



Staurosporine-induced neuronal death: multiple mechanisms and methodological implications

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Abstract

To examine whether multiple pathways of cell death exist in sympathetic neurons, we studied the cell death pathway induced by staurosporine (STS) in sympathetic neurons and compared it with the well-characterized NGF deprivation-induced death pathway. Increasing concentrations of STS were found to induce sympathetic neuronal death with different biochemical and morphological characteristics. One hundred nM STS induced metabolic changes, loss of cytochrome *c*, and caspase-dependent morphological degeneration which closely resembled the apoptotic death induced by NGF deprivation. In contrast, sympathetic neurons treated with 1 μ M STS showed no loss of cytochrome *c* but exhibited extensive, caspase-independent, chromatin changes that were not TUNEL positive. One μ M STS-treated sympathetic neurons had greatly reduced metabolic activities and became committed to die rapidly, yet maintained soma structure and appeared viable by other criteria even up to 48 h after STS treatment, illustrating the need to assess cell death by multiple criteria. Lastly, in contrast to the cell death-inducing activities of 100 nM STS or 1 μ M STS, very low concentrations of STS (1 nM STS) inhibited sympathetic neuronal death by acting either at or prior to *c-jun* phosphorylation in the NGF deprivation-induced PCD pathway. *Cell Death and Differentiation* (2000) 7, 250–261.

Keywords: sympathetic neurons; apoptosis; staurosporine; programmed cell death; neuronal death

Abbreviations: BAF, boc-aspartyl(OMe)-fluoromethylketone; JNK, *c-jun*-N-terminal kinase; NGF, nerve growth factor; PCD, programmed cell death; STS, staurosporine; TUNEL, Tdt-mediated dUTP-biotin nick-end labeling assay

Introduction

Extensive programmed cell death (PCD) occurs during normal nervous system development.¹ Dying cells exhibit

characteristic morphological features of apoptosis, which include shrinking of the cytoplasm, plasma membrane blebbing, and chromatin condensation.² Recent data indicate that cell death in pathological situations such as stroke, spinal cord injury, and also in certain neurodegenerative disease occurs, at least in part, by apoptosis.^{3–5} Therefore, understanding how neurons undergo apoptosis is important from both a developmental as well as a clinical perspective for developing rational therapies that may prevent neuronal death after injury or disease.

Neuronal PCD has been extensively studied in cultures of sympathetic neurons that undergo apoptosis after deprivation of nerve growth factor (NGF).⁶ The NGF deprivation-induced death of sympathetic neurons, which occurs within 24–48 h, is prevented by inhibitors of macromolecular synthesis such as cycloheximide,^{7–9} by cyclic AMP (CPT-cAMP),¹⁰ and depolarizing concentrations of potassium.^{11–13} Sympathetic neuronal death after NGF deprivation is also dependent on the proapoptotic BCL-2 family protein, BAX, and on caspase function. Sympathetic neurons from BAX-deficient mice do not undergo apoptosis after NGF deprivation.¹⁴ Likewise, pan-caspase inhibitors, such as boc-aspartyl(OMe)-fluoromethylketone (BAF), prevent apoptosis of sympathetic neurons after NGF removal.^{15,16}

NGF deprivation induces numerous biochemical and molecular events in sympathetic neurons. For example, the rates of glucose uptake and of RNA and protein synthesis decrease rapidly, with rates falling to less than 40% of NGF-maintained control cultures within 12 h of NGF removal.⁸ Similarly, *c-jun*-N-terminal kinase (JNK) activity and *c-jun* phosphorylation increase dramatically in cells by 6–12 h after NGF deprivation.^{17–19} *c-Jun* is important in mediating neuronal apoptosis because microinjection of either a *c-jun* neutralizing antibody or a dominant negative *c-jun* construct, inhibits sympathetic neuronal death after NGF withdrawal.^{20,21} NGF-deprived sympathetic neurons also exhibit a BAX-dependent loss of cytochrome *c* from mitochondria prior to caspase activation and cell death.^{22–24} The translocation of cytochrome *c* from the mitochondria to the cytosol occurs in several models of apoptosis and appears to be one of the critical events that result in caspase activation during apoptosis.²⁵ Once activated, caspases cleave specific cellular proteins and bring about rapid cell death.²⁶

Much of our understanding of cell death in sympathetic neurons comes from studies that have focused on the apoptotic pathway induced by NGF deprivation. A question that remains unclear is whether there is one or many pathways leading to apoptotic death in these neurons. Whereas different death-inducing signals initiate multiple pathways, these may converge into one final common pathway of apoptotic death in all cells. Alternatively, multiple pathways of apoptotic death may exist in cells. A

recent report indicates that cell death after NGF deprivation, DNA-damaging agents, and oxidative stress appears to be mediated by different caspases in sympathetic neurons.²⁷ However, the specific pathways that cause activation of different cysteine proteases under these conditions remain unknown.

In this study, we examined the pathway of neuronal death induced by staurosporine (STS) in sympathetic neurons. STS is a broad-spectrum protein kinase inhibitor isolated from *Streptomyces* and has been used extensively to induce apoptosis in a variety of cells. These include tumor cell lines,^{28–31} lymphocytes,^{32,34} neurons,^{16,35–38} and other primary cells.³⁹ Despite its widespread use to study apoptosis, the mechanism by which STS induces cell death is not clear. STS may activate some central component of the apoptotic machinery since it induces a rapid, macromolecular synthesis-independent death in a variety of cell types.^{39,40} Consistent with this model, STS-induced death is inhibited by BCL-2 overexpression,⁴⁰ involves the release of cytochrome *c*,^{41,42} and results in caspase activation⁴³ in several cell types. However, a wide range of STS concentrations (100 nM to 100 μ M) have been used to induce cell death in different cell types, and cell death has been quantitated by several methods.^{35,39,44,45} Therefore, the mechanism of STS-induced death may vary according to the cell type and the STS concentration used.

The objective of this study was to compare sympathetic neuronal death induced by STS treatment with that induced by NGF deprivation. We found that sympathetic neurons exhibited different biochemical and morphological characteristics of death depending on the concentration of STS used. Whereas the events induced by 100 nM STS were similar to those induced by NGF deprivation, the death induced by 1 μ M STS was different in several important aspects. Our data illustrate the complexity of cell death pathways and point to the necessity to assess cell death by a variety of criteria. Lastly, we report that in contrast to the cell death-inducing activity of 100 nM or 1 μ M STS, very low concentrations of STS (1 nM STS) inhibited sympathetic neuronal death after NGF deprivation. The survival-promoting effect of 1 nM STS appeared to be mediated by its ability to prevent sympathetic neuronal death either at or prior to *c-jun* phosphorylation in the NGF deprivation-induced sympathetic neuronal apoptotic pathway.

Results

Morphology of dying neurons changes with the concentration of STS

Sympathetic neurons undergoing PCD after NGF deprivation exhibit several characteristic morphological changes, including cytoplasmic shrinkage, soma degeneration, neurite fragmentation, and chromatin condensation.^{7,8} To compare cell death induced by STS with NGF deprivation-induced apoptosis in these neurons, we first examined the morphology of sympathetic neurons treated with increasing concentrations of STS. One hundred nM STS induced soma and neurite degeneration in most neurons by 48 h after STS treatment;

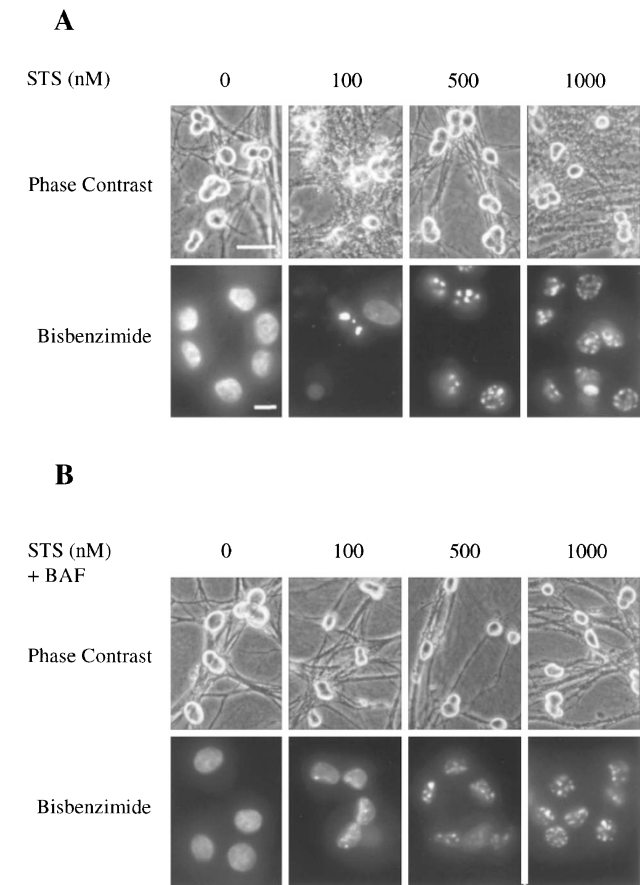


Figure 1 Increasing concentrations of STS induce sympathetic neuronal death with different morphological characteristics. **(A)** Phase-contrast photographs of NGF-maintained sympathetic neurons treated with increasing concentrations of STS (0, 100, 500, 1000 nM) for 48 h (upper panels). Bisbenzimidide-stained photographs of the nuclei of STS-treated sympathetic neurons from same cultures as described above are shown in the corresponding lower panels; note that the phase-contrast and bisbenzimidide images do not show the same cells. The effect of adding the caspase inhibitor BAF to STS-treated sympathetic neurons is shown in **(B)**. Phase-contrast photographs of NGF-maintained sympathetic neurons treated with increasing concentrations of STS (0, 100, 500, 1000 nM) for 48 h in the presence of 100 μ M BAF (upper panels). Bisbenzimidide-stained photographs of the nuclei of STS-treated sympathetic neurons in the presence of BAF from same cultures are shown in the corresponding lower panels; note that the phase-contrast and bisbenzimidide images are not matched to show the same cells. Scale bars (for both **A** and **B**): phase contrast photographs, 50 μ m; bisbenzimidide-stained photographs of nuclei, 10 μ m

the chromatin of these neurons was either condensed or lacked staining (Figure 1A). Similar loss of nuclear staining is also observed in NGF-deprived, dying sympathetic neurons and presumably reflects complete DNA degradation over time. The pattern of neuronal degeneration and chromatin condensation induced by 100 nM STS was similar to that seen with NGF deprivation in these neurons.⁸

In contrast, higher concentrations of STS induced a distinctly different pattern of morphological changes in sympathetic neurons. Sympathetic neurons treated with 500 nM or 1 μ M STS showed neurite fragmentation but remarkably little soma degeneration, as assessed by phase-contrast microscopy. The cell bodies remained

intact and appeared phase bright even 48 h after treatment (Figure 1A). Thereafter, the STS-treated cells became detached from the collagen substratum, most likely because of the extensive neuritic disintegration (data not shown). Also, 500 nM or 1 μ M STS induced changes in chromatin structure as early as 6 h after STS treatment (Figure 1A, data not shown). However, the pattern of chromatin changes was different from that seen in 100 nM STS-treated and NGF-deprived sympathetic neurons. One hundred nM STS-treated neurons, like NGF-deprived neurons, had fewer (approximately 1–3) chromatin clumps per cell that completely degraded with time. In contrast, the nuclei of 500 nM or 1 μ M STS-treated neurons exhibited many (approximately 5–10), smaller chromatin clumps that, surprisingly, did not degrade with time (Figure 1A, data not shown).

Caspase inhibitors, such as BAF and zVAD-FMK, prevent sympathetic neuronal death induced by NGF deprivation.^{15,16,46} Therefore, we examined whether BAF prevented the morphological and nuclear changes induced by STS treatment in sympathetic neurons. BAF (100 μ M) inhibited both neuronal degeneration and chromatin condensation in 100 nM STS-treated neurons 48 h after STS treatment (Figure 1B). In contrast, BAF addition did not prevent the chromatin changes induced by 500 nM or 1 μ M STS (Figure 1B). Similarly, addition of another pan caspase inhibitor, zVAD-FMK (100 μ M), did not have any effect on the changes induced by 500 nM or 1 μ M STS in sympathetic neurons (data not shown). These results indicate that whereas the morphological changes induced by 100 nM STS in sympathetic neurons, like those seen after NGF deprivation, were caspase dependent, higher concentrations of STS (500 nM and 1 μ M) induced different, caspase-independent, degenerative changes in these neurons.

Chromatin changes induced by 1 μ M STS in sympathetic neurons are not TUNEL positive

The morphological differences in the pattern of chromatin changes induced by low (100 nM) and high (500 nM and 1 μ M) concentrations of STS in sympathetic neurons, along with differences in their susceptibility to caspase inhibition, suggested that different concentrations of STS induced nuclear changes by different mechanisms. Therefore, we examined whether the chromatin changes induced by low (100 nM) or high (1 μ M) concentrations of STS in sympathetic neurons labeled positively in the Tdt-mediated dUTP-biotin nick-end labeling (TUNEL) assay; TUNEL reagent labels nicks in double-stranded DNA *in situ*.⁴⁷ Chromatin changes induced by NGF deprivation in these neurons were examined in parallel. NGF deprivation or treatment with 100 nM STS for 24 h induced chromatin changes that labeled with the TUNEL reagent (Figure 2). However, chromatin changes induced by 1 μ M STS treatment for 24 h did not label positively with the TUNEL reagent (Figure 2), even when examined at earlier (12 h) or later (36 h) times after STS treatment (data not shown). The single TUNEL-positive cell that is seen in the 1 μ M STS-treated cultures (Figure 2) corresponds to a nonneuronal cell (as assessed by phase-contrast microscopy); the ability of 1 μ M STS to induce a TUNEL-positive

cell death in nonneuronal cells has been described previously.^{30,39}

Timecourse of loss of viability of sympathetic neurons exposed to STS

The degenerative changes induced by 100 nM STS in sympathetic neurons resembled those seen after NGF deprivation and were clearly indicative of dying neurons. However, the assessment of cell death was less clear in sympathetic neurons exposed to 500 nM or 1 μ M STS, since these neurons maintained apparent soma integrity despite extensive nuclear changes. To assess the timecourse of loss of viability in sympathetic neurons treated with low (100 nM) and high (1 μ M) STS concentrations, we examined the status of 100 nM and 1 μ M STS-treated neurons by using a variety of morphological and metabolic parameters. NGF-deprived sympathetic neurons were examined in parallel as an internal control for cell death in these assays.

Our standard method of assessing sympathetic neuronal viability after various treatments is to fix cells with paraformaldehyde and stain with crystal violet; crystal violet stains nissl substance (nucleic acids) in cells.⁸ After 48 h, 100 nM STS-treated neurons, like the NGF-deprived sympathetic neurons, appeared degenerated and did not stain with crystal violet (Figure 3A). Since sympathetic neurons treated with 1 μ M STS maintained soma integrity despite extensive chromatin fragmentation, we anticipated that the crystal violet-staining method may not provide a reliable assessment of viability in this situation. Indeed, we found that 1 μ M STS-treated neurons, like the NGF-maintained neurons, stained positively with crystal violet even 48 h after STS treatment (Figure 3A). At longer times, however, as the 1 μ M STS-treated cells become partially degenerated and detached from the collagen matrix, fewer

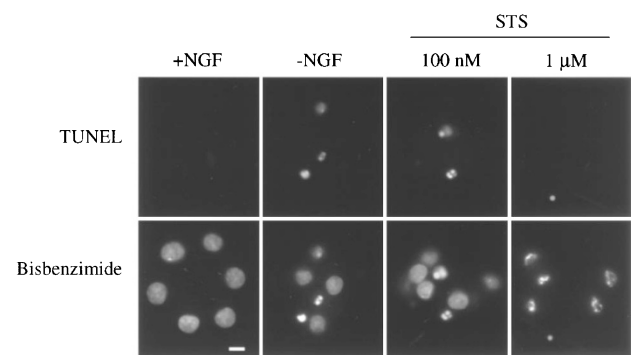


Figure 2 Chromatin changes in sympathetic neurons induced by NGF deprivation or exposure to 100 nM STS are TUNEL positive whereas those induced by 1 μ M STS are not. Shown are representative photographs of sympathetic neurons either maintained in NGF (+NGF), deprived of NGF for 24 h (–NGF), or maintained in NGF in the presence of 100 nM or 1 μ M STS for 24 h. Cells were then fixed with paraformaldehyde and stained with the TUNEL reagent (upper panel). Bisbenzimidide staining (lower panels) shows the corresponding nuclei of the same cells as shown in the upper panels. Note that the condensed nuclei in the –NGF and 100 nM STS panels are TUNEL positive whereas those in the 1 μ M STS are not; the single TUNEL-positive nucleus in the 1 μ M STS-treated panel corresponds to a nonneuronal cell (as assessed by phase-contrast microscopy). Scale bar: 10 μ m

crystal violet-positive cells were visible in the culture (data not shown).

We also used calcein AM staining as another indicator of cell viability in STS-treated neurons.⁴⁸ Calcein AM is an acetomethoxy ester fluorescein derivative that passively crosses cell membranes. Once inside the cell, it is cleaved by nonspecific intracellular esterases and the resulting fluorescent salts are retained in cells that have intact plasma membranes. Most 100 nM STS-treated sympathetic neurons, like the NGF-deprived neurons, lost calcein AM staining by 48 h after treatment (Figure 3B). In contrast, most 1 μ M STS-treated sympathetic neurons that maintained intact and phase-bright cell bodies even 48 h after treatment, also retained calcein AM staining (Figure 3B). The 1 μ M STS-treated sympathetic neurons also excluded trypan blue, another indicator of plasma membrane integrity, at least up to 48 h after STS treatment whereas the 100 nM STS-treated neurons did (data not shown).

As an alternate indicator of cell viability, we assessed the metabolic status of STS-treated sympathetic neurons. The rate of protein synthesis is dramatically reduced in sympathetic neurons undergoing PCD after NGF deprivation. Protein synthesis rates fall to 10% of control levels by 24 h and to undetectable levels by 48 h after NGF removal⁸

(Figure 4A). We examined whether STS-treated sympathetic neurons exhibited a similar decrease in protein synthesis. Protein synthesis rates decreased to 30% within 24 h and to less than 10% by 48 h after treatment with 100 nM STS (Figure 4A). The fall in protein synthesis was even more dramatic in 1 μ M STS-treated sympathetic neurons, despite the overall maintenance of soma structure in these neurons. Protein synthesis rates decreased to less than 10% of control levels by 12 h and to undetectable levels by 48 h after treatment with 1 μ M STS (Figure 4A).

Another indicator of metabolic activity that is used frequently as a marker of viability is the rate by which cells reduce the tetrazolium dye 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The rate of MTT reduction decreases rapidly after NGF deprivation in sympathetic neurons⁸ (Figure 4B). Comparable decreases in the rate of MTT reduction were seen in STS-treated sympathetic neurons (Figure 4B). One μ M STS-treated neurons reduced MTT at 20% of control levels 24 h after STS addition and to undetectable levels by 72 h after 1 μ M STS treatment. The decrease in the rate of MTT reduction was slower in 100 nM STS-treated neurons: MTT reduction decreased to less than 30% of control levels by 72 h after 100 nM STS treatment (Figure 4B).

Thus, although sympathetic neuronal death induced by 100 nM STS resembles that induced by NGF deprivation, sympathetic neuronal death induced by 1 μ M STS presents a paradox in the assessment of cell viability. After 48 h of treatment with 1 μ M STS, neurons would be considered alive by certain criteria (morphological assessment, crystal violet staining, calcein AM staining), but dead by others (nuclear changes, protein synthesis, MTT reduction) (Table 1).

Sympathetic neurons become committed to die within 30 min of treatment with 1 μ M STS

The results thus far suggest that 100 nM STS and 1 μ M STS induced sympathetic neuronal death with different characteristics. We next examined the timecourse with which neurons became committed to die after exposure to 100 nM and 1 μ M STS. To examine when cells became committed to die, sympathetic neurons maintained in NGF were treated with

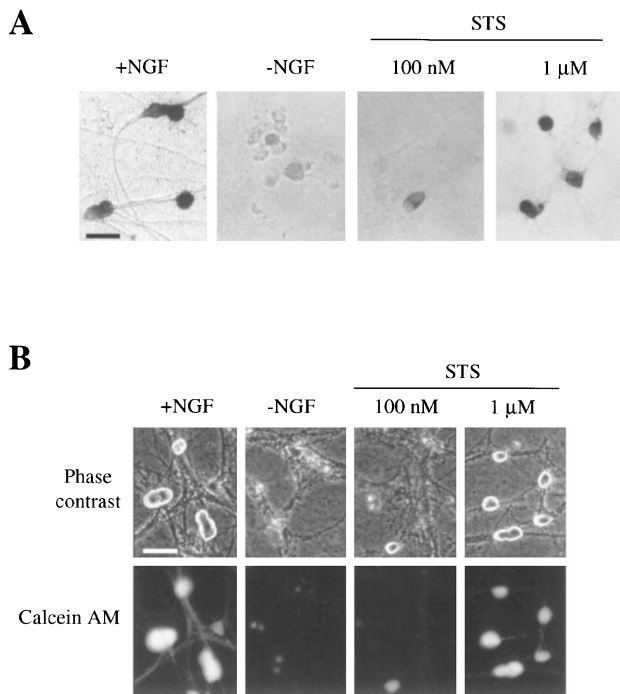


Figure 3 One μ M STS-treated sympathetic neurons appear viable by crystal violet staining and calcein AM uptake. **(A)** Crystal violet-stained photographs of sympathetic neurons either maintained in NGF (+NGF) or treated for 48 h with NGF deprivation (-NGF), 100 nM STS or 1 μ M STS. Scale bar: 40 μ m. **(B)** Sympathetic neurons were either maintained in NGF (+NGF) or treated for 48 h with NGF deprivation (-NGF), 100 nM STS, or 1 μ M STS. Cells were then treated with Calcein AM and photographed. Upper panels show phase-contrast photographs of cells, and lower panels show the Calcein AM staining for the same cells. Whereas NGF-deprived cells or 100 nM STS-treated cells showed minimal staining with crystal violet or Calcein AM, 1 μ M STS-treated cells stained with both crystal violet and Calcein AM. Scale bar: 40 μ m

Table 1 Conclusions regarding 'alive vs dead' if based upon commonly used measures of cell viability

Cell death assay (assessment at 48 h)	NGF deprivation	100 nM STS	1 μ M STS
Somal morphology	Dead	Dead	Alive
Calcein AM staining	Dead	Dead	Alive
Trypan blue exclusion	Dead	Dead	Alive
Crystal violet staining	Dead	Dead	Alive
MTT reduction	Dead	Dead	Dead
Protein synthesis	Dead	Dead	Dead
Commitment to die	Dead	Dead	Dead
Nuclear morphology	Condensed*	Condensed*	Condensed‡
TUNEL assay	Positive	Positive	negative

*Fewer (1–3) chromatin clumps per cell; caspase-dependent (Figure 1).

‡Several (5–10) chromatin clumps per cell; caspase-independent (Figure 1)

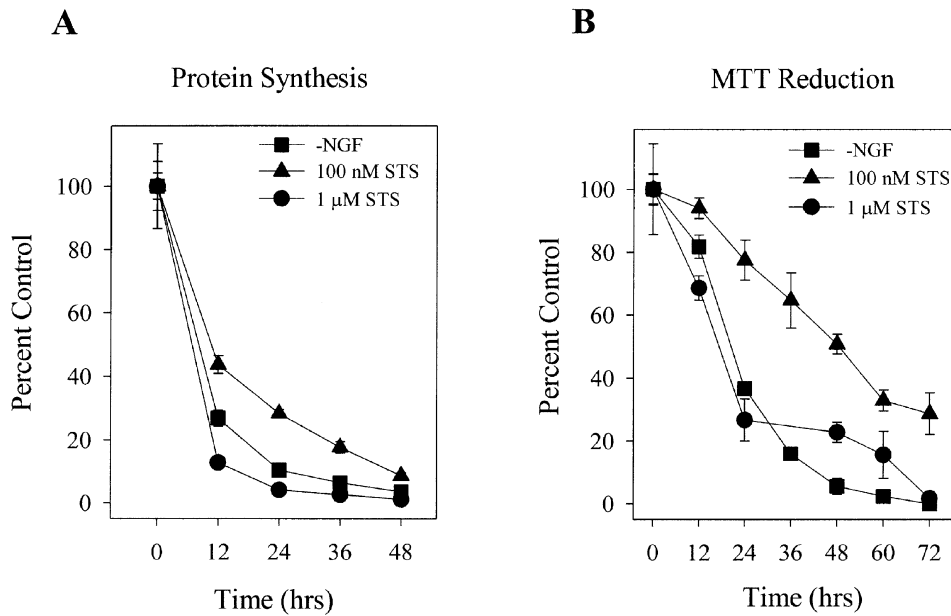


Figure 4 Metabolic parameters are markedly reduced in both 100 nM and 1 μ M STS-treated sympathetic neurons. Sympathetic neuronal cultures were deprived of NGF (-NGF; squares), or treated with 100 nM STS (triangles) or 1 μ M STS (circles). (A) Shows the timecourse of the rate of protein synthesis measured in these cultures at the indicated times after treatment. Data are represented as percentage of the protein synthesis rate of the NGF-maintained control neurons. (B) Shows the timecourse of the rate of MTT reduction in these cultures at the indicated times after treatment. Data are represented as percentage of the MTT reduction rate of the NGF-maintained control neurons. STS-treated neurons, like NGF-deprived neurons, show decreases in the rates of protein synthesis and MTT reduction after treatment. Mean \pm S.D., $n=3$

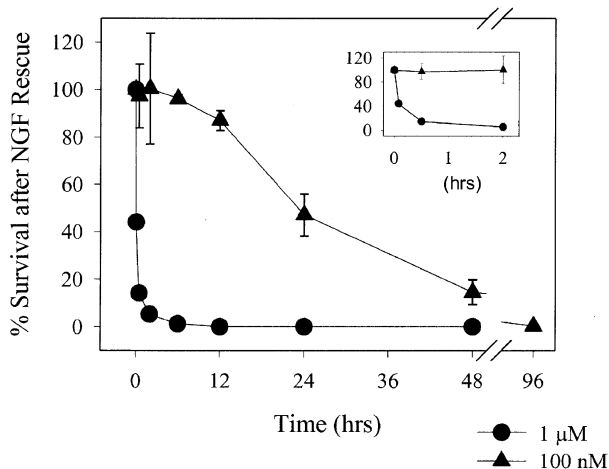


Figure 5 Timecourse of commitment to death in STS-treated sympathetic neurons. NGF-maintained sympathetic neurons were treated with 100 nM STS (triangles) or 1 μ M STS (circles). At various times after STS treatment, cells were washed extensively and fresh NGF-containing medium was added. The viability of cells was then determined 7 days later by crystal violet staining and counting. Data for the early timepoints (0, 15 min, 30 min, 2 h) are also shown in an inset panel to highlight the rapid commitment to death of 1 μ M STS-treated neurons. Results are mean \pm range of two independent experiments

either 100 nM STS or 1 μ M STS. At various times after STS treatment, cells were washed extensively and incubated in fresh NGF-containing medium, and the number of surviving neurons was then determined 7 days later by staining with crystal violet and counting the viable cells. This long-term

rescue period with NGF readdition was needed to assess survival unambiguously, especially for the 1 μ M STS-treated sympathetic neurons, because of difficulty in assessing viability at shorter times (48 h) after treatments (Figure 3). Sympathetic neurons treated with 100 nM STS did not start to become irreversibly committed to die until 12 h after STS addition since greater than 80% of the 100 nM STS-treated neurons could be rescued if STS were washed away within the first 12 h of STS treatment (Figure 5). By 24 h after 100 nM STS treatment, only 50% of the neurons could be rescued, and less than 10% of these neurons were rescued by 48–96 h after 100 nM STS treatment. This timecourse of commitment to death with 100 nM STS treatment is similar to that reported previously for sympathetic neurons deprived of NGF.⁸

In contrast, sympathetic neurons treated with 1 μ M STS rapidly became committed to die; within 15 min of 1 μ M STS treatment, 50% of the neurons could no longer be rescued with NGF readdition, and all neurons became committed to die after 30 min of STS treatment (Figure 5). This rapid, irreversible commitment to death induced by 1 μ M STS is particularly intriguing since soma degeneration typically associated with cell death did not become apparent even up to 48 h after treatment.

Low and high concentrations of STS induce distinct pathways of sympathetic neuronal death

The PCD pathway of sympathetic neurons induced by NGF deprivation is extensively characterized (reviewed in⁶). To determine whether STS induced sympathetic neuronal

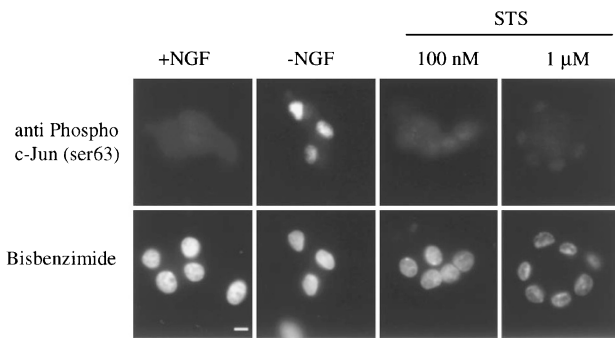


Figure 6 Increase in *c-jun* phosphorylation is not seen during STS-induced sympathetic neuronal death. Sympathetic neuronal cultures were either maintained in NGF (+NGF), subjected to NGF deprivation (–NGF), or treated with 100 nM STS or 1 μ M STS for 12 h. Cultures were then immunostained with an anti-phospho *c-jun* (ser 63) antibody. Whereas the NGF-deprived cells show a strong increase in *c-jun* phosphorylation, neither the 100 nM STS nor the 1 μ M STS-treated cells exhibit any increase in *c-jun* phosphorylation. Scale bar: 10 μ m. Identical results were obtained in multiple experiments

death by a similar or different mechanism, we examined whether STS treatment induced the same events that are seen in the NGF deprivation-induced PCD pathway.

Sympathetic neurons undergoing NGF deprivation-induced PCD exhibit a prominent increase in *c-jun* phosphorylation by 12 h after NGF removal^{18,19} (Figure 6). We examined whether 100 nM or 1 μ M STS-induced sympathetic neuronal death involved an increase in *c-jun* phosphorylation. Sympathetic neurons treated with either 100 nM or 1 μ M STS showed no increase in *c-jun* phosphorylation 12 h after STS treatment (Figure 6). No increase in *c-jun* phosphorylation was seen at earlier (2 h) or later (36 h) times after STS treatment (data not shown).

Another event that occurs in NGF-deprived sympathetic neurons, subsequent to the increase in *c-jun* phosphorylation, is the release of cytochrome *c* from the mitochondria to the cytosol.^{22–24} In NGF-maintained sympathetic neurons, cytochrome *c* exhibits a punctate staining pattern, consistent with its mitochondrial localization. NGF deprivation induces a loss of this punctate staining pattern, consistent with its release from the mitochondria and subsequent degradation in the cytosol.^{22–24} We examined whether the sympathetic neuronal death induced by STS treatment involved a similar loss of cytochrome *c* from mitochondria. Sympathetic neurons were treated with 100 nM or 1 μ M STS and, at various times after treatment, the localization of cytochrome *c* was assessed in these cells. Parallel cultures were deprived of NGF and examined likewise. BAF (50 μ M) was added to the NGF-deprived and 100 nM STS-treated cultures in these experiments to prevent any cell loss that would otherwise complicate quantitation of results. One hundred nM STS induced the loss of cytochrome *c* from mitochondria with a timecourse similar to that seen after NGF deprivation (Figure 7A). Fifty per cent of 100 nM STS-treated neurons showed loss of cytochrome *c* by 24 h after STS treatment. In contrast, no loss of cytochrome *c* was observed in sympathetic neurons treated with 1 μ M STS; cytochrome *c*

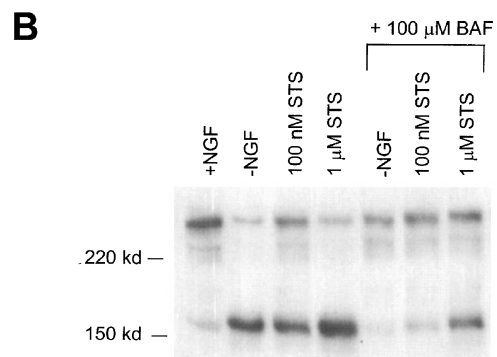
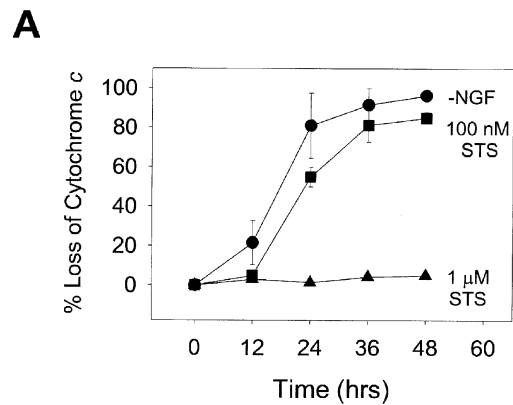


Figure 7 (A) Cytochrome *c* is lost from the mitochondria during sympathetic neuronal death induced by 100 nM STS but not by 1 μ M STS. Sympathetic neuronal cultures were treated with 100 nM (squares) or 1 μ M STS (triangles) or deprived of NGF (circles); 50 μ M BAF was added to the cultures of 100 nM STS-treated or NGF-deprived cells to prevent any cell loss that would otherwise affect the quantitation. At the indicated times after treatment, cells were immunostained with anti-cytochrome *c* antibody and of the total number of cells assessed (by counting the bisbenzimidide-stained nuclei) the number of cells that had lost the punctate cytochrome *c* staining was determined. Data shown are mean \pm S.D. of three independent experiments. (B) Cleavage of α -spectrin during STS-induced sympathetic neuronal death. Extracts of sympathetic neurons maintained in NGF (+NGF) or deprived of NGF (–NGF) or treated with 100 nM STS or 1 μ M STS for 24 h, with or without 100 μ M BAF, were subjected to SDS–PAGE and Western analysis with anti- α -spectrin antibodies. Although an indistinguishable pattern of α -spectrin cleavage was seen in cells treated with all three conditions, the mechanisms of cleavage appeared to be different since BAF treatment blocked this cleavage fully in NGF-deprived and 100 nM STS-treated cells but only partially in 1 μ M STS-treated cells. Identical results were obtained in multiple experiments

maintained its punctate staining pattern even after 48 h of 1 μ M STS treatment (Figure 7A).

We also examined whether α -spectrin was cleaved during STS-induced sympathetic neuronal death. α -spectrin undergoes a characteristic, caspase-dependent cleavage from a 240-kDa to a 150-kDa fragment in sympathetic neurons after NGF deprivation¹⁹ (Figure 7B). One hundred nM STS treatment induced a similar cleavage of α -spectrin in sympathetic neurons (Figure 7B). Surprisingly, α -spectrin cleavage to a 150-kDa fragment was also detected in 1 μ M STS-treated neurons by 24 h after STS treatment (Figure 7B). However, whereas the cleavage of α -spectrin in NGF-deprived and

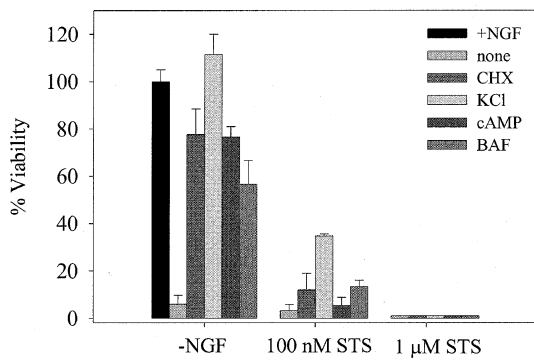


Figure 8 Effect of various neuroprotective agents on sympathetic neuronal death induced by STS. Cultures of NGF-maintained sympathetic neurons were deprived of NGF (-NGF) or treated with 100 nM STS or 1 μM STS for 4 days either alone (none) or in the presence of various neuroprotective agents: CHX, 1 μg/ml cycloheximide; KCl, 50 mM KCl; cAMP, 400 μM CPTcAMP; BAF, 100 μM boc-aspartyl(OMe)fluoromethylketone. After 4 days, cultures were washed extensively and allowed to recover in NGF-containing medium for 7 additional days. The number of viable neurons remaining after this 7-day rescue period was then determined by staining the cultures with crystal violet and counting. Results are mean ± range of two independent experiments

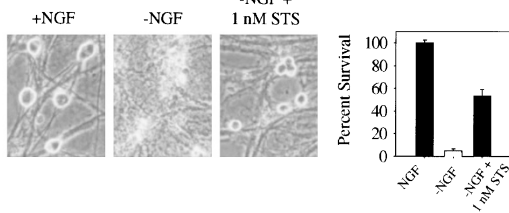
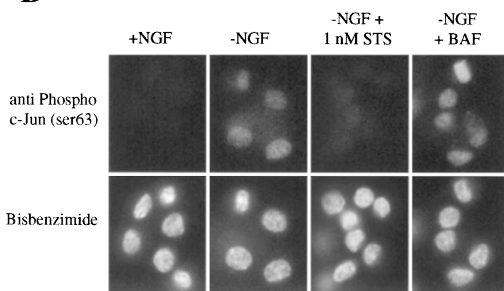
A

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Figure 9 One nM STS prevents NGF deprivation-induced sympathetic neuronal death. **(A)** Phase-contrast photographs of sympathetic neurons either maintained in NGF (+NGF), or deprived of NGF for 4 days (-NGF), or deprived of NGF for 4 days in the presence of 1 nM STS (-NGF+1 nM STS). The number of surviving cells after 4 days of treatment was determined by fixing the cells and staining with crystal violet and counting. Data are means ± S.D. of four independent experiments. **(B)** NGF-deprived, 1 nM STS-saved sympathetic neurons do not show an increase in *c-jun* phosphorylation. Sympathetic neuronal cultures were maintained in NGF (+NGF), or deprived of NGF for 12 h either without (-NGF) or with 1 nM STS (-NGF+1 nM STS). Parallel cultures were also deprived of NGF for 12 h in the presence of 50 μM BAF (-NGF+BAF). Cultures were then immunostained with anti-phospho-*c-jun* (ser 63) antibodies. Identical results were obtained in at least three independent experiments

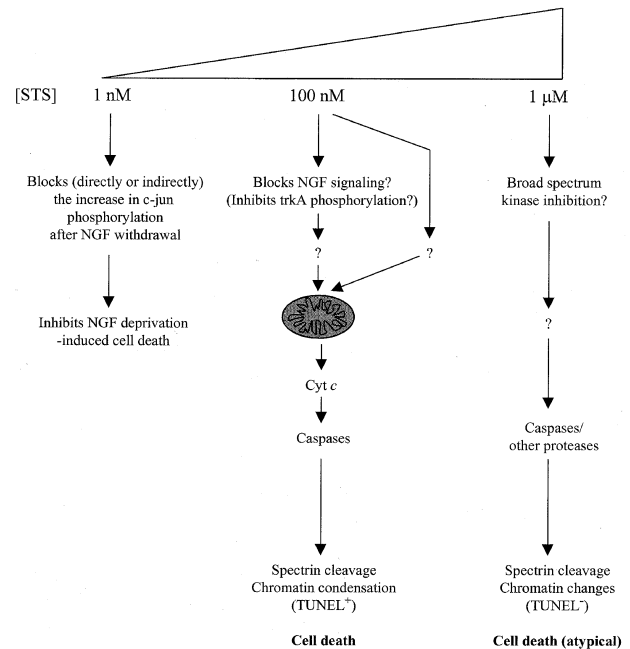


Figure 10 Model of the dose-response effect of STS on sympathetic neurons. Very low concentration of STS, 1 nM STS, prevented sympathetic neuronal death induced by NGF deprivation presumably by blocking (directly or indirectly) the increase in *c-jun* phosphorylation. One hundred nM STS induced cell death in sympathetic neurons with characteristics resembling those induced by NGF deprivation in these neurons. One hundred nM STS is proposed to activate the sympathetic neuronal death pathway either by inhibiting *trkA*-mediated NGF signaling, or by activating a parallel upstream pathway that converges with the NGF deprivation-induced death pathway of the mitochondrial events. In contrast, higher concentrations of STS, 1 μM STS, induced sympathetic neuronal death by a different mechanism. These diverse effects of increasing STS concentrations on sympathetic neurons may be a consequence of different kinases being inhibited with increasing STS concentrations. Cyt *c*, cytochrome *c*

100 nM STS-treated neurons was prevented by the caspase inhibitor BAF, BAF addition only partially inhibited α -spectrin cleavage seen in 1 μM STS-treated neurons (Figure 7B). These results indicate that although the pattern of α -spectrin cleavage was similar in both 100 nM and 1 μM STS-treated neurons, the mechanism of cleavage appeared to be different.

Lastly, we examined whether the neuroprotective agents that prevent NGF deprivation-induced sympathetic neuronal death had any effect on sympathetic neuronal death induced by 100 nM or 1 μM STS. Sympathetic neurons were treated with either NGF deprivation or 100 nM or 1 μM STS addition in the presence of various neuroprotective agents for 4 days. After this, cultures were washed and allowed to recover in NGF-containing medium for 7 additional days. The cells were then stained with crystal violet and counted to assess the number of surviving neurons. The extent of neuroprotection was examined by using this long-term rescue paradigm because of difficulty in assessing cell death reliably in other short-term assays for the 1 μM STS-treated neurons (see Figures 1–5). Cycloheximide, KCl, CPT-cAMP, or the caspase inhibitor

BAF was remarkably effective in preventing neuronal death after NGF deprivation, as described previously (reviewed in⁶; Figure 8). These neuroprotective agents, however, were only partially effective in preventing sympathetic neuronal death induced by 100 nM STS in such long-term rescue assays. Treatment with KCl provided the maximum efficacy (38%) in preventing cell death in 100 nM STS-treated sympathetic neurons in these experiments (Figure 8). Any of the four neuroprotective agents appeared to be effective in promoting the short-term survival (maintaining intact neurites and soma structure) of the 100 nM STS-treated neurons, even 3–4 days after STS treatment (data not shown). At longer times, the 100 nM STS-treated cells eventually underwent cell death even in the presence of these neuroprotective agents (Figure 8). However, 100 nM STS-treated cells that died in the presence of these neuroprotective agents exhibited atypical patterns of chromatin changes that appeared similar to that induced by 1 μ M STS (data not shown; see Discussion). None of the neuroprotective agents tested (cycloheximide, KCl, CPT-cAMP, BAF) had any effect on sympathetic neuronal death induced by 1 μ M STS treatment (Figure 8).

Very low concentrations of STS (1 nM) prevent sympathetic neuronal death

A surprising outcome of the dose response studies with STS was the finding that very low concentration of STS (1 nM) inhibited sympathetic neuronal death induced by NGF deprivation (Figure 9A). One nM STS was effective in preventing approximately 50% sympathetic neuronal death after 4 days of NGF deprivation (Figure 9A). The 1 nM STS-saved neurons maintained intact neurites and phase-bright cell bodies (Figure 9A). Nuclear morphology was also normal in 1 nM STS-saved sympathetic neurons (Figure 9B, data not shown).

Since STS is a broad-spectrum kinase inhibitor, we considered the possibility that 1 nM STS might have inhibited sympathetic neuronal death by preventing JNK activity and the phosphorylation of *c-jun*. Several studies point to the importance of JNK signaling and *c-jun* function in promoting neuronal death.^{18,20,21,49} To test this hypothesis, we examined whether 1 nM STS treatment prevented *c-jun* phosphorylation after NGF deprivation. Addition of 1 nM STS completely prevented the increase in *c-jun* phosphorylation that is normally seen by 12 h after NGF deprivation (Figure 9B). As described previously the caspase inhibitor BAF (50 μ M) had no effect on *c-jun* phosphorylation since it prevents sympathetic neuronal death subsequent to this event in the PCD pathway¹⁵ (Figure 9B). These data indicate that 1 nM STS prevented NGF deprivation-induced cell death in sympathetic neurons by inhibiting an event either at or prior to the increase in *c-jun* phosphorylation in this pathway.

Discussion

In this study we examined the mechanism of sympathetic neuronal death induced by STS. We compared the pathway of STS-induced sympathetic neuronal death with the well-

characterized pathway of sympathetic neuronal death induced by NGF deprivation. We have three main conclusions from this study. First, different concentrations of STS induced very different pathways of cell death in these neurons. Specifically, whereas the sympathetic neuronal death induced by 100 nM STS resembled the apoptotic cell death that is induced by NGF deprivation, cell death induced by 1 μ M STS was clearly different and, presumably, not even apoptotic in these cells (Figure 10). Second, our studies with 1 μ M STS-treated sympathetic neurons highlights the limitations of some commonly used cell death assays that rely on one or two features for assessing cell death. Our results show that assessment of cell death can vary dramatically depending on the assay used. Third, we report that in contrast to the death-inducing effect of 100 nM or 1 μ M STS, very low concentration of STS (1 nM) inhibited apoptosis induced by NGF deprivation in sympathetic neurons. One nM STS appeared to prevent sympathetic neuronal death by acting either at or prior to *c-jun* phosphorylation in the NGF deprivation-induced PCD pathway.

Multiple pathways of sympathetic neuronal death induced by STS

Our data show that low (100 nM) and high (1 μ M) concentrations of STS induced distinct pathways of death in sympathetic neurons. Whereas the cell death induced by 100 nM STS in sympathetic neurons resembled the pathway of death induced by NGF deprivation, the death induced by 1 μ M STS was clearly different. Both NGF deprivation and 100 nM STS treatment induced similar morphological changes of soma degeneration and chromatin condensation. In contrast, 1 μ M STS-treated sympathetic neurons showed remarkably little soma degeneration despite an extensive but different pattern of chromatin alteration (Figures 1 and 3). The chromatin changes induced by NGF deprivation and 100 nM STS treatment were TUNEL positive whereas those induced by 1 μ M STS were not (Figure 2). Membrane blebbing during apoptosis in PC12 cells is mediated by kinases that phosphorylate myosin light chain and is inhibited by 1 μ M STS treatment.⁵⁰ Whether the maintenance of soma structure seen in 1 μ M STS-treated sympathetic neurons is caused by inhibition of certain kinases by 1 μ M STS is unclear.

Our data indicate that the death induced by 100 nM STS treatment, like NGF deprivation, is mediated by caspases. First, the caspase inhibitors of BAF or zVAD-FMK blocked the morphological and nuclear changes induced by 100 nM STS treatment, at least up to 48 h after STS treatment (Figure 1). Second, α -spectrin, a caspase substrate, was cleaved in a BAF-inhibitable manner during 100 nM STS-induced death (Figure 7B). Third, 100 nM STS-treated neurons, like the NGF-deprived neurons, exhibited loss of cytochrome *c* from the mitochondria (Figure 7A); translocation of cytochrome *c* results in caspase activation in many cell types, including sympathetic neurons.^{22,23} Furthermore, the 100 nM STS-treated neurons stained positively with an antibody (CM1) that detects the activated form of caspases 3 or 7⁵¹ (data not shown). In contrast, BAF addition had no effect on the morphological changes induced by 1 μ M STS in sympathetic neurons (Figure 1). Preincubation of cells with BAF or zVAD-

FMK even 4 h prior to 1 μ M STS treatment also had no effect on sympathetic neuronal death induced by 1 μ M STS treatment (data not shown).

We were surprised to find that despite the differences between sympathetic neuronal death induced by 100 nM or 1 μ M STS, the pattern of α -spectrin cleavage in NGF-deprived neurons or in neurons treated with either 100 nM or 1 μ M STS was indistinguishable (Figure 7B). However, the mechanism of α -spectrin cleavage appeared to be different. Whereas α -spectrin cleavage in NGF-deprived neurons or 100 nM STS-treated neurons was caspase-dependent, BAF only partially inhibited α -spectrin cleavage in 1 μ M STS-treated neurons. Thus, multiple proteases appear to mediate α -spectrin cleavage in 1 μ M STS-treated sympathetic neurons. In several models of death, α -spectrin cleavage is mediated by calpains.^{52–55} Whether calpains are activated and contribute to α -spectrin cleavage in 1 μ M STS-treated sympathetic neurons is unclear. Nevertheless, the observation that BAF addition prevented partial α -spectrin cleavage suggests that some caspase activation occurred during 1 μ M STS-mediated sympathetic neuronal death. Since cytochrome *c* maintained a punctate distribution during 1 μ M STS-induced sympathetic neuronal death, the mechanism of caspase activation in this pathway is likely to be independent of cytochrome *c* pathway. Which specific caspases mediate α -spectrin cleavage in 1 μ M STS-treated neurons is unknown. One μ M STS-treated sympathetic neurons did not stain positively with the CM1 antibodies (data not shown), suggesting that caspases 3 or 7 were unlikely to be activated in this death pathway.

What is the mechanism by which 100 nM or 1 μ M STS induces cell death in sympathetic neurons? NGF deprivation and 100 nM STS treatment both show loss of cytochrome *c*, caspase mediated cleavage of α -spectrin, and exhibit similar, BAF-inhibitable morphological and chromatin changes (Figure 10). The simplest explanation for why the 100 nM STS and NGF deprivation pathways appear similar may be that 100 nM STS treatment blocks NGF signaling by inhibiting phosphorylation of the NGF receptor, *trkA*, or other downstream adaptors in this pathway, thereby mimicking NGF deprivation. One hundred nM STS, or similar concentrations of other structurally related K252 compounds, inhibit *trkA* phosphorylation in several cell lines including PC12 cells.^{56–58} If this hypothesis were correct, then the death pathways induced by NGF deprivation and 100 nM STS treatment should be identical. However, some important differences were observed. For example, the increase in *c-jun* phosphorylation seen after NGF deprivation was not observed in neurons undergoing death after 100 nM STS treatment (Figure 6). Although *c-jun*-dependent functions are required for the NGF deprivation-induced sympathetic neuronal death,^{20,21} exactly how *c-jun* functions to promote neuronal death remains unclear. One hundred nM STS treatment may block the increase in *c-jun* phosphorylation in sympathetic neurons by inhibiting *c-jun* N-terminal kinases (JNKs) but leave the death-promoting, *c-jun*-dependent functions intact.

Another difference between the 100 nM STS and the NGF deprivation-induced death pathway is that neuroprotective agents such as KCl, CPTcAMP, cycloheximide, and BAF,

which prevent death long term after NGF deprivation, were only partially capable of preventing death long term after 100 nM STS treatment (Figure 8). However, in addition to activating a predominantly NGF deprivation-like apoptotic pathway, 100 nM STS may also activate other slow-acting toxic events in these cells. Such slow-acting toxic events may become apparent only when the predominant, NGF deprivation-like pathway is blocked, such as upon addition of the neuroprotective agents. The observation that the 100 nM STS-treated neurons dying in the presence of these neuroprotective agents exhibited some atypical morphological features of death (data not shown) is consistent with this hypothesis. Alternately, 100 nM STS may induce sympathetic neuronal death by a completely different upstream parallel pathway. Our data do not exclude the possibility that a separate pathway activated by 100 nM STS ultimately converges with the NGF deprivation-induced pathway at a point after the increase in *c-jun* phosphorylation but upstream of the mitochondrial events (Figure 10). Mitochondrial events, such as loss of cytochrome *c*, induced by either NGF deprivation or 100 nM STS treatment are BAX-dependent. Sympathetic neurons from BAX-deficient mice did not release cytochrome *c* or undergo apoptosis after 100 nM STS treatment (unpublished observations). In contrast, the nuclear changes induced by 1 μ M STS were not BAX-dependent (unpublished observations).

The mechanism by which 1 μ M STS induces sympathetic neuronal death is unknown. Our data show that in primary sympathetic neurons, death induced by 1 μ M STS was different from that induced by NGF deprivation (Figures 1–8). These results are in contrast to those from a recent report that concluded that sympathetic neuronal death induced by 1 μ M STS closely resembles that produced by NGF withdrawal and is apoptotic.³⁶ Indeed, based on certain death assays, such as loss of protein synthesis or MTT reduction (Figure 4), or a live/dead assay based on calcein AM staining,³⁶ the timecourses of death appear comparable in both NGF-deprived and 1 μ M STS-treated neurons. Also, both NGF-deprived neurons and 1 μ M STS-treated neurons, exhibited alterations in chromatin structure (Figure 1). However, only upon closer examination by using multiple assays were clear differences in the morphological features of death induced by NGF deprivation or treatment with 1 μ M STS revealed (Figures 1–8). Thus, we conclude that sympathetic neuronal death induced by 1 μ M STS is distinct from that induced by NGF deprivation and does not appear apoptotic.

Exposure to 1 μ M STS rapidly and irreversibly commits sympathetic neurons to undergo a death that has few or no properties of apoptosis. Since no similarities (with the exception of limited caspase involvement in α -spectrin cleavage) were observed between the NGF deprivation-induced PCD pathway and the death induced by 1 μ M STS, the components involved in mediating sympathetic neuronal death after 1 μ M STS treatment remain unidentified.

Assessment of cell death

Our studies with 1 μ M STS have nevertheless provided important lessons regarding assessment of cell death in general. Sympathetic neurons treated with 1 μ M STS had

virtually undetectable levels of protein synthesis and MTT reduction by 24 h after STS treatment (Figure 4). These cells also showed a dramatically altered chromatin structure as early as 6 h after STS treatment. Yet the cells maintained soma structure and stained positively with calcein AM or crystal violet even 48 h after 1 μ M STS treatment (Figures 1 and 3). If one examined how long after exposure to 1 μ M STS these cells became committed to die, our data indicated that even a 30 min exposure was sufficient to commit all sympathetic neurons to undergo cell death (Figure 5). Assessment of whether the 1 μ M STS-treated sympathetic neurons were alive or dead at a particular timepoint, therefore, clearly depended on the criterion used to assay cell viability (summarized in Table 1).

Furthermore, our experiments with STS revealed at least two distinct patterns of chromatin changes in sympathetic neurons that reacted differently with the TUNEL reagent (Figures 1 and 2). Also, although cleavage of α -spectrin appeared indistinguishable in extracts of sympathetic neurons treated with 1 μ M STS or NGF deprivation, mechanisms of cleavage were apparently different (Figure 7B). Therefore, these results illustrate the need to use multiple criteria for assessing cell death or concluding whether two pathways of death are identical.

Neuroprotection with 1 nM STS

In contrast to the cell death-inducing effect of STS at concentrations of 100 nM or higher, we report that very low concentrations of STS (1 nM) protected sympathetic neurons from NGF deprivation-induced death (Figures 9 and 10). Addition of 1 nM STS promoted the survival of approximately 50% of sympathetic neurons after 4 days of NGF deprivation (Figure 9A). Low STS concentrations promote neurite outgrowth and survival of other neuronal applications as well: STS (10–50 nM) promotes neurite outgrowth in PC12 cells.^{59,60} STS (10 fM–10 nM) also promotes the survival of hippocampal, septal, and cortical cells after glucose deprivation⁶¹ and prevents death of hippocampal neurons after amyloid β toxicity or oxidative injury.⁶² STS administration in rats also facilitates recovery from deficits induced by basal forebrain lesions⁶³ and prevents neuronal damage after ischemic injury.⁶⁴

The exact mechanism by which 1 nM STS inhibits the sympathetic neuronal PCD pathway is not known. Since the NGF-deprived, 1 nM STS-saved neurons did not show an increase in *c-jun* phosphorylation (Figure 9B), 1 nM STS presumably inhibited the sympathetic neuronal PCD pathway either at or prior to increase in *c-jun* phosphorylation. Perhaps 1 nM STS promotes sympathetic neuronal survival by activating some upstream, survival-promoting signaling pathways. In contrast to kinase-inhibiting activities of high STS concentrations (\geq 100 nM), low nanomolar concentration of STS promote tyrosine phosphorylation of several proteins in hippocampal cell extracts.⁶¹

The dose-dependent complexity in STS signaling that can result in either survival or death of neurons, as reported in this and previous studies, clearly highlights the therapeutic limitations of using STS *per se*. However, future studies that focus on examining exactly how low

nanomolar concentrations of STS prevent neuronal death may aid in the development of rational strategies that could specifically prevent neuronal death after injury or disease.

Materials and Methods

Reagents

All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Collagenase and trypsin were purchased from Worthington Biochemical Corporation (Freehold, NJ, USA). Caspase inhibitor boc-aspartyl(OMe)-fluoromethylketone (BAF) was purchased from Enzyme Systems Products (Livermore, CA, USA).

Sympathetic neuronal cultures and media

Primary cultures of sympathetic neurons from the superior cervical ganglion were prepared from embryonic day-21 rats by a modification¹⁵ of a previously described method.⁶⁵ Briefly, the dissected ganglia were treated with collagenase (1 mg/ml), then trypsin (2.5 mg/ml) for 30 min each at 37°C. The ganglia were triturated and the dissociated cells were plated on collagen-coated dishes in NGF-containing medium (AM50); NGF was purchased from Harlan (Indianapolis, IN, USA). This medium contained Eagle's minimum essential medium with Earle's salts (Life Technologies Inc., Gaithersburg, MD, USA) with the addition of 50 ng/ml 2.5S NGF, 10% fetal calf serum, 2 mM glutamine, 100 μ g/ml penicillin, and 100 μ g/ml streptomycin; 20 μ M fluorodeoxyuridine, 20 μ M uridine, and 3.3 μ g/ml aphidicolin were also included to reduce the number of nonneuronal cells. After 5 days of growth in this medium, the cultures were treated with various conditions.

For NGF deprivation, cultures were rinsed twice with medium lacking NGF (AM0: AM50 medium without NGF), followed by addition of AM0 containing goat anti-NGF neutralizing antibody. For treatment with staurosporine, the appropriate concentration of staurosporine was added to sympathetic neurons maintained in AM50.

TUNEL assay

TUNEL staining was assayed with the In Situ Cell Death Detection Kit (Fluorescein; Roche Biochemicals, Indianapolis, IN, USA) following manufacturer's instructions. Cells were fixed for 30 min in 4% paraformaldehyde/phosphate-buffered saline (PBS) at room temperature, washed once with PBS, and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate for 20 min at 4°C. After two more washes with PBS, the cells were incubated with 50 μ l of the TUNEL reagent for 1 h at 37°C. Cells were then washed again with PBS, stained for 15 min with the nuclear dye bisbenzimidazole (1 μ g/ml Hoechst 33258, Molecular Probes Inc., Eugene, OR, USA), and examined by fluorescence microscopy.

Metabolic measurements

Experiments were performed in 24-well dishes with approximately 10 000 cells per well. After 5 days *in vitro*, sympathetic neuronal cultures were treated with various conditions and assayed for metabolic activity essentially as described previously.⁸

Rate of protein synthesis Neuronal cultures were labeled for 4 h at 37°C with 10 μ Ci/ml Trans label (³⁵S-methionine and ³⁵S-cysteine; ICN, Costa Mesa, CA, USA) in modified AM0 medium lacking methionine and cysteine. After labeling, cultures were washed once with PBS and lysed

in 500 μ l of 0.5% SDS, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5. Total proteins were then precipitated with 10% trichloroacetic acid (TCA) on ice and filtered through 0.45 μ m nitrocellulose. After washing the precipitate with cold 10% TCA, its radioactivity was measured in a liquid scintillation counter.

Rate of MTT reduction Culture medium was removed and 500 μ l of 0.4 mg/ml 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in Leibovitz L-15 medium (Life Technologies Inc.) containing 10% fetal calf serum was added to the cells for 9 min at 37°C. The MTT solution was aspirated and cells were lysed with 200 μ l dimethylsulfoxide. The amount of MTT formazan was quantitated by determining its absorbance at 510 nm in a Titertek Multiscan plate reader.

Calcein AM staining After removal of the culture medium, cells were washed once with Lockes solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2.7 mM CaCl₂, 1.2 mM MgCl₂, 5.6 mM D-glucose, 5 mM HEPES, pH 7.4) and then labeled with 1 μ M Calcein AM (Molecular Probes, Eugene, OR, USA) in Lockes solution for 20 min in the dark. Cells were then examined by fluorescence microscopy.

Immunohistochemistry

The method for immunostaining with anti-phospho-c-Jun (Ser 63) (New England Biolabs, Beverly, MA, USA) antibodies has been described previously.⁶⁶ Immunostaining with anti-cytochrome *c* antibodies (Pharmingen, San Diego, CA, USA) and the cell counts for loss of cytochrome *c* were performed as described previously.²³

Western blot analysis

Protein lysates from neuronal cultures, approximately 30 000 cells, grown in 35-mm dishes were separated on 6% SDS-polyacrylamide gels and electrophoretically transferred to a PVDF membrane (Immobilon P, Millipore, Bedford, MA, USA). The membrane was subjected to immunoblot analysis with an anti- α -spectrin antibody (1:3000 dilution of MAB1622 from Chemicon International Inc., Temecula, CA, USA) by using a previously described procedure.¹⁵

Cell survival counts

Viability of sympathetic neurons was quantitated after fixing cultures with 4% paraformaldehyde and staining with crystal violet as described earlier.⁸ Neurons were scored as viable if the crystal violet-positive cells had a defined cellular outline.

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