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The Neurotransmitter Noradrenaline Rescues Septal Cholinergic Neurons in Culture from Degeneration Caused by Low-Level Oxidative Stress

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ABSTRACT

We have developed a model system in which rat basal forebrain cholinergic neurons degenerate progressively when maintained in culture conditions that make them susceptible to low-level oxidative stress. In this study, we showed that cholinergic neurons identified by acetylcholinesterase cytochemistry or choline acetyl transferase immunocytochemistry are rescued efficiently by the neurotransmitter noradrenaline (NA). The effect of NA required neither adrenoceptor activation nor intracellular accumulation. NA operated via a mechanism that precluded activation of a cell death pathway in which reactive oxygen species (ROS) and proapoptotic caspases were crucially involved. It is noteworthy that NA remained protective even when applied late in the degenerative process but before intracellular ROS began to increase. The high efficacy of iron chelators and catalase in preventing the death of cholinergic neurons in this model suggested that NA neutralized the effects of hydroxyl radicals produced through a Fenton-type reaction. Pyrocatechol [the diphenolic moiety of NA] was sufficient in itself to prevent ROS production and cholinergic cell demise, indicating that the catechol structure was instrumental for the neuroprotective function of NA. Therefore, the noncatecholic neurotransmitter GABA failed to prevent neurodegeneration. Nerve growth factor and brain derived neurotrophic factor, two trophic peptides for septal cholinergic neurons, did not afford protection by themselves and did not improve neuroprotection provided by NA. However, in the presence of NA, they both retained their efficacy to stimulate cholinergic parameters. These data indicate that NA-based therapeutic strategies may be of interest in such neurodegenerative conditions as Alzheimer's disease, where progressive cholinergic deficits occur.

The locus ceruleus (LC) provides the major source of noradrenaline (NA)-containing axons that are distributed widely throughout the central nervous system (Berridge and Waterhouse, 2003). This ubiquitous distribution is consistent with a prominent role for NA in a variety of central nervous system functions and behaviors that include locomotor control, cognition, motivation, and attention (Berridge and Waterhouse, 2003). In addition to these established transmitter signaling functions, NA evidently operates as an endogenous neurotrophic substance during development and adulthood (Meier et al., 1991; Colpaert, 1994; Goldstein, 2000; Marien et al., 2004). For instance, pharmacological agents that mimic or increase NA release, such as α_2 adrenoceptor antagonists and amphetamine-related compounds, reduced neuronal death in experimental models of cerebral ischemia and improved behavioral and functional recovery after brain injury (Goldstein, 2000). Furthermore, increasing extracellular levels of NA by genetic invalidation of the NA transporter or by treatment with specific uptake inhibitors conferred protection to dopaminergic neurons in a mouse model of Parkinson's disease (Rommelfanger et al., 2004). On the other hand, lesions of the LC-NA system were shown to induce synaptic loss (Matsukawa et al., 2003), aggravate postischemic cerebral damage (Goldstein, 2000), increase brain inflammatory responses (Galea et al., 2003), or worsen neurological deficits in animal models of Parkinson's disease (Marien et al., 1993).

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ABBREVIATIONS: LC, locus ceruleus; NA, noradrenaline; AD, Alzheimer's disease; ChAT, choline acetyltransferase; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; Z-, carbobenzoxy; FMK, fluoromethylketone; Z-DEVD-FMK, Z-Asp-Glu-Val-Asp FMK; Z-IETD-FMK, Z-Ile-Glu-Thr-Asp-FMK; Z-LEHD-FMK, Z-Leu-Glu-His-Asp-FMK; FITC, fluorescein isothiocyanate; FITC-VAD-FMK, FITC-Val-Ala-Asp-FMK; MK-801, dizocilpine; AChE, acetylcholinesterase; D-PBS, Dulbecco's phosphate-buffered saline; ACh, acetylcholine; ROS, reactive oxygen species; DHR-123, dihydrorhodamine 123; UK14,304; NAC, *N*-acetyl cysteine; araC, cytosine arabinoside; DIV, day in vitro.

Although numerous theories for the cause of Alzheimer's disease (AD) and its symptoms have been postulated, it has most often been characterized as a disorder of progressive cholinergic deterioration. Studies of postmortem human brain have revealed a severe depletion of choline acetyltransferase (ChAT), the biosynthetic enzyme for acetylcholine, in the cortex and hippocampus, concomitant with the reduction in the number of basal forebrain cholinergic neurons (Mufson et al., 2003). A number of observations suggest that the progressive nature of these cholinergic deficits might be influenced by the LC-NA system: 1) synapses between noradrenergic nerve terminals and cholinergic neurons are frequently encountered throughout basal forebrain areas, including the vertical and horizontal limb of the diagonal band nuclei and the nucleus basalis magnocellularis (Fort et al., 1995); 2) there is a remarkably early and profound loss of NA cells in the LC in AD (Zarow et al., 2003); 3) stimulation of LC-NA output by the α_2 adrenoceptor antagonist, dexefaroxan, affords protection in a retrograde lesion paradigm of basal forebrain cholinergic degeneration (Debeir et al., 2002; Chopin et al., 2004).

To examine the neuroprotective potential of NA on basal forebrain cholinergic neurons, we have developed a model system in which rat septal cholinergic neurons in culture degenerate progressively when maintained in conditions that make them susceptible to low-level oxidative stress. In particular, our aim was to use this model 1) to determine whether NA can prevent cholinergic cell death in vitro and to quantify its effects, 2) to explore the possible mechanisms underlying this protection, and 3) to examine whether trophic factors that are known to stimulate cholinergic differentiation and survival could modulate these effects.

Materials and Methods

Peptides and Pharmacological Agents. The L and D stereoisomers of NA (tartrate salts) were from Fluka (Saint Quentin Fallavier, France). Nerve growth factor 2.5S (NGF; Grade II) and brain-derived neurotrophic factor (BDNF) were purchased from Alomone Labs (Jerusalem, Israel) and R&D Systems (Minneapolis, MN), respectively. Synthetic peptide inhibitors of caspases, including carbobenzoxy-Asp-Glu-Val-Asp fluoromethylketone (Z-DEVD-FMK), Z-Ile-Glu-Thr-Asp-FMK (Z-IETD-FMK), and Z-Leu-Glu-His-Asp-FMK (Z-LEHD-FMK) were obtained from VWR International (Fontenay/Bois, France). The fluorogenic caspase substrate CaspACE-fluorescein-isothiocyanate-Val-Ala-Asp-FMK (FITC-VAD-FMK) was obtained from Promega (Madison, WI). Unless specified, other pharmacological and biochemical agents were purchased from Sigma/RBI-Aldrich (Saint Quentin Fallavier, France). [1-^{14}C]-acetyl-coenzyme-A and L-[7,8-^{3}H]-NA were obtained from Amersham Biosciences (Orsay, France).

Cultures of Septal Cholinergic and LC Noradrenergic Neurons. Animals were treated in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*, European Directive no. 86/609, and the guidelines of the local institutional animal care and use committee. Septal cultures were prepared from embryonic day 15.5 Wistar rat embryos (Janvier Breeding Center, Le Genest St. Isles, France). The septal area was dissected as described by Hartikka and Hefti (1988). The dissected area contained cholinergic neurons that give rise to the medial septal nucleus and the nucleus of the diagonal band of Brocca (Hartikka and Hefti, 1988). Tissue pieces were triturated mechanically without enzymatic treatment using modified L15 medium (Hartikka and Hefti, 1988). Cultures were plated at a density of 2.5 to 3 $\times$ 10^5 cells/cm^2 and were maintained initially in 500 μl of N5 medium (Kawamoto and Barrett, 1986) supplemented with 5 mM glucose, 5% horse serum, and 0.5% fetal calf serum. Glial cell proliferation was prevented by gradually increasing the concentration of the antimitotic cytosine arabinoside (araC) according to the culture age [day in vitro (DIV)] as follows: 1.5 μM (DIV 0), 3 μM (DIV 1–2), and 5 μM (DIV 3–5). To generate low-level oxidative stress, the N5 medium was replaced at DIV 10 with a chemically defined serum-free medium containing a trace amount of ferrous iron (0.5 μM). This medium consisted of minimal essential medium with Earle's salts/Ham's F-12 nutrient mixture (2:1; Invitrogen, Cergy Pontoise, France) supplemented with insulin (10 μg/ml), glucose (30 mM), and penicillin/streptomycin (100 U/ml). Because neurons were grown in an astrocyte-poor environment that favors excitotoxic stress, the noncompetitive NMDA receptor antagonist dizocilpine (MK-801; 3 μM) was also added to the culture medium from DIV 3 until the end of the culture period. Unless specified, 50 ng/ml NGF was used to supplement the culture media to stimulate cholinergic differentiation. Some experiments were also performed with BDNF at a final concentration of 20 ng/ml. The cultures were fed daily by replacing 350 μl of the iron-containing culture medium. They were then supplemented with appropriate pharmacological treatments including NA and adrenoceptor agonists/antagonists. Cholinergic neurons in control cultures represented ~1% of all septal neurons.

LC cultures were obtained from embryonic day 14 Wistar rat embryos. The area dissected corresponded to the whole proximal rhombencephalic ring between the distal part of the mesencephalic flexure and the proximal part of the pontine flexure (Holm et al., 2003). After trituration, the cells were plated at a density of 3 to 4 $\times$ 10^5 cells/cm^2 and then maintained for 9 to 10 days in a culture medium consisting of minimal essential medium with Earle's salts /Ham's F-12 nutrient mixture (1:1) supplemented with fetal calf serum (0.5%), N2 cocktail (1$\times$; Invitrogen), BDNF (20 ng/ml), glucose (30 mM), penicillin/streptomycin (100 U/ml), and 3 μM MK-801. araC (1.5 μM) was added between DIV 1 and 3 to limit glial cell proliferation.

Acetylcholine Esterase Cytochemistry. Acetylcholinesterase (AChE) cytochemistry was performed as described previously (Michel and Agid, 1995). In brief, after fixation with 4% formaldehyde and three washes with Dulbecco's phosphate-buffered saline (D-PBS) the cultures were incubated at 4°C in 50 mM acetate buffer, pH 5.0, containing 4 mM acetylthiocholine iodide, 2 mM copper sulfate, 10 mM glycine, and 10 mg/ml gelatin to prevent diffusion of the reaction products. Nonspecific choline esterases were inhibited by 0.2 mM ethopropazine. After 3 to 4 days, the gelatin was dissolved by briefly incubating the cultures at 37°C. The cultures were rinsed with distilled water and exposed for 30 min in 10% potassium ferricyanide. The reaction was stopped by washing with distilled water.

Choline Acetyltransferase Immunodetection. Cultures fixed with 4% formaldehyde were incubated for 4 days at 4°C with a ChAT monoclonal antibody (1:50 in D-PBS) derived from a rat hybridoma (Diasorin, Stillwater, MN). Subsequent incubations were performed at room temperature with a biotinylated anti-rat Ig antibody from goat (1:100 in D-PBS; Amersham Biosciences, Orsay, France), followed by amplification with a preformed avidin-biotin horseradish peroxidase complex (Vectastain; Vector Laboratories, Burlingame, CA). The peroxidase was revealed by incubation with a solution of diaminobenzidine (0.5 mg/ml) in D-PBS containing 0.0075% hydrogen peroxide (H_2O_2). The reaction was stopped by two rapid washes with D-PBS.

Measurement of ChAT Activity. ChAT activity was measured by the method described by Hartikka and Hefti (1988) with minor modifications. In brief, the cultures were washed three times with D-PBS and lysed at 4°C with 250 μl/well of 50 mM Tris-HCl buffer, pH 6, containing 0.3% Triton-X-100. Aliquots (50 μl) of the lysate were mixed with 100 μl of a solution containing 30 mM choline chloride, 0.6 mM eserine hemisulfate, 900 mM NaCl, and 150 mM Na_2HPO_4. The reaction was initiated by addition of 50 μl of a 10 mM sodium phosphate buffer, pH 7.4, containing [1-^{14}C]acetyl-coenzyme-A diluted with unlabeled acetyl-CoA to give a final substrate concen-

tration of 20 μM (specific activity, 4 mCi/mmol). Radioactivity corresponding to the reaction product ([^{14}C]ACh) was measured by organic liquid scintillation counting. Note that the rate of ChAT activity was linear with time and amount of cell homogenate added.

Quantification of Reactive Oxygen Species. The production of mitochondrial reactive oxygen species (ROS) was quantified using dihydrorhodamine 123 (DHR-123; Molecular Probes, Montluçon, France) according to the method described by Troadec et al. (2002). In brief, septal cultures were exposed for 30 min to 50 μM DHR-123, washed three times, and then maintained in serum-free medium supplemented with the test treatments. The fluorescent signal, visualized by epifluorescence microscopy (excitation at 488 nm; emission at 515 nm), was quantified using the Simple-PCI software from C-Imaging Systems (Cranberry Township, PA) and a Nikon TE-300 inverted microscope equipped with an ORCA-ER digital camera (Hamamatsu Photonics, Massy, France). Fluorescent images of randomly chosen fields (six to eight in each culture condition) were acquired with a 63× fluorescent objective. The average pixel intensity over the surface of each cell body was determined under the different test conditions. Background fluorescence was subtracted from raw data, and the results were expressed as a percentage of the mean fluorescence intensity per cell in control cultures. A minimum of 60 cells was analyzed under each test condition. In some experiments, ROS were measured specifically in the population of magnocellular cholinergic neurons identified on the basis of their larger size under phase contrast microscopy optics.

Detection of Caspase Activation. CaspACE-FITC-VAD-FMK in situ marker was added directly to the cultures at a final concentration of 10 μM in the presence of the test treatments. After incubation for 20 min at 37°C, the cultures were washed three times and then maintained with the test treatments during examination. The fluorescent signal was visualized by epifluorescence microscopy using the same filters as for DHR-123. Fluorescent cells were counted in 20 to 30 fields using a 40× objective.

Quantification and Visualization of [^{3}H]NA Uptake. Intraneuronal accumulation of NA via active transport was monitored in septal and LC cultures using a protocol adapted from Troadec et al. (2002). In brief, after preincubation for 10 min in 500 μl of PBS containing 5 mM glucose and 100 μM ascorbic acid, the uptake was initiated by addition of 50 nM [^{3}H]NA (37 Ci/mmol) to the cultures and terminated after 15 min by removal of the incubation medium followed by two rapid washes with ice-cold PBS. Cells were scraped off the culture wells and counted by liquid scintillation counting. When the accumulation of [^{3}H]NA was visualized by microautoradiography, the incubation time with [^{3}H]NA was extended to 30 min. After two rapid washes with PBS, the cultures were fixed for 20 min in PBS containing 0.5% glutaraldehyde and 4% formaldehyde, and dehydrated with ethanol. The incorporation of the tritiated label was detected with the Hypercoat LM-1 emulsion (Amersham Biosciences) after an exposure of 8 to 10 days in the dark at 4°C. Blockade of NA saturable transport was obtained in the presence of 0.5 μM desipramine.

Statistical Analysis. Comparisons between two groups were performed with Student's t test. Multiple comparisons against a single reference group were made by one-way analysis of variance followed by Dunnett's test. When all pairwise comparisons were made, the Student-Newman-Keuls test was used. S.E.M. values were derived from at least three independent experiments.

Results

NA Provides Long-Lasting Protection to Cholinergic Neurons. In the model system used here, rat septal cholinergic neurons were maintained initially for 10 days in a culture environment that favored their maturation and differentiation, and then exposed to trace amounts of ferrous iron to stimulate low-level oxidative stress. In the first 3 to 4 days after the initiation of the iron treatment, no noticeable morphological changes were detectable, and the number of cholinergic neurons assessed by AChE cytochemistry remained virtually unchanged (Fig. 1A). After DIV 14, however, AChE$^+$ neurons began to degenerate progressively such that >90% had died by DIV 16 (Fig. 1A). When NA (5 μM) was applied from DIV 10 onward, the loss of AChE$^+$ neurons was completely prevented (Figs. 1A and 2, top). The survival-promoting effects of NA could be extended to at least DIV 20. When the addition of iron was made at DIV 13 instead of DIV 10, the loss of cholinergic neurons did not occur between DIV 14 and 16, as observed previously, but between DIV 18 and 20, which suggested that 1) neuronal demise was not dependent on a specific stage of maturation of the cultures and 2) a period of at least 3 to 4 days was required to initiate the degenerative process in the presence of the transition metal. In this paradigm, NA again provided robust protection to cholinergic neurons (Fig. 1A). Note that in subsequent experiments made with septal cultures, treatments with iron were initiated at DIV 10.

AChE cytochemistry is a reliable and sensitive marker to detect and quantify cholinergic neurons in septal cultures (Hartikka and Hefti, 1988; Michel and Agid, 1995). However, to confirm and extend our initial observation, we

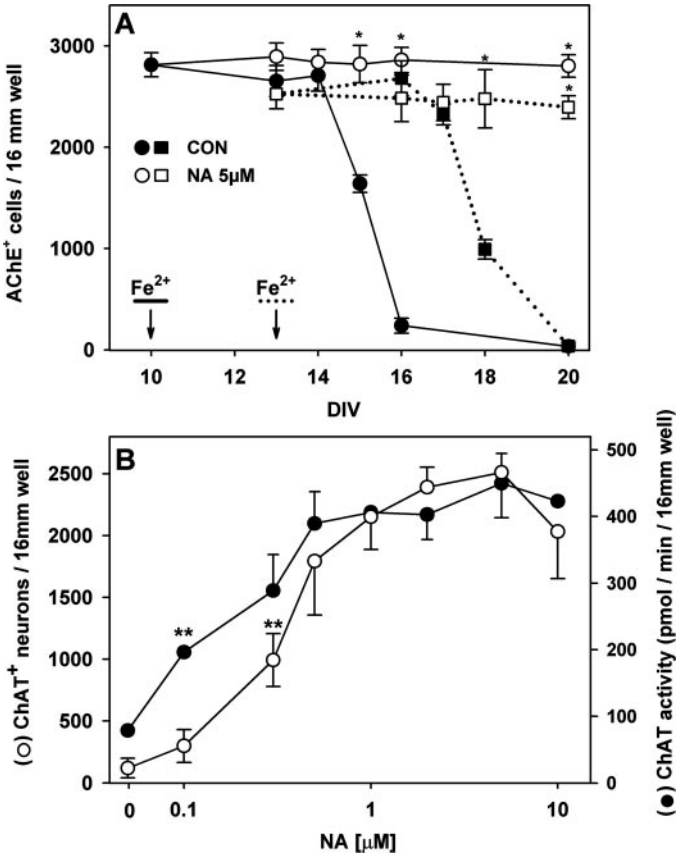

Fig. 1. Protection of cholinergic neurons by NA in septal cultures. A, number of AChE$^+$ neurons in control and NA-treated (5 μM) cultures as a function of the age of the cultures (DIV). The arrows indicate that the initiation of a continuous exposure to medium containing iron (0.5 μM) was initiated from DIV 10 (plain line) or DIV 13 (dotted line) onward. B, number of ChAT$^+$ neurons (left axis) and quantification of ChAT enzymatic activity (right axis) as a function of the concentration of NA (0–10 μM) in DIV 17 cultures. *, $p < 0.05$ versus corresponding aged-matched control cultures. **, $p < 0.05$, lowest concentration at which the test parameter is significantly increased.

also counted ChAT$^+$ cells at DIV 17 (i.e., at a time when extensive cell loss was occurring in control cultures exposed to iron first at DIV 10). ChAT$^+$ cell counts revealed that the survival promoting effect of NA was concentration dependent with an EC_{50} of ~0.3 μM (Fig. 1B). Maximal effects were observed in the range of concentrations between 1 and 5 μM (Fig. 1B). ChAT$^+$ neurons exposed to NA were well differentiated (Fig. 2, bottom) and could be distinguished from other septal neurons on the basis of their size; i.e., the widest diameter of cholinergic neurons was, on average, 22.7 ± 0.4 μm, whereas that of noncholinergic cells was 12.8 ± 0.4 μm. Thus, NA preserved not only the survival but also the key morphological features of this population of neurons. In comparison degenerating cholinergic cells remaining in control cultures had shrunken cell bodies, and their neurites were either fragmented or absent (Fig. 2). Consistent with the results obtained by cell counting, NA prevented the loss in ChAT enzymatic activity associated with neuronal demise (Fig. 1B), indicating that cholinergic neurons rescued by NA remained functional. Finally, phase contrast examination of the cultures revealed that NA was also effective in providing neuroprotection to noncholinergic (mostly GABAergic) neurons in septal cultures (data not shown).

The Effects of NA on Cholinergic Neuronal Survival Are Independent of Adrenoceptors. We compared the neuroprotective potential of NA with that of selective agonists of adrenoceptors. Neither α_1, α_2 (phenylephrine and UK14,304, respectively), β_1, nor β_2 (xamoterol and procaterol, respectively) adrenoceptor agonists, each used at 5 μM, were able to substitute for NA treatment (Fig. 3A). In addition, no survival-promoting effect was detectable when phenylephrine and UK14,304 or xamoterol and procaterol treatments were combined. Therefore, the protective effect of NA (2 μM) was antagonized neither by phen-

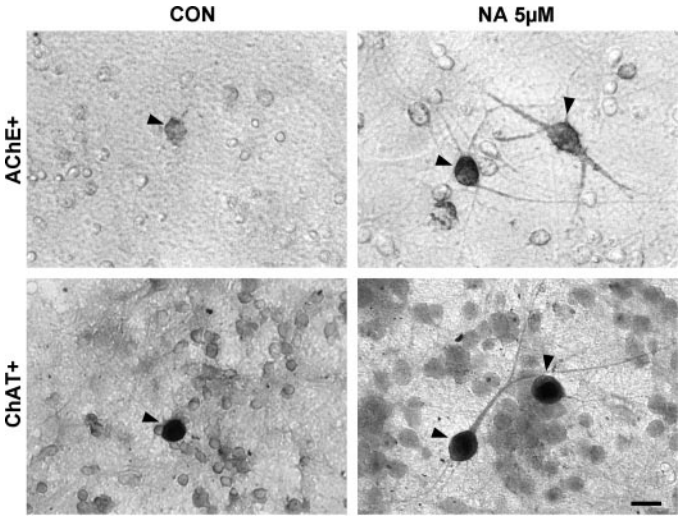

Fig. 2. Photomicrographs illustrating the survival promoting effects of NA on cholinergic neurons in septal cultures at DIV 17. Top, cholinergic neurons visualized by AChE cytochemistry. Bottom, ChAT immunodetection. Left, control cultures. Right, cultures treated with NA (5 μM) during DIV 10 to 17. Arrowheads indicate cholinergic cell bodies. Scale bar, 20 μm.

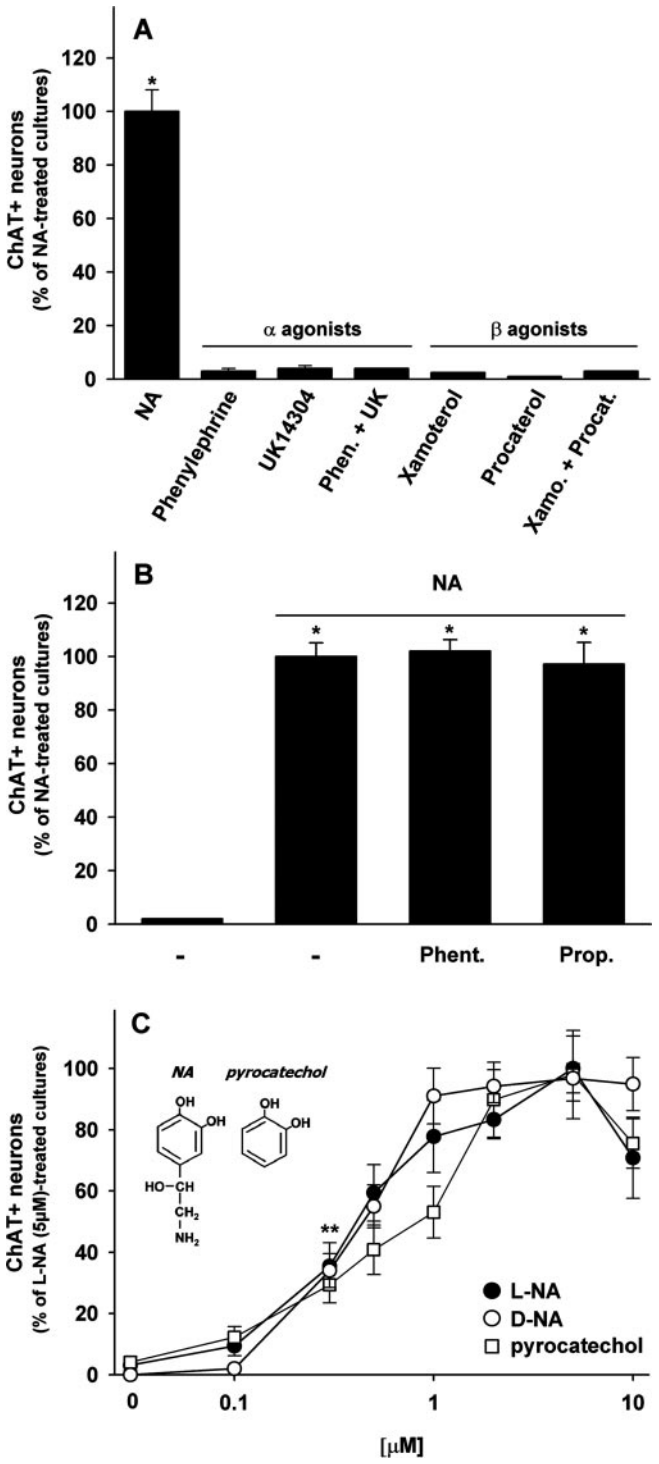

Fig. 3. Role of adrenoceptors in the protective effect of NA. A, number of ChAT$^+$ neurons in cultures exposed to NA alone (2 μM) or to α_1 (phenylephrine), α_2 (UK14,304), β_1 (xamoterol), or β_2 (procaterol) adrenoceptor agonists, each used at 5 μM. Note that none of these agonists could substitute for NA treatment. A combination of treatments with phenylephrine and UK14,304 or xamoterol and procaterol gave similar results. B, number of ChAT$^+$ neurons in cultures exposed to NA (2 μM) alone or in the presence of α (phentolamine) or β (propranolol) adrenoceptor antagonists, each used at 2 μM. C, the neuroprotective effect of NA (i.e., L-NA) was mimicked in a concentration-dependent manner by its D stereoisomer (D-NA) and by pyrocatechol, a compound that reproduces the structure of the diphenolic moiety of NA. Note that neither D-NA nor pyrocatechol was active at the adrenoceptor site. *, $p < 0.05$ versus nontreated control cultures. **, $p < 0.05$, lowest concentration producing a significant increase in ChAT$^+$ cell number.

tolamine (2 μM) nor by propanolol (2 μM), two prototypical antagonists of α- and β-adrenoceptors, respectively, indicating that NA acted by a mechanism that presumably circumvented adrenoceptors (Fig. 3B). There were two other strong arguments in favor of this hypothesis: 1) the D stereoisomer of the neurotransmitter, which is not active at the adrenoceptor site (Hearse and Sutherland, 1999), was as protective as the naturally occurring L form (i.e., the form used throughout the manuscript except when specified), and 2) pyrocatechol, a compound that reproduces the structure of the diphenolic moiety of NA but lacks significant binding affinity for adrenoceptors (Vauquelin et al., 1979), mimicked in a concentration-dependent manner the protective effects of the neurotransmitter for cholinergic neurons (Fig. 3C).

NA Rescues Cholinergic Neurons by Preventing Oxidative Stress. Because the neuroprotective effect of NA for cholinergic neurons seemed to be independent of adrenoceptor activation, we examined the nature of other putative mechanisms of action. The survival promoting activity of NA was mimicked by Trolox (10 μM; RBI/Sigma), a cell-permeable, water-soluble derivative of vitamin E, suggesting that NA itself behaved as an antioxidant (Fig. 4A). The use of the fluorescent probe DHR-123 revealed that NA was indeed as potent as Trolox in reducing ROS production in degenerating septal neurons (Fig. 4B) and in particular in the population of magnocellular cholinergic neurons (Fig. 4C). Pyrocatechol and the D stereoisomer of NA, which were as potent as NA in protecting cholinergic neurons (Figs. 3 and 4A) were also highly effective in reducing intracellular ROS production in our culture model (Fig. 4B). However, two other antioxidants, vitamin C 2-phosphate (100 μM), a stable form of vitamin C, and *N*-acetyl cysteine (NAC; 5 mM), the precursor of reduced glutathione, both failed to provide neuroprotection (Fig. 4A). In addition, vitamin C and NAC were also totally ineffective in reducing oxidative stress in the present paradigm (Fig. 4B). Finally, GABA, a neurotransmitter that is structurally unrelated to NA in that it does not possess a catechol ring, was totally ineffective in reducing ROS levels and in rescuing cholinergic neurons from degeneration.

Besides Trolox and pyrocatechol, iron chelators such as desferrioxamine (10 μM) and apotransferrin (100 μg/ml) reproduced the neuroprotective action of NA (Fig. 5A). Neuroprotection with desferrioxamine and apotransferrin was associated with a decrease in the production of intracellular ROS similar to that obtained with NA (5 μM) (Fig. 5B). This demonstrates that NA prevented a degenerative process initiated by iron, which is present at a low concentration in our culture medium. Consistent with this observation, cholinergic neurons were resistant to degeneration when maintained with the same culture medium formulated without iron (data not shown). It is interesting that catalase (300 IU/ml), an enzyme known to prevent iron-catalyzed Fenton-type chemistry, afforded a degree of protection and a reduction in the emission of intracellular ROS similar to that provided by NA (Fig. 5).

NA Provides Neuroprotection but Is Not Accumulated Intracellularly. Blocking NA uptake with desipramine, a selective inhibitor of the high-affinity NA transport system, was reported previously to prevent the trophic effects of NA in another model system (Zhang et al., 1997). This led us to speculate that the neuroprotective and thus antioxidant-like effect of NA might result from its accumulation into cholinergic neurons by active transport. Such a mechanism, however, was apparently not involved in the present model system. Indeed, in septal cultures, there was no detectable desipramine-sensitive NA uptake, in contrast to what we observed in a culture system that contains NA neurons (i.e., LC cultures) (Fig. 6A). Consistent with these observations, microautoradiographic studies revealed that the tritiated label was virtually absent from cholinergic neurons and other neurons in septal cultures (Fig. 6, B and C) but readily detectable in LC NA

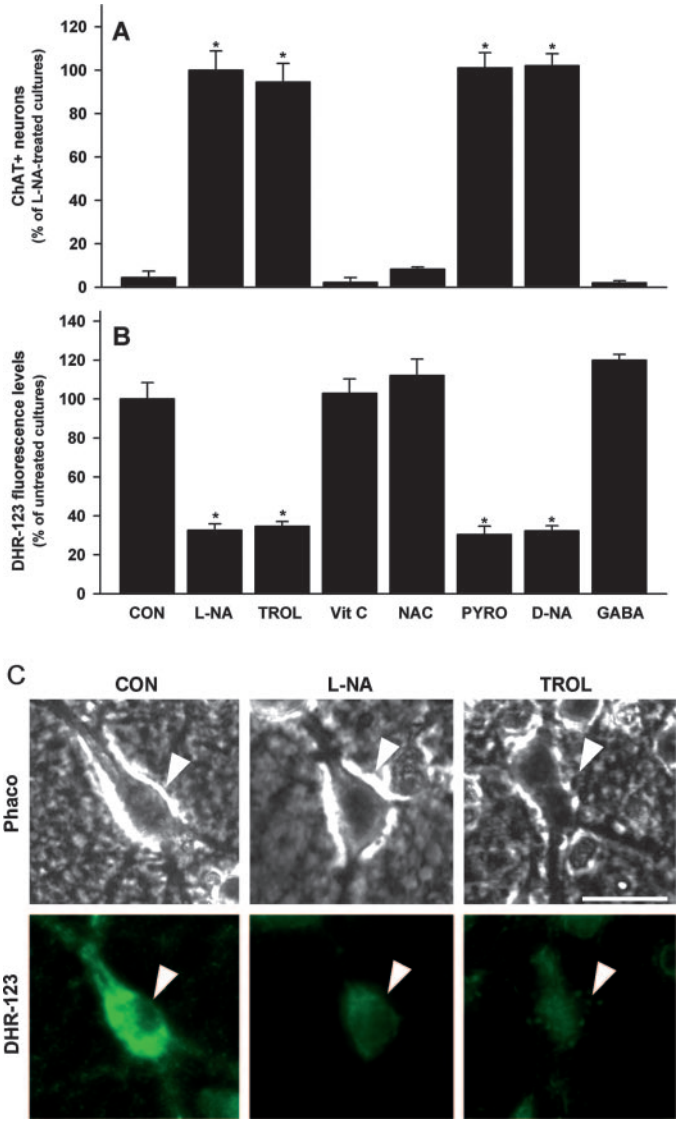

Fig. 4. The survival promoting effect of NA on cholinergic neurons is mimicked by antioxidants. A, number of ChAT$^+$ neurons in DIV 17 cultures treated with NA (5 μM; L and D forms), Trolox (TROL, 10 μM), 2P-vitamin C (VIT-C, 100 μM), NAC (5 mM), pyrocatechol (PYRO, 5 μM), or GABA (5 μM). B, detection of intracellular ROS production with the fluorescent dye DHR-123 in DIV 15 to 16 septal cultures treated as in A. *, $p < 0.05$ versus corresponding nontreated control (CON) cultures. C, photomicrographs illustrating the antioxidant effect of NA on cholinergic neurons. Top, cholinergic neurons identified on the basis of their size by examination under phase contrast (Phaco) microscopy. Bottom, intracellular ROS detection in the same neurons using the fluorescence probe DHR-123. Cultures were treated with NA (5 μM) or Trolox (10 μM). Scale bar, 20 μm.

neurons and their processes (Fig. 6D). Not surprisingly, desipramine (0.5 μM) was unable to reduce the survival promoting action of NA (1 μM) for cholinergic neurons (Fig. 6E). No positive label was detectable in cholinergic neurons or in other neurons if the treatment with 50 nM [^{3}H]NA was applied persistently (DIV 10–17) to septal cultures maintained concomitantly with Trolox (10 μM) to prevent neuronal cell demise (data not shown).

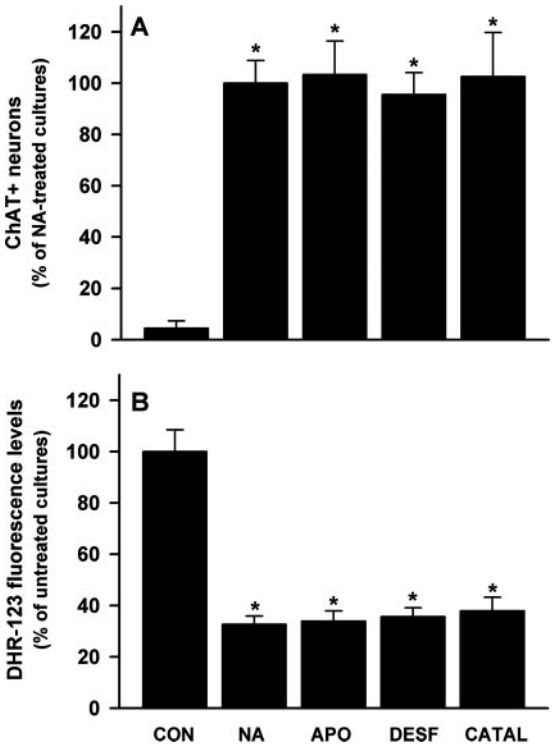

Fig. 5. NA prevents a degenerative process mediated by an iron-catalyzed Fenton-type reaction. A, number of ChAT$^+$ neurons in DIV 17 cultures in the presence of NA (5 μM), apotransferrin (APO, 100 μg/ml), desferrioxamine (DESF, 10 μm) or catalase (CATAL, 300 IU/ml). B, measurement of intracellular ROS production using the fluorescence probe DHR-123 in sister cultures at DIV 15 exposed to the same treatments as in A. *, $p <$ 0.05 versus corresponding nontreated control (CON) cultures.

At What Stage of the Degenerative Process Does the Presence of NA Become Critical As a Protective Factor? Experiments were carried out to determine whether the onset of cholinergic cell loss was preceded by a progressive accumulation of intracellular ROS (i.e., before DIV 14) and whether the antioxidant effect of NA was detectable during this time. ROS were measured in DIV 13 and 15 cultures in the absence and presence of NA (5 μM). An increased production of ROS was observed only when the cell death process was truly engaged and not before (Fig. 7A); therefore, the antioxidant effect of NA was evident only during the stage when cell demise was occurring in the cultures. This might explain why NA could be protective to cholinergic neurons even if its application was delayed up to DIV 15 (Fig. 7B).

Protection of Cholinergic Neurons by NA Occurs via Inhibition of a Caspase-Dependent Mechanism. To further characterize the neuroprotective mechanisms of NA in our model, a possible involvement of proapoptotic caspases in cholinergic neuronal death was examined. For that reason, we compared the survival promoting effect of NA to that of several caspase inhibitors; Z-DEVD-FMK (100 μM), a broad-range cell permeable caspase inhibitor; Z-IETD-FMK (50 μM), a specific inhibitor of caspase-8; and Z-LEHD-FMK (100 μM), an inhibitor that is relatively specific for caspase-9. Z-IETD-FMK mimicked completely the neuroprotective effect of NA, whereas Z-DEVD-FMK was partially protective and Z-LEHD-FMK was completely ineffective (Fig. 8A). It is noteworthy that the neuroprotective action of Z-IETD-FMK was not additive to that produced by NA (data not shown), suggesting that both compounds acted by preventing a common cell death pathway.

A more direct approach to test for caspase involvement was

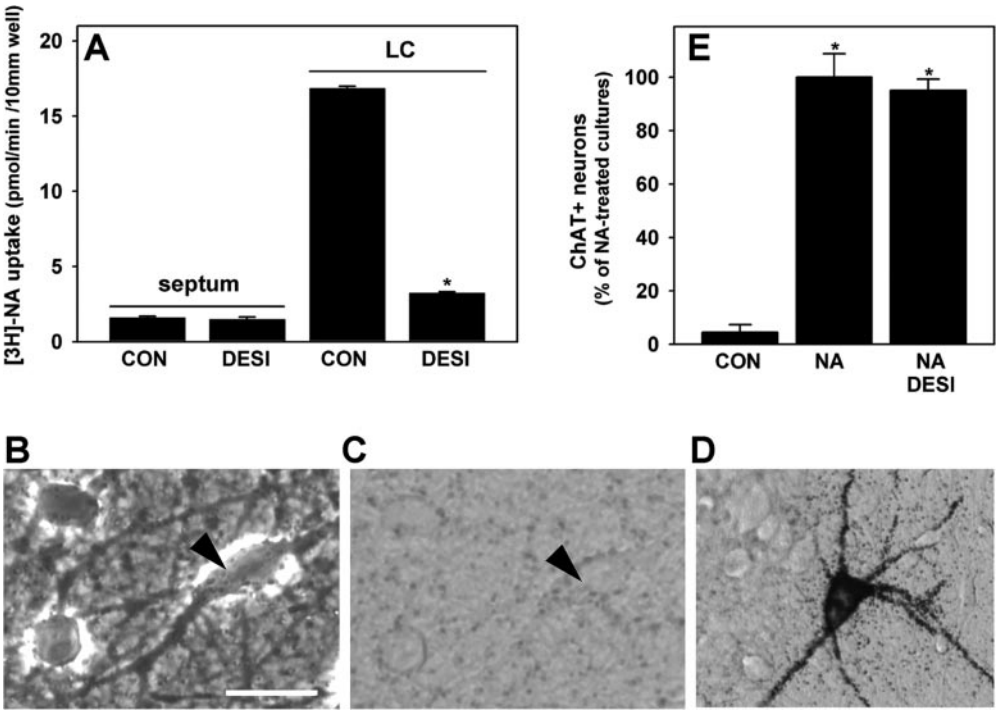

Fig. 6. NA protects cholinergic neurons by a mechanism taking place extracellularly. A, quantification of the uptake of [^{3}H]NA in septal and LC cultures treated or not with the NA uptake inhibitor desipramine (DESI, 0.5 μM). B–D, microautoradiographic visualization of NA uptake in septal (B and C) and LC (D) cultures exposed to [^{3}H]NA for 30 min. B and C correspond to the same visual field visualized by phase contrast and bright field microscopy, respectively. The black arrowhead points to a magnocellular cholinergic neuron identified on the basis of its size. E, number of ChAT$^+$ neurons in DIV 17 cultures undergoing long-term treatment or not with a protective concentration of unlabeled NA (1 μM), alone or in the presence of 0.5 μM desipramine. Scale bars, 20 μm. *, $p <$ 0.05 versus corresponding untreated cultures.

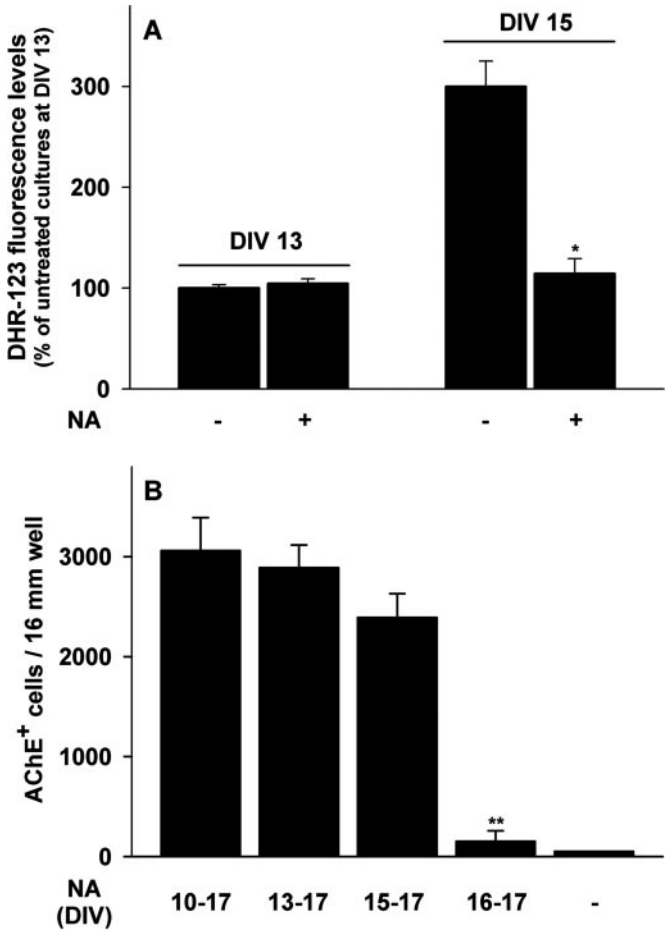

Fig. 7. Cholinergic neurons require the presence of NA only at a late stage of degenerative cell loss. A, effect of NA on ROS production at an early (DIV 13) and a late stage (DIV 15) of cell loss. B, the protective effect of NA on ChAT$^+$ neurons is still observed if NA is applied with a delay of up to but not later than DIV 15. *, $p < 0.05$ versus aged-matched control cultures. **, $p < 0.05$ versus cultures treated with NA at DIV 10 to 17.

taken by exposing the cultures to CaspACE FITC-VAD-FMK (10 μM), a cell-permeable pan-caspase fluorogenic substrate. Visual examination of control cultures revealed the presence of a strong fluorescent signal in 8 to 12% of neuronal cells (Fig. 8, B and C), particularly in those neurons exhibiting evident morphological changes. The number of cell bodies labeled by CaspACE FITC-VAD-FMK was dramatically reduced by the presence of NA or Z-IETD-FMK, attesting to the reduction of caspase activity by the neurotransmitter (Fig. 8, B and C). It is interesting that the use of DHR-123 revealed that caspase inhibition by Z-IETD-FMK was also very effective in preventing ROS production in septal neurons and more specifically so in the population of magnocellular neurons exhibiting the typical morphology of cholinergic neurons (Fig. 8, D and E).

Is the Neuroprotective Action of NA Modulated by NGF? In our model, the neurotrophin NGF was provided under basal conditions to stimulate the differentiation of cholinergic neurons. To determine whether NGF influenced the neuroprotective action of NA, we assessed the efficacy of a treatment with NA (DIV 10–17) on cholinergic neurons maintained throughout their lifespan in the absence of the neurotrophin. NA had the same efficacy in preserving cholinergic neuronal numbers in the absence of NGF (Fig. 9A), suggesting that the survival promoting action of NA was not dependent on NGF. It is noteworthy that when NA was absent from the culture medium, NGF was totally ineffective in protecting cholinergic neurons from degeneration. However, NGF dramatically increased the proportion of cholinergic neurons expressing high levels of ChAT in NA-treated cultures, indicating that the capacity of this factor to stimulate ChAT expression was dependent on the presence of the neurotransmitter. The proportion of cells expressing high levels of ChAT was the same regardless of whether the NA treatment in the absence of NGF was initiated at DIV 1 or DIV 10 (data not shown). In line with these findings, ChAT

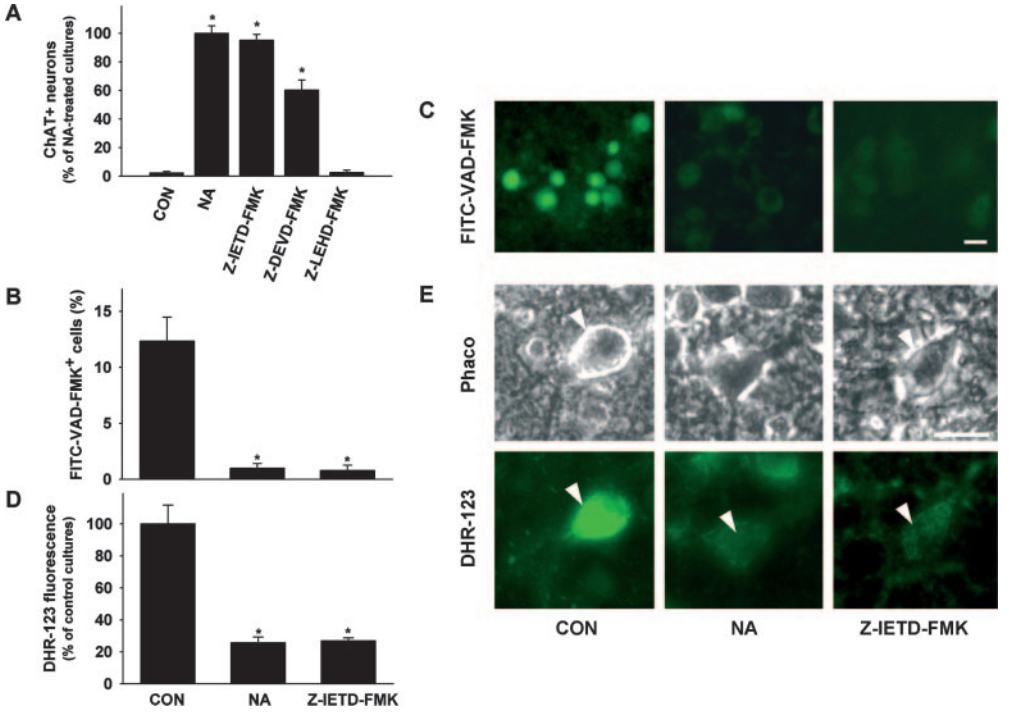

Fig. 8. NA prevents a cell death process that is mediated by caspases. A, number of ChAT$^+$ neurons in the presence of NA (5 μM) or the cell permeable caspase inhibitors Z-IETD-FMK (50 μM), Z-DEVD-FMK (100 μM), and Z-LEHD-FMK (100 μM). B, percentage of cells positively labeled with the in situ marker of activated caspases CaspACE FITC-VAD-FMK (10 μM) in cultures receiving NA, Z-IETD-FMK, or no treatment. C, visualization of the effects of the same treatments as in B. D, detection of ROS using DHR-123 in cultures receiving NA, Z-IETD-FMK, or no treatment. E, top, identification of cholinergic neurons under phase contrast (Phaco) microscopy. Bottom, detection of ROS in the same neurons using DHR-123. Scale bars, 20 μm. *, $p < 0.05$ versus corresponding control cultures.

enzymatic activity was at least 3-fold higher in the presence of NGF and NA compared with NA alone (Fig. 9B). Furthermore, the cell bodies of cholinergic neurons treated with NGF and NA were larger than those treated with NA alone (Fig. 9C).

BDNF, another neurotrophin known to promote the development and maintenance of cholinergic neurons (Knusel et al., 1992), was examined under similar experimental conditions to determine whether it, too, could influence the protective effects of NA. Similar to what was observed in NGF/NA-treated cultures, the large majority of cholinergic neurons rescued by cotreatment with BDNF and NA expressed high levels of the ChAT enzyme (Fig. 9, A and C). These neurons, however, were smaller than those treated with NGF and NA (Fig. 9C). Consistent with this finding, BDNF was also less effective than NGF in stimulating ChAT activity in the presence of NA (Fig. 9B). It is noteworthy that BDNF, like NGF, failed to increase cholinergic cell survival in the absence or the presence of NA (Fig. 9A).

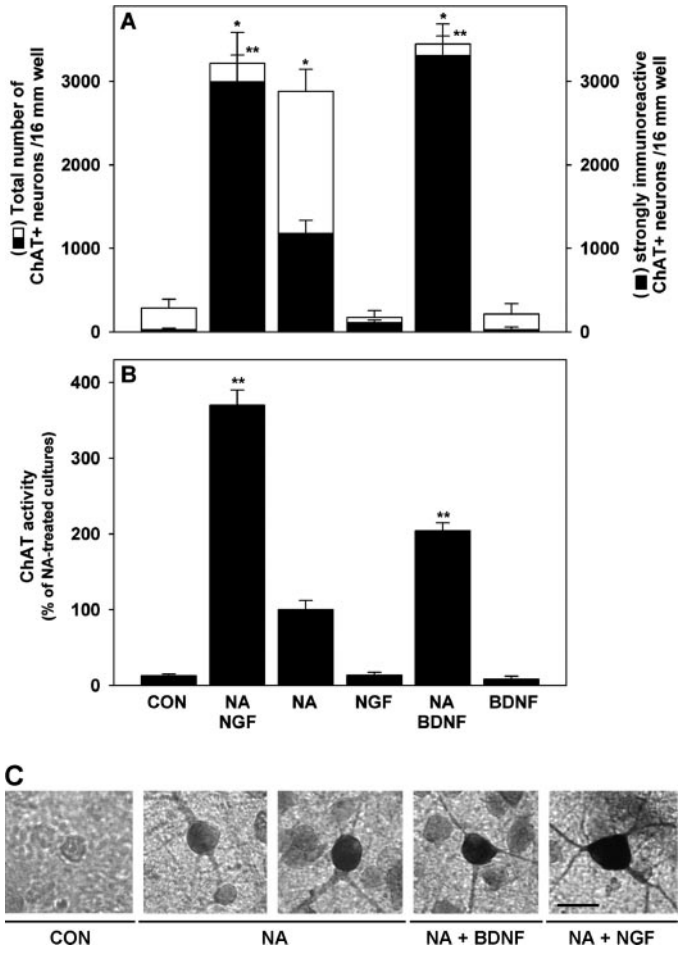

Fig. 9. Modulation of the neuroprotective effects of NA by NGF and BDNF. A, septal cultures exposed or not to NA in the presence or the absence of NGF (50 ng/ml) or BDNF (20 ng/ml). For each treatment condition, the entire bar represents the total number of cholinergic neurons per 16-mm well. The filled portions of the bars represent the proportion of neurons that were strongly immunoreactive for ChAT. Note that NGF and BDNF failed to improve cholinergic cell survival either alone or in the presence of NA. However, in the presence of NA, both factors strongly increased the proportion of cholinergic neurons expressing high levels of ChAT. B, ChAT enzymatic activity for the same treatments as in A. C, photomicrographs illustrating the effects of NA alone, NA+NGF, and NA+BDNF on the morphology of cholinergic cells and intensity of ChAT immunostaining. Scale bar, 25 μm. *, $p < 0.05$ versus control (CON) cultures. **, $p < 0.05$ versus cultures treated with NA alone.

Discussion

In this study, we demonstrated that NA provided sustained and robust neuroprotection in a model system of septal cholinergic cell death in vitro. It is noteworthy that rescued cholinergic neurons were functional and well preserved morphologically. Neuroprotection by NA was atypical in that it did not require adrenoceptor activation. NA evidently operated through a mechanism that precludes activation of a ROS-dependent cell death pathway. Neither NGF nor BDNF alone was able to mimic the protective action of NA, although both neurotrophins seemed dependent on the presence of the neurotransmitter to reinforce the expression of the cholinergic phenotype.

NA Protects Cholinergic Neurons from Oxidative Stress Caused by a Fenton-Type Reaction. Several observations indicate that NA operates in our model by reducing oxidative stress: 1) cholinergic neurons were protected efficiently by an analog of vitamin E Trolox and by pyrocatechol, a diphenolic compound with a strong antioxidant capacity (Troadec et al., 2002); 2) the rescue of cholinergic neurons was also achieved by desferrioxamine and apotransferrin, two molecules known to sequester iron, a transition metal catalyst for the Fenton reaction that allows the conversion of H_2O_2 into highly reactive hydroxyl radicals (Halliwell and Gutteridge, 1984); and 3) detoxification of H_2O_2 by catalase was strongly neuroprotective. The finding that NA reduced ROS emission to a large extent within septal neurons, particularly the population of cholinergic neurons, confirmed that NA was operating by preventing oxidative stress or its consequences. The antioxidant function of NA was apparently not mediated by activation of membrane located adrenoceptors. It seemed to be related, however, to the presence of a catechol moiety in its chemical structure because 1) its effects were reproduced in a concentration-dependent manner by the structural congener pyrocatechol (the *o*-catechol moiety of NA), and 2) GABA, a neurotransmitter with no catechol ring, was totally ineffective in reducing ROS levels and rescuing cholinergic neurons.

NA Operates via an Extracellular Mechanism. Both the protective and antioxidant effects of NA for cholinergic neurons were mimicked by long-term treatment with catalase, an enzyme that neutralizes ROS exclusively in the extracellular compartment because it cannot penetrate the plasma membrane of intact cells (Muzykantov, 2001). Several observations would suggest that the site of the antioxidant effect of NA was, like that of catalase, extracellular. First, neuroprotection by NA was independent of adrenoceptors, ruling out an antioxidant effect resulting from the activation of intracellular signaling pathways. Second, accumulation of NA via active transport has been described in peripheral nervous system developing cholinergic neurons (Habecker et al., 2000), but we found that this mode of transport was absent in basal forebrain cholinergic neurons. Third, the possibility that the accumulation of NA or its potential metabolites occurred by passive transport is also unlikely because we failed to detect substantial amounts of radioactive label within cholinergic neurons as well as other

septal neurons, even after long-term exposure to [^{3}H]NA. Therefore, we may surmise that the action of the neurotransmitter consisted in preventing the propagation of oxidative stress at the level of the outer plasma membrane, thus precluding ROS from being generated intracellularly. Consistent with this view, NA has been shown to prevent the peroxidation of membrane lipids in cell-free systems (Andorn and Pappolla, 2001). We cannot exclude the possibility that iron scavenging by the neurotransmitter represented the underlying chemical mechanism. Based on previous studies performed with other catechol derivatives (Stites et al., 2000), however, it is also conceivable that redox-cycling between NA and its autoxidation product NA-quinone participated in neuroprotection.

When Do Cholinergic Neurons Require the Presence of NA to Survive? We were surprised to find that the death of cholinergic neurons did not occur immediately after addition of iron to the cultures at DIV 10 but only after a delay of 3 to 4 days. It may be that cholinergic neurons become susceptible to oxidative stress upon reaching some critical stage just before DIV 15 to 16 in culture, although this seems unlikely because a comparable delay before neurodegeneration was observed when the treatment with iron was postponed by several days. On the other hand, as a consequence of the treatment with iron, intracellular ROS might accumulate progressively before reaching levels sufficient to kill cholinergic cells. If this were the case, one would expect NA to suppress intracellular ROS production during that time. Arguing against this hypothesis, ROS levels were identical in NA-treated and control cultures before the onset of cholinergic cell degeneration. It cannot be excluded that NA protected the plasma membrane of cholinergic neurons from oxidative stress-mediated damage long before an increase in ROS levels was detectable intracellularly. The fact that NA was still able to effectively interrupt the degenerative process even when applied several days after placing the cultures in a ROS-producing environment indicates that these alterations if they occurred were still reversible in the presence of the neurotransmitter.

NA Reduces Intracellular ROS Production via Inhibition of a Caspase-Dependent Mechanism. Two arguments suggest that caspases [i.e., proteases that mediate apoptotic cell death (Hengartner, 2000)] were crucially involved in the death pathway inhibited by NA: 1) Z-IETD-FMK, a selective inhibitor of caspase-8, and Z-DEVD-FMK, a broad-range caspase inhibitor reproduced totally and partially the protective effect of NA, respectively; and 2) NA itself prevented caspase activation. Given that the site of action of NA was presumably extracellular, caspase inhibition produced by the neurotransmitter was most probably indirect. Activation of caspase-8 and other caspases is frequently caused by stimulation of the death domain receptor Fas (FasR) by its cognate ligand FasL (Qiu et al., 2002), suggesting that NA might prevent a FasR-related mechanism. This seems unlikely, however, because cholinergic neurons were resistant to a soluble recombinant FasL protein, and a FasL recombinant neutralizing antibody failed to reproduce neuroprotection by NA (data not shown). It is worth noting that the reduction of oxidative stress produced by the inhibitor of caspase-8 in cholinergic neurons was similar to that obtained with NA, which indicates that intracellular ROS were probably generated downstream of the level at which the caspase-dependent mechanism intervenes. It is interesting that a recent study has linked mitochondrial ROS production during apoptosis to caspase-dependent cleavage of a complex I subunit of the mitochondrial electron transport chain (Ricci et al., 2004).

NA Provides Optimal Neuroprotection Even in the Absence of NGF. Unlike dopaminergic neurons, which were protected only partially against oxidative stress by NA [~40% of them were rescued after 10 days of treatment (Troadec et al., 2001)], virtually all septal cholinergic neurons survived to ROS-mediated damage in the presence of the neurotransmitter. Such a difference may lie in the fact that NGF present continuously for the differentiation and maturation of cholinergic neurons may have already enhanced the survival promoting effects of NA in the same way that cAMP-elevating agents improved the effects of the neurotransmitter for dopaminergic neurons in mesencephalic cultures (Troadec et al., 2002). Supporting this view, NGF can attenuate the deleterious consequences of oxidative stress-mediated neuronal injuries in some culture models (Salinas et al., 2003); on the other hand, mitochondrial-derived ROS are produced in NGF-responsive neurons that are deprived of the neurotrophin (Kirkland and Franklin, 2003). To determine whether NGF improved the neuroprotective action of NA on cholinergic neurons, we performed experiments with cultures maintained throughout their lifespan without the neurotrophin. Despite the absence of NGF, NA alone was sufficient to rescue virtually all cholinergic neurons susceptible to degeneration. This indicates that NGF was not essential for survival promotion by NA. Consistent with this observation, NGF was unable to promote cholinergic cell survival or to reduce ROS production in the absence of NA. Therefore, the presence of NGF cannot account for the high efficacy of NA to rescue septal cholinergic neurons.

NGF and BDNF Retain Their Capacity to Stimulate Cholinergic Parameters in the Presence of NA. Even though NGF lacked survival-promoting effects either alone or in the presence of NA, it did improve the impact of NA by increasing ChAT expression and cell size in surviving cholinergic neurons. BDNF, another neurotrophin known to function as a trophic factor for cholinergic neurons (Knusel et al., 1992), had an effect that was similar to but somewhat less pronounced than that of NGF. That exogenous NGF and BDNF were able to stimulate cholinergic parameters exclusively in the presence of NA is noteworthy, because a number of diphenolic antioxidants have been shown to interfere with the function of some regulatory peptides or cytokines (Zi et al., 1998). Our results are more consistent with studies showing that NA can act cooperatively with trophic peptides, such as glial cell line-derived neurotrophic factor and ciliary neurotrophic factor on specific populations of neurons in the brain (Louis et al., 1993; our unpublished data).

Recent studies support the idea that proapoptotic caspases (Rohn et al., 2001; Cribbs et al., 2004) and ROS-mediated reactions (Casadesus et al., 2004; Pratico and Sung, 2004) are crucially involved in neurodegeneration in AD. Our results showing that NA can substantially reduce both caspase activation and ROS production in cholinergic neurons, one of the neuronal populations most at risk in AD, provide support to the concept that compounds that stimulate NA output may have therapeutic utility in the treatment of this pathology.

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