes after i.p. treatment with 50 mg/kg of 7-NI the mean spontaneous firing rate was not significantly different from the spontanous firing rate of untreated controls. To demonstrate that the NMDA channels were still functional after treatment with 7-NI, animals were then given the NMDA antagonist MK-801. This produced a decrease in mean firing rate which was rapid and profound (Fig. 10), suggesting that the effects of 7-NI were not mediated by an interaction with excitatory amino acid receptors.

Discussion

The role of nitric oxide in neuronal injury both *in vitro* and *in vivo* has been controversial (Dawson and Snyder, 1994). Much of this controversy may be due to the nonspecificity of NOS inhibitors for various isoforms of NOS. Three major isoforms have been indentified which are the constituative neuronal and endothelial forms, and the inducible form produced by macrophages. 7-Nitroindazole has a high degree of specificity for the neuronal isoform of NOS *in vivo* (Babbedge et al., 1993; Moore et al., 1993; Yoshida et al., 1994). It produces no effects on

blood pressure yet it has antinociceptive effects, and it inhibits brain NOS activity (Babbedge et al., 1993; Connop et al., 1994; Yoshida et al., 1994). *In vitro* studies show that it will inhibit both endothelial NOS and macrophage inducible NOS (Wolff et al., 1994), suggesting that compartmentalization or metabolism may contribute to its selectivity *in vivo*. It acts as an inhibitor by competing with L-arginine for binding to the prosthetic heme group of NOS, and it additionally affect the pteridine site of the enzyme (Mayer et al., 1994).

Recent evidence has implicated neuronal NOS in both focal ischemic lesions (Huang et al., 1994; Yoshida et al., 1994), and in MPTP induced toxicity to dopaminergic neurons (Schulz et al., 1995b). In the present study we examined the role of NO• in excitotoxic neuronal injury *in vivo* by determining whether inhibition of neuronal NOS with 7-NI could attenuate lesions produced by NMDA, kainic acid or AMPA. Lesions produced by NMDA were significantly attenuated whereas there was no effect on lesions produced by either kainic acid or AMPA. These results are consistent with those of Dawson and colleagues *in vitro* (Dawson et al., 1991; 1993). They found that nitric oxide synthase inhibitors, and hemoglobin, which binds NO•, could protect against both glutamate and NMDA neurotoxicity, yet there was no effect on kainate toxicity. A small protection was observed against quisqualate, but it was thought that this might relate to a portion of quisqualate toxicity occurring via NMDA receptor activation. Similarly another study showed no effect of NOS inhibitiors on KA and AMPA neurotoxicity *in vitro* (Garthwaite and Garthwaite, 1994). Subsequent studies showed that treatment of cultures with low doses of quisqualate, which preferentially kills nitric oxide synthase containing neurons, blocked glutamate neurotoxicity in cultured cortical and striatal neurons (Dawson et al., 1993). These results have been replicated in some studies (Izumi et al., 1992; Vigé et al., 1993) but not in others (Demerlé-Pallardy et al., 1991; Pauwels and Leysen, 1992; Hewett et al., 1993; Regan et al., 1993; Zinkand et al., 1993; Garthwaite and Garthwaite, 1994) Results *in vivo* were also inconsistent in protecting hippocampal neurons from NMDA neurotoxicity (Haberny et al., 1992; Lerner-Natoli et al., 1992; Moncada et al., 1992).

The present results show that an inhibitor of neuronal NOS can produce significant protection against NMDA mediated excitotoxic lesions, but not against those produced by kainic acid or AMPA, consistent with results *in vitro* (Dawson et al., 1991; Dawson et al., 1993) The selectivity for NMDA mediated excitotoxic lesions may be related to the calcium conductance of the NMDA receptor. The entry of calcium through NMDA receptor channels into cells stimulates NOS by binding to calmodulin, which is a cofactor for NOS (Bredt and Snyder, 1990).

In the present study we provide the first evidence that inhibition of neuronal NOS can attenuate striatal lesions produced by either intrastriatal administration of malonate or systemic administration of 3-NP. We and others found that both malonate and 3-NP produce striatal lesions by a secondary excitotoxic mechanism as a consequence of impairment of energy metabolism (Beal et al., 1993a; Greene et al., 1993; Henshaw et al., 1994). Interestingly 7-NI was much more effective against these secondary excitotoxic lesions than it was against the excitotoxin NMDA. 7-NI produced almost complete protection against 3-NP neurotoxicity, which is the best neuroprotection we have achieved with any compound against this type of lesion (Schulz and Beal, unpublished results). To further investigate the specificity of the effects of 7-NI we showed that L-arginine but not
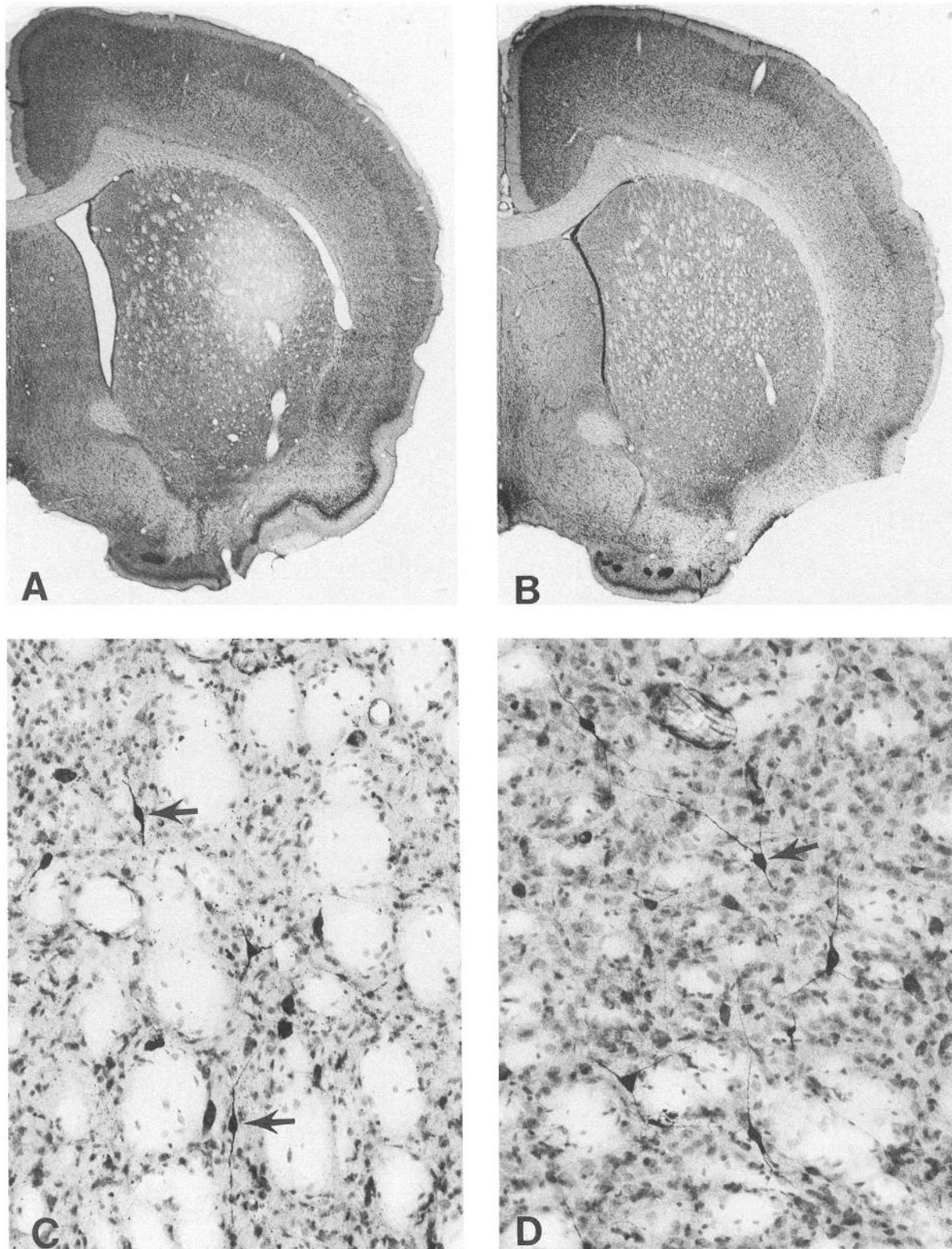
Figure 6. Photomicrographs of the neostriatal neurotoxic lesion as a consequence of the subcutaneous administration of the succinate dehydrogenase inhibitor, 3-NP, in *A* and *C*. Tissue sections are double stained with Nissl and for NADPH-diaphorase. The striatal lesion is represented by staining pallor in the dorso-lateral quadrant (*A*). The lesion is characterized by marked neuronal loss and gliosis with a relative preservation of NADPH-diaphorase neurons (*arrows*) (*C*). Concomitant subcutaneous injection of the nitric oxide inhibitor, 7-NI, with 3-NP resulted in complete protection against striatal lesions (*B* and *D*) as compared to the 3-NP lesion.
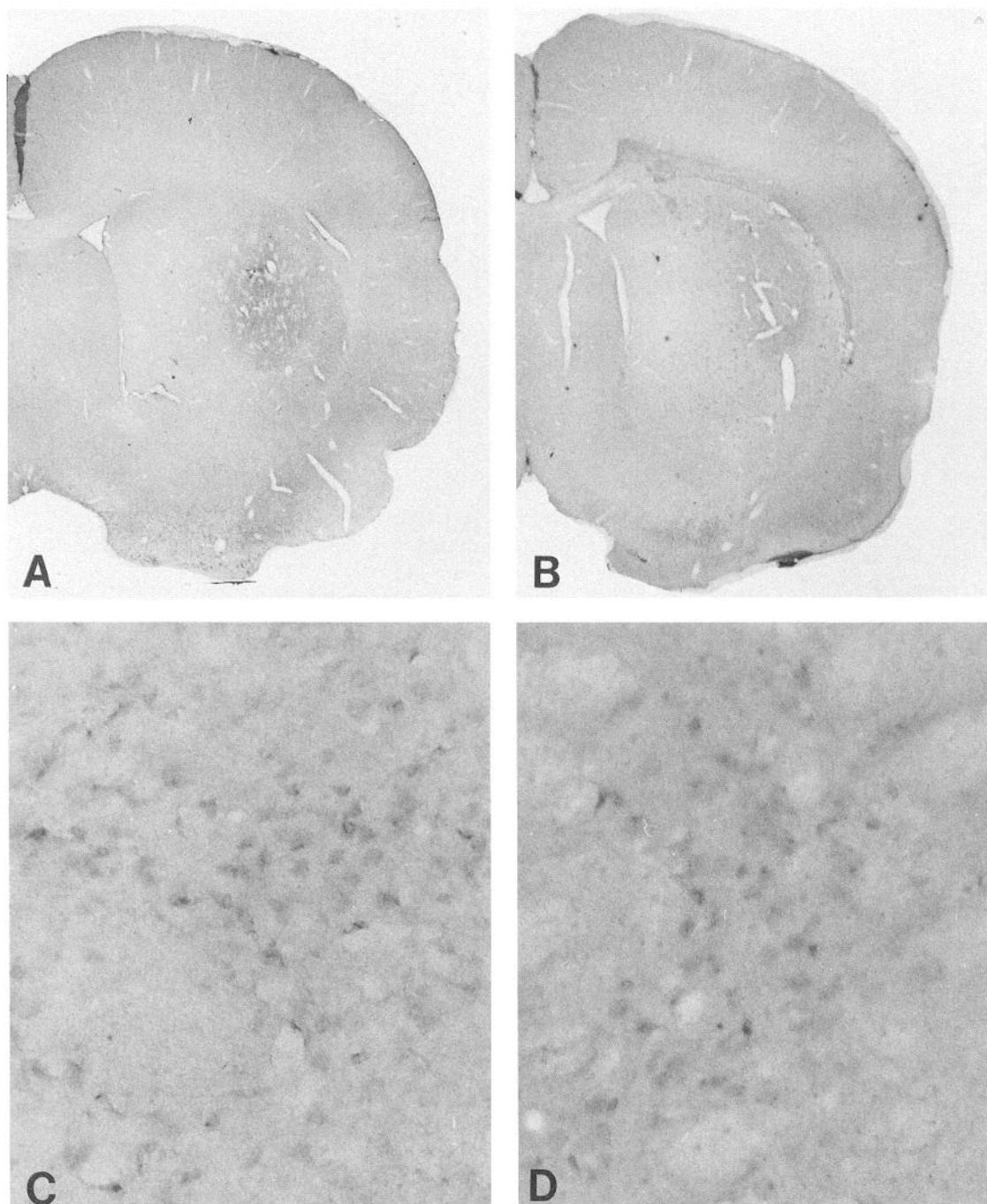
Figure 7. Photomicrograph of 3-NP treated rats which resulted in increased immunocytochemical activity for 8-hydroxy-2-deoxyguanosine in *A* and *C* and nitrotyrosine in *B* and *D*. The increased immunohistochemical activity corresponds to the lesion area observed in Figure 6*A* in both *A* and *B*, with cellular localization in *C* and *D*.

D-arginine would reverse its protective effects against malonate lesions. This is consistent with the findings of Yoshida et al. (1994) in focal ischemic lesions. We also found that 7-NI had no effects on blood pressure and heart rate at the doses used in the present experiments, consistent with a relatively selective effect on neuronal NOS *in vivo*.

We investigated the mechanism of the neuroprotective effects of 7-NI. 7-NI had no effect on spontaneous electrophysiologic activity in rat striatum *in vivo*, while MK-801 inhibited activity. This suggests that 7-NI does not act at NMDA receptors. We

also examined whether 7-NI could attenuate ATP depletions and increases in lactate concentrations produced by intrastriatal administration of malonate. Interestingly 7-NI attenuated both ATP depletions and increases in lactate. The latter result was also confirmed using ^{1}H magnetic resonance spectroscopy *in vivo*. This result contrasts with those of a free radical spin trap which exerted neuroprotective effects but had no effect on malonate induced depletions of ATP (Schulz et al., 1995). One possible explanation for this is that an inhibition of NO˙ production may prevent DNA damage, and activation of poly ADP-ribose poly-
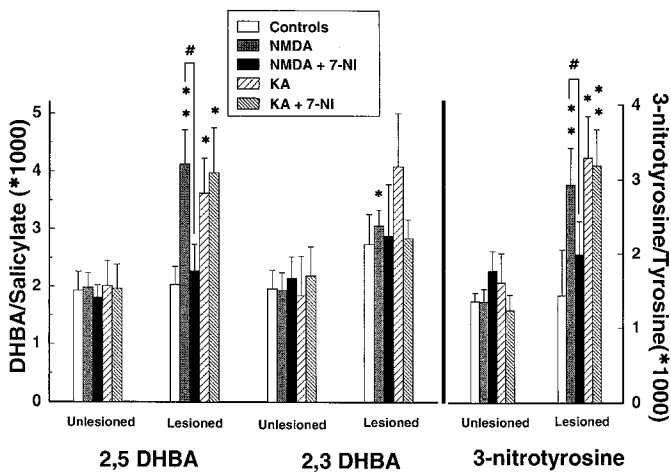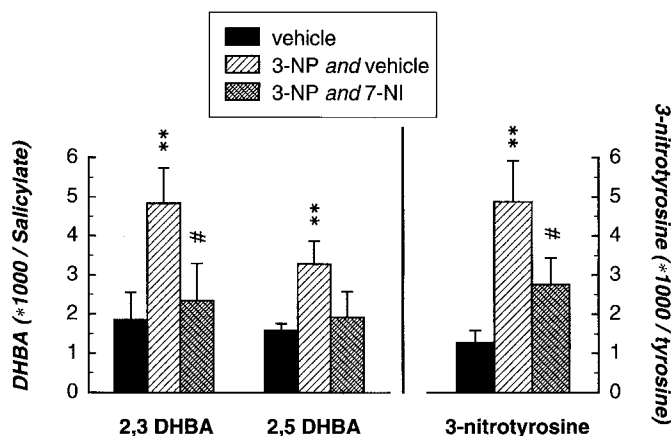
Figure 8. Production of 2,3 and 2,5 DHBA and 3-nitrotyrosine following intrastriatal injection of NMDA and KA and effects of 7-NI. NMDA significantly increased 2,3 and 2,5 DHBA and 3-nitrotyrosine as compared with controls, while KA significantly increased both 2,5 DHBA and 3-nitrotyrosine ($*$, $p < 0.05$; $**$, $p < 0.01$ as compared with saline controls, ANOVA). An increase of 2,3 DHBA produced by KA was not quite significant ($p < 0.07$). 7-NI significantly attenuated increases in 2,5 DHBA and 3-nitrotyrosine produced by NMDA but not by KA (#, $p < 0.05$ as compared with NMDA treated animals, ANOVA).

merase which then leads to both ATP and NAD depletion, followed by cell death (Zhang et al., 1994). Consistent with this possibility we found that 3-NP treatment leads to oxidative damage to DNA as shown by increases in 8-hydroxy-2-deoxyguanosine measurements and 8-hydroxy-2-deoxyguanosine immunohistochemistry. NO$^-$ can inhibit cytochrome c oxidase *in vitro,* and deenergize mitochondria (Bolanos et al., 1994; Cleeter et al., 1994; Schweizer and Richter, 1994). Peroxynitrite also inhibits the mitochondrial respiratory chain in cultured neurons (Bolanos et al., 1995).

We examined the effects of 7-NI on increases in 2,3 and 2,5 DHBA and 3-nitrotyrosine *in vivo.* ·OH radical generation results in an increase in the conversion of salicylate to 2,3 and 2,5

Figure 9. Production of 2,3 and 2,5 DHBA and 3-nitrotyrosine by systemic treatment with 3-NP and effects of 7-NI. Intraperitoneal injections of 3-NP significantly increased concentrations of 2,3 and 2,5 DHBA and of 3-nitrotyrosine ($**$, $p < 0.01$ as compared to saline treated controls, ANOVA). These increases were significantly attenuated by 7-NI (#, $p < 0.05$ as compared to 3-NP and vehicle treated animals, ANOVA). Ten animals were used per group.
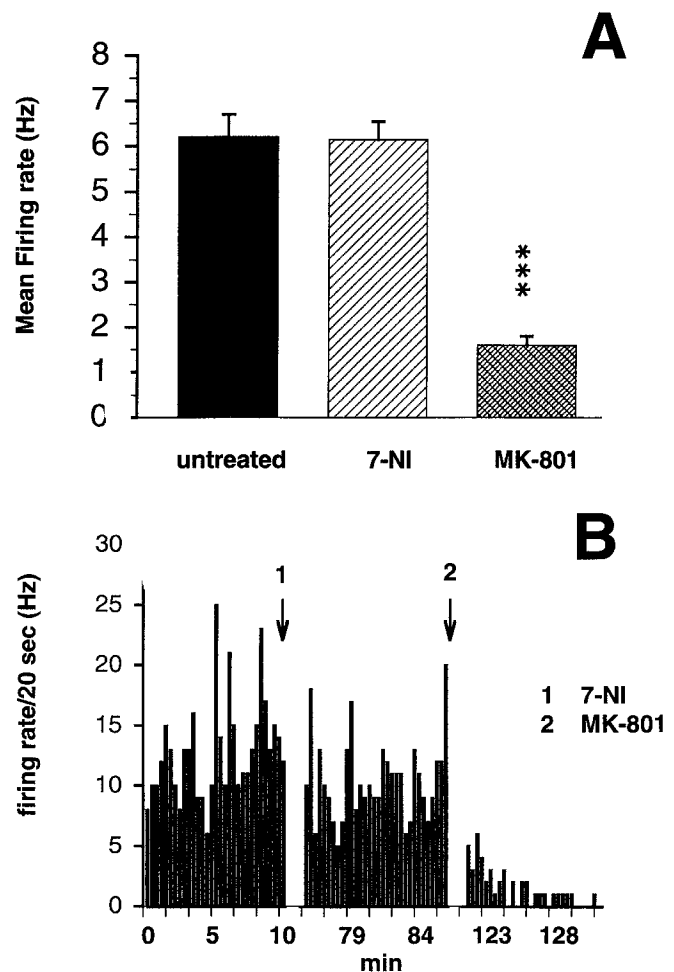
Figure 10. Electrophysiologic effects of 7-NI in the striatum *in vivo.* A, The graph illustrates the mean spontaneous firing rate, after injection of 7-NI (50 mg/kg i.p.), and after injection of MK-801 (4 mg/kg i.p.). Four animals received 7-NI and three of these also received MK-801. The spike counts were averaged. $***$, $p < 0.001$ compared to the spontaneous firing rate and after treatment with 7-NI (ANOVA). B, Actual firing rate of neurons in the striatum from one of these animals. The baseline spontaneous activity was recorded between 0 and 16 min. The first gap in the graph represents 60 min which elapsed from the time when the animal was injected with 7-NI (*arrow 1*). During this gap no change in activity occurred. The second gap indicates the 30 min that passed after administering the MK-801 (*arrow 2*).

DHBA (Floyd et al., 1984; Hall et al., 1993). In the present study we found that both NMDA and KA produced increases in ·OH radical generation and 3-nitrotyrosine. The increases produced by NMDA were attenuated by pretreatment with 7-NI, consistent with its protective effect, whereas 7-NI had no effect on increases produced by KA. The explanation for the lack of an effect of KA induced increases is unclear, but may be due to compartmentalization of calcium fluxes, as suggested *in vitro* (Tymianski et al., 1993). 3-NP produced increases in both 2,3 and 2,5 DHBA/salicylate, which were attenuated by pretreatment with 7-NI. Furthermore we found that 3-NP produced increases in 3-nitrotyrosine concentrations and in immunocytochemical staining for 3-nitrotyrosine which were attenuated by prior treatment with 7-NI.

These results suggest that peroxynitrite may play a role in both NMDA and 3-NP induced neurotoxicity *in vivo.* Peroxynitrite is formed by the reaction of NO$^-$ with superoxide radical

(Beckman et al., 1992; Ischiropoulos et al., 1992). This reaction occurs at an extremely fast rate of 6.7×10^9 M^{-1} sec^{-1} and does not require transition metals. Peroxynitrite may decompose to form ·OH radical, nitrogen dioxide, and nitronium ions which can nitrate tyrosines (Beckman et al., 1990, 1992; Ischiropoulos et al., 1992; Crow et al., 1994; van der Vliet et al., 1994). Our findings that both NMDA and 3-NP produce an increase in ·OH radical generation and in 3-nitrotyrosine are therefore consistent with a role of peroxynitrite in NMDA and 3-NP neurotoxicity. The histologic results showing that 3-NP produces increased staining for 8-hydroxy-2-deoxyguanosine, a marker of OH· radical damage to DNA, and 3-nitrotyrosine also suggests a role of peroxynitrite in the pathogenesis of the lesions.

The present results provide evidence that NO· plays a role in NMDA, malonate and 3-NP neurotoxicity *in vivo*. We recently reported that 7-NI protects against MPTP neurotoxicity in mice, which is a model for Parkinson's disease (PD) (Schulz et al., 1995b). Similarly lesions produced by malonate and 3-NP in both rats and non-human primates closely resemble the histologic, neurochemical and clinical features of HD (Beal et al., 1993a,b; Brouillet et al., 1995; Greene et al., 1993; Henshaw et al., 1994). The present results implicate NO· in the pathogenesis of these lesions. As such these results suggest that treatment with specific inhibitors of neuronal NOS may prove efficacious in the treatment of neurodegenerative diseases, such as HD and PD.

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