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Mechanisms of Nitric Oxide–mediated Neurotoxicity in Primary Brain Cultures

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In addition to mediating several physiological functions, nitric oxide (NO) has been implicated in the cytotoxicities observed following activation of macrophages or excess stimulation of neurons by glutamate. We extend our previous observations of glutamate-stimulated, NO-mediated neurotoxicity in primary cultures of rat fetal cortical, striatal, and hippocampal neurons. Neurotoxicity elicited by either NMDA or sodium nitroprusside (SNP) exhibits a similar concentration–effect relationship and time course. The concentration–effect curve of NMDA-induced neurotoxicity is shifted to the right in the presence of nitro-L-arginine and farther to the right in arginine-free media. The rank order of potency of several NO synthase (NOS) inhibitors in preventing neurotoxicity is the same as the rank order of these compounds in inhibiting NOS, and this inhibition is stereospecific. NMDA neurotoxicity is also prevented by flavoprotein inhibitors and calmodulin inhibitors, fitting with the roles of flavoproteins and calmodulin as NOS regulators. 8-Bromo-cGMP and guanylyl cyclase inhibitors do not affect neurotoxicity, while superoxide dismutase attenuates neurotoxicity. NOS neurons appear to be the source of neurotoxic NO in culture, as lesions of these neurons with 20 μM quisqualate diminish subsequent NMDA neurotoxicity. Moreover, NMDA neurotoxicity develops over time in culture coincident with the expression of NOS. Immunohistochemical localization of NOS in cultures and intact brain demonstrates widespread distribution of the cell processes suggesting that NOS neurons contact the majority of cortical neurons and so could mediate widespread neurotoxicity.

[Key words: NADPH-diaphorase, glutamate, NMDA, nitric oxide synthase, excitotoxicity, neurodegeneration]

Besides its roles as endothelial-derived relaxing factor (Furchgott, 1990; Ignarro, 1990; Moncada et al., 1991) and a mediator of macrophage cytotoxicity (Hibbs et al., 1987; Marletta, 1989; Ignarro, 1990), nitric oxide (NO) appears to be a neuronal messenger in the brain and PNS, satisfying many criteria of a neurotransmitter (Garthwaite, 1991; Snyder and Bredt, 1991; Dawson et al., 1992; Snyder, 1992). NO synthase (NOS) is localized to the same discrete neuronal populations in the brain that stain for NADPH-diaphorase (T. M. Dawson et al., 1991; Hope et al., 1991), a histochemical marker for neurons that resist certain forms of neurotoxicity (Beal et al., 1986; Koh et al., 1986; Koh and Choi, 1988). NOS catalytic activity accounts for NADPH-diaphorase staining, as transfection of human kidney 293 cells with NOS cDNA provides NADPH-diaphorase and NOS staining in the same proportions as in neurons (T. M. Dawson et al., 1991).

Glutamate neurotoxicity elicited via NMDA subtypes of glutamate receptors appears to mediate much of the neurotoxicity in focal ischemia, as NMDA antagonists block such neurotoxicity (Choi, 1988, 1990). Glutamate neurotoxicity may also play a role in neurodegenerative diseases such as Alzheimer's and Huntington's diseases (Choi, 1988; Meldrum and Garthwaite, 1990). At NMDA receptors, glutamate triggers the opening of cation-permeable channels. The entry of calcium through these channels into cells stimulates NOS activity (Bredt and Snyder, 1989) by binding to calmodulin, which is a cofactor for NOS (Bredt and Snyder, 1990).

Recently we demonstrated that NMDA neurotoxicity is attenuated in primary cerebral cortical cultures by the coapplication of NOS inhibitors or removal of the precursor of NO, L-arginine (V. L. Dawson et al., 1991; T. M. Dawson et al., 1993). These protective effects are reversed by addition of excess L-arginine. In addition, sodium nitroprusside (SNP), which generates NO, mimics NMDA-induced neurotoxicity. Both SNP and NMDA neurotoxicities are also blocked by hemoglobin, which binds NO. Together, these results implicate NO as a potential mediator of NMDA neurotoxicity. In the present study we examine the detailed mechanisms regulating NO mediation of glutamate neurotoxicity.

Materials and Methods

Cell cultures

Primary cell cultures were prepared from fetal rats (gestation day 14 for cortex and caudate-putamen cultures, gestation day 17 for hippocampal cultures). The various brain regions were dissected under a microscope, incubated for 20 min in 0.027% trypsin/saline solution (5% phosphate-buffered saline, 40 mM sucrose, 30 mM glucose, 10 mM HEPES, pH 7.4), and transferred to modified Eagle's medium (MEM), 10% horse serum, 10% fetal bovine serum, 2 mM glutamine. Cells were dissociated by trituration, counted, and plated in 15 mm multiwell (Nunc) plates

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coated with polyornithine at a density of 3–4 × 10^5 cells per well. Four days after plating, the cells were treated with 10 μg/ml of 5-fluoro-2'-deoxyuridine for 3 d to inhibit proliferation of non-neuronal cells. Cells were maintained in MEM, 5% horse serum, 2 mM glutamine in 8% CO_2, humidified, 37°C atmosphere. The medium was changed twice a week. Mature neurons (more than 21 d in culture) were used in all experiments except for the experiments examining the ontogeny and development of NMDA neurotoxicity in relation to NMDA currents and expression of NOS. In the mature cultures the percentage of neurons is approximately 70–90% of the total number of cells as assessed by neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) immunocytochemical staining of neurons and astrocytes, respectively (V. L. Dawson, T. M. Dawson, D. A. Bartley, G. R. Uhl, and S. H. Snyder, unpublished observations).

Cytotoxicity

Cells were exposed to test solutions as previously described (V. L. Dawson et al., 1991). The cells were washed three times with a Tris-buffered control salt solution (CSS) containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM Tris-HCl, and 15 mM glucose, pH 7.4 at room temperature. Except for exposures to kainate, all other drug solutions were applied to the cells for 5 min and then washed away with CSS replaced with MEM containing 21 mM glucose and the cells were put back in the incubator. Exposures to kainate were performed in MEM, 21 mM glucose for 24 hr in the incubator. Twenty to twenty-four hours after exposure to drug solutions the cells were exposed to 0.4% trypan blue in CSS to stain the residue of nonviable cells. Two to four photoprints at 10–20× were made of each well. Viable versus nonviable cells were counted, with approximately 500–1500 cells counted per well. At least two separate experiments utilizing four separate wells were performed so that a minimum of 4000–12,000 neurons were counted for each data point. Ten percent of the photomicrographs were counted by an additional observer blinded to the arrangement of photomicrographs, study design, and treatment protocol. An inter-rater reliability of greater than 90% was consistently observed for the cell counting. In some of the experiments photomicrographs were made before and after treatment using a transparent grid at the bottom of each culture plate. Viable and nonviable neurons in identical fields were counted by an observer blinded to study design and treatment protocol. Under the conditions used for assessment of neurotoxicity, no appreciable loss of neurons after the various treatment conditions was identified when comparing "before" and "after" photomicrographs. Furthermore, there was no significant difference between the results obtained with either method of assessing neurotoxicity.

Electrophysiology

Cortical neurons in sister cultures were voltage clamped by whole-cell patch clamp as previously described (Hamill et al., 1981). Cells were bathed in an extracellular solution containing 137 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.3. Micropipettes were pulled from 1.5 mm fiber-fill capillary tubing and filled with an intracellular solution containing 140 mM KCl, 2 mM $MgCl_2$, 11 mM EGTA, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.2. Micropipette tip internal diameters were approximately 1 μm with resistances of approximately 7 MΩ. The target cell was microsuperfused (Demirgoren et al., 1991) with extracellular solution containing 0.3 μM tetrodotoxin with or without 200 μM NMDA, 10 μM glycine. Recordings were made in the whole-cell voltage-clamp mode (Hamill et al., 1981) using a List EPC-7 amplifier (Adams and List Associates, Ltd., Great Neck, NY). Signals were recorded simultaneously on an FM tape recorder and a Gould strip chart recorder.

Diaphorase staining

Cells were washed three times with CSS and fixed for 30 min at 4°C in a 4% paraformaldehyde (PF), 0.1 M phosphate buffer (PB). The PF solution was washed away with Tris-buffered saline (TBS) 50 mM Tris-HCl, 1.5% NaCl, pH 7.4. The reaction solution containing 1 mM NADPH, 0.2 mM nitroblue tetrazolium, 0.2% Triton X-100 (TX-100), 1.2 mM sodium azide, 0.1 M Tris-HCl, pH 7.2, was applied to the fixed cell cultures for 1 hr at 37°C. The reaction was terminated by washing away the reaction solution with TBS. All diaphorase-positive cells in each well were counted utilizing an inverted microscope.

NOS immunohistochemistry.

Cells were washed three times with CSS and fixed for 30 min at 4°C in a 4% PF, 0.1 M PB. The cells were then washed with TBS. The cells were then permeabilized with 0.2% TX-100 in TBS for 5 min, followed by blocking with 4% normal goat serum (NGS), 0.1% TX-100 in TBS for 1 hr. This was followed by incubating the cells with affinity-purified anti-NOS antibodies overnight at 4°C (Bredt et al., 1990, 1991; T. M. Dawson et al., 1991). The cells were then rinsed three times in TBS for 10 min each. After rinsing, the cells were incubated with biotin-conjugated secondary antibody (goat anti-rabbit; Vector Laboratories) for 1 hr at room temperature in 1.5% NGS, TBS, 0.1% TX-100. After an additional three washes in TBS, the cells were incubated with an avidin-biotin-peroxidase complex (1:50; Vector Elite, Vector Laboratories) in TBS for 45 min at room temperature. The cells were again rinsed three times for 10 min each in TBS and were developed with a substrate solution consisting of 0.01% H_2O_2 and 0.5 mg/ml diaminobenzidine in TBS. Cells were then rinsed in TBS containing 0.02% sodium azide. All NOS-positive cells in each well were counted utilizing an inverted microscope.

Immunohistochemistry for NSE (Incstar) and GFAP (Incstar) was performed as described above with substitution of NSE antiserum or GFAP antiserum for anti-NOS antibody.

NOS immunohistochemistry of rat brain was performed as described (Bredt et al., 1990, 1991; T. M. Dawson et al., 1991). Briefly, male Sprague–Dawley rats (150–250 gm) were perfused with phosphate-buffered saline (PBS; 50 mM PB, 0.9% NaCl, pH 7.4, 4°C) followed by perfusion with 4% PF in 0.1 M PB, pH 7.4, 4°C. The brains were removed and postfixed for 2 hr in 4% PF in 0.1 M PB, pH 7.4, 4°C. This was followed by cryoprotection in 20% glycerol in 0.1 M PB, pH 7.4, 4°C (v/v) overnight. Thick (40 μm) sections were cut with a sliding microtome (Microm) and sections were stained with the anti-NOS antibody as described for the cortical cultures.

Measurement of NOS catalytic activity.

Mature cortical neurons were treated with 500 μM NMDA for 5 min utilizing identical conditions as in the cytotoxicity assay. At 2, 4, 8, and 24 hr after NMDA administration, the cells were scraped from the culture wells and homogenized in 50 mM Tris-HCl buffer containing 1 mM EDTA and 1 mM EGTA, pH 7.4. Cortical cultures were also treated with 3.5 ng/ml lipopolysaccharide (LPS) and 5 U/ml γ-interferon for 24 hr and then homogenized in the above buffer. NOS catalytic activity was assessed by measuring the conversion of ^{3}H-arginine to ^{3}H-citrulline as previously described (Bredt and Snyder, 1989, 1990) in the presence and absence of calcium as well as NADPH. After incubation for 15 min at room temperature, the assays were quenched with two 2 ml aliquots of 20 mM HEPES, pH 5.5, 2 mM EDTA applied to 1 ml columns of Dowex AG50WX-8 (Na^+ form). ^{3}H-citrulline was quantified by liquid scintillation counting of the 4 ml flow through. All values were compared to control vehicle-treated cells.

Materials

NMDA and MK801 were purchased from Research Biochemicals Incorporated. A23187 was obtained from Calbiochem. Diphenyleniodonium (DPI) was obtained from Kodak. DPI and A23187 were dissolved in dimethyl sulfoxide (DMSO) and diluted to a final concentration of 0.1% DMSO. All other compounds were dissolved in CSS. LPS was obtained from GIBCO, and recombinant murine γ-interferon, from Upstate Biotechnology, Inc. ^{3}H-arginine was obtained from New England Nuclear/DuPont. L-Iminoethylornithine (NIO) was a generous gift from Dr. Salvador Moncada, The Wellcome Foundation, Ltd.; SIN-1 (3-morpholino-sydnonimine-hydrochloride) and C88-3934 were gifts of Cassella AG. Unless otherwise noted, all other chemicals were purchased from Sigma (St. Louis, MO). Reduced hemoglobin was prepared as described by Martin et al. (1985). Solutions of SNP, SIN-1, and C88-3934 were prepared in the dark immediately before application to the cultures to minimize photolysis. Cell culture media and arginine-free media were obtained from GIBCO. Arginine can be depleted from cultures by growing them in arginine-free media for 24 hr in the presence of glutamine, which inhibits argininosuccinate synthetase (Sessa et al., 1990). Cortical neurons remain viable in arginine-free media for up to 72 hr (Dawson, Dawson, Bartley, Uhl, and Snyder, unpublished observations).

Results

Effects of NOS inhibitors on NMDA neurotoxicity. To evaluate the relationship of NO generation to NMDA neurotoxicity, we examined concentration–response relationships and the time course of cell death for both SNP and NMDA in cortical cultures (Fig. 1). The shapes of the concentration–response curves for NMDA and SNP are essentially the same. Neurotoxicity increases from about 10% to more than 80% cell death with both SNP and NMDA over about 2 orders of magnitude, suggesting the absence of cooperativity. The time course of toxicity for NMDA and SNP is comparable with minimal effects over the first 4 hr proceeding to almost maximal neurotoxicity after 12 hr. This lag phase, following a brief 5 min application of NMDA, described previously by others (Choi, 1987; Choi et al., 1988; Koh et al., 1990), is relatively unique to this form of neurotoxicity; for example, it is not evident with kainate toxicity. Thus, the close similarity of SNP and NMDA neurotoxic effects both in concentration–response relationships and time course is consistent with NO mediating NMDA toxicity. However, since SNP breaks down to NO and $Fe(CN_5)^{2-}$ it is possible that SNP kills neurons by a process unrelated to NO, as SNP has nonspecific actions that include blockade of NMDA receptors (East et al., 1991; Kiedrowski et al., 1991; Manzoni et al., 1992a). It has been proposed that the nonspecific action is due to the $Fe(CN_5)^{2-}$ moiety of SNP, as $K_4[Fe(CN)_6]$ had similar effects (East et al., 1991; Kiedrowski et al., 1991, 1992; Manzoni et al., 1992a). To determine whether SNP toxicity could involve $Fe(CN_5)^{2-}$, we applied $K_4[Fe(CN)_6]$ at concentrations up to 3 mM under identical exposure conditions and found no significant neuronal injury. Interestingly, concentrations as low as 100 μM $K_4[Fe(CN)_6]$ are completely neuroprotective against NMDA toxicity (data not shown). Further evidence that NO is neurotoxic is that SIN-1, another agent that releases NO in aqueous solutions, also elicits neuronal injury, while C88-3934, a structurally related compound that does not release NO, does not elicit neuronal toxicity (data not shown). Other NO releasers elicit neurotoxicity in cortical cultures (Lustig et al., 1992b), fitting with our observations.

Confirming our previous observations (V. L. Dawson et al., 1991), the NOS inhibitor N^G-nitro-L-arginine (N-Arg) blocks NMDA cell death (Fig. 2). The concentration–response relationship for NMDA is shifted to the right in the presence of 100 μM N-Arg. This protective effect is only overcome by an NMDA concentration of 7 mM. Arginine-free medium also attenuates NMDA neurotoxicity, producing a rightward shift of the NMDA concentration–response curve. Arginine-free medium has a greater effect on NMDA neurotoxicity than coapplication of 100 μM N-Arg, a maximally effective concentration in culture. At 7 mM NMDA, neither N-Arg nor arginine-free medium prevented toxicity. Additionally, the NMDA antagonist MK801, which blocks the neurotoxicity of 1 mM NMDA, fails to block the neurotoxicity elicited by 7 mM NMDA. Thus, the toxicity observed at concentrations of NMDA at or exceeding 7 mM NMDA appears not to involve NMDA receptors or NO.

Previously we reported that a single high concentration of L-arginine can reverse the protective effects of N-Arg (V. L. Dawson et al., 1991). In the present study we have explored a detailed concentration–response relationship for L-arginine (Fig. 2, inset). Half-maximal reversal of the protective effects of arginine-free medium is evident at 25 μM L-arginine, with maximal effects near 100 μM.

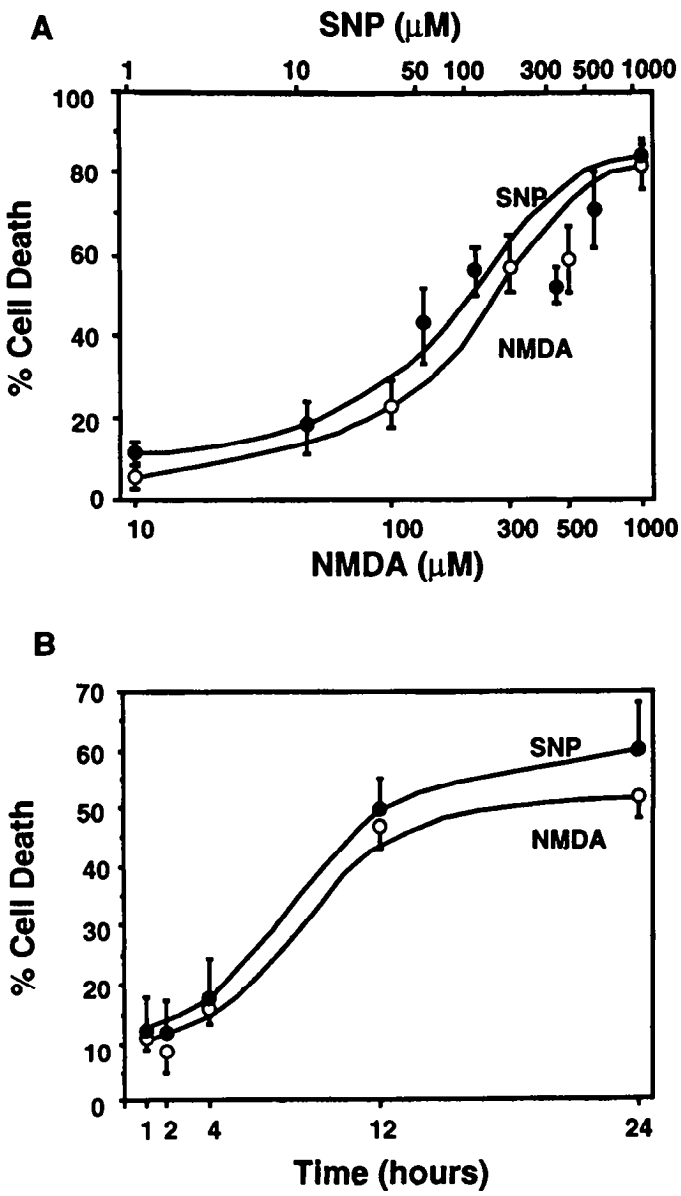

Figure 1. Dose dependence and time course of NMDA and SNP neurotoxicity. In primary cortical cultures SNP elicits cell death in a dose-dependent fashion that parallels NMDA dose dependence (*A*). $Fe(CN_5)^{2-}$, produced when SNP releases NO, does not elicit toxicity when applied to cells in concentrations equivalent to SNP (data not shown). The time course of cell death following a 5 min application of SNP mimics NMDA cell death (*B*). Each data point represents the mean ± SEM of at least two separate experiments in which at least four wells were treated with the various agents per experiment; 500–1500 neurons were counted per well.

Various arginine derivatives differ in their potencies as NOS inhibitors as well as their selectivity for brain as compared to macrophage NOS (Moncada et al., 1991). Accordingly, we examined concentration–response relationships for a series of NOS inhibitors (Fig. 3). N-Arg is most potent with 50% protective effects at 20 μM. *N*-methyl-arginine (NMA), a weaker inhibitory of brain NOS than N-Arg, is about one-eighth as potent as N-Arg in protecting against toxicity. NIO, which has a greater affinity for macrophage NOS and has intermediate potency between N-Arg and NMA in inhibiting brain NOS (McCall et al., 1991), also displays intermediate potency in blocking NMDA toxicity.

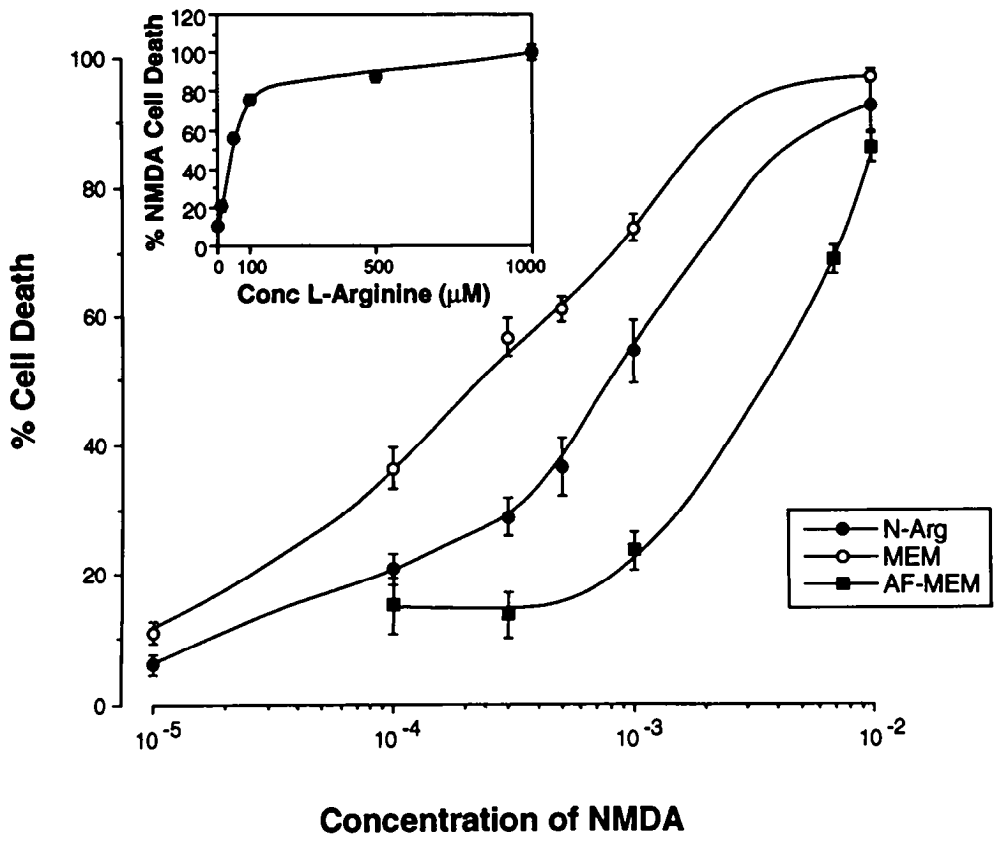

Figure 2. NMDA neurotoxicity is inhibited by N-Arg or arginine-free media (*AF-MEM*). The dose–response of cortical cultures to increasing concentrations of NMDA is shifted to the right by N-Arg and further to the right in arginine-free media with a more than 20-fold increase in the 50% lethal concentration of NMDA. Adding graded concentrations of L-arginine to 300 μM NMDA in arginine-free media reveals a requirement for L-arginine with an EC_{50} = 25 μM (*inset*). All data points represent the mean ± SEM of at least two separate experiments in which at least four wells were treated with various agents per experiment; 500–1500 neurons were counted per well. The concentration of NMDA is molar.

The methyl ester of N-Arg (NAME) also has an intermediate potency in inhibiting NOS activity and NMDA neurotoxicity, with an EC_{50} of 310 μM. The effects of NMA are stereospecific, as 500 μM *N*-methyl-D-arginine provides no protection against NMDA neurotoxicity.

In addition to arginine analogs, NOS can also be inhibited by DPI, which binds the flavin moiety critical to NOS activity (Stuehr et al., 1991). DPI inhibits NMDA toxicity with an EC_{50} of 30 nM (Fig. 4). DPI also blocks NADPH-diaphorase staining of cortical neurons, an effect not observed with NOS inhibitors that are arginine derivatives.

We also compared effects of NOS inhibitors on neurotoxicity in cultures from different brain regions. In these studies we evaluated neurotoxicity elicited by glutamate, quisqualate, and kainate in addition to NMDA (Table 1). Glutamate and its three analogs produce substantial neurotoxicity in all three brain regions. In all the brain regions N-Arg produces the most protection against glutamate and NMDA, with somewhat less pro-

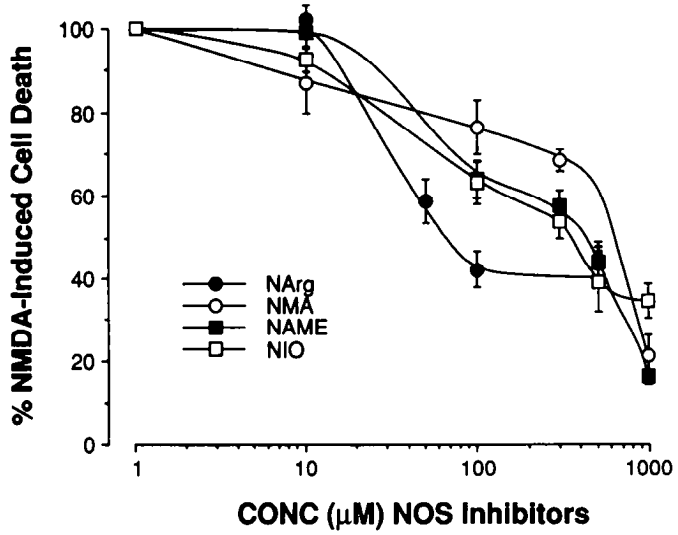

Figure 3. Concentration–response of NOS inhibitors in attenuating 300 μM NMDA toxicity. All data points represent the mean ± SEM of at least two separate experiments in which at least four wells were treated with various agents per experiment and 500–1500 neurons were counted per well.

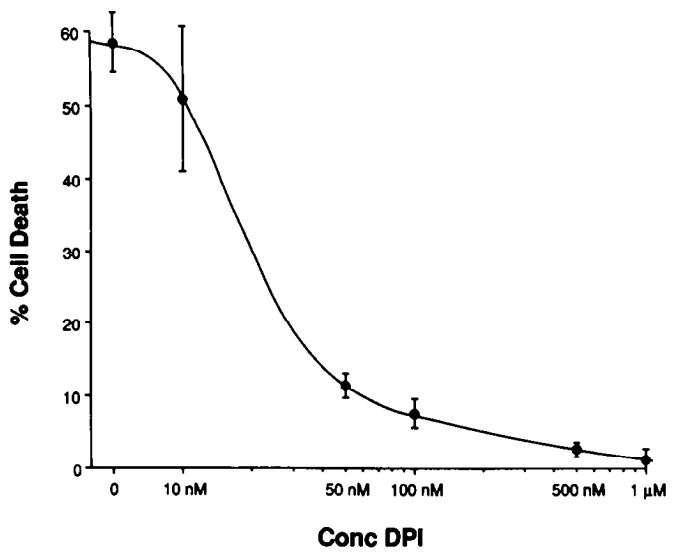

Figure 4. DPI attenuates NMDA neurotoxicity and NADPH-diaphorase staining. All data points represent the mean ± SEM ($n \geq 8$) in which approximately 4000–12,000 neurons were counted for each data point.

Table 1. Inhibition of neurotoxicity by N-Arg and reversal by L-arginine

Treatment	Cortex (% killed ± SEM)	Caudate-putamen (% killed ± SEM)	Hippocampus (% killed ± SEM)
500 μM glutamate	49.2 ± 3.7	74.5 ± 5.1	58.0 ± 3.1
+100 μM N-Arg	14.5 ± 3.7*	25.9 ± 2.9*	2.7 ± 1.8*
+N-Arg + 1 mM L-Arg	50.1 ± 1.8	74.4 ± 5.3	52.8 ± 1.9
300 μM NMDA	62.0 ± 2.7	63.5 ± 3.0	62.8 ± 3.2
+100 μM N-Arg	20.3 ± 3.3*	15.5 ± 2.4*	18.7 ± 1.3*
+N-Arg + 1 mM L-Arg	64.6 ± 5.0	60.8 ± 2.6	56.7 ± 2.7
500 μM quisqualate	60.7 ± 4.7	64.9 ± 3.5	60.3 ± 2.0
+500 μM N-Arg	41.5 ± 4.1*	25.6 ± 3.4*	49.2 ± 4.5
+N-Arg + 5 mM L-Arg	63.0 ± 4.5	67.1 ± 4.0	56.2 ± 2.2
100 μM kainate	83.9 ± 4.0	85.6 ± 2.5	83.2 ± 3.9
+500 μM N-Arg	83.0 ± 4.0	67.6 ± 5.5*	73.9 ± 4.9

Data are the means ± SEM (n = 6–26). Each data point represents a minimum of 4000–12,000 neurons counted. In some experiments up to 40,000 neurons were counted. Toxicity was assessed by trypan blue exclusion as described in Materials and Methods. Significant overall values were obtained using a one-way between-groups ANOVA. Specific comparisons on all possible pairwise combinations were made with the Student's t test for independent means; *, $p < 0.05$.

tection against quisqualate and negligible protection in the cerebral cortex and hippocampus against kainate. In the caudate putamen, N-Arg produces modest but statistically significant protection against kainate. In all brain regions excess arginine reverses the effects of N-Arg.

The role of calcium, cGMP, and superoxide anion in NMDA neurotoxicity. Large increases in intracellular calcium following NMDA receptor activation have been implicated as a primary mediator of NMDA neurotoxicity (Choi, 1987, 1988; Choi et al., 1988; Meldrum and Garthwaite, 1990). To ascertain whether cell death mediated by increased intracellular calcium involves NO, we examined effects of agents influencing NO disposition following calcium influx (Fig. 5). The calcium ionophore A23187 (30 μM, applied for 5 min) kills about 70% of cortical cells, as assessed by trypan blue exclusion 20–24 hr later, similar to the effects of NMDA (Choi, 1987). This neurotoxicity is blocked by N-Arg to a similar extent as the inhibition of NMDA toxicity. Moreover, L-arginine reverses the protective effects of N-Arg. Reduced hemoglobin, which binds extracellular NO, markedly prevents A23187 toxicity, similar to its protection against NMDA toxicity (V. L. Dawson et al., 1991). A23187 toxicity is also prevented in arginine-free medium.

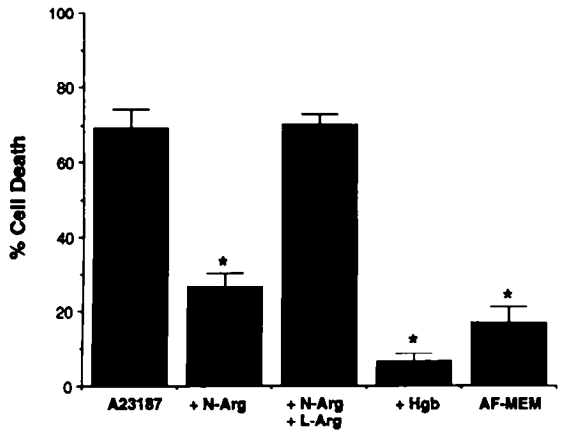

Figure 5. Neurotoxicity elicited by the calcium ionophore A23187. A23187 is affected by the same agents that influence NMDA neurotoxicity. The NOS inhibitor N-Arg (100 μM) inhibits A23187 (30 μM) neurotoxicity, which can be overcome with excess (1 mM) substrate, L-arginine (L-Arg). Toxicity is also attenuated in cultures exposed to 30 μM A23187 in arginine-free media (*AF-MEM*) or in the presence of 500 μM reduced hemoglobin (*Hgb*). Data are means ± SEM ($n \geq 8$); approximately 4000–12,000 neurons were counted for each data point. Toxicity was assessed by trypan blue exclusion. Significance was determined by Student's t test for independent means; *, $p < 0.01$.

Calcium activation of NOS involves calmodulin. To evaluate the role of calmodulin in NMDA toxicity, we examined the effects of calmidazolium, a calmodulin antagonist (Table 2). Calmidazolium blocks NMDA neurotoxicity to a similar extent as N-Arg. W7, which binds calmodulin, also blocks NMDA toxicity.

The concentration–response curve for SNP toxicity in cortical cultures parallels its effects in stimulating cGMP concentrations in these cultures (V. L. Dawson et al., 1991). Additionally, the same conditions that modulate NMDA toxicity alter cGMP formation in a similar manner (Dawson et al., 1991a). To ascertain whether cGMP mediates NMDA- and NO-mediated neurotoxicity, we examined the effects of methylene blue, an inhibitor of guanylyl cyclase (Table 3). Methylene blue fails to block both NMDA- and SNP-induced toxicity. Moreover, 8-bromo-cGMP, a derivative of cGMP that penetrates into cells, has no effect upon neurotoxicity elicited by NMDA or SNP. Exposure to cultures with 8-bromo-cGMP alone does not elicit neurotoxicity. These observations confirm recent findings of Greenberg and collaborators (Lustig et al., 1992a), who explored a variety of guanylyl cyclase inhibitors and cGMP derivatives.

NO can combine with the superoxide anion to form peroxynitrite, which decomposes into the hydroxyl free radical ($OH^{\cdot}$) and the nitrogen dioxide free radical ($NO_2^{\cdot}$), both of which are highly reactive and potentially toxic (Beckman et al., 1990; Radi et al., 1991a,b). To evaluate the role of the superoxide anion in NMDA and NO neurotoxicity, we examined the effects of su-

Table 2. Calmodulin inhibitors attenuate NMDA neurotoxicity

Treatment	% cell death ± SEM
300 μM NMDA	63.8 ± 4.2
+10 μM calmidazolium	20.9 ± 5.0*
+200 μM W7	23.3 ± 6.6*

Data are means ± SEM ($n \geq 8$). Each data point represents approximately 4000–12,000 counted neurons. Toxicity was assessed by trypan blue exclusion as described in Materials and Methods. Significance was determined by the Student's t test for independent means; *, $p < 0.01$.

Table 3. Superoxide dismutase but not guanylyl cyclase modulates NMDA and SNP neurotoxicity

Treatment	% cell death (± SEM)
300 μM NMDA	60.6 ± 5.4
+100 μM methylene blue	53.9 ± 2.2
+1 mM 8-bromo-cGMP	69.3 ± 6.4
+100 U SOD	32.1 ± 4.0*
300 μM SNP	51.3 ± 3.7
+100 μM methylene blue	48.8 ± 4.9
+1 mM 8-bromo-cGMP	48.4 ± 4.4
+100 U SOD	18.0 ± 3.7*

Data are the means ± SEM ($n \geq 8$). Each data point represents 4000–12,000 counted neurons. Toxicity was assessed by trypan blue exclusion as described in Materials and Methods. Significance was determined by Student's t test for independent means; *, $p < 0.05$.

peroxide dismutase (SOD), an enzyme that scavenges the superoxide anion (Table 3). Addition of SOD to the exposure solution reduces the toxicity elicited by NMDA, SNP, and A23187.

Evidence that NOS neurons are the source of NO that mediates neurotoxicity. NO can be formed by NOS in macrophages and endothelial cells as well as neurons expressing NOS. In the brain, microglia function as macrophages. To determine whether NOS neurons in the cultures are the source of the neurotoxic NO, we took advantage of the differential sensitivity of NOS neurons and other neurons to various toxins (Fig. 6). While about 60% of the total neuronal population of cortical cultures are killed by 300 μM NMDA or 300 μM SNP, only about 25% and 10%, respectively, of NOS neurons die with the two treatments. In contrast, 20 μM quisqualate kills less than 20% of the total neuronal population, but kills about 85–95% of NOS neurons. These results resemble those of Choi and co-workers, who contrasted the degree of cell death elicited in NADPH-diaphorase–

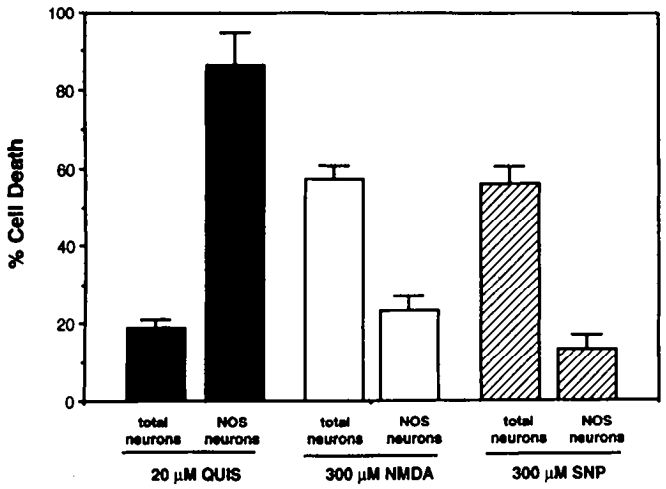

Figure 6. Selective sensitivity of NOS neurons to quisqualate. NOS neurons in primary cortical cultures resist NMDA and SNP neurotoxicity but are more sensitive than other neurons to quisqualate. Low-dose, 20 μM quisqualate (*QUIS*) produces substantial loss of NOS neurons without much decrease in the total cell population. The total cell counts include NOS neurons. NMDA (300 μM) and SNP (300 μM) elicit more than 60% cell death in the total neuronal population but only 25% and 13% loss of NOS neurons, respectively. Data are means ± SEM ($n \geq 8$).

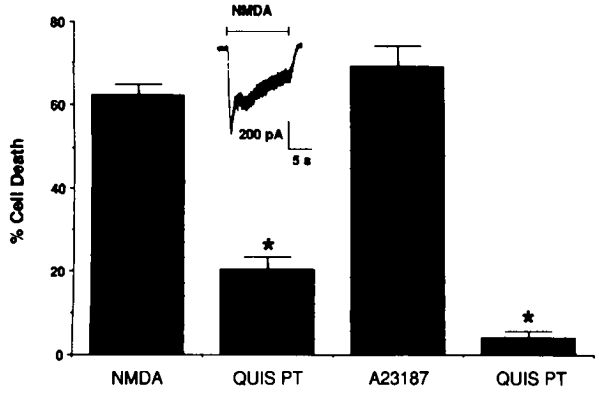

Figure 7. Selectively killing NOS neurons prior to NMDA or A23187 exposure reduces toxicity. Treating primary cortical neurons with 20 μM quisqualate, 24 hr prior to treating these neurons with 300 μM NMDA or 30 μM A23187 (*QUIS PT*), reduces NMDA neurotoxicity 71% and A23187 neurotoxicity 94%. Neurons in sister cultures treated with 20 μM quisqualate still produce currents on application of 200 μM NMDA (*trace inset*). Data are means ± SEM ($n \geq 8$). Significance was determined by Student's t test for independent means; *, $p < 0.01$.

positive neurons versus the total neuronal population following exposure to graded concentrations of NMDA or quisqualate (Koh et al., 1986; Koh and Choi, 1988). NOS neurons are also resistant to the toxic effects of exogenously applied NO (Fig. 6). We examined the neurotoxicity of 300 μM NMDA in cultures treated 24 hr earlier with 20 μM quisqualate, a concentration that destroys 85–95% of NOS neurons (Fig. 7). In these cultures NMDA toxicity is profoundly attenuated. In sister cultures pretreated in the same manner with 20 μM quisqualate, 200 μM NMDA still elicits calcium currents, indicating the persistence of functional NMDA receptors. Toxicity elicited by the calcium ionophore A23187 is also attenuated by quisqualate pretreatment (Fig. 7). In addition, a critical number of NOS neurons (approximately 100 NOS-positive cells per 15 mm well) were required to observe an NO component to NMDA neurotoxicity (data not shown). These experiments strongly implicate NOS neurons as the source of NO that mediates NMDA toxicity.

To examine the role of NOS neurons in NMDA toxicity by another paradigm, we evaluated NMDA toxicity at different days in culture while monitoring the percentage of cells that stain positive for NADPH-diaphorase (Fig. 8). NMDA toxicity is not evident for the first 16 d in culture, and then develops gradually, reaching full effects at 20 d in culture. NADPH-diaphorase staining of the cells displays a virtually identical time course. Despite the absence of NMDA toxicity in early stages of the culture, NMDA-mediated calcium currents are evident at day 7 and day 14, though they are somewhat smaller than at day 21. Although young cultures are resistant to NMDA toxicity, they are sensitive to 300 μM SNP, which produces the same extent of cell killing at 7 and 14 d as at 21 d (data not shown).

Inducible macrophage NOS activity is calcium independent, while neuronal NOS requires added Ca^{2+} (Marletta, 1989). We assessed a potential role for microglial, inducible NOS by examining whether there was any calcium-independent NOS catalytic activity 2, 4, 8, and 24 hr after a 5 min application of NMDA. Despite the presence of abundant calcium-dependent NOS catalytic activity, no appreciable calcium-independent NOS catalytic activity was detected (data not shown). In addition, application of LPS and γ-interferon, potent inducers of mac-

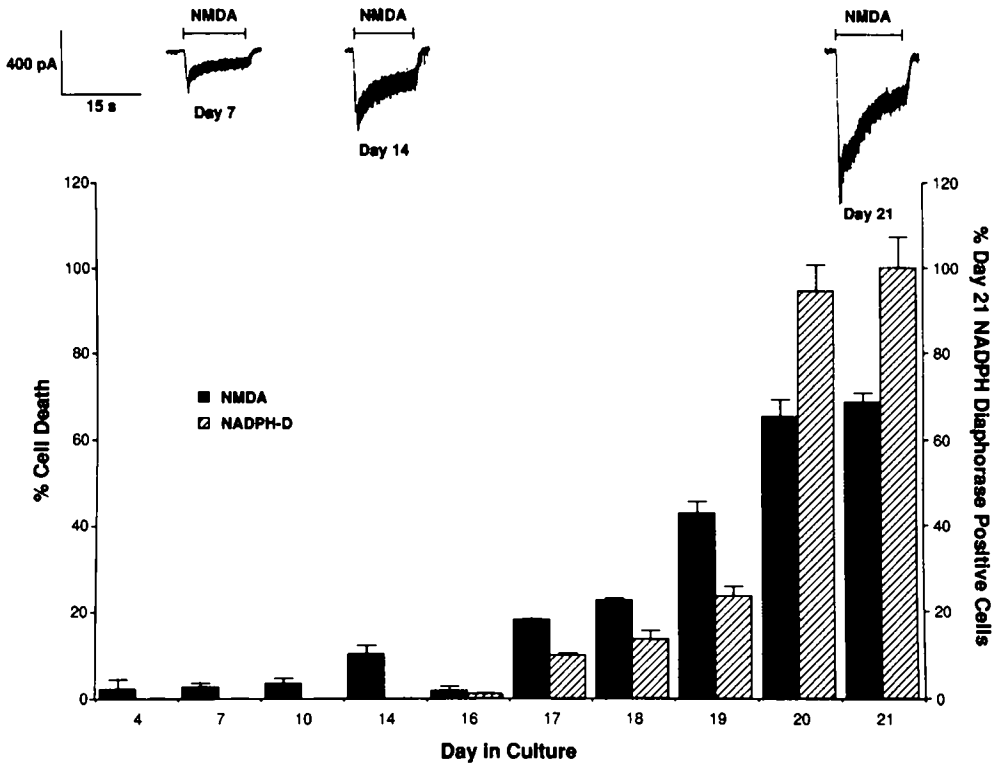

Figure 8. The development of NMDA neurotoxicity corresponds with the appearance of NOS neurons. After dissociated cortical cells were plated (day 0), cells were periodically assessed for NMDA neurotoxicity and NADPH-diaphorase stain. Neurotoxicity and NADPH-diaphorase staining were first observed on day 17 in culture, with development of full expression by day 20. NMDA-induced currents were observed as early as day 7 in culture. Neurotoxicity to 300 μM SNP was observed at day 7 and did not vary over time. Data are means ± SEM ($n \geq 9$).

rophage NOS, fails to induce a calcium-independent NOS. Thus, neuronal NOS seems to be the primary source of neurotoxic NO in primary cortical cultures.

Because NOS occurs in only 1–2% of cerebral cortical cells, we might ask how NO is able to kill the majority of cells in cultures. To examine this question, we conducted both dark- and bright-field microscopy of sections of intact adult rat cerebral cortex (Fig. 9) and cortical cultures (Fig. 10 and data not shown). While the number of NOS cells in a given field may be few, processes of the cells ramify extensively to cover virtually all the surface of the culture (Fig. 10). Since the extensive ramification of NOS processes in culture is difficult to illustrate, we show the extensive amount of NOS processes in a tissue section of intact adult rat cerebral cortex (Fig. 9). Dead cells following NMDA treatment occur in patches. These patches are largely concentrated about processes and cell bodies of NOS neurons (Fig. 10).

Discussion

In the present study we confirm and extend our previous observations that NO is responsible, at least in part, for NMDA-type glutamate neurotoxicity. The relative potencies of several NOS inhibitors in blocking neurotoxicity parallel their potencies as NOS inhibitors. The ability of NOS inhibitors to protect against NMDA neurotoxicity in brain cultures as well as in brain slices has now been confirmed by several groups (Izumi et al., 1992; Lustig et al., 1992b; Moncada et al., 1992; Wallis et al., 1992). NO has also been implicated in neuronal injury in intact animals, as N-Arg (1 mg/kg, i.p., repeated doses) blocks neurotoxicity elicited by ligation of the middle cerebral artery more effectively than the noncompetitive NMDA antagonist MK801 (Nowicki et al., 1991). Additionally, N-Arg is completely neuroprotective against focal stroke in the 7-d-old rat (Trifiletti, 1992). In one report the NOS inhibitor N-Arg did not block neuronal injury in whole rat brain cultures (Demerle-Pallardy et al., 1991). Whether the use of whole brain cultures or other factors accounted for the failure of NOS inhibitors to block neurotoxicity is unclear.

Besides the cerebral cortex, we observed blockade of toxicity by NOS inhibitors in caudate-putamen and hippocampal cultures. N-Arg blocks glutamate and NMDA toxicity to a greater extent than quisqualate toxicity. At the concentrations of quisqualate and the mode of application that we have employed, it is likely that quisqualate may act in part via NMDA receptors (Koh et al., 1990), which may account for the portion of quisqualate toxicity prevented by N-Arg. N-Arg does not block kainate toxicity in the cerebral and hippocampal cultures, which fits with observations of Coyle and associates (Puttfarcken et al., 1992) that kainate toxicity involves free radicals and not NO. N-Arg does prevent a portion of kainate neurotoxicity in the striatal cultures. Perhaps kainate-sensitive calcium channels (Murphy et al., 1987) cause excessive release of NO from NOS neurons and account in part for the neuronal injury associated with kainate in the caudate-putamen. Interestingly, NOS inhibitors do not prevent glutamate neurotoxicity in cerebellar granule cells (Puttfarcken et al., 1992). All cerebellar granule cells are enriched in NOS (Bredt et al., 1991). Their resistance to NMDA toxicity fits with our observation that NOS neurons are uniquely resistant to NMDA and SNP toxicity. On the other hand, R. J. Miller and colleagues (personal communication) have shown that in cultures of cerebellar enriched Purkinje cells, kainate-induced killing of cerebellar Purkinje cells, which lack NOS, is attenuated by NOS inhibitors.

Application of SNP, which spontaneously releases NO, elicits cell death in a concentration- and time-dependent fashion that parallels NMDA toxicity, observations also made by Chen et al. (1991) in striatal cultures. SNP elicits neuronal injury in intact animals when injected directly into the hippocampus

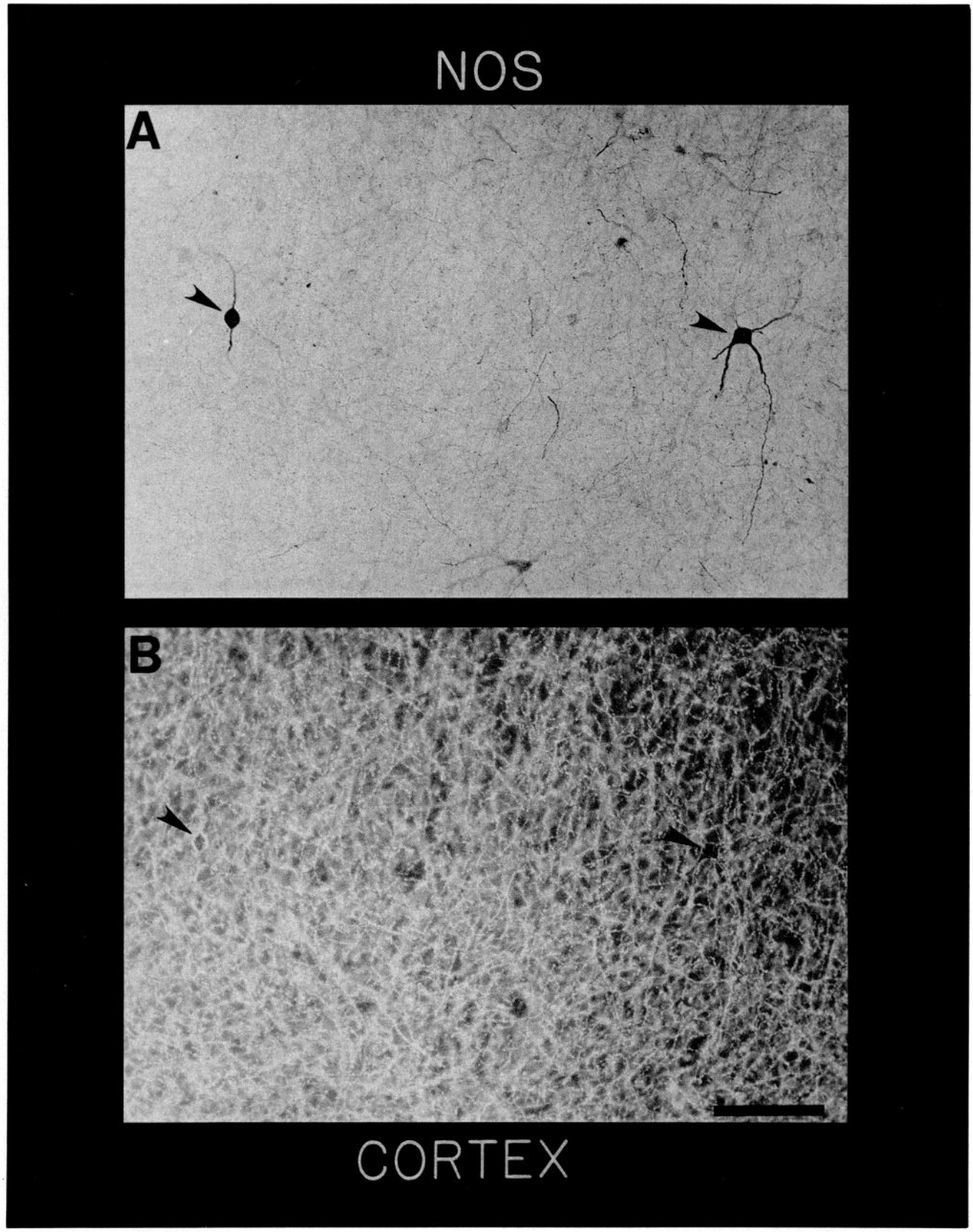

Figure 9. NOS neurons and processes in cerebral cortex. *A*, Bright-field photomicrograph illustrating the distribution and density of NOS neurons (*arrowheads*) in the cerebral cortex from intact adult rat brain. *B*, Dark-field photomicrograph of *A*, illustrating the dense plexus of NOS fibers throughout the cortex. Scale bar, 100 μm.

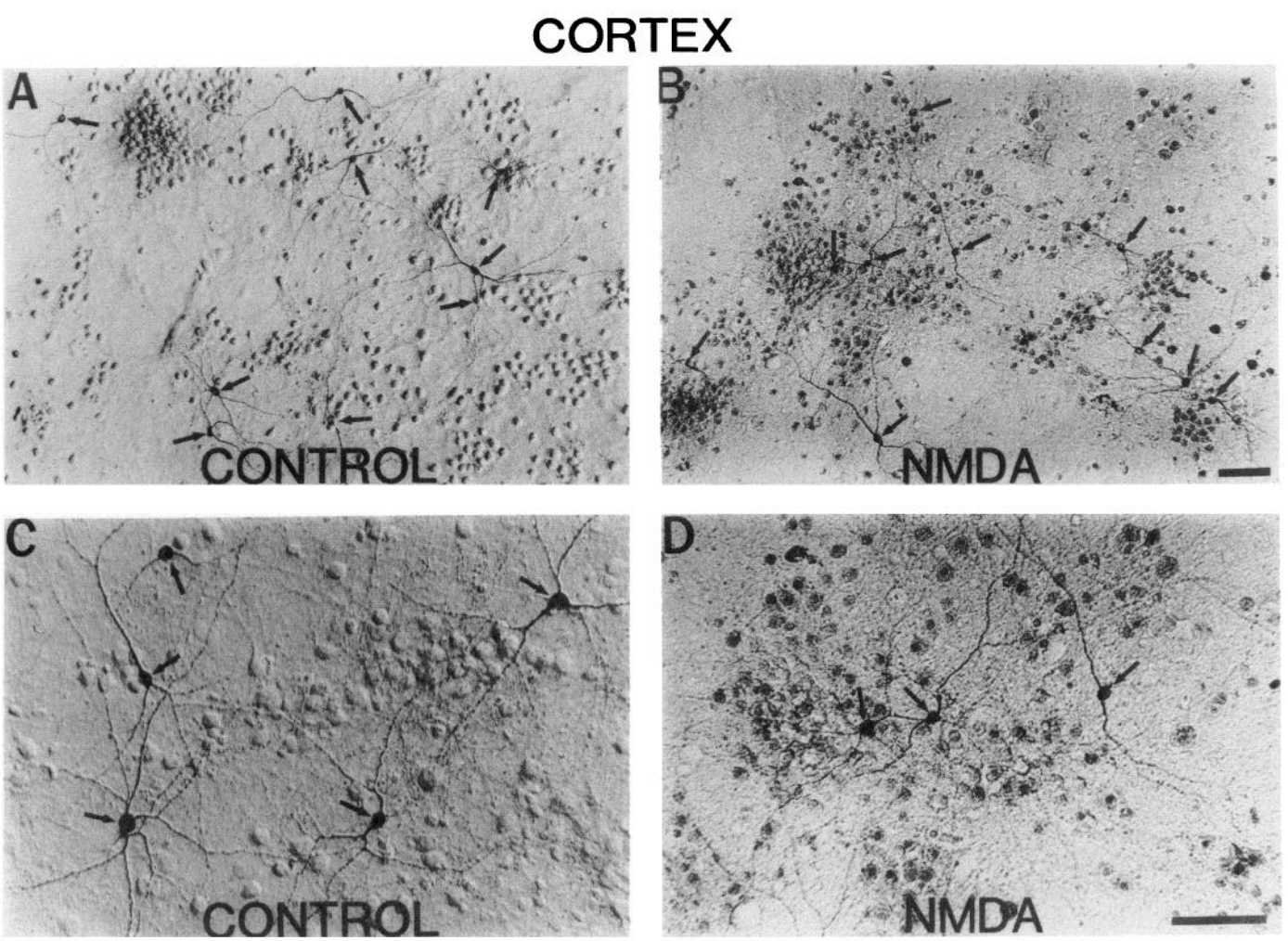

Figure 10. NMDA kills neurons within the vicinity of NOS neurons and/or processes. *A,* Low-power Hoffman modulation photomicrography of NOS neurons (*arrows*) in cortical cultures. *B,* NMDA kills neurons in the vicinity of NOS neurons. Dead neurons appear *black* in these photomicrographs. *C,* High-power view of cortical cultures stained with anti-NOS antibody. *D,* High-power view of NMDA neurotoxicity. Scale bar, 100 μm.

(Loiacono and Beart, 1992). $K_4[(Fe(CN)_6]$, which elicits the same nonspecific effects as the $Fe(CN_5)^{2-}$ moiety of SNP, does not cause neuronal injury. Several other NO releasers also cause neuronal injury both in primary cultures (Chen et al., 1991; Lustig et al., 1992b) and in intact animals (Smith et al., 1991). In addition, 40 nM authentic NO can cause cell death in hippocampal cultures (O'Dell et al., 1991). Thus, whether NO is from exogenous sources, such as SNP or other NO releasers, or is endogenously generated, it may function as a neurotoxin under conditions of excessive NO production or release.

Calcium has been implicated as a major mediator of glutamate neurotoxicity (Choi, 1988; Meldrum and Garthwaite, 1990). However, some evidence indicates that intracellular calcium levels do not directly correlate with such toxicity (Michaels and Rothman, 1990; Dubinsky and Rothman, 1991). For instance, brief (5 min) glutamate exposure elicits a transient elevation in intracellular calcium that recovers to the basal level in the majority of neurons in culture (Randall and Thayer, 1992). Despite normal intracellular calcium levels after the initial glutamate exposure, irreversible processes are started that no longer require a sustained elevation of intracellular calcium (Randall and Thayer, 1992). These early irreversible processes lead to the initiation of "delayed neurotoxicity" associated with calcium overload (Choi, 1988, 1990; Randall and Thayer, 1992). Our studies indicate that activation of NOS leads to these lethal processes, as neurotoxicity directly elicited by a calcium ionophore A23187 is mediated via NO, being blocked by N-Arg, hemoglobin, and arginine-free medium. Moreover, NMDA toxicity is blocked by calmodulin antagonists, indicating that the toxicity elicited by calcium involves calmodulin and, presumably, the calmodulin-induced activation of NOS. In contrast to the crucial role for calcium and calmodulin, cGMP does not appear to mediate the toxicity directly. In our experiments as well as those of Greenberg and colleagues (Lustig et al., 1992a), the guanylyl cyclase inhibitor methylene blue does not block neurotoxicity, and 8-bromo-cGMP does not influence toxicity. Garthwaite and Garthwaite (1988) obtained evidence that cGMP may protect against neurotoxicity in cerebellar slices.

Evidence for participation of superoxide anions in neurotoxicity elicited by NMDA, the calcium ionophore, as well as the direct generation of NO from SNP is evident from the protective effects of SOD. The importance of superoxide anions in NMDA neurotoxicity is emphasized by findings that cortical cultures from transgenic mice overexpressing SOD are resistant to NMDA

neurotoxicity (Chan et al., 1990) and these same animals are relatively resistant to focal ischemia (Kinouchi et al., 1991). SOD protection might relate to superoxides directly generated by NOS, as purified NOS directly forms superoxide anions (Pou et al., 1992). The combination of the superoxide anion and NO gives rise to peroxynitrite, which in turn decomposes into $OH^{\cdot}$ and $NO_2^{\cdot}$ free radicals that are highly reactive and might be the final common mediators of NO toxicity (Beckman et al., 1990; Beckman, 1991; Radi et al., 1991a,b; Dawson et al., 1992, 1993). Extracellular SOD would not be expected to penetrate cells, suggesting that the superoxide anion may be released upon glutamate exposure. However, Bennett and colleagues (Saez et al., 1987) have shown that exogenously administered ^{125}I-labeled SOD can attain high intracellular levels under depolarizing conditions. Both NO (Lei et al., 1992; Manzoni et al., 1992b) and the superoxide anion (Aizenman et al., 1989, 1990) block both NMDA receptor currents and the associated increase in intracellular calcium. Thus, both potential toxic agents might exert feedback inhibition on NMDA receptors under physiologic conditions. With excessive release of both NO and the superoxide anion, normal regulatory mechanisms might be overwhelmed, leading to cell death. As suggested by Lei et al. (1992), NO is necessary but not sufficient for neuronal injury and is toxic only in the presence of other factors, such as the superoxide anion.

Why NOS neurons are selectively resistant to various forms of neurotoxicity is not known. SOD could, in principle, protect these cells against such toxicity. Conceivably NOS neurons are enriched in SOD, which would account in part for their resistance to toxicity. In the cerebral cortex and in the caudate-putamen all somatostatin neurons are positive for NOS (T. M. Dawson et al., 1991). Inagaki et al. (1991) have observed high levels of SOD staining in somatostatin neurons of the caudate-putamen. In the caudate-putamen large ACh-containing interneurons as well as NADPH-diaphorase neurons are resistant to degeneration in Huntington's disease (Ferrante et al., 1985), and though the ACh-containing interneurons do not stain for NOS (T. M. Dawson et al., 1991), these cells do display high densities of SOD staining (Inagaki et al., 1991).

Using several experimental approaches, we have established that the NO that mediates neurotoxicity derives from NOS neurons. Thus, destruction of 85–95% of NOS neurons but less than 10–20% of the total neuronal population in cortical cultures attenuates the ability of NMDA to elicit toxicity. Moreover, NMDA toxicity in cortical cultures does not appear until day 17 and peaks at day 20, coincident with the development of NOS-positive cells. Finally, although they comprise only approximately 1–2% of the total neuronal population, NOS neurons have extensive processes and ramify to influence the majority of neurons in culture as well as in intact animals. Thus, under conditions of excessive NO production, this small population of neurons could kill a large population of surrounding neurons.

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