Temporal Analysis of Events Associated with Programmed Cell Death (Apoptosis) of Sympathetic Neurons Deprived of Nerve Growth Factor

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Abstract. The time course of molecular events that accompany degeneration and death after nerve growth factor (NGF) deprivation and neuroprotection by NGF and other agents was examined in cultures of NGF-dependent neonatal rat sympathetic neurons and compared to death by apoptosis. Within 12 h after onset of NGF deprivation, glucose uptake, protein synthesis, and RNA synthesis fell precipitously followed by a moderate decrease of mitochondrial function. The molecular mechanisms underlying the NGF deprivation–induced decrease of protein synthesis and neuronal death were compared and found to be different, demonstrating that this decrease of protein synthesis is insufficient to cause death subsequently. After these early changes and during the onset of neuronal atrophy, inhibition of protein synthesis ceased to halt neuronal degeneration while readdition of NGF or a cAMP analogue remained neuroprotective for 6 h. This suggests a model in which a putative killer protein reaches lethal levels several hours before the neurons cease to respond to readdition of NGF with survival and become committed to die. Preceding loss of viability by 5 h and concurrent with commitment to die, the neuronal DNA fragmented into oligonucleosomes. The temporal and pharmacological characteristics of DNA fragmentation is consistent with DNA fragmentation being part of the mechanism that commits the neuron to die. The antimitotic and neurotoxin cytosine arabinoside induced DNA fragmentation in the presence of NGF, supporting previous evidence that it mimicked NGF deprivation–induced death closely. Thus trophic factor deprivation–induced death occurs by apoptosis and is an example of programmed cell death.

E XTENSIVE cell death occurs during the normal development and maintenance of the tissues and organs of vertebrates (Glücksmann, 1951). Naturally occurring cell death helps to establish and maintain the functional properties of tissues and organs (Saunders, 1966; Kerr et al., 1972) and presumably developed because it conferred an evolutionary advantage to the organism (Umansky, 1982). Cell death is often controlled by survival-promoting signals from other cells (for concise review see Raff, 1992), but is mostly executed in a cell-autonomous manner. The cell corpse or fragments thereof are eliminated by proximal phagocytosing cells or infiltrating tissue macrophages and do not elicit an inflammatory response (Kerr et al., 1972; reviewed by Savill et al., 1993).

The molecular mechanisms underlying naturally occurring cell death are not well understood. Based upon morphological criteria, several different modes of cell death have been defined (Schweichel and Merker, 1973; Beaulaton and Lockshin, 1982; Clarke, 1990) for which different underlying molecular mechanisms are believed to exist. Among the different modes, the mode of apoptosis (Kerr et al., 1972) has received much attention since many non-neuronal cell types show this mode of death during development and adulthood and several non-neuronal model systems are available that allow the study of the underlying molecular mechanisms (reviewed by Arends and Wyllie, 1991). At early stages, morphologically visible cytoplasmic changes are rather subtle. Polyribosomes disintegrate and may form semicrystalline arrays concurrent with the decrease of protein synthesis. The cell atrophies, but mitochondria and other organelles remain physically intact for an extended period. Among the principal criteria that characterize cell death as apoptotic are the condensation of the nuclear chromatin (Kerr et al., 1972) and the fragmentation of the nuclear DNA into oligonucleosomes before cell death (Wyllie, 1980); both phenomena appear to be closely correlated (Arends et al., 1990). Transiently, cells show blebbing of the plasma membrane. Thereafter, all cytoplasmic organelles start to degenerate and both nucleus and cytoplasm become compartmentalized into membrane-bound apoptotic bodies which degenerate by secondary necrosis or are engulfed by phagocytic cells.

Extensive neuronal death occurs during the normal development of the nervous system (reviewed in detail by Oppenheim, 1991). Supernumerary neurons generated by cell division from neuroblasts die while the neurons make functional connections with their target. This elimination serves to match the number of innervating neurons to the size of their target. Among the signals that control neuronal survival are trophic factors upon which the neurons depend for survival during the period of neuronal death (Purves, 1986) and, to a lesser extent, also thereafter (Gorin and Johnson, 1980; Otto et al., 1987; Rich et al., 1987). Classic trophic factors are secreted in minute amounts by the target, bind to receptors on the innervating nerve terminals, are internalized, and retrogradely transported to the cell body. A yet unidentified retrogradely transported signal activates mechanisms that maintain the survival of and trigger trophic responses in the neuronal soma (Barde, 1989; Snider and Johnson, 1989). Insufficient access to trophic factor initiates a protein and RNA synthesis–dependent degenerative process that terminates in neuronal death (Martin et al., 1988; Oppenheim et al., 1990; Scott et al., 1990).

A well-studied model system of trophic factor deprivation-induced death is based on the physiological role that the neurotrophic factor NGF has in maintaining sympathetic neurons during development (Levi-Montalcini and Booker, 1960; Gorin and Johnson, 1979) and in the adult (Gorin and Johnson, 1980) in vivo and during development in vitro (Levi-Montalcini and Angeletti, 1963; Martin et al., 1988). The current understanding of the molecular mechanisms underlying NGF deprivation–induced death of sympathetic neurons has been reviewed recently (Johnson and Deckwerth, 1993). The present work examines the temporal relationships of several biochemical changes that accompany degeneration and death of sympathetic neurons deprived of NGF in vitro and studies the causal relationship of some of these processes to death by examining the effect of neuroprotective agents on these processes. It is found that immediately after NGF deprivation and preceding any morphologically discernable change, energy and macromolecular metabolism decrease rapidly. The neurons lose the ability to be rescued by inhibition of protein synthesis while the somata atrophy and the neurites disintegrate. The neuronal genomic DNA becomes fragmented concurrent with the loss of the neurons' ability to respond to readdition of NGF with survival consistent with DNA fragmentation being part of the mechanism that commits the neuron to die. A few hours later, the neuron loses its viability. Taken together with previous ultrastructural data, this sequence of events identifies trophic factor deprivation–induced neuronal death as apoptotic and as an example of programmed cell death. Much of this work has been reported in abstract form (Deckwerth, T. L., and E. M. Johnson, Jr. 1991. *Soc. Neurosci. Abs.* 17:228a; Deckwerth, T. L., and E. M. Johnson, Jr. 1992. *Eur. J. Neurosci. Suppl.* 5:308a; Deckwerth, T. L., and E. M. Johnson, Jr. 1992. *Soc. Neurosci. Abs.* 18:49a).

Materials and Methods

Materials

Timed pregnant Sprague Dawley rats and female Sprague Dawley rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Collagenase

P, dispase, cyclic 8-(4-chlorophenythio)adenosine-3'5'-monophosphate (CPTcAMP)[1], and the restriction enzyme EcoRI were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Mouse 2.5S NGF was prepared using the method of Bocchini and Angeletti (1969). The goat anti-NGF antiserum has been characterized previously (Ruit et al., 1992) and had increased in titer to more than 100,000 against 5 ng/ml NGF in the chick dorsal root ganglion bioassay (Fenton, 1970). Rat tail collagen was prepared and the tissue culture dishes coated by the method of Johnson and Argiro (1983). Glassware was silanized with a 5% solution of dichlorodimethylsilane in chloroform (Sambrook et al., 1989). All other reagents, whose suppliers are not mentioned explicitly, were obtained from Sigma Immunochemicals (St. Louis, MO).

Cell Culture

Primary cultures of sympathetic superior cervical ganglion (SCG) neurons were prepared using a modification of a previously published procedure (Johnson and Argiro, 1983). SCGs were dissected from E21 rat embryos into Leibovitz L15 medium (GIBCO-BRL, Gaithersburg, MD), digested for 30 min with 1 mg/ml collagenase and 1 mg/ml dispase in L15 medium at 37°C, washed in PBS, and dissociated into a single cell suspension using a silanized and flame-polished pasteur pipet. After filtration of the suspension through a nitex filter (size 3-20/14; Tetko Inc., Elmsford, NY), the number of viable cells was determined by trypan blue exclusion. Cells were allowed to attach for 45 to 90 min in a drop of medium S (Eagle's MEM with Earle's salts from GIBCO-BRL or Sigma Immunochemicals supplemented with 10% FBS from Hyclone Laboratories, Logan, UT) on ammoniated collagen-coated 24-well plates (Costar Corp., Cambridge, MA) at 20,000 living cells per well or on ammoniated and air-dried collagen-coated 2-well chamber slides (Nunc, Naperville, IL) at 9,000 living cells per well. Cultures of sympathetic neurons were established by incubation for 4 d at 35°C in SNaPS (S supplemented with 50 ng/ml mouse 2.5S NGF, 3.3 μg/ml aphidicolin, 100 U/ml penicillin, and 100 μg/ml streptomycin) inside a humidified desiccator with a 5% $CO_2/95\%$ air atmosphere. Cultures devoid of neurons were generated by substituting 0.05% anti-NGF antiserum for NGF. After 4 d, two thirds of the medium were removed and replaced with SNa (S supplemented with 50 ng/ml NGF and 3.3 μg/ml aphidicolin).

To reduce the number of contaminating non-neuronal cells in the established cultures by ~30-fold, up to 10^6 living freshly dissociated cells were preplated in 10 ml medium S at 37°C for 2-3 h onto a 100 mm petri dish (Falcon Brand, Becton Dickinson, Lincoln Park, NJ) and incubated in a humidified incubator with a 5% $CO_2/95\%$ air atmosphere. Non-adhering neurons were collected from the supernatant in silanized conical glass tubes by centrifugation at 500 g for 10 min, resuspended in PBS, and counted by trypan blue exclusion. These purified neurons were plated as described above.

NGF Deprivation

SCG neuronal cultures were deprived of NGF by exchanging the NGF-containing medium for SAa (S supplemented with 0.05% goat anti-NGF antiserum and 3.3 μg/ml aphidicolin). To generate a set of 8-d-old in vitro cultures deprived of NGF for various periods between 0 and 96 h, the onset of NGF deprivation was varied accordingly. To control for the effect of exchanging the medium on metabolic measurements, control cultures, whose medium was exchanged for SNa at identical times, were processed in parallel.

Analysis of Morphological Changes

The morphological changes visible by phase contrast microscopy were derived from a longitudinal study of identified neurons. Serial phase contrast micrographs of fields of neurons maintained in culture for 6 d were taken before and at various times after onset of NGF deprivation using a Nikon Diaphot inverted microscope equipped with a heated stage. One field was selected per individual neuronal culture. Neuronal cell bodies growing in

isolation or in small groups were selected on the micrographs of the fields taken before NGF deprivation and the neurons' fate was followed on the micrographs taken at various times after onset of NGF deprivation. Only neurons that were visible at each of the monitoring points were included in the analysis. Soma degeneration was indicated when the neuronal soma was reduced in size, showed swellings, contained vacuoles, or had lost refractility. A field of neurites was scored as showing signs of disintegration when swellings and blebs appeared along the neurite bundles. The observer was blinded as to the length of NGF deprivation to which a field of neurons had been exposed.

Viability (Nissl Staining). Neuronal cultures on chamber slides were fixed with 4% paraformaldehyde in PBS for more than 12 h, stained with crystal violet (EM Diagnostic, Gibbstown, NY), destained in water, dehydrated in increasing ethanol concentrations, transferred to toluene, and mounted in a toluene-based mounting solution (Pro-Texx; Baxter Diagnostics, Deerfield, IL). Neurons were scored as viable if they had a defined cellular outline and stained stronger than debris.

Nuclear Staining. Neuronal cultures on chamber slides were fixed with 4% paraformaldehyde in PBS for more than 12 h, exposed to 1 µg/ml 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride pentahydrate (Bisbenzimide, Hoechst 33258; Molecular Probes, Eugene, OR) in PBS for 15 min at 22°C, and washed with PBS. The cultures were mounted in 0.1% p-phenylene diamine and 90% glycerol in PBS (Johnson and Noguiera Araujo, 1981) and examined under UV illumination on a Nikon Microphot fluorescence microscope. The morphological features visualized by bisbenzimide represent an extrapolation from neuronal populations.

Rescue with NGF and Other Neuroprotective Agents

To measure the ability of NGF-deprived neurons to respond to NGF with survival, neuronal cultures deprived of NGF for periods ranging from zero to 48 h were washed once with SNa and then incubated in SNa for a week. Rescue with CPTcAMP or cycloheximide was accomplished by replacing the medium with SAa containing 400 µM CPTcAMP or 1 µg/ml cycloheximide, respectively, and incubating the cultures for 48 h. To examine the effect of protein synthesis inhibition on the neuroprotective effect of NGF, neuronal cultures deprived of NGF for 18 or 24 h were washed once with SNa and rescued with 1 µg/ml cycloheximide in SNa, SNa alone, or 1 µg/ml cycloheximide in SAa for 55 h. A rescue period of at least two days was chosen such that neurons incapable of responding to the rescuing agent with survival would have sufficient time to degenerate and die. For NGF, this minimal rescue period can be extended since NGF maintains sympathetic neurons indefinitely, while this is not possible for neuroprotective agents with toxic side effects, such as cycloheximide. To determine the number of surviving neurons, the rescued cultures were fixed, Nissl stained, and counted by a blinded observer as described above. The time course of commitment to die was calculated as 100% minus the percentage of neurons capable of responding to NGF with survival at each time point.

Metabolic Parameters

Cultures of sympathetic neurons were maintained in 24-well plates. All assays measuring metabolic rates were linear with time throughout the measuring period.

Total Neuronal Protein. Neuronal cultures were washed once with PBS and lysed into 200 µl of reagent A of the BCA protein assay from Pierce (Rockford, IL). The color reaction was adapted to a reaction volume of 200 µl and the absorbance of the Cu^+-bicinchoninic acid complex was read with a Titertek Multiscan MC 96-well plate reader. The protein concentration was determined from an internal BSA standard curve. The alkaline lysis conditions prevent the contamination of the neuronal lysate with collagen.

Rate of Protein Synthesis. Neuronal cultures were labeled for 4 h at 35°C with 10 µCi/ml [^{35}S]-L-methionine (>1,000 Ci/mmol; ICN, Costa Mesa, CA) in media SNa or SAa containing 10 µM unlabeled L-methionine. Cultures were washed once with medium S and lysed with 500 µl of 0.5% SDS, 1 mM EDTA, 10 mM Tris, pH 7.5 (HCl). After addition of 10 µg of BSA to each sample, the protein was precipitated with 10% TCA on ice and retained by filtration through a 0.45 µm nitrocellulose filter (BA-85; Schleicher & Schüll, Keene, NH). The filter was washed twice with cold 10% TCA, dried, and its radioactivity measured in a liquid scintillation counter.

Rate of RNA Synthesis. Neuronal cultures were labeled with 10 µCi/ml [5,6-^{3}H]-uridine (44 Ci/mmole; ICN, Costa Mesa, CA) in medium SNa or SAa for 4 h at 35°C. The cultures were washed once with medium S and lysed with 500 µl of 0.5% N-lauroylsarcosine, 1 mM EDTA, 10 mM Tris pH 7.5 (HCl) on ice. After addition of 10 µg yeast tRNA to each sample, the RNA was precipitated with 10% TCA, 1% sodium pyrophosphate on ice and retained by filtration through a 0.45 µm nitrocellulose filter. The filter was washed twice with cold 10% TCA, 1% sodium pyrophosphate, dried, and its radioactivity was measured in a liquid scintillation counter.

Rate of 2-deoxyglucose Uptake. Neuronal cultures were labeled for 10 min at 35°C with 2.5 µCi/ml 2-deoxy-D-[2,6-^{3}H]-glucose (34 Ci/mmole; Amersham Corp., Arlington Heights, IL) in medium S modified to contain 500 µM D-glucose and 20 mM Hepes, pH 7.0. The cultures were washed three times with Hepes-buffered S and lysed with 500 µl of 1% SDS, 1 mM EDTA, 10 mM Tris pH 7.5 (HCl). The radioactivity of the lysate was measured in a liquid scintillation counter.

Rate of MTT Reduction. A modification of a previously described procedure was used (Carmichael et al., 1987). Neuronal cultures were exposed to 500 µl of 0.4 mg/ml 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in L15 containing 10% FCS for 9 min at 35°C. The MTT solution was aspirated and the cells were lysed into 200 µl DMSO. The amount of MTT formazan was quantitated by determining its absorbance at 510 nm in a Titertek Multiscan MC 96-well plate reader.

Rate of Rhodamine 123 Uptake. Neuronal cultures were exposed to 500 µl of 10 µg/ml rhodamine 123 (Molecular Probes, Inc., Eugene, OR) in L15 supplemented with 10% FCS for 9 min at 35°C. The cultures were washed once with Locke's solution (Didier et al., 1990). The fluorescent dye was recovered by lysis of the cultures into 200 µl DMSO and was quantitated by measuring the fluorescence of the lysate at 538 nm upon excitation at 485 nm in a Titertek Fluoroscan II 96-well plate reader.

Activity of Cytochrome Oxidase (Ferrocytochrome C/Oxygen Oxidoreductase, EC 1.9.3.1). Neuronal cultures were washed once with PBS and lysed with 150 µl of 0.75% deoxycholate on ice. The method of Hess and Pope (1953) was used to measure the cytochrome oxidase activity of the lysate. Briefly, 300 µg/ml cytochrome C was reduced stepwise to over 90% with small aliquots of 30 mM sodium hydrosulfite. The initial rate of oxidation of reduced cytochrome C was measured with a spectrophotometer (DU-8; Beckman Instruments) at 22°C and a wavelength of 550 nm after addition of an aliquot of the cell lysate. The rate was normalized to the difference of the absorbance between maximally reduced and oxidized cytochrome C.

Dexamethasone-induced Death of Thymocytes

Female rats (200 g body weight) were injected i.p. with 5 mg/kg dexamethasone in PBS containing 10% DMSO or with vehicle alone. After 10 h, the rats were killed and the thymi removed. Thymocytes were isolated by extruding the loosely bound cells through a steel mesh into cold PBS. Large tissue fragments were allowed to settle for 10 min on ice and the thymocytes were collected from the supernatant by centrifugation at 400 g for 5 min at 4°C. The thymocytes were resuspended in PBS and counted. Aliquots of thymocytes were frozen at −70°C to serve as controls during neuronal DNA isolation.

DNA Fragmentation

Genomic DNA was prepared by a modification of the method of Davis et al. (1986). Neuronal cultures (20,000 neurons) were washed once with cold PBS and lysed with 400 µl of lysis buffer (0.5% SDS, 1 mM EDTA, 100 mM NaCl, 10 mM Tris HCl, pH 8.0). An aliquot of control and dexamethasone-exposed thymocytes was thawed in 400 µl of lysis buffer. Carrier DNA (5 µg Escherichia coli DNA from strain K12) and 40 µg proteinase K (Life Technologies, Gaithersburg, MD) were added and the lysates were incubated for 60 min at 50°C. Proteinase K (40 µg) was added again and the incubation was continued overnight. RNase A (40 µg) was added and the solution incubated for 60 min at 37°C. The lysates were extracted with phenol/chloroform and the DNA was ethanol precipitated. The DNA was collected by centrifugation at 13,000 g for 20 min at 4°C, the pellet was washed with 70% ethanol, dried superficially, and redissolved in TE buffer (Sambrook et al., 1989) overnight.

To isolate rat thymocyte DNA for the probe from 5×10^7 thymocytes of the vehicle-injected rat, the preceding protocol was scaled up and the addition of carrier DNA omitted. An aliquot of the thymocyte DNA was completely digested with the restriction enzyme EcoRI, the DNA purified by phenol/chloroform extraction and ethanol precipitation. The probe was prepared by labeling the EcoRI-digested rat thymocyte DNA with α[^{32}P]-dCTP (>3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by random priming using a kit from Boehringer Mannheim Biochemicals (Indi-

anapolis, IN). Unincorporated nucleotides were removed with a spin column (Chroma Spin-10 Column; Clontech Laboratories, Palo Alto, CA) before hybridization. Using total rat genomic DNA as a probe will favor the detection of the relatively abundant high and medium repetitive DNA sequences and thus reflect the state of integrity of such sequences. It has been demonstrated however in thymocytes dying of apoptosis after γ irradiation that the coding sequences of specific genes become fragmented into oligonucleosomes (Umansky, 1991) suggesting that the whole genome is susceptible to DNA fragmentation.

To detect DNA fragments, purified DNA from about 5,000 neurons (araC experiment: 10,000 neurons) or thymocytes was separated on a 3% agarose gel (75% NuSieve GTG agarose, 25% SeaKem GTG agarose; FMC BioProducts, Rockland, ME) in TAE buffer (Sambrook et al., 1989). To check the recovery of DNA after DNA isolation and the equality of loading, the gel was stained with ethidium bromide and the intensity of the carrier DNA band examined. The DNA was denatured by alkali, neutralized, transferred to a nylon membrane (Genescreen Plus; NEN Dupont, Boston, MA), and fixed to the membrane by the method of Southern (1975). The nicking of the DNA with acid before transfer (Sambrook et al., 1989) was avoided to enhance the transfer of low molecular weight DNA relative to intact DNA. After the transfer, the gel was stained with ethidium bromide again which revealed a distinct high molecular weight DNA band confirming that high molecular weight DNA was transferred incompletely (Southern, 1975). To detect the transferred neuronal DNA, the membrane was hybridized with [^{35}P]-labeled EcoRI-digested rat thymocyte DNA, washed, and an autoradiogram prepared.

Data Presentation

The neuronal counts for the time courses of rescue and viability and the raw data of the metabolic measurements were normalized to the mean of the values measured for the NGF-maintained zero-hour control cultures (100%) and the background signal calculated from the mean of the values obtained from cultures devoid of neurons (0%). For all biochemical measurements, this background signal was <15% of the value of the NGF-maintained control cultures. The normalized data from several identical experiments conducted with cultures from different neuronal preparations were pooled and are presented as mean ± SEM. For the metabolic measurements, NGF-maintained cultures were processed in parallel for each time point to examine a possible drift of the parameters due to the ongoing growth processes

in the cultures. This drift was found to be <15% for all parameters (data not shown). To measure soma degeneration and neurite disintegration, the scores for each time point were normalized to the number of neurons in the NGF-maintained zero-hour control cultures. The normalized scores from four independent experiments were averaged and are presented as mean ± SEM.

Empirical smooth curves connecting the normalized datapoints of the time courses of viability and of rescue with NGF, CPTcAMP, and cycloheximide (see Fig. 3 a) were calculated by fitting the means to the asymmetric logistic equation $f(t) = (a_1 - a_2)/(1 + (t/t_i)^r)^s + a_2$ with the asymptotes set at $f(0) = a_1 = 100\%$ and $\lim f(t \to \infty) = a_2 = 0\%$. The variables t_i, r, and s were varied systematically to minimize the sum of the squared residuals using the SigmaPlot 5.0 software (Jandel Scientific, Corte Madeira, CA). For all time courses, the asymmetric ($s < 1$) logistic equation allowed a better fit than the best symmetric ($s = 1$) solution. The fitted curve for the time course of commitment to die was calculated as 100% minus the fitted curve of the time course of rescue with NGF and was differentiated numerically to derive the time course of the rate of commitment to die (see Fig. 8 b).

Results

Analysis of Morphological Changes

To generate a temporal reference frame of morphological markers and allow the comparison with quantitative biochemical parameters, light microscopically visible changes such as the onset of degeneration of the soma, the onset of neurite disintegration, and loss of viability were measured. The onset of soma degeneration and neurite disintegration were quantitated from serial phase contrast micrographs of 6-d-old established cultures of sympathetic neurons deprived of NGF (Figs. 1 and 2). No consistent morphological change was apparent during the first 12 h of NGF deprivation. At 19 h, atrophy was detectable in half of the neuronal somas. In many neurons the nucleus moved to one side of the soma.
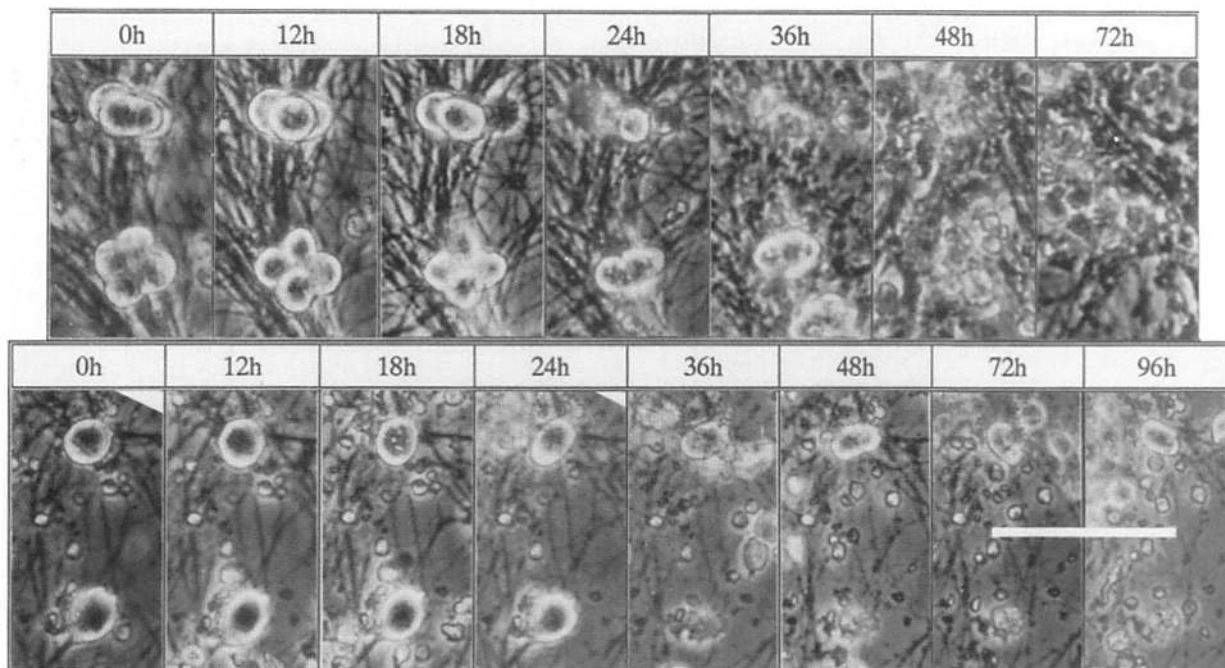

Figure 1. Serial phase contrast photomicrographs of rat sympathetic neurons undergoing NGF deprivation–induced death. After an initial 12-h period without consistent morphological change, neuronal somas began to atrophy and some neurons displayed large swellings or small vacuoles. Eventually the neurites disintegrate and the neurons lose their refractile appearance. Bar, 100 μm.
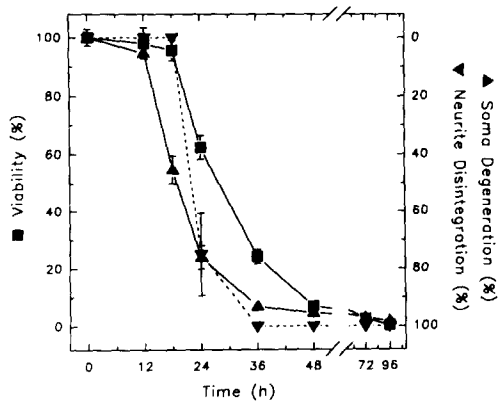
Figure 2. Time courses of the onset of soma degeneration (▲), neurite disintegration (--▼--), and loss of viability as measured by Nissl staining (■) of sympathetic neurons after onset of NGF deprivation. Half of the neuronal somata show signs of degeneration after 19 h, half the cultures show fragmented neurites after 22 h and half of the neurons have died after 27 h. The quantitation is described in Materials and Methods. To examine soma degeneration, the fate of 407 neurons from four individual platings was followed. Neurite disintegration was scored in 34 neuronal fields from four different platings. The measurement of the time course of viability was based on between 6 and 14 Nissl-stained cultures from three individual platings that were counted for each time point.

A minority of cells contained small vacuoles or displayed large translucent swellings of delicate and fragile appearance. The neuronal plasma membrane lost its smooth appearance and the cellular outline became increasingly irregular. After 36 h, 95% of the neurons had atrophied. The onset of the disintegration of the neurite network was evaluated from serial photomicrographs showing the neuritic network in focus (Martin et al., 1988; Deckwerth and Johnson, submitted). After 22 h, in half of the cultures the neurite bundles had lost their normally smooth appearance and developed small swellings reminiscent of beads on a string. With degeneration proceeding, the remaining intact neurites disintegrated progressively while the amount of debris increased leaving tracks of neuritic debris on the dish. Neuronal viability was measured by counting the number of Nissl-stained neurons in separate cultures fixed after various periods of NGF deprivation (Fig. 2). In a series of separate experiments it was determined that counting Nissl-stained neurons provided a reliable and reproducible way of determining neuronal viability in this in vitro system (data not shown). After a lag period of 18 h after onset of NGF deprivation during which 95% of the neurons remained viable, the period of cell death set in with half of the neurons being dead after 27 h and 95% having died after 48 h.

Time Course of Commitment to Die

A critical capability that a dying neuron loses eventually after trophic factor deprivation is the ability to respond to readdition of its physiological trophic factor with long-term survival. Upon loss of this ability, the neuron is committed to die. To quantitate the time course of commitment to die, neurons were deprived of NGF for variable times followed by readdition of NGF for one week. At the end of the rescue period, the cultures were fixed, Nissl-stained, and the num-
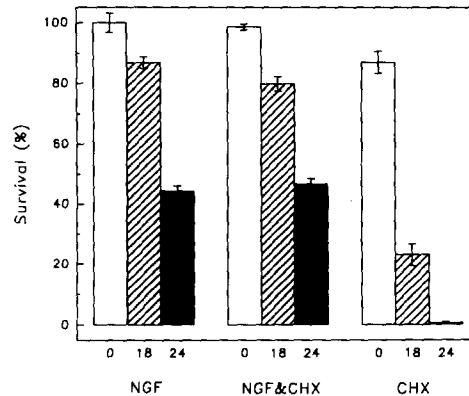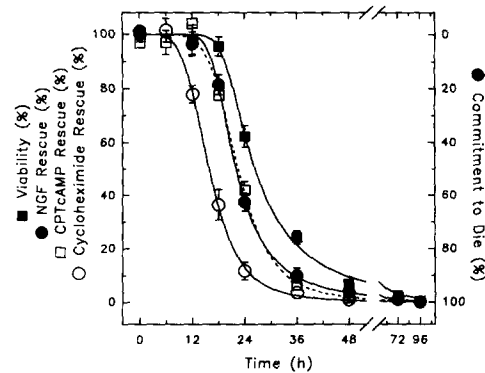

Figure 3. (*a*) Comparison of the time courses of rescue with NGF (●), rescue with the cyclic AMP analogue CPTcAMP (--□--), rescue with the protein synthesis inhibitor cycloheximide (○), and loss of viability (■). Half of the neurons lose the ability to be rescued by cycloheximide after 16 h while CPTcAMP and NGF protect half of the neurons for 22 h. The time course of commitment to die (●) is given by the percentile of neurons that have lost the ability to respond to NGF with long-term survival. Experimental details and the generation of the fitted curves are described in Materials and Methods. For the time course of rescue with NGF, rescued neurons of between 7 and 20 cultures from four individual platings were counted for each time point. For the time course of rescue with cycloheximide, between 4 and 16 cultures from three individual platings were counted for each time point. For the time course of rescue with CPTcAMP, between 6 and 16 cultures from three individual platings were counted. The data for the time course of viability were taken from Fig. 2 for easier comparison. (*b*) Rescue of sympathetic neurons deprived of NGF for 18 or 24 h by NGF, cycloheximide, or both. Simultaneous application of NGF and cycloheximide saves the same fraction of neurons as NGF alone, suggesting that protein synthesis is not required for NGF to protect NGF-deprived neurons from death. For each experimental condition, the rescued neurons from between three and six cultures were counted.

ber of neurons counted. For up to 12 h of NGF deprivation, all neurons could be rescued by readdition of NGF (Fig. 3 *a*). Half of the neurons were committed to die after 22 h of NGF deprivation, and >95% after 48 h. The time course of rescue with NGF was preceded by the time course of soma degeneration by 3 h (Fig. 2), and preceded the time course of loss of viability by 5 h. This indicates that there is a period in the life of a neuron during which the degenerative processes have become irreversible but have not yet caused the death of the neuron.

Time Courses of Rescue with Other
Neuroprotective Agents

To examine whether the time courses of rescue with neuroprotective agents other than NGF were similar to the time course of rescue with NGF, NGF was substituted by 400 μM of the cyclic AMP analogue CPTcAMP (Rydel and Greene, 1988) or 1 μg/ml of the inhibitor of protein synthesis cycloheximide during the rescue period (upon addition of 1 μg/ml cycloheximide protein synthesis is inhibited completely within a few minutes [data not shown]). After rescue for 48 h, the number of neurons was determined subsequent to fixation and Nissl staining. The time course of rescue with CPTcAMP was indistinguishable from the time course of rescue with NGF while the time course of rescue with cycloheximide preceded both time courses by about 6 h (Fig. 3 a). This finding suggests that the rate-limiting process by which CPTcAMP and NGF protect from NGF deprivation–induced death may be identical but certainly different from the rate-limiting step, by which cycloheximide exerts its neuroprotective effect. To eliminate the possibility that a toxic side effect of cycloheximide might be responsible for the reduced neuroprotective effect of cycloheximide relative to NGF or CPTcAMP, we examined whether the presence of cycloheximide would affect the temporal characteristics of neuroprotection by NGF. Neuronal cultures deprived of NGF for 18 and 24 h were exposed to 50 ng/ml NGF, 1 μg/ml cycloheximide, or both for 55 h after which the number of rescued neurons was determined (Fig. 3 b). The fraction of rescued neurons after 18 or 24 h of NGF deprivation was identical in cultures rescued with NGF or a combination of cycloheximide and NGF. Since cycloheximide did not reduce the efficiency of the neuroprotective effect of NGF, a toxic side effect of cycloheximide can be excluded.

Effect on Protein and RNA Metabolism

The morphological manifestation of the degenerative process initiated by trophic factor deprivation has underlying molecular correlates. The atrophy of the neuronal soma indicates a reduction of cell body mass that may be caused at least in part by a decrease of total neuronal protein. Total neuronal protein was measured using the bicinchoninic acid method at various times after NGF deprivation (Fig. 4 a). After 18 h of NGF deprivation, the protein content decreased by ∼20% concurrent with the onset of soma degeneration, but before any significant loss of viability. At this time about half of the neuronal cell bodies showed detectable signs of soma degeneration. At 24 h and later, neuronal protein decreased slower than the fraction of living neurons. This retardation is apparently caused by the contamination of the protein from viable neurons with protein associated with dead cells and debris, which could not be separated completely from the remaining living neurons by the washing procedure used. Because of the inability to measure the amount of protein associated with living neurons over the whole time course, the biochemical parameters were not normalized to the amount of neuronal protein but care was taken to establish cultures with equal numbers of neurons for a given experiment.

A likely reason for the decrease of total protein prior to any cell death is a decrease of protein synthesis. The rate of protein synthesis as measured by the rate of incorporation of
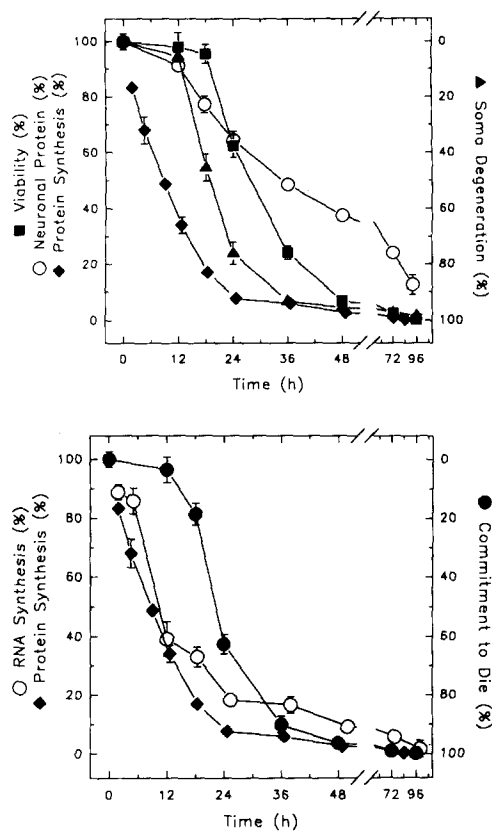

Figure 4. (a) Time courses of total neuronal protein (O), rate of protein synthesis (◆), onset of soma degeneration (▲), and loss of viability (■). NGF deprivation triggers the immediate fall of the rate of incorporation of [³⁵S]-L-methionine into protein (10% remaining after 24 h). Concurrent with the onset of soma degeneration at 18 h, total neuronal protein decreases prior to onset of loss of viability. Beyond 24 hours, the increasing amount of protein associated with debris and dead cells, leads to a retardation of the decline relative to the time course of viability. Between 9 and 18 cultures form three different platings were examined for each time point for their total protein content and between 3 and 19 cultures from four different platings were examined to measure the time course of protein synthesis. The time courses of degeneration and loss of viability were taken from Fig. 2. (b) Time courses of the rate of RNA synthesis (O), rate of protein synthesis (◆), and commitment to die (●). The rate of incorporation of [³H]uridine into RNA decreases with a time course slightly delayed relative to the time course of incorporation of [³⁵S]-L-methionine into protein (20% remaining after 24 h). For each time point between 3 and 9 cultures from three different platings were examined for the rate of incorporation of [³H]uridine into RNA. The time courses of protein synthesis and of commitment to die were taken from Figs. 4 a and 3 a, respectively.

[³⁵S]-L-methionine into protein decreased rapidly after removal of NGF and fell to one third after only 12 h and to 10% after 24 h (Fig. 4 a). Thus the decrease of total protein content and the concurrent degenerative processes may in part be caused by the dramatic reduction of protein synthesis.

Protein synthesis is dependent upon the availability of mRNA for translation. To test whether removal of NGF also affected the rate of RNA synthesis, the time course of incorporation of [³H]uridine into RNA was measured. The rate

of RNA synthesis decreased with a time course slightly delayed relative to the rate of protein synthesis reaching 20% of control levels after 24 h (Fig. 4 b). Taken together, these findings suggest that anabolic macromolecular metabolism is rapidly reduced upon removal of NGF.

To examine whether the decrease of protein synthesis after 12 h of NGF deprivation was triggered by the same molecular mechanism that leads to commitment to die and death, the effect of three neuroprotective agents other than NGF was examined. Cultures of sympathetic neurons were deprived of NGF for 12 h followed by measuring the rate of protein synthesis during the subsequent four hour period in the absence of NGF. Control cultures were maintained in the presence of NGF during the 12-h period, but NGF was withdrawn during the four hours of measuring the rate of protein synthesis. As seen before (Fig. 4 a), 12 h of NGF deprivation reduced the rate of protein synthesis to ~30% while in the control cultures the rate of protein synthesis was 85% compared to cultures which had NGF present during the period of protein synthesis measurement (Fig. 5 a). Hence, no other neuroprotective agent would be expected to maintain >85% of the rate of normal protein synthesis in this paradigm. To test the influence of neuroprotective agents on the decrease of protein synthesis induced by NGF deprivation, neuronal cultures were exposed to 400 μM CPTcAMP, 35 mM potassium chloride, or 1 μg/ml cycloheximide during the 12-h period.

Subsequently, the drugs were removed completely and the rate of protein synthesis was measured. (If present during the measurement of protein synthesis, 1 μg/ml cycloheximide completely inhibited incorporation of [^{35}S]-L-methionine into protein both in the presence of NGF and after removal of NGF for 12 h, while 400 μM CPTcAMP or 35 mM potassium chloride had no inhibitory effect on protein synthesis [data not shown].) In the presence of NGF, the three agents had only a minor effect on the rate of protein synthesis during the subsequent measuring period (Fig. 5 b). In the absence of NGF, both CPTcAMP and potassium depolarization retarded the fall of protein synthesis caused by 12 h of NGF deprivation (to 75 and 60%, respectively), however cycloheximide had no protective effect on the NGF deprivation-induced reduction of the rate of protein synthesis (Fig. 5 b). Also, the time course of protein synthesis during the first 12 h of NGF deprivation is not affected by the presence of cycloheximide (Fig. 5 c). These experiments demonstrate that the mechanism underlying the NGF deprivation-induced decrease of protein synthesis does not require protein synthesis and is different from the mechanism underlying commitment to die and death, since these latter events are prevented by cycloheximide.

Effect on Energy Metabolism

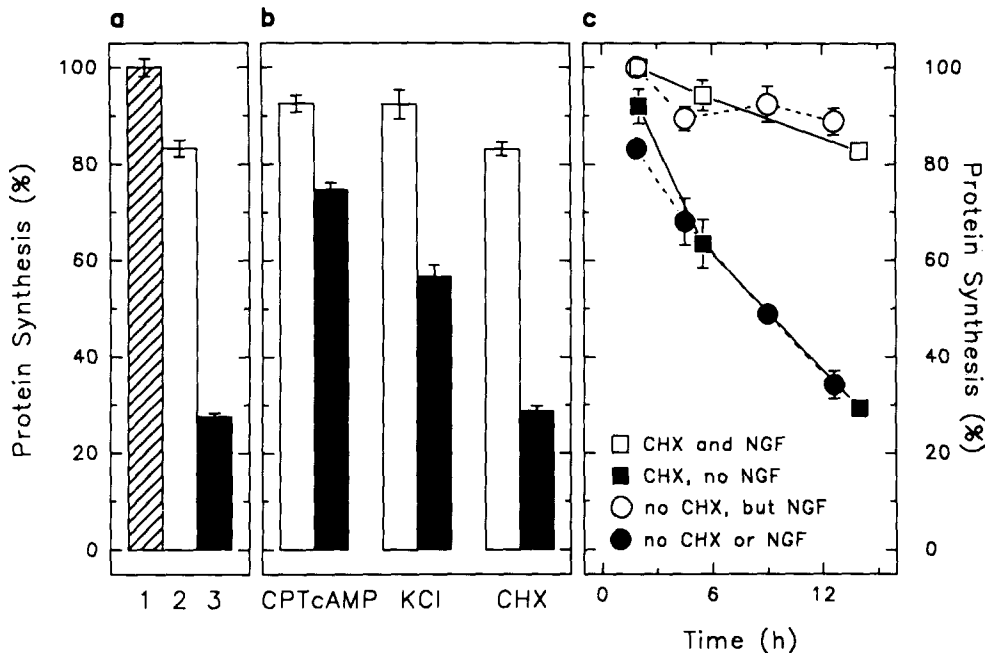To examine whether the rate of neuronal energy metabolism

Figure 5. The effect of neuroprotective agents on the decrease of protein synthesis caused by NGF deprivation. (a) Control experiments defining the range of the response. Sympathetic neuronal cultures were deprived of NGF (3) or maintained in the presence of NGF (2) for 12 h followed by measurement of the rate of protein synthesis in the absence of NGF. The response was normalized to the rate of protein synthesis seen in cultures maintained permanently in the presence of NGF (1). NGF deprivation for 12 h leads to a decrease of protein synthesis to ~30%, the presence of NGF during this period decreases the rate of protein synthesis to 85%, which defines the maximal protective effect that a neuroprotective agent has in this paradigm. (b) Effect of neuroprotective agents on the decrease of the rate of protein synthesis induced by 12 h of NGF deprivation. Sympathetic neurons were deprived of NGF for 12 h (■) or maintained in NGF (□) as control, followed by measuring the rate of protein synthesis in the absence of NGF. During the 12-h incubation period, 400 μM CPTcAMP, 35 mM potassium chloride (KCl), or 1 μg/ml cycloheximide (CHX) were present. The drugs were washed out before measuring the rate of protein synthesis. While potassium depolarization and CPTcAMP are capable of retarding the NGF deprivation-induced fall of the rate of protein synthesis, cycloheximide is not. Each experimental condition was examined in 6 to 12 cultures for two to four different platings. (c) Time courses of the rate of protein synthesis in the presence (□,■) or in the absence (○,●) of cycloheximide (CHX) in response to NGF deprivation (●,■) or while maintained with NGF (○,□). The time course in the absence of NGF and cycloheximide (CHX) was taken from Fig. 4 a. The experimental paradigm was identical to the cycloheximide experiment in Fig. 5b, except that the length of the incubation period prior to measurement of the rate of protein synthesis was varied. The data were plotted such that the time coordinate is centered onto the middle of the four-hour L-methionine incorporation period. Each data point in the presence of cycloheximide is derived from 6 samples of two different neuronal platings.
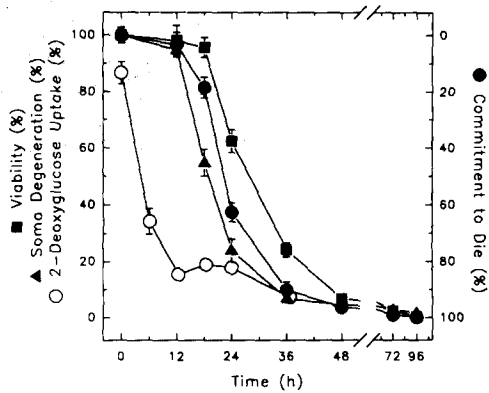
Figure 6. Time course of the rate of [2,6-³H]-2-deoxy-D-glucose (DOG) uptake (O). Sympathetic neurons were deprived of NGF and the rate of DOG uptake was measured at different times thereafter. The rate of DOG uptake falls to 20% within 12 h. The fall precedes the time courses of onset of soma degeneration (▲), commitment to die (●), and loss of viability (■). Nine different cultures from three different platings were examined for each time point. The time courses of soma degeneration, commitment to die, and loss of viability were taken from Figs. 2 and 3 a.

was affected by trophic factor deprivation, glucose uptake and mitochondrial function of NGF-deprived sympathetic neurons in culture was studied. The rate of glucose uptake was determined by measuring the rate of uptake of the glucose analogue 2-deoxy-D-[2,6-³H]-glucose (DOG; Sokoloff et al., 1977). After NGF withdrawal, the rate of DOG uptake declined faster than any other parameter examined, being reduced to 35% by six and to 20% within 12 h (Fig. 6). After remaining at this level for several hours, residual DOG uptake decreased concurrent with neuronal death. The rapid decrease during the first 12 h occurred before the onset of any morphological change or commitment to die. The decline in glucose uptake is consistent with a decrease of energy available for cellular processes and may well be responsible for the down-regulation of macromolecular synthesis.

The decrease of glucose uptake after 12 h of NGF deprivation may lead to a decrease of mitochondrial energy metabolism which is the major source for ATP and reducing equivalents (NADH, NADPH) in neurons (Erecińska and Silver, 1989). The reduction of the tetrazolium dye 1-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its formazan is commonly used as a measure of cellular viability (Mosmann, 1983). More recently it has been realized that in a living cell MTT reduction is modulated by the cellular metabolic status (Jabbar et al., 1989; Huet et al., 1992; Janjic and Wollheim, 1992) and a measure of mitochondrial function, since it varies with the cellular amounts of reducing equivalents and is largely, though not exclusively, reduced by electron transfer complexes of the mitochondrial respiratory chain (Slater et al., 1963; Vistica et al., 1991; Huet et al., 1992). In sympathetic neurons, MTT reduction reflects the cellular metabolic status as well, since inhibition of energy metabolism by a 2-h exposure to 5 mM sodium azide and 5 mM 2-deoxyglucose led to the almost complete inhibition of MTT reduction while all neurons were viable at that time (data not shown). Upon NGF withdrawal for
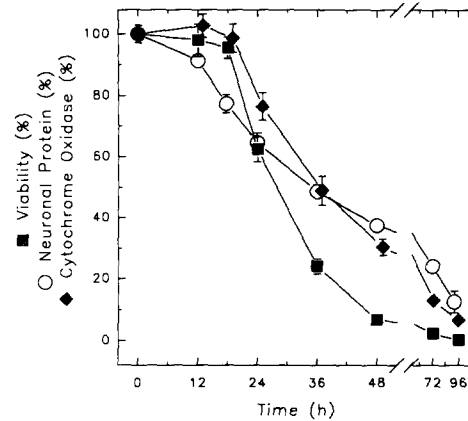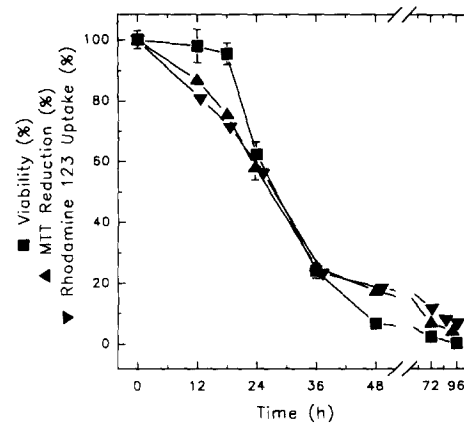

Figure 7. (a) Time courses of the rate of MTT reduction (▲) and rhodamine 123 uptake (▼). The rates of MTT reduction and rhodamine 123 uptake were measured at various times after removal of NGF from sympathetic cultures. MTT reduction and rhodamine 123 uptake decrease with similar time courses by 20% after 18 hours prior to loss of viability (■). Between 24 and 36 h, the time courses of the mitochondrial parameters and loss of viability are similar. Between 9 and 17 or three and 17 cultures from three different platings were examined for each time point of the time courses of the rate of MTT reduction or rhodamine 123 uptake, respectively. The time course of loss of viability was taken from Fig. 2. (b) Time course of neuronal cytochrome oxidase. The time course of the mitochondrial marker cytochrome oxidase (♦) does not change prior to the onset of loss of viability at 18 h (■) suggesting that the mitochondria remain intact while the neurons are viable. The time course of total neuronal protein (O) is shown for comparison. Between 11 and 24 cultures from four different platings were examined for each time point. The time courses of neuronal protein and loss of viability were taken from Figs. 4 a and 2, respectively.

18 h, the initial rate of MTT reduction was decreased by 25% (Fig. 7 a). This suggests that NGF deprivation leads to a decrease of mitochondrial function and reduced metabolic activity before any significant loss of viability.

The size of the electrochemical gradient across the inner mitochondrial membrane was assessed by examining the staining of cells with the lipophilic cationic dye rhodamine 123 (Johnson et al., 1981). Sympathetic neurons accumulated rhodamine 123 in tubular cytoplasmic organelles reminiscent of mitochondria as imaged by confocal micros-

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copy (data not shown). The initial rate of rhodamine 123 uptake decreased with a time course virtually identical to the rate of MTT reduction and preceded the onset of neuronal death (Fig. 7 a). This decrease is consistent with a decrease of the mitochondrial membrane potential. Beyond 24 h after onset of NGF deprivation, the time courses of MTT reduction and rhodamine 123 uptake decreased concurrent with loss of viability illustrating the close relationship between viability and mitochondrial function.

The early decline of mitochondrial function observed prior to death might be the result of the elimination of mitochondria from the cells. To examine this possibility, the time course of the activity of the mitochondrial marker enzyme cytochrome oxidase was measured. In contrast to the rates of MTT reduction and rhodamine 123 uptake, cytochrome oxidase activity in sympathetic neuronal lysates remained unchanged for the first 18 h of NGF deprivation and decreased only with the onset of loss of viability (Fig. 7 b). Beyond 24 h of NGF deprivation, the time course of cytochrome oxidase lagged behind the time course of viability, presumably for reasons identical to those discussed for the slow decrease of neuronal protein (see above). Hence there

is no indication for the early elimination of mitochondria from the neurons.

NGF Deprivation Causes Nuclear Morphological Changes and DNA Fragmentation

Nuclear changes, such as the condensation of the nuclear chromatin, fragmentation of the nucleus, and degradation of the nuclear genome into oligonucleosome-sized fragments before the loss of viability, have been observed in many instances of physiological and drug-induced apoptotic death (reviewed by Arends and Wyllie, 1991). To examine changes in the nuclear morphology of NGF-deprived sympathetic neurons, neuronal cultures were deprived of NGF for 18 h, fixed, and stained with the nuclear probe bisbenzimide. While the nuclei of NGF-maintained neurons appeared oval in shape and were rather homogeneously stained with moderate intensity, starting at 18 h of NGF deprivation neurons displayed several different nuclear morphologies consistent with apoptotic death (Fig. 8). Upon moving to the cellular periphery, the nuclei lost their oval shape and became indented on the cytoplasmic side. More intensely stained
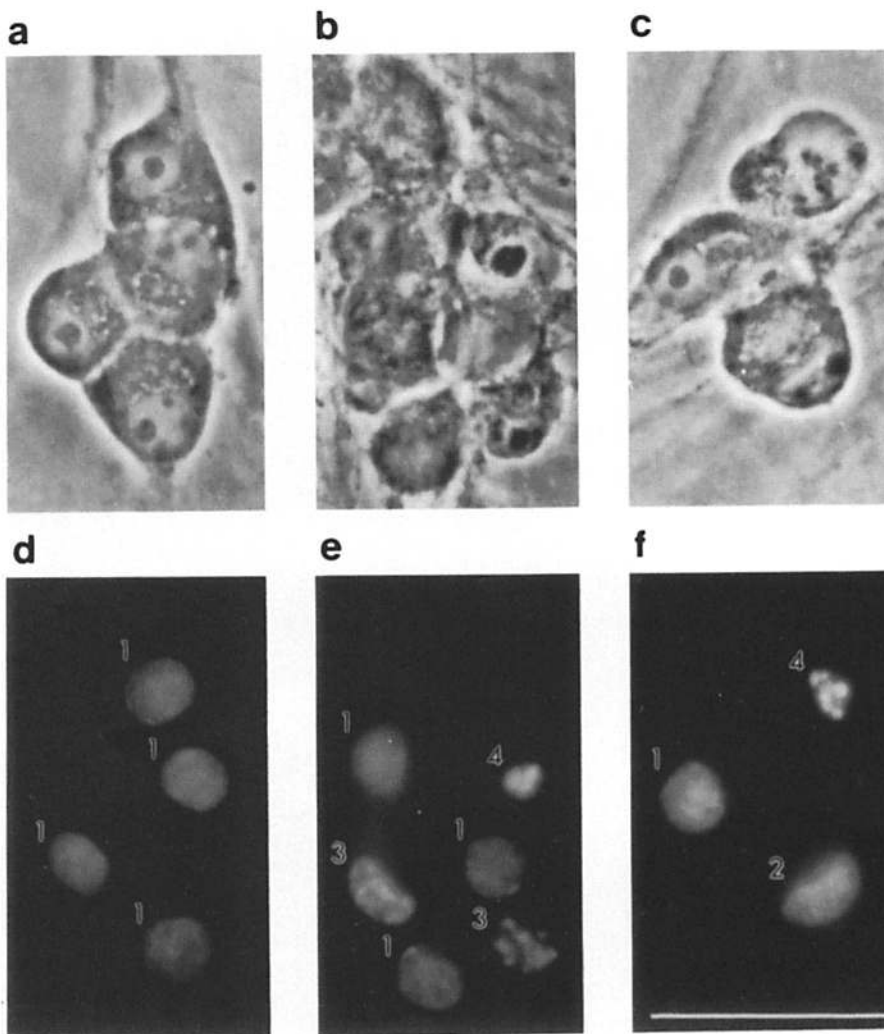

Figure 8. Nuclear staining of NGF-deprived sympathetic neurons with bisbenzimide. Phase contrast (a-c) and corresponding fluorescence (d-f) micrographs of sympathetic neurons maintained with NGF (a and d) or deprived of NGF for 18 h (b, c, e, f) illustrate the change of the nuclear morphology upon NGF deprivation: normal nucleus (1), eccentrically located and indented nucleus (2), nucleus with brighter stained branched filaments and spots of irregular shape (3), and nucleus fragmented into brightly stained round compartments (4). Bar, 50 μm.

branched filaments and spots of irregular shape appeared which coalesced into bright round spots. This indicates massive changes of the chromatin structure (condensation) that allowed for a greatly enhanced bisbenzimide fluorescence and finally led to the breakdown of the nucleus into individual fragments.

To examine whether sympathetic neurons in vitro fragment their DNA upon NGF deprivation, genomic DNA was prepared from NGF-deprived neurons after different times of NGF deprivation, separated by nondenaturing agarose gel electrophoresis, transferred to a nylon membrane under conditions that favor the transfer of low molecular weight DNA (Southern, 1975), hybridized with radiolabeled total rat genomic DNA, and visualized by autoradiography (Fig. 9 a). Fragmented DNA was almost completely absent in neuronal cultures maintained with NGF or deprived of NGF for 12 h, increased to its maximum at 19 h and decreased in intensity at 25 and 35 h. In other experiments, DNA fragmentation was observed as early as 17 h after onset of NGF deprivation but not at 14 h (data not shown). DNA prepared from control thymocytes and thymocytes dying by apoptosis after exposure to the glucocorticoid dexamethasone showed a similar pattern of high molecular weight DNA and oligonucleosomal fragments (Fig. 9 a). The maximal amount of neuronal DNA fragments occurred close to the maximum of the rate of commitment to die at about 20 hours (Fig. 9 b). DNA fragmentation at 18 and 24 h after NGF deprivation was prevented by CPTcAMP, potassium depolarization, inhibition of protein synthesis with cycloheximide (Fig. 10 a), and aurintricarboxylic acid (Fig. 10 b). Furthermore, 1-β-D-arabinofuranosylcytosine (araC), which kills NGF-maintained sympathetic neurons by a mechanism that mimics NGF deprivation by morphological and pharmacological criteria (Wallace and Johnson, 1989; Martin et al., 1990), produced the apoptotic nuclear morphology seen by
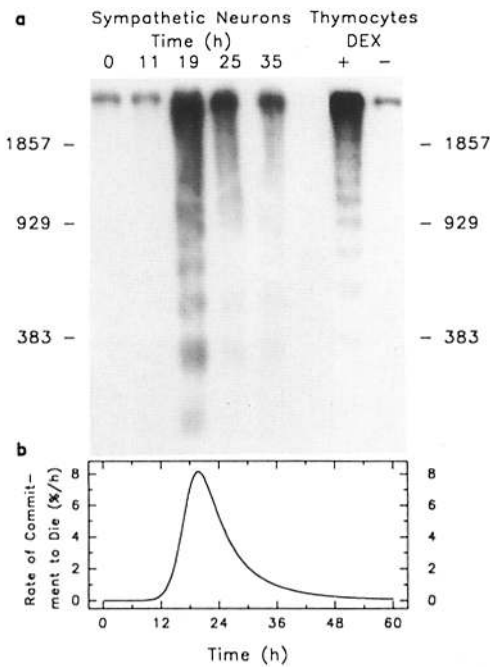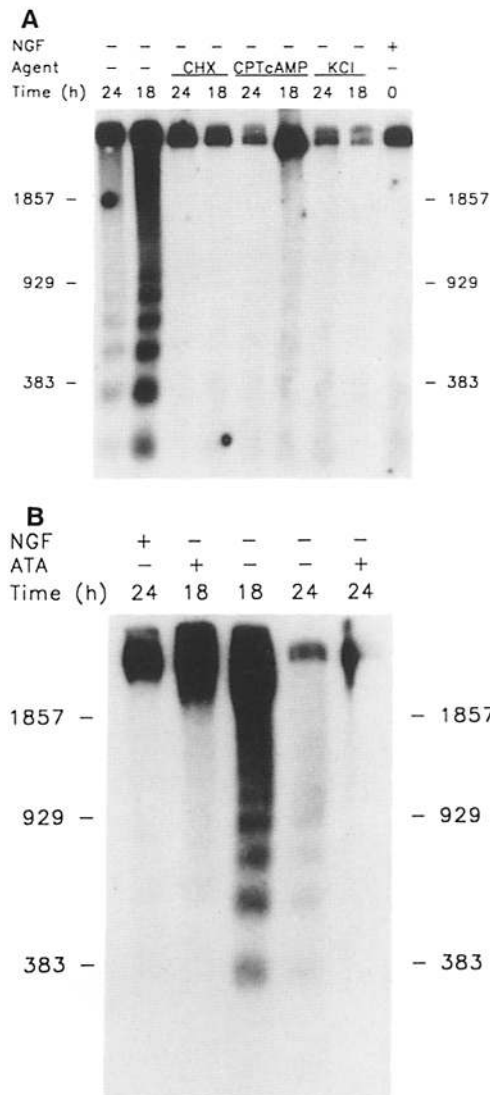

Figure 9. (a) Time course of DNA fragmentation into oligonucleosomal fragments. Genomic DNA was isolated from equal numbers of sympathetic neurons deprived of NGF for 12, 19, 25, and 35 h as well as from NGF-maintained cultures. The DNA was separated by nondenaturing agarose gel electrophoresis, transferred to nylon under conditions that favor the transfer of low molecular weight DNA, hybridized with radiolabeled rat genomic DNA and visualized by autoradiography. Oligonucleosomal fragments are most prominent at 19 h after onset of NGF deprivation. For comparison, DNA from thymocytes exposed to dexamethasone (DEX) or vehicle is shown in the two rightmost lanes. (b) Time course of the rate of commitment to die. The curve was calculated by numerical differentiation of the asymmetric logistic equation fitted to the time course of commitment to die (see Materials and Methods). The peak of the rate of commitment corresponds closely to the peak of the amount of fragmented DNA (a).

Figure 10. Neuroprotective agents prevent NGF deprivation-induced death. (a) Effect of 400 μM CPTcAMP, 35 mM potassium chloride (KCl), and 1 μg/ml cycloheximide (CHX) on DNA fragmentation induced by NGF deprivation. The drugs were added at the time of NGF deprivation to the neuronal cultures and genomic DNA was prepared at 18 and 24 h thereafter. DNA fragmentation is suppressed by all three neuroprotective agents. (b) Exposure to 200 μM aurintricarboxylic acid (ATA) during the time of NGF deprivation suppresses DNA fragmentation at 18 and 24 h as well. This concentration was found to convey maximal protection of sympathetic neurons (data not shown).
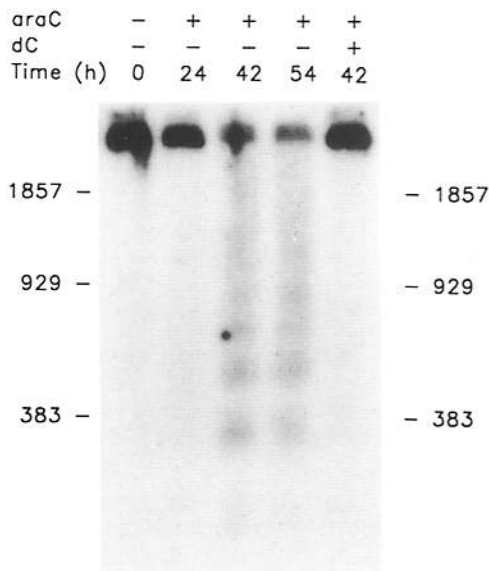
araC	−	+	+	+	+
dC	−	−	−	−	+
Time (h)	0	24	42	54	42

Figure 11. Exposure of sympathetic neurons to 1-β-D-arabino-furanosylcytosine (araC) leads to DNA fragmentation. Sympathetic neuronal cultures maintained in NGF were exposed to 100 μM araC for 24, 42, and 54 h, or not at all. A separate culture was exposed to 100 μM araC and 100 μM deoxycytidine (*dC*) for 42 h. NGF-maintained sympathetic neurons fragment their genomic DNA into oligonucleosomal fragments after exposure to araC for 42 and 54 h. Deoxycytidine prevents the araC-induced DNA fragmentation.

bisbenzimide staining after NGF deprivation (data not shown) and caused fragmentation of the neuronal DNA into oligonucleosomal fragments after 42 h (Fig. 11) consistent with a delay of approximately 24 h in the onset of toxicity compared to the onset of NGF deprivation–induced degeneration. Deoxycytidine, which prevents neuronal death caused by araC, prevented DNA fragmentation. The temporal correlation between DNA fragmentation and the time course of commitment to die as well as the pharmacological evidence suggest that DNA fragmentation may be part of the mechanism that commits the neurons to die.

Discussion

Morphological Changes

The degeneration of sympathetic neurons in response to neurotrophic factor deprivation has been characterized qualitatively by both light and electron microscopy in vivo (Levi-Montalcini et al., 1969; Levi-Montalcini, 1972; Wright et al., 1983) and in vitro (Martin et al., 1988). The relative timing of the morphological changes quantitated here (onset of soma degeneration, onset of neurite disintegration, and loss of viability) is largely in agreement with these qualitative observations. The swellings and vacuoles (Fig. 1) observed from 18 to 24 h of NGF deprivation were present on only a few neurons at any time. The frequency of their incidence is unknown but could be high if the average lifetime of these structures is short.

Neuroprotective Agents and Commitment to Die

A variety of neuroprotective compounds have been found to prevent or at least retard death of NGF-deprived SCG neurons: inhibitors of protein and RNA synthesis (Martin et al., 1988; Edwards et al., 1991; Martin et al., 1992), agents that increase or mimic cAMP (Rydel and Greene, 1988; Martin et al., 1992; Tanaka and Koike, 1992), agents that increase intracellular calcium (Koike et al., 1989; Koike and Tanaka, 1991; the cytokine interferon γ (Chang et al., 1990), aurintricarboxylic acid (Batistatou and Greene, 1991), and the ganglioside G_{M1} (Ferrari et al., 1993). The time course of rescue with the inhibitor of protein synthesis cycloheximide preceded the time course of rescue with NGF and the cAMP analogue CPTcAMP (Fig. 3 *a*). This result qualitatively confirms the observation by Edwards et al. (1991). A difference is observed for the time interval between the time courses of rescue with cycloheximide and NGF which was found to be 6 h in this study compared to 12 h as reported previously (Edwards et al., 1991). Both of these observations differ from the results obtained previously in this laboratory (Martin et al., 1992), which did not detect any difference in the time courses of rescue with cycloheximide, NGF, or CPTcAMP. This discrepancy may be due to differences in the methodology used to determine the fraction of living or dead neurons. This work and the studies by Edwards et al. (1991) relied on counting living neurons, while the study by Martin et al. (1992) used the release of the soluble enzyme adenylate kinase by dead cells at a fixed time after onset of NGF deprivation. Because of the indirect nature of their viability assay, Martin et al. (1992) concluded that the temporal resolution of their data would not allow the discrimination between the time courses of rescue with cycloheximide and NGF, as seen in this study.

The time course of rescue with the cAMP analogue CPTcAMP was identical to the time course of rescue with NGF (Fig. 3 *a*) suggesting that the rate-limiting mechanisms underlying rescue by NGF and CPTcAMP may be identical. Since NGF does not appear to act by a cAMP-dependent mechanism to promote survival (Rydel and Greene, 1988), the signaling chains started by NGF and CPTcAMP are likely to converge at or before the rate-limiting process that prevents neuronal death.

The existence of an interval of six hours, during which NGF and CPTcAMP, but not an inhibitor of protein synthesis can prevent neuronal death, suggests, that different rate-limiting processes underlie neuroprotection by cycloheximide and NGF. While this finding does not argue against the hypothesis that NGF may suppress the accumulation of a killer protein termed "thanatin" (Martin et al., 1988; Johnson et al., 1989), it lends no further support to it either, which it otherwise would have if the time courses of rescue had been identical. The most straightforward interpretation of the current finding is that the inhibition of protein synthesis by cycloheximide prevents the accumulation of a killer protein, which reaches critical levels in half of the neurons after 16 h of NGF deprivation. Thanatin accumulation may be caused by increased gene transcription which should occur before this time point. Consistent with this general idea, increased transcription of several identified genes before and at this time has indeed been observed in this laboratory (Estus, S., R. S. Freeman, and E. M. Johnson, Jr., manu-

script submitted for publication; Freeman, R. S., S. Estus, and E. M. Johnson, Jr., manuscript submitted for publication). Alternatively, thanatin may accumulate by several other molecular mechanisms, which we have discussed previously in detail (Johnson and Deckwerth, 1993).

The time course of rescue with NGF preceded the time course of loss of viability by 5 h (compare Figs. 2 and 3 *a*). This suggests that there exists a period during which the degenerating neuron is committed to die, but has not yet lost viability. This period in the life of a cell has previously not been resolvable. The delay of 11 h between the loss of neuroprotection by cycloheximide and death (Figs. 2 and 3 *a*) suggests that the ultimate manifestation of thanatin activity (death) is not instantaneous. Several mechanisms are consistent with such a lag period. The life-endangering mechanism may be silent during this time, be suddenly activated after 11 h, and kill the neuron. Alternatively, a thanatin with weak constitutive activity may inflict damage which accumulates slowly during the following period, triggers other degenerative processes, and finally reaches a lethal threshold after 11 h. The latter hypothesis appears more consistent with the slow progression of the morphological degeneration of soma and neurites and the activation of DNA fragmentation during this time period. Six hours after critical levels of thanatin have been reached and 5 h before death, NGF can still reverse the degenerative changes and prevent loss of viability. NGF may accomplish this by inactivating the thanatin before it has inflicted irreversible cellular damage, or by activating a compensatory life-sustaining cellular mechanism capable of neutralizing the degenerative events induced by NGF deprivation, or both.

The observation that the time course of rescue with a combination of NGF and cycloheximide was identical to the time course of rescue with NGF alone (Fig. 3 *b*) indicates that cycloheximide does not exert any toxic effect which affects the timing of rescue. Furthermore, this observation addresses the mechanism by which NGF acts to either inactivate thanatin or activate the life-sustaining mechanism. Rescue by NGF is obviously independent of ongoing protein synthesis during the 55-h rescue period. Thus thanatin may be inactivated by a posttranslational mechanism as has been suggested previously (Edwards et al., 1991). Alternatively, NGF may activate by a posttranslational modification some life-sustaining mechanism as mentioned above. The time course of these events and the thanatin model is summarized schematically in Fig. 12.

Macromolecular Metabolism

Preceding any morphologically visible change, NGF deprivation caused the rapid and dramatic reduction of the incorporation of methionine and uridine into protein and RNA, respectively (Fig. 4). While these methods are most commonly used to assess the rates of total protein and RNA synthesis, decreased rates of uptake of the monomeric precursors or enhanced rates of degradation of the polymers may also cause a reduction of these measures. However both the disintegration of polyribosomes and alterations of the fine structure of the nucleoli early after NGF deprivation in vivo (Levi-Montalcini et al., 1969; Levi-Montalcini, 1972) indicate that the synthesis of protein and rRNA may indeed be decreased by NGF deprivation. The decrease of protein and
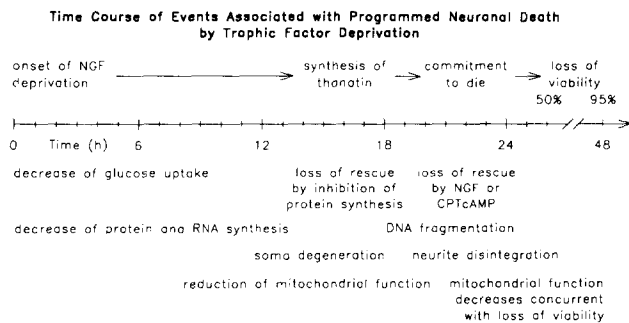

Figure 12. Time course of events associated with NGF deprivation–induced death of sympathetic neurons. The killer protein model indicating the proposed timing of thanatin synthesis and commitment to die is shown above the time axis.

RNA synthesis explains the observed decrease of total protein (Fig. 4 *a*) and RNA (Estus, S., and R. S. Freeman, unpublished data) associated with living neurons. The temporal correlation between the onset of soma degeneration (Fig. 2) and the decrease of total protein suggests that at least part of the neuronal atrophy may be caused by the decreased amount of protein associated with living neurons.

The rapid reduction of protein and RNA synthesis may at first appear contradictory to the finding that trophic factor deprivation–induced death is dependent upon protein and RNA synthesis. However the time course of rescue with cycloheximide (Fig. 3 *a*) is delayed by only 8 h relative to the time course of the rate of protein synthesis (Fig. 4 *a*) providing considerable overlap between the two time courses. Hence, even if the falling rate of global protein synthesis was rate limiting for the rate of thanatin synthesis, thanatin could still be synthesized at a time when cycloheximide can no longer prevent death.

Rapid decreases in the rates of RNA and protein synthesis have also been observed in thymocytes dying after exposure to glucocorticoids (Makman et al., 1966; Mendelsohn et al., 1977), one of the best studied systems of apoptotic death (Arends and Wyllie, 1991). This suggests that similar underlying molecular mechanisms may regulate cellular degeneration in both cell types.

The NGF deprivation–induced decrease of protein synthesis occurred long before the neurons became committed to die (Fig. 4, *A* and *B*) and was prevented by all neuroprotective agents examined except by cycloheximide (Fig. 5, *b* and *c*). This lack of effect of cycloheximide indicates that the molecular mechanisms underlying the decrease of protein synthesis must differ from those underlying commitment to die and death; in other words, interpreted in the context of the thanatin model, it cannot be the thanatin itself, which mediates the NGF deprivation–induced decrease of protein synthesis. Therefore, the NGF deprivation–induced decrease of protein synthesis is not sufficient for commitment to die and death. However it cannot be excluded that the fall of protein synthesis is required for degeneration and death, possibly by sensitizing the neuron to damage inflicted by the thanatin, e.g., by causing the fall of the levels of a labile inhibitor of thanatin production or action.

Since inhibition of protein synthesis by cycloheximide did not inhibit the NGF deprivation–induced down-regulation of

protein synthesis, NGF deprivation probably uses a post-translational mechanism to shut off translation. NGF is known to affect the phosphorylation status of initiation (Frederickson et al., 1992) and elongation factors (Koizumi et al., 1989) as well as of ribosomal protein S6 (Halegoua and Patrick, 1980). Whether any of these mechanisms is operative in the present setting is unknown.

Energy Metabolism

All vital processes including macromolecular synthesis are dependent upon the supply with energy. Glucose is the most important high-energy source for neurons (Erecińska and Silver, 1989). The rate of glucose uptake decreased precipitously upon NGF removal (Fig. 6). A similar phenomenon has been observed in thymocytes dying of apoptosis after exposure to glucocorticoids (Munck, 1968). This indicates a further similarity between the mechanism underlying trophic factor deprivation-induced death and apoptosis.

The decrease of glucose uptake suggests that the utilization of this extracellular fuel source decreases upon NGF deprivation. It is currently unknown whether other energy sources such as glutamine or fatty acids are used more intensely to compensate for this change. The accumulation of lipid droplets in NGF-deprived sympathetic neurons (Martin et al., 1988) however is more consistent with a decrease of mitochondrial β-oxidation rather than an increase. It cannot be excluded that intracellular energy reserves such as creatine phosphate or glycogen become mobilized; the small size of these reserves however suggests that they are unlikely to retard the fall of energy production significantly (Kauppinen and Nicholls, 1986; Erecińska and Silver, 1989).

Two independent measures of mitochondrial function, the reduction of the tetrazolium dye MTT and the uptake of the mitochondrial stain rhodamine 123, showed a modest but reproducible decrease before loss of viability (Fig. 7 a) suggesting that the rates of production of reduction equivalents (NADH) and high energy phosphates (ATP, GTP) are diminished. Consistent with a decrease of high-energy phosphate turnover is the observation that NGF deprivation causes a significant enhancement of the efflux of purines from sympathetic neurons long before loss of viability (Tolkovsky and Buckmaster, 1989). It is currently unknown if the absolute levels of these metabolites change after NGF deprivation.

During the phase of major cell death, both measures of mitochondrial function decreased concurrent with loss of viability. This is consistent with the notion that mitochondrial energy metabolism ceases upon loss of viability. Alternatively, the terminal event which results in death may be the shut down of residual mitochondrial function. Which of the two possibilities applies remains subject to further studies.

It cannot be excluded that the rate of uptake of rhodamine 123 partially reflects a weak depolarization of the plasmamembrane potential (Scott and Nicholls, 1980) which is seen in brain synaptosomes in response to glucose deprivation or inhibition of glycolysis (Kauppinen and Nicholls, 1986). Whether sympathetic neurons depolarize upon NGF withdrawal is not known.

The decrease of mitochondrial function is not simply caused by the destruction of mitochondria since cytochrome oxidase activity remained unchanged as long as viability was not impaired (Fig. 7 b). In neurons, the amount of cyto-

chrome oxidase is regulated according to the metabolic demand and cytochrome oxidase activity in vitro has been shown to correlate well with the amount of the enzyme (Hevner and Wong-Riley, 1989, 1990). Notably, mitochondria appear to be protected from the early degenerative processes or have a slower rate of turnover than the bulk of the protein since the time course of cytochrome oxidase did not decrease concurrent with time course of total neuronal protein during the first 18 h of NGF deprivation. One might speculate that some aspect of mitochondrial function, e.g., the supply of energy and reducing equivalents, is required during NGF deprivation-induced degeneration and death. The biochemically detectable preservation of the mitochondria is consistent with the ultrastructural preservation of mitochondria of NGF-deprived sympathetic neurons (Levi-Montalcini et al., 1969, 1972; Wright et al., 1983; Martin et al., 1988). Since mitochondria are largely preserved during the early stages of apoptotic death (Arends and Wyllie, 1991), this points to another similarity between the mechanisms underlying death by apoptosis and trophic factor deprivation-induced neuronal death.

Nuclear Changes and DNA Fragmentation

NGF deprivation-induced pronounced changes in the nuclear chromatin and led to the breakdown of the nuclei into nuclear fragments (Fig. 8). Such nuclear changes have been observed in several non-neuronal cells dying of apoptosis (McConkey et al., 1988; Yanagihara and Tsumuraya, 1992; Lindhout et al., 1993) and indicate a further similarity between apoptotic death and NGF deprivation-induced death. Furthermore, aurintricarboxylic acid has been found to inhibit NGF deprivation-induced death of sympathetic neurons (Batistatou and Greene, 1991; data not shown) as well as apoptosis of pheochromocytoma cells (Batistatou and Greene, 1991) and thymocytes (McConkey et al., 1989) and is believed to act by inhibiting an endonuclease, though inhibitory effects on numerous other enzymes have been reported as well (Bina-Stein and Tritton, 1976; Gonzáles et al., 1979). NGF deprivation caused the fragmentation of sympathetic neuronal DNA into oligonucleosomal fragments during the time when the neurons became committed to die (Fig. 9). The maximum of DNA fragmention occurred close to the point in time when the neuronal rate of commitment to die was maximal. Agents that prevented neuronal death inhibited DNA fragmentation (Fig. 10). This suggests that the destruction of the neuronal genome by an endonuclease may be part of the molecular mechanism that prevents NGF from rescuing the NGF-deprived neuron thus committing the neuron to die.

Using a similar technique, Edwards et al. (1991) detected the presence of DNA fragments in sympathetic neurons deprived of NGF for 18 h, while Mesner et al. (1992) did not confirm this observation. Since the oligonucleosomal DNA fragments are intermediates between fully intact and randomly degraded DNA, their absolute levels are determined by their relative rates of production and degradation integrated over time. For a first approximation one may assume that the rates of production and degradation be proportional to the rates of commitment to die (Fig. 9 b) and death, respectively (cell and nuclear lysis is accompanied by nucleosome disintegration exposing remaining intact DNA

and nucleosomal DNA fragments to random degradation by nucleases). This is supported by the time course of DNA fragmentation which consistently showed decreased amounts of oligonucleosomal fragments with the onset of cell loss at 24 h. Hence, the accumulation of oligosomal DNA fragments will be greatly influenced by the synchronization of the degenerative changes in the neuronal population and the stability of the neurons that are committed to die; minor differences in the tissue culture conditions can be expected to affect the stability of these fragile degenerating cells and thus influence the detectability of oligonucleosomal DNA fragments.

It was seen consistently that the size of the oligonucleosomal fragments from neuronal DNA was slightly larger than the corresponding oligonucleosomal fragments from glucocorticoid-exposed thymocytes (Fig. 9 a). This may be due to cell-specific differences in the length of the linker region between two oligonucleosomes or point to differences in the action of cell-specific exonucleases to which the ends of the unprotected linker DNA are susceptible.

The apoptotic morphology of the neuronal nuclei (Fig. 8) and the occurrence of DNA fragmentation (Fig. 9) complements previous ultrastructural data that suggest that chromatin condensation may indeed occur in NGF-deprived sympathetic neurons. A nuclear morphology reminiscent of apoptotic death was observed in previous studies examining the cytoplasmic and nuclear morphology of sympathetic neurons dying after exposure to anti-NGF antiserum in vivo (Levi-Montalcini et al., 1969, 1972) and the morphology of sympathetic (Wright et al., 1983) and ciliary neurons (Pilar and Landmesser, 1976) during the period of neuronal death. Martin et al. (1988), examining NGF deprivation-induced death of sympathetic neurons in vitro, show some images of dying neurons consistent with apoptotic death. Taken together, this strongly suggests that trophic factor deprivation-induced death and apoptosis share common underlying mechanisms.

The antimitotic agent araC has been shown to mimic NGF deprivation-induced death by morphological and pharmacological criteria (Martin et al., 1990). AraC kills NGF-maintained sympathetic neurons with a time course slower than NGF deprivation and its toxicity can be completely prevented by deoxycytidine, suggesting that araC interferes with a deoxycytidine-dependent cellular process. AraC induces an apoptotic nuclear morphology and leads to DNA fragmentation in NGF-maintained neurons with a time course consistent with its delayed onset of toxicity (Fig. 11). Deoxycytidine inhibited DNA fragmentation consistent with its neuroprotective action. While the site of action of araC in sympathetic neurons is not understood, this result supports the observation that araC toxicity uses at least parts of the mechanism by which NGF-deprivation triggers neuronal death. AraC has also been found to induce DNA fragmentation in dividing human myeloid leukemia cells (Gunji et al., 1991). This suggests that araC can trigger apoptosis in both mitotic and postmitotic cells. Whether the mechanisms by which araC elicits apoptosis in myeloid leukemia cells and in sympathetic neurons are similar remains to be determined.

The accumulated evidence that araC produces neuronal death by a mechanism similar or identical to NGF deprivation argues strongly that insults other than trophic factor deprivation are able to trigger apoptosis in postmitotic neu-

rons. It is of interest that araC is neurotoxic in humans (Winkelman and Hines, 1983; Sylvester et al., 1987; Vogel and Horoupian, 1993). It is reasonable to suggest that araC triggers neuronal death by apoptosis in these clinical settings. This lends further credence to the speculation that the study of the mechanisms underlying trophic factor deprivation-induced death of sympathetic neurons in vitro may be relevant to neuronal death seen in human neurodegenerative conditions.

Apoptosis and Programmed Cell Death

The time course of events following trophic factor deprivation-induced death and the thanatin model are summarized in Fig. 12. The time courses of the biochemical and metabolic changes demonstrate that trophic factor deprivation-induced neuronal death shares most of the features commonly associated with death by apoptosis. The existence of a well-defined sequence of degenerative events and the requirement of death for macromolecular synthesis indicates the existence of a coordinated program which is triggered upon NGF deprivation and underlies neuronal degeneration and death. This program consists of a series of reversible and irreversible steps and may involve the controlled expression of one or more killer protein(s). To qualify for a thanatin, a protein should be physiologically induced or activated during cellular degeneration, its inactivation should prevent death, and its mode of action should reveal the critical mechanism underlying cell destruction. Furthermore, there should exist physiological negative regulators of cell death, which should be inactivated or eliminated during cell degeneration and death, be induced or activated by neuroprotective agents and physiological protective stimuli, and act by inactivating or eliminating the thanatin or at least by neutralizing its lethal action. While candidates for both killer proteins and negative regulators have been found in this and other systems of programmed cell death on the basis that they fulfil one of the criteria listed above, a protein that unites all the criteria for a thanatin or a physiological negative regulator has not yet been identified.

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