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Alternating metabolic pathways in NGF-deprived sympathetic neurons affect caspase-independent death

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Mitochondrial release of cytochrome *c* in apoptotic cells activates caspases, which execute apoptotic cell death. However, the events themselves that culminate in caspase activation can have deleterious effects because caspase inhibitor–saved cells ultimately die in a caspase-independent manner. To determine what events may underlie this form of cell death, we examined bioenergetic changes in sympathetic neurons deprived of NGF in the presence of a broad-spectrum caspase inhibitor, boc-aspartyl-(OMe)-fluoromethylketone. Here, we report that NGF-deprived, boc-aspartyl-(OMe)-fluoromethylketone–saved neurons rely heavily on glycolysis for ATP generation and for survival. Second, the activity of F_0F_1 contributes to caspase-independent death, but has only a minor role in the maintenance of mitochondrial membrane potential, which is maintained primarily by electron transport. Third, permeability transition pore inhibition by cyclosporin A attenuates NGF deprivation–induced loss of mitochondrial proteins, suggesting that permeability transition pore opening may have a function in regulating the degradation of mitochondria after cytochrome *c* release. Identification of changes in caspase inhibitor–saved cells may provide the basis for rational strategies to augment the effectiveness of the therapeutic use of postmitochondrial interventions.

Introduction

Apoptosis is a form of cell death that occurs during normal development and in pathological situations. Cells undergoing apoptosis exhibit certain characteristics, including cytoplasmic shrinkage, nuclear blebbing, and chromatin condensation (Kerr et al., 1972). The caspase family of cysteine proteases has a critical role in executing apoptosis (Cryns and Yuan, 1998). In the intrinsic, or mitochondria-dependent, pathway of apoptosis, caspase activation is regulated mainly by the release of cytochrome *c* from the intermembrane space of mitochondria (Li et al., 1997). The Bcl-2 family of proteins contributes to regulating cytochrome *c* release by integrating and conveying pro- and antiapoptotic signals to the mitochondria (Adams and Cory, 1998). Once in the cytosol, cytochrome *c* initiates a cascade of caspase activation by promoting the oligomerization of APAF-1 and the activation of procaspase-9 (Li et al., 1997). Other mitochondrial proteins, such as Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000) and HtrA2 (Suzuki et al., 2001), are also released into the cytosol during apoptosis and may contribute to the regulation of caspase activity.

The critical function of caspases in apoptosis is underscored by observations that caspase inhibition prevents the appearance of many markers of apoptosis in neurons, including certain biochemical (Miller et al., 1997; Stefanis et al., 1999) and ultrastructural changes (Oppenheim et al., 2001). However, in many, if not all, systems, caspase inhibition does not prevent the ultimate death of these cells. This caspase-independent cell death often occurs with a delayed time course (Miller et al., 1997; Stefanis et al., 1999). The initiation of the apoptotic cell death pathway leads to a number of caspase-independent changes, including release of death-promoting factors, such as apoptosis-inducing factor (Susin et al., 1999), Endo G (Li et al., 2001), and HtrA2 (Suzuki et al., 2001), and changes in mitochondrial structure (Mootha et al., 2001). In fact, microinjection of neutralizing antibodies to AIF protects cortical neurons from some forms of caspase-independent death (Cregan et al., 2002). However, which of these changes critically regulate caspase-independent death, and whether these mechanisms vary in different models, is not known.

Here, we examined the bioenergetic status of NGF-deprived sympathetic neurons that were prevented from completing apoptosis by a caspase inhibitor. Removal of NGF from these neurons in vitro triggers a classic apoptotic death that recapitulates naturally occurring cell death that ensues within the superior cervical ganglion in vivo during the first week of life. This apoptotic death requires macromolecular

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synthesis (Martin et al., 1988), BAX expression (Deckwerth et al., 1996), cytochrome *c* release (Neame et al., 1998), and caspase activity (Deshmukh et al., 1996; Troy et al., 1996). Pharmacologic or genetic inhibition of caspase activity delays, but does not prevent, the death of NGF-deprived sympathetic neurons (Deshmukh et al., 2000).

Progression along this cell death pathway can be aborted by readdition of NGF to an NGF-deprived sympathetic neuron before a cell has reached the commitment-to-die (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). After a cell has committed to die, it can no longer be rescued by NGF and will die even in the presence of trophic factor. In NGF-deprived sympathetic neurons, the time course of commitment-to-die in the absence of a caspase inhibitor, termed Commitment 1, is virtually identical to the time course of cytochrome *c* release and rapidly ensuing caspase activation (Putcha et al., 1999). However, trophic factor–deprived sympathetic neurons in which caspase activity has been inhibited by pharmacologic (Martinou et al., 1999) or genetic (Deshmukh et al., 2000) means can be rescued by NGF after release of cytochrome *c*, arguing that caspase activation is the critical event that normally commits a cell to die. However, caspase inhibitor–saved cells eventually die in a caspase-independent manner. The commitment-to-die in the presence of a caspase inhibitor, termed Commitment 2, occurs several days after cytochrome *c* release (Deshmukh et al., 2000).

The mechanisms that regulate Commitment 2 in sympathetic neurons are not well understood. However, several lines of evidence support the hypothesis that mitochondria are key sites of regulation of this event in sympathetic neurons. First, in contrast to the delayed death of caspase inhibitor–saved cells, sympathetic neurons from BAX-deficient mice remain viable even after 1 mo of NGF deprivation (Deckwerth et al., 1996). This striking difference suggests that the "mitochondrial hit," or the mitochondrial events that occur downstream of BAX but upstream of caspase activation, is required for caspase-independent death. Second, commitment-to-die in the presence of a caspase inhibitor is temporally correlated with the loss of mitochondrial membrane potential ($\Delta\Psi$m;* Deshmukh et al., 2000). The loss of $\Delta\Psi$m in NGF-deprived, caspase inhibitor–saved neurons occurs much later than the release of cytochrome *c* from the mitochondria, suggesting that the mitochondrial events that regulate Commitment 2 are distinct from those that regulate Commitment 1. Third, the mitochondrial permeability transition pore (PTP) has an important role in Commitment 2 in rat sympathetic neurons (Chang and Johnson, 2002). The physiologic function of the PTP, a channel that transiently connects the cytosol with the mitochondrial matrix, is not known, but it appears to damage mitochondria in a number of models of cell death (for review see Crompton, 1999), including cerebral ischemia (Matsumoto et al., 1999) and hypoglycemia-induced hippocampal damage in vivo (Ferrand-Drake et al., 1999). Cyclosporin A (CsA), a PTP inhibitor (Crompton et al., 1998), dramatically decreases the rate of Commitment 2 and the drop in $\Delta\Psi$m in NGF-deprived, boc-aspartyl-(OMe)-fluoromethylketone (BAF)–saved rat sympathetic neurons (Chang and Johnson, 2002). Importantly, CsA has no effect on cytochrome *c* release or Commitment 1, arguing that opening of the PTP is an event that is required specifically for Commitment 2 in this model system. Together, these findings suggest that events associated with the mitochondrial hit are required for caspase-independent cell death when apoptotic cell death is prevented by caspase inhibition.

Here, we report that NGF-deprived, caspase inhibitor–saved sympathetic neurons rely on glycolysis, but not oxidative phosphorylation, to generate ATP. The reliance on glycolysis confers sensitivity to glucose deprivation and resistance to oligomycin treatment in NGF-deprived, BAF-saved neurons. Remarkably, oligomycin protects NGF-deprived, BAF-saved cells, suggesting that reverse operation of the F_0F_1 ATPase may contribute to caspase-independent death. However, although reversal of the F_0F_1 ATPase has a minor role in maintaining $\Delta\Psi$m after cytochrome *c* release in NGF-deprived, BAF-saved neurons, electron transport remains the primary mechanism by which this electrochemical gradient is formed in these cells. Finally, CsA does not affect these changes, but does attenuate loss of mitochondria that occurs in NGF-deprived, BAF-saved neurons. These findings may have important implications for strategies that target caspases for therapeutic intervention.

Results

NGF-deprived, BAF-saved neurons maintain slightly lower levels of ATP

NGF-deprived sympathetic neurons that are prevented from completing apoptosis by caspase inhibition exist in a depressed metabolic state, as determined by a number of morphological and biochemical parameters (Deshmukh et al., 1996; Chang and Johnson, 2002). To determine the effect of caspase inhibition on the energetic status of neurons, we measured total cellular ATP by the luciferase/luciferin method in sympathetic neuronal cultures that were either maintained in NGF or deprived of NGF in the presence of BAF. As seen in Fig. 1 A, the total amount of ATP increased in NGF-maintained cultures over 9 d, whereas it decreased in NGF-deprived, BAF-saved cells. Most of the decrease in ATP in NGF-deprived cells occurred within the first 3 d, because total ATP was 79% of that of cells at the time of deprivation after 3 d and 65% after 9 d. However, cells maintained in NGF increased in size during this time, whereas NGF-deprived, BAF-saved neurons atrophied and had a decreased rate of protein synthesis (Deshmukh et al., 1996). When normalized to total protein of each sample, NGF-deprived, BAF-saved cells actually had higher levels of ATP than NGF-maintained neurons (Fig. 1 B). Total cellular ATP is only one measure of overall energy balance. However, maintenance of relatively normal amounts of intracel-

*Abbreviations used in this paper: ANT, adenine nucleotide translocase; BAF, boc-aspartyl-(OMe)-fluoromethylketone; Cc, cytochrome *c*; CCCP, carbonyl cyanide m-chlorophenylhydrazone; COX IV, cytochrome oxidase subunit IV; CsA, cyclosporin A; JC-1,5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraetylbenzimidazolylcarbocyanine iodide; $\Delta\Psi$m, mitochondrial membrane potential; PTP, permeability transition pore; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel.

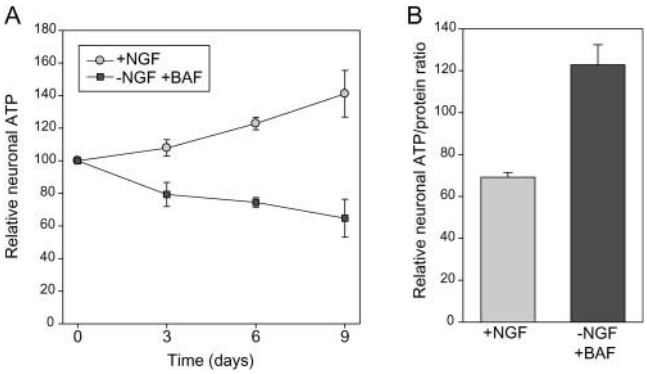

Figure 1. **NGF-deprived, BAF-saved cells maintain high levels of ATP.** (A) ATP was measured in NGF-maintained and -deprived, BAF-saved sister cultures at the times indicated on the x axis by a luciferase-based assay. (B) ATP levels after 3 d of treatment were normalized to the total amount of protein in each sample. Values were normalized to the time 0 value for each experiment and represent mean ± SD from three independent experiments performed in quadruplicate.

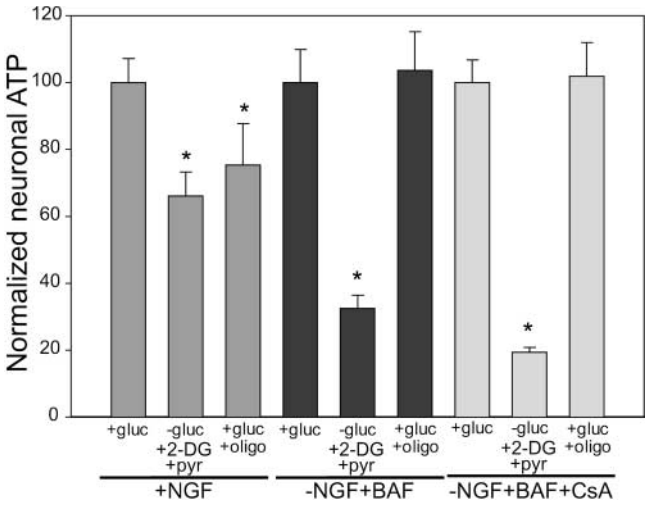

Figure 2. **NGF-deprived, BAF-saved cells rely on glycolysis for ATP production.** Cells were maintained in NGF or deprived of NGF in the presence of BAF or BAF and CsA for 3 d. ATP was measured after 2-h exposure to control the medium (gluc), glucose-free medium with 2-deoxyglucose and pyruvate (2-DG + pyr) to inhibit glycolysis, or oligomycin (5 μg/ml) to inhibit oxidative phosphorylation. Values are mean ± SD of the control level from three independent experiments performed in quadruplicate. Asterisk indicates statistical significance ($P < 0.05$) versus ATP in control medium for that culture condition.

lular ATP suggests ongoing metabolism in NGF-deprived, BAF-saved cells consistent with their ability to survive long periods of NGF deprivation (Chang and Johnson, 2002).

NGF-deprived, BAF-saved neurons require glycolysis, but not oxidative phosphorylation, to generate ATP

To identify which bioenergetic pathways are used to generate ATP within these cells, we determined whether acutely inhibiting either glycolysis or oxidative phosphorylation altered cellular ATP levels in NGF-maintained and NGF-deprived, BAF-saved neurons. We used total ATP, which is only a gross measure of cellular energy charge, to identify large perturbations in energy balance that were likely to affect cell survival. As seen in Fig. 2, inhibition of glycolysis, achieved by incubating cells in glucose-free medium supplemented with 5 mM 2-deoxyglucose and 1 mM pyruvate, for 2 h in NGF-maintained cells decreased total ATP to 66% of control. Treatment of cells with the F_0F_1 ATPase inhibitor, oligomycin, in standard, glucose-containing medium was used to inhibit ATP production by oxidative phosphorylation. In NGF-maintained cells, 5 μg/ml oligomycin caused a decrease in ATP levels to 75% of the control. These data indicate that both glycolysis and oxidative phosphorylation contribute to ATP production in NGF-maintained neurons. To determine the contributions of these pathways to ATP production in NGF-deprived, BAF-saved cells, cultures were synchronized by depriving them of NGF in the presence of BAF for 3 d, such that essentially all cells have released cytochrome *c* but few cells have become committed to die (Chang and Johnson, 2002). In NGF-deprived, BAF-saved cells, inhibiting glycolysis for 2 h caused a decrease in ATP levels to 32% of control, which is much greater than in NGF-maintained neurons ($P < 10^{-7}$). In contrast, inhibiting oxidative phosphorylation in NGF-deprived, BAF-saved cells had no effect on cellular ATP (Fig. 2). Thus, unlike NGF-maintained neurons, NGF-deprived, BAF-saved neurons do not require oxidative phosphorylation to maintain maximal ATP levels, but instead rely more heavily, if not solely, on glycolysis for ATP production.

CsA delays Commitment 2 in rat sympathetic neurons, presumably by inhibiting mitochondrial PTP opening (Chang and Johnson, 2002). To determine whether CsA affected mitochondrial function in NGF-deprived, BAF-saved cells, we examined the effect of oligomycin on ATP levels within NGF-deprived cells treated with BAF and 10 μM CsA. Inhibiting glycolysis in NGF-deprived cells treated with BAF and CsA caused a dramatic decrease in ATP levels, whereas oligomycin had no effect on ATP levels in the presence of CsA, similar to cells treated with BAF alone (Fig. 2). This suggests that the protective effects of CsA on NGF-deprived, BAF-saved neurons are not mediated by preserving oxidative phosphorylation.

Glucose deprivation kills NGF-deprived, BAF-saved, but not NGF-maintained, neurons

One prediction from the finding that NGF-deprived, BAF-saved cells rely more heavily on glycolysis for ATP generation than NGF-maintained neurons (Fig. 2) is that NGF-deprived, BAF-saved cells should be more dependent on glycolysis for survival and more sensitive to glucose deprivation. To test this hypothesis, the effects of altering the concentration of glucose in the medium on NGF-maintained and NGF-deprived, BAF-saved cells on survival were determined. As schematized in Fig. 3 A, the latter was performed by first depriving cultures of NGF in the presence of BAF for 2 d, to generate a synchronized population of neurons that had released cytochrome *c* but had not committed to die. At this time, cells were switched from the standard medium, which contains 5 mM glucose, to a medium containing 0, 5, or 25 mM glucose for 8 d, after which the proportion that had committed-to-die was determined by readdition of a medium containing NGF and 5 mM glucose for 7 d. As seen

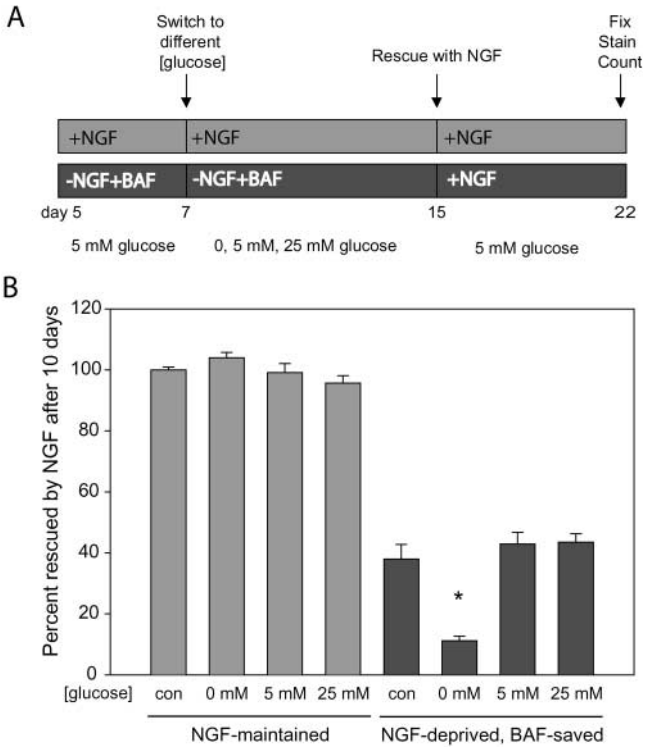

Figure 3. **NGF deprivation increases sensitivity to glucose deprivation.** Cultures were deprived of NGF or maintained in NGF for 2 d in a standard medium, and then for an additional 8 d in a standard medium (con) or glucose-free medium supplemented with 0, 5, or 25 mM glucose as schematized in A. The cells were rescued with NGF for an additional 7 d and counted. Values shown in B are mean ± SD of three independent experiments performed in triplicate of the proportion of cells that could be rescued by NGF after this 10-d treatment. Asterisk indicates statistical significance of $P < 0.05$ compared with survival in a control medium.

in Fig. 3 B, survival in the presence of NGF was identical in a medium containing 0, 5, or 25 mM glucose medium. Because NGF-deprived, BAF-saved cells become committed-to-die during the course of the experiment, only roughly 40% of NGF-deprived, BAF-saved neurons incubated in 5 mM glucose after 10 d can be rescued by NGF (Chang and Johnson, 2002). In contrast, only 11% of NGF-deprived, BAF-saved cells maintained in a medium lacking glucose for the final 8 d of the 10-d period could be rescued. Thus, glucose deprivation decreases by roughly 75% the number of BAF-saved cells that are rescued by NGF. Increasing the glucose concentration to 25 mM did not increase the proportion of cells that could be rescued, suggesting that insufficient glucose in the standard culture medium does not underlie caspase-independent death of NGF-deprived sympathetic neurons. These data demonstrate that NGF-deprived, BAF-saved neurons were more sensitive to glucose deprivation than were NGF-maintained neurons, which were remarkably resistant to this insult.

Oligomycin kills NGF-maintained neurons, but protects NGF-deprived, BAF-saved neurons

Because NGF-maintained neurons, but not NGF-deprived, BAF-saved cells, require oxidative phosphorylation to maintain maximal ATP levels, a second prediction from the data in Fig. 2 is that inhibition of oxidative phosphorylation should be more detrimental to NGF-maintained neurons than to NGF-deprived, BAF-saved neurons. To examine the effect of inhibiting oxidative phosphorylation on Commitment 2, cells were deprived of NGF in the presence of BAF for 2 d and maintained for an additional 8 d in the presence of 5 μg/ml oligomycin to block oxidative phosphorylation, and subsequently rescued with NGF (Fig. 4 A). Consistent with our previous report (Chang and Johnson, 2002) and data in Fig. 3, the number of NGF-deprived, BAF-saved cells that could be rescued after 2 and 10 d of NGF deprivation decreased from roughly 85% of control to 35%, indicating that caspase-independent death occurred during this period. After 8 d of treatment with oligomycin, only 10% of NGF-maintained neurons survived (Fig. 4 B). Surprisingly, treatment with oligomycin increased the number of BAF-

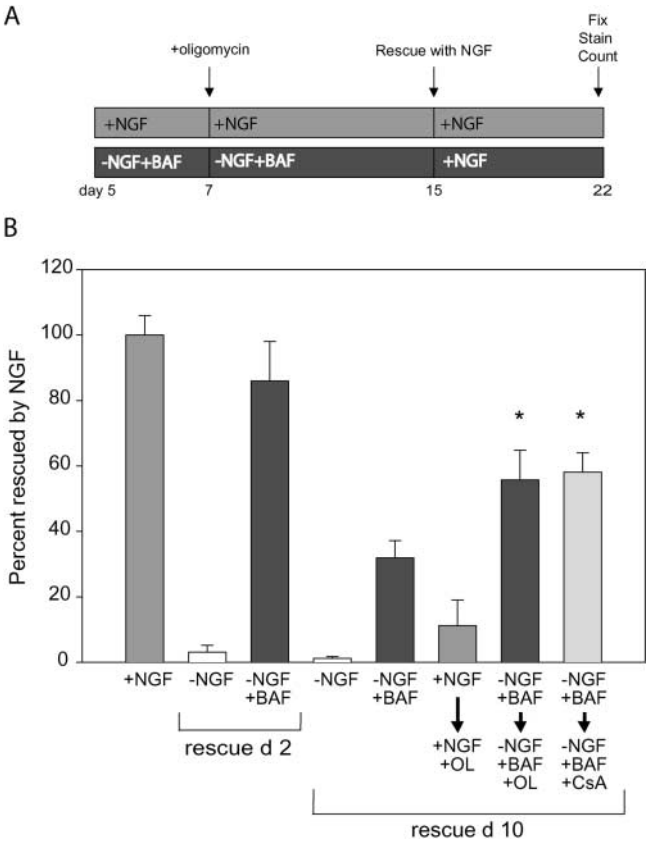

Figure 4. **Oligomycin kills NGF-maintained neurons, but protects NGF-deprived, BAF-saved neurons.** (A) To determine the effect of oligomycin on Commitment 2, oligomycin (OL) was added to cultures after they were maintained in NGF or deprived of NGF in the presence of BAF for 2 d. (B) After 8 d of exposure to oligomycin, cells were rescued with NGF for 7 d and counted. At the time of oligomycin addition, nearly all cells had released cytochrome *c*, because <5% of NGF-deprived cells could be rescued. Asterisk indicates statistical significance of $P < 10^{-4}$ compared with −NGF +BAF. For comparison, cyclosporin A (CsA) added after 2 d of deprivation had a similar effect on Commitment 2 at 10 d as oligomycin. This value was not different from that of the −NGF +BAF + OL condition. Values shown in B are mean ± SD of three independent experiments performed in quadruplicate for each condition.

saved neurons that could be rescued by NGF, from 35 to roughly 55% of control. Because only 85% of NGF-deprived, BAF-saved cells could be rescued at the time of oligomycin treatment, almost half of the cells that could be saved were protected by oligomycin. To illustrate the magnitude of this effect, CsA, a robust inhibitor of Commitment 2 (Chang and Johnson, 2002), increased the proportion of cells that were rescued to a similar degree when added after 2 d of NGF deprivation (Fig. 4 B). Thus, although oligomycin is toxic to NGF-maintained sympathetic neurons, it protects NGF-deprived, BAF-saved cells. These findings strongly suggest that activity of the F_0F_1 ATPase contributes to caspase-independent death of sympathetic neurons.

Electron transport maintains $\Delta\Psi_m$ after cytochrome *c* release

Inhibition of F_0F_1 does not decrease ATP levels in cells that have released cytochrome *c* (Fig. 2), arguing that it is not generating ATP at the expense of the mitochondrial proton gradient. However, the F_0F_1 ATPase inhibitor, oligomycin, prevents caspase-independent death, suggesting that the activity of this enzyme complex has a role in the death of these cells. As its name suggests, the F_0F_1 ATPase can hydrolyze ATP to transport protons actively against the electrochemical gradient. In this mode of operation, F_0F_1 activity would contribute to $\Delta\Psi$m, which is maintained, albeit to a lesser degree, in NGF-deprived, BAF-saved neurons after cytochrome *c* has been released (Chang and Johnson, 2002). Because cytochrome *c* mediates electron transport from complexes III to IV, the loss of mitochondrial cytochrome *c* would be expected to impair electron transport chain–mediated maintenance of $\Delta\Psi$m. To determine the mechanism by which caspase inhibitor–saved cells maintain $\Delta\Psi$m, we examined the effect of inhibiting either electron transport or F_0F_1 on $\Delta\Psi$m in NGF-deprived, BAF-saved neurons by using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraetylbenzimidazolylcarbocyanine iodide (JC-1), a cell-permeable, $\Delta\Psi$m-sensitive dye (Smiley et al., 1991). JC-1 exists as a fluorescent monomer, but reversibly forms aggregates, which have different spectral properties than the monomeric form, in the matrix of polarized mitochondria (Nicholls and Ward, 2000). Sympathetic neurons that were either maintained in NGF or deprived of NGF in the presence of BAF for 3 d were loaded with JC-1 and treated with the 2 μM rotenone and 2 μM antimycin A , to block electron transport at sites I and II, respectively, and/or 5 μg/ml oligomycin, to block both forward and reverse operation of F_0F_1. The effects of these treatments on $\Delta\Psi$m were determined by comparing the JC-1 ratios obtained immediately before and 15 min after drug or vehicle addition. Exposure to the protonophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP), decreased the JC-1 ratio in NGF-maintained cells, reflecting complete mitochondrial depolarization (Fig. 5). Oligomycin had very little effect on $\Delta\Psi$m in NGF-maintained neurons after oligomycin treatment. Treatment of NGF-maintained neurons with rotenone and antimycin A caused complete mitochondrial depolarization, demonstrating that electron transport is responsible for maintenance of $\Delta\Psi$m in NGF-maintained neurons. Inhibition of electron transport largely,

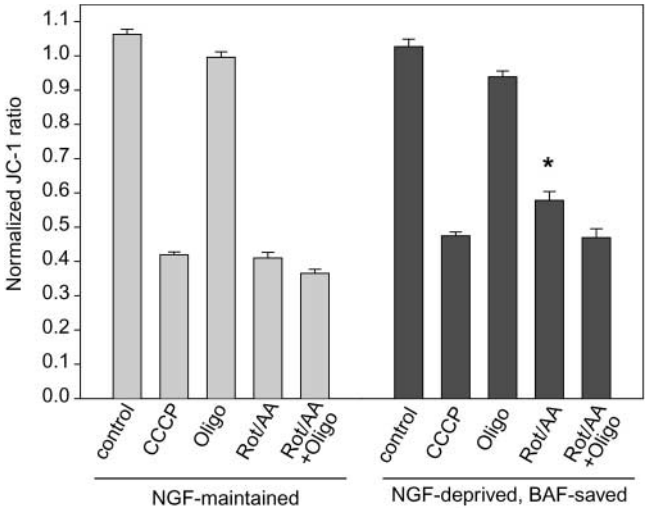

Figure 5. **Electron transport maintains ΔΨm in NGF-deprived, BAF-saved cells.** To determine the mechanism by which ΔΨm is generated, cells were maintained in NGF or deprived of NGF in the presence of BAF for 2 d. ΔΨm was determined by JC-1 staining before and after a 15-min exposure to CCCP (50 μM), rotenone, and antimycin A (Rot/AA, 2 μM each) to block electron transport, or oligomycin (5 μg/ml) to inhibit the F_0F_1 ATPase. Values are normalized to the JC-1 ratio of each culture before treatment. Values represent mean ± SD of 22–23 wells from four independent experiments. Asterisk indicates a statistically significant increase ($P < 0.05$) from CCCP treatment.

but not completely, depolarized mitochondria in NGF-deprived, BAF-saved neurons because the degree of mitochondrial depolarization achieved by treatment with antimycin and rotenone was less than that with CCCP. This electron transport inhibitor–insensitive contribution to $\Delta\Psi$m persisted for at least 45 min after drug addition, arguing against the possibility that inhibition of electron transport dissipates $\Delta\Psi$m more slowly in NGF-deprived, BAF-saved cells (unpublished data). A combination of rotenone, antimycin A, and oligomycin completely depolarized mitochondria in NGF-deprived, BAF-saved cells, suggesting that the residual $\Delta\Psi$m after inhibition of electron transport was maintained by reversal of F_0F_1 and hydrolysis of ATP (Fig. 5). Thus, although reversal of F_0F_1 has a minor function in the maintenance of $\Delta\Psi$m in NGF-deprived, BAF-saved, but not NGF-maintained, neurons, electron transport is the primary mechanism by which $\Delta\Psi$m is maintained before and after cytochrome *c* release in sympathetic neurons.

CsA attenuates loss of mitochondrial proteins

As seen in Fig. 2, CsA did not preserve the ability of mitochondria in NGF-deprived, BAF-saved neurons to generate ATP. Therefore, to investigate alternate mechanisms by which PTP inhibition by CsA might block Commitment 2, we asked whether CsA attenuated the loss of mitochondria observed in caspase inhibitor–saved neurons that may correlate with commitment-to-die (Xue et al., 2001). Western blots (Fig. 6 A) demonstrate that the levels of two mitochondrial proteins, voltage-dependent anion channel (VDAC) and cytochrome oxidase subunit IV (COX IV) decreased over time in NGF-deprived, BAF-saved cells, but less dra-

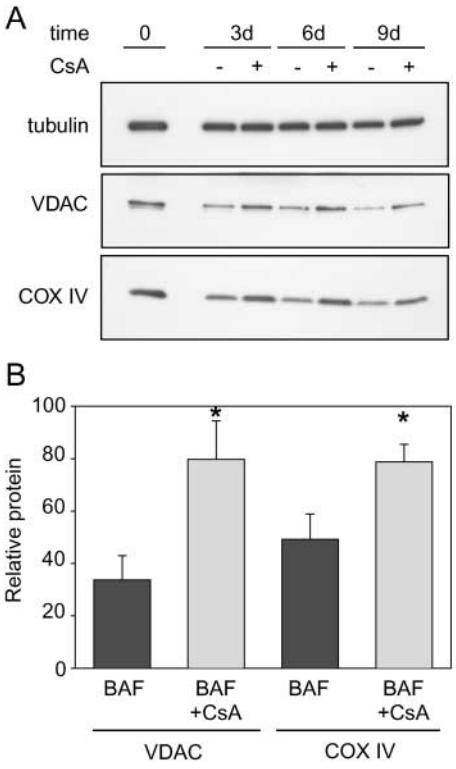

Figure 6. **CsA attenuates NGF deprivation–induced loss of mitochondrial proteins.** Mitochondrial proteins in NGF deprivation in the presence of BAF or BAF + 10 μM CsA were examined by Western blot. Blots were simultaneously probed with antibodies directed against tubulin, VDAC, and cytoCOX IV. (B) Levels of VDAC and COX IV after 9 d of NGF deprivation were normalized to the amount of tubulin in each sample. Data shown are mean ± SD of three independent experiments. Asterisks denote that differences between BAF and BAF + CsA are statistically significant ($P < 0.05$).

matically in cells also treated with CsA. When normalized to tubulin levels as a loading control, cultures in the presence of CsA had nearly twice the amount of VDAC and COX IV of cultures treated with BAF alone (Fig. 6 B). These findings demonstrate that CsA prevents the loss of mitochondrial proteins that occurs in NGF-deprived, BAF-saved neurons.

Mitochondria can be selectively eliminated by autophagy, a process involving the formation of degradative vesicles derived from lysosomes, endoplasmic reticulum, and Golgi apparatus (Dunn, 1990). Next, we examined the effects of CsA on the ultrastructural appearance of NGF-deprived, BAF-saved sympathetic neurons. We examined neurons after 10 d of NGF deprivation in the presence of BAF or BAF and CsA for two reasons. First, NGF-deprived, BAF-saved neurons reach Commitment 2 over a course of several days, with ~65% committing to die over the first 10 d, after which the rate of onset of Commitment 2 decreases dramatically, increasing to ~75% after 36 d of NGF deprivation (Chang and Johnson, 2002). Thus, NGF-deprived, BAF-saved cells at this time point are a relatively homogenous and stable population of cells. Second, at this time exists the largest quantitative effect of CsA on Commitment 2 because only ~10% of NGF-deprived, BAF-saved cells treated with CsA are committed-to-die (Chang and Johnson, 2002).

Control neurons maintained in NGF (Fig. 7 A) had large, round nuclei (N) with prominent nucleoli. The cytosol contained many mitochondria (Fig. 7 B, m) and occasional electron-dense, membrane-limited late autophagic vesicles. Neurons deprived of NGF in the presence of BAF for 10 d displayed extensive atrophy (Fig. 7 C), as expected from their light microscopic appearance. The most prominent change was the appearance of numerous electron-dense bodies in the cytosol (Fig. 7 D, black arrows), which may represent lipid droplets (Martin et al., 1988) or autolysosomes that have engulfed a large amount of lipid membranes or other electron-dense material (Xue et al., 1999). Similar structures were also occasionally seen in NGF-maintained neurons, although these were often smaller and had limiting membranes (Fig. 7 B, white arrows). These structures were present at a much greater frequency in sections of NGF-deprived, BAF-saved neurons (0.51 ± 0.08 per μm^2 of cytosol, ± SEM, in 11 sections from different neurons), than in NGF-maintained neurons (0.07 ± 0.04 per μm^2, $n = 6$). Cells deprived of NGF in the presence of BAF and CsA also displayed cytoplasmic atrophy and convolution of the nuclear membrane (Fig. 7 E). However, two key differences were observed between the appearance of these cells and those treated with BAF alone. First, these electron-dense bodies were much less abundant in cells treated with BAF and CsA (0.15 ± 0.04 per μm^2, $n = 11$) than in neurons saved with BAF only (Fig. 7 E). Second, abundant multilamellar vesicles were present throughout the cytosol of these cells (Fig. 7, E and F, black arrowheads). These multilamellar vesicles were abundant throughout multiple sections of NGF-deprived neurons treated with BAF and CsA (0.59 ± 0.11 per μm^2, $n = 11$), but rarely present in NGF-deprived, BAF-saved cells (0.09 ± 0.03 per μm^2, $n = 11$) and completely absent in NGF-maintained neurons ($n = 6$). Although the precise nature of these structures is uncertain, they resemble autolysosomes seen in NGF-deprived, caspase inhibitor–saved neurons (Xue et al., 1999). When normalized to surface area, NGF-deprived, BAF-saved cells with (0.22 ± 0.08, ± SEM) or without CsA treatment (0.17 ± 0.08) had slightly fewer mitochondria than NGF-maintained neurons (0.33 ± 0.09), but this difference was not statistically significant. In NGF-deprived, BAF-saved cells, there was a trend toward more mitochondria in CsA-treated cells, but this difference was not statistically significant.

Discussion

Here, we examined changes in metabolism in sympathetic neurons prevented from completing apoptosis by inhibition of caspases and how these changes influenced caspase-independent death. We present three major findings. First, NGF-deprived, BAF-saved neurons rely on glycolysis for ATP generation and for survival. Second, activity of F_0F_1 contributes to caspase-independent death, but has only a minor role in the maintenance of $\Delta\Psi m$, which is maintained primarily by electron transport. Finally, CsA preserves mitochondrial proteins, suggesting that PTP opening has a function in regulating the degradation of mitochondria after cytochrome *c* release.

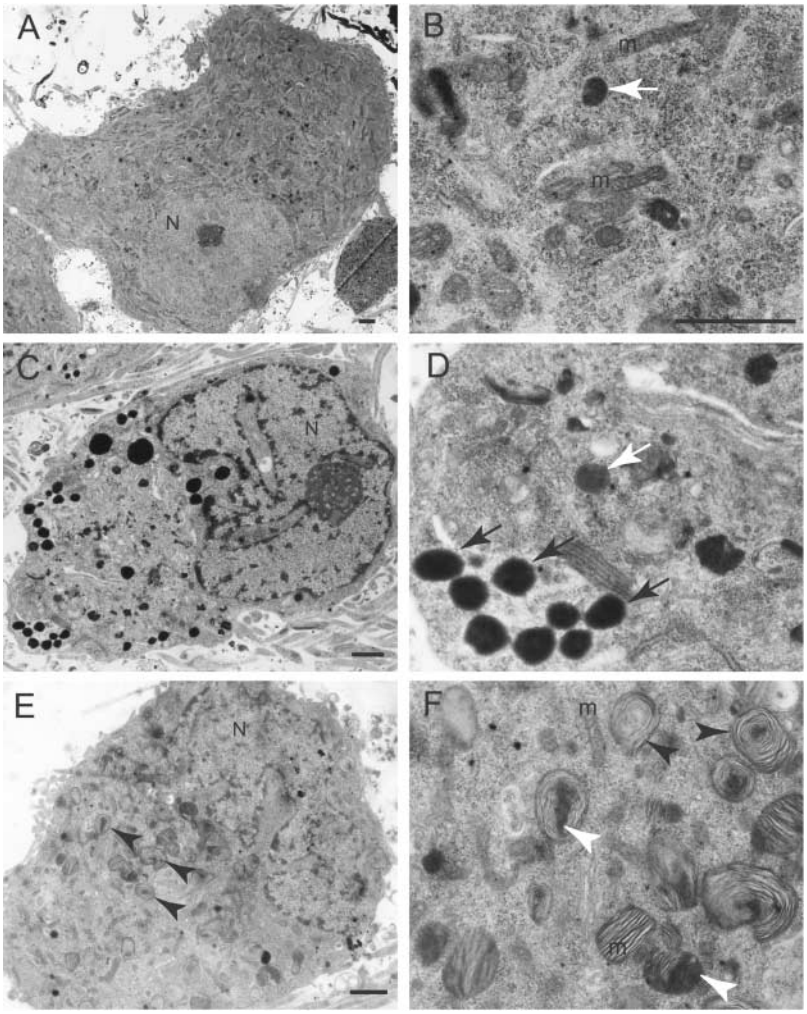

Figure 7. **CsA alters ultrastructure of NGF-deprived, BAF-saved cells.** Electron micrographs of neurons that were maintained in NGF (A and B), deprived of NGF in the presence of BAF (C and D), or deprived of NGF in the presence of BAF and CsA (E and F) for 10 d. (A) This representative NGF-maintained neuron has a large, round nucleus (N) that contains a nucleolus. (B) At a higher magnification, abundant mitochondria (m) and occasional electron-dense, membrane-limited autophagic vesicles (white arrow) can be seen. (C) NGF-deprived, BAF-saved cells display decreased cytoplasmic volume, nuclear irregularity, and chromatin margination, but retain a prominent nucleolus. Note the numerous electron-dense lipid droplets throughout the cytosol. (D) At higher magnification, lipid droplets (black arrows) lack limiting membranes and internal structure, whereas late autophagosomes (white arrow) are membrane limited. (E) CsA-treated neurons appear similar to NGF-deprived, BAF-saved cells. Note the absence of lipid droplets and the abundant multilamellar structures (black arrowheads), which are likely to be autophagosomes. (F) At higher magnification, the multilamellar structure of these autophagic vesicles (black arrowheads) is evident. Some of these vesicles contain electron-dense material (white arrowheads). Normal mitochondria (m) can be seen in this section. Boxes in low power views in left column indicate the area of magnification in high power views shown in the right column. Bars: 1 μm; bar in B also applies to D and F.

NGF-deprived, BAF-saved cells rely largely on glycolysis to generate ATP

NGF-maintained neurons are remarkably resistant to glucose deprivation (Fig. 3 B), despite the fact that acute inhibition of glycolysis decreased ATP levels within these cells (Fig. 2). The medium used in this experiment lacks pyruvate, but contains nine amino acids that can serve as carbon sources for intermediates in the citric acid cycle. This argues that in sympathetic neurons, the citric acid cycle and oxidative phosphorylation are able to generate sufficient ATP for survival. However, NGF-deprived, BAF-saved sympathetic neurons rely on glycolysis to generate ATP (Fig. 2) and have a marked sensitivity to glucose deprivation. In these cells, blocking glycolysis for 2 h decreased ATP levels by over 70%. This increased reliance on glycolysis renders NGF-deprived, BAF-saved neurons vulnerable to glucose deprivation, an insult to which NGF-maintained neurons are completely insensitive. Thus, unlike in NGF-maintained cells, mitochondria within NGF-deprived, BAF-saved cells cannot generate ATP to compensate for the loss of glycolysis, arguing that the mitochondrial hit has compromised certain mitochondrial functions.

Given the striking effect of glucose deprivation on ATP levels, that even a small proportion of NGF-deprived, BAF-saved cells survive prolonged glucose deprivation is surprising. These surviving cells may be a subpopulation of cells that are able to maintain oxidative phosphorylation. That no change in total ATP was detected after treatment of NGF-deprived, BAF-saved cells with oligomycin is likely a reflection of the small size of this subpopulation.

This increased sensitivity to glucose deprivation suggests that insufficient ability to generate ATP underlies caspase-independent death of NGF-deprived sympathetic neurons. In fact, a decrease in glucose transport occurs soon after removal of NGF from sympathetic neurons (Deckwerth and Johnson, 1993). However, increasing the concentration of glucose to 25 mM had no effect on Commitment 2, suggesting that availability of glucose in the culture medium was not the factor that limited survival.

Interestingly, oxidative phosphorylation continues to generate ATP in UV-irradiated HeLa cells that have released cytochrome *c* from their mitochondria (Waterhouse et al., 2001). In this model system, although mitochondrial cytochrome *c* is lost, ATP levels are initially maintained but decrease ~10 h after cytochrome *c* release (Waterhouse et al., 2001). Because caspase-independent death in our model occurred with a much slower time course, the time points used in our experiments might not have detected a similar short-term phenomenon. Alternatively, these different findings could reflect the cell type– and stimulus-specific nature of

caspase-independent events, such as degradation of cytosolic cytochrome *c* or its equilibration between the cytosol and mitochondrial intermembrane space.

F_0F_1 ATPase does not contribute to ATP generation after cytochrome *c* release

Oligomycin protected NGF-deprived, BAF-saved cells (Fig. 4 B), suggesting that activity of F_0F_1 contributes to caspase-independent death. Although we cannot rule out the possibility that oligomycin may have additional activities, it is striking that the toxicity was selective for NGF-maintained cells. Inhibition of oxidative phosphorylation did not decrease the amount of ATP within NGF-deprived, BAF-saved sympathetic neurons (Fig. 2), arguing that F_0F_1 activity does not generate ATP in these cells. Therefore, reverse operation of F_0F_1 to hydrolyze ATP may underlie its role in caspase-independent death. Consistent with this mode of operation, oligomycin dissipated the antimycin, rotenone-insensitive portion of $\Delta\Psi$m in NGF-deprived, BAF-saved cells (Fig. 5).

Inhibition of complexes I and II was sufficient to disrupt the majority of $\Delta\Psi$m in NGF-deprived, BAF-saved sympathetic neurons (Fig. 5), suggesting that electron transport is the primary mechanism by which $\Delta\Psi$m is maintained in cells that have released cytochrome *c*. How does electron transport continue after the loss of mitochondrial cytochrome *c*, which is a required component of the electron transport chain? At least two conceivable mechanisms exist. First, enough residual cytochrome *c* may exist in the intermembrane space after it is "released" from the mitochondrion to continue electron transport. In apoptotic HeLa cells saved with a caspase inhibitor, cytochrome *c* equilibrates throughout the cytosol and intermembrane space to a concentration sufficient to mediate electron transport and maintenance of $\Delta\Psi$m (Waterhouse et al., 2001). However, in sympathetic neurons, cytochrome *c* appears to be rapidly degraded in the cytosol after its release from the mitochondria, as determined by immunocytochemistry and Western blot of subcellular fractions of cell lysates (Putcha et al., 2000). A second possibility is that an alternate electron carrier substitutes for cytochrome *c*. This electron carrier could act as an intermediate electron carrier to recapitulate the electron transport chain, or as a terminal electron recipient to form a truncated electron transport chain. Although the nature of this hypothetical electron carrier is not known, precursors to reactive oxygen species (ROS) can act as terminal electron acceptors (Cai and Jones, 1998), allowing continued electron transport and generation of a proton gradient by complexes I and III. ROS, generated by abnormal electron transport, could be directly damaging to caspase inhibitor–saved neurons. The involvement of oxidative damage in caspase-independent death is a topic of current investigation.

Oligomycin collapsed the residual $\Delta\Psi$m remaining after inhibition of electron transport (Fig. 5). However, oligomycin alone did not increase the JC-1 ratio in NGF-maintained neurons (Fig. 5). Under conditions of oxidative phosphorylation, oligomycin would normally be expected to cause a slight mitochondrial hyperpolarization because it prevents dissipation of the proton gradient by inhibiting F_0F_1 (Scott and Nicholls, 1980). We did not observe this in

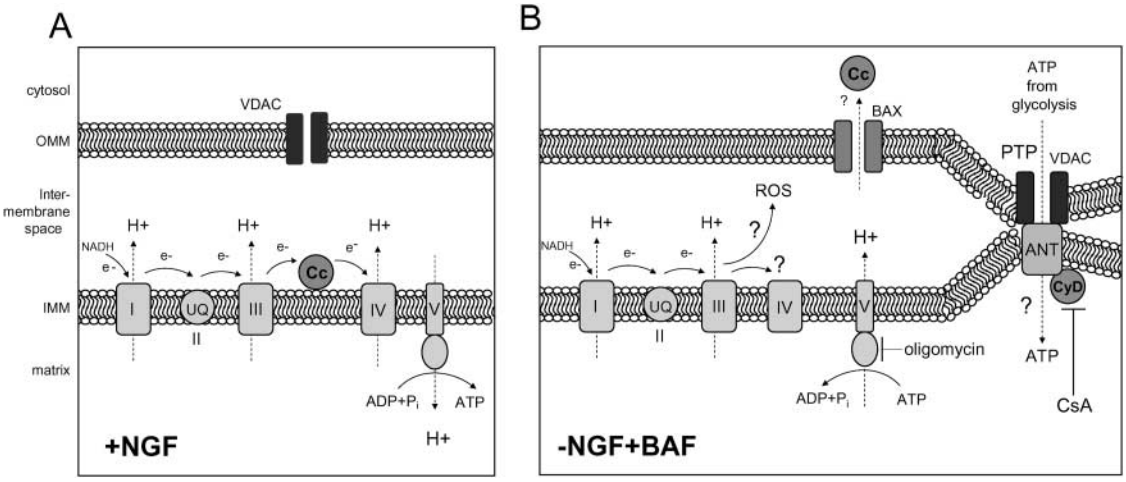

Figure 8. **Proposed events that account for the protective effect of oligomycin and CsA in neurons subsequent to cytochrome *c* release by the "mitochondrial hit."** In NGF-maintained sympathetic neurons (A), electron transport generates $\Delta\Psi$m, which is used by the $\Delta\Psi$m ATPase to generate ATP. In NGF-deprived, BAF-saved cells (B), mitochondrial cytochrome *c* (Cc) is lost by permeabilization of the outer mitochondrial membrane, possibly by a channel that includes the proapoptotic Bcl-2 family member BAX. Despite this, electron transport continues, at least through complexes I and III, contributing to $\Delta\Psi$m. Oxidation of electron transport intermediates could be mediated by residual mitochondrial cytochrome *c*, or by the generation of reactive oxygen species (ROS), which could themselves be detrimental. Reverse operation of F_0F_1 also contributes to $\Delta\Psi$m by hydrolyzing ATP. The importance of the permeability transition pore (PTP), which is composed of the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophilin D (CyD), is evidenced by the ability of CsA to inhibit caspase-independent death (Chang and Johnson, 2002; Fig. 4). Although precisely how PTP opening contributes to caspase-independent cell death is not known, it is possible, but purely speculative, that the opening of the PTP could allow the F_0F_1 to hydrolyze cytosolic ATP generated by glycolysis, on which the cell depends for survival. Although this model can account for inhibition of caspase-independent death by both oligomycin and CsA, it supposes that mechanisms to equilibrate adenine nucleotides are compromised in NGF-deprived, BAF-saved cells.

our experiments on NGF-maintained cells, even though we were able to detect mitochondrial hyperpolarization caused by exposure to nigericin, which permeabilizes the plasma membrane to protons and increases the proton gradient across the mitochondrial inner membrane, in both NGF-maintained and NGF-deprived, BAF-saved neurons (unpublished data). The failure to observe the predicted effect of oligomycin alone may be because of insensitivity in the upper ranges of this assay, because one cannot rigorously demonstrate the linearity of this assay in this setting. However, clear qualitative differences occur between the nature of $\Delta\Psi$m in NGF-maintained and NGF-deprived, BAF-saved neurons.

If reverse operation of F_0F_1 contributes to caspase-independent death, how does it do so? Circumstantial evidence suggests that cytochrome *c* release, and the events leading up to it, have effects on mitochondria that are detrimental to cell survival, even in models of cell death in which cytochrome *c* release occurs by selective permeabilization of the outer mitochondrial membrane (Von Ahsen et al., 2000). The F_0F_1 ATPase generates ATP under normal conditions, but hydrolyzes ATP in an attempt to maintain mitochondrial membrane potential ($\Delta\Psi$m) after cytochrome *c* release in apoptotic GT1–7 cells (Rego et al., 2001). The continued ATP hydrolysis by F_0F_1 is likely to be detrimental to a cell because it would deplete cellular ATP.

The polarity of the F_0F_1 ATPase in the inner mitochondrial membrane dictates that it can only hydrolyze ATP within the mitochondrial matrix. Under normal conditions, ATP and ADP are freely exchanged between the mitochondrial matrix and the cytosol via the adenine nucleotide translocase (ANT). However, this may not hold true during cell death, as ANT function is compromised in lymphocytes undergoing growth factor deprivation–induced apoptosis (Vander Heiden et al., 1999). If adenine nucleotide equilibration is compromised in NGF-deprived, BAF-saved sympathetic neurons, it is possible that opening of the PTP renews the pool of ATP within the mitochondrial matrix by providing equilibration of ATP levels between the cytosol and the matrix, schematized in Fig. 8. In such a scenario, PTP opening allows the F_0F_1 ATPase access to ATP that has been generated in the cytosol by glycolysis, which is required by these cells for survival (Fig. 3 B). Thus, inhibiting PTP opening with CsA or directly inhibiting ATP hydrolysis by reverse operation of F_0F_1 with oligomycin limits the deleterious effects of the mitochondrial hit by preserving glycolytic ATP. Consistent with this hypothesis, CsA and oligomycin inhibited Commitment 2 to virtually the same degree (Fig. 4 B). Although purely speculative at this point, because it rests on the supposition that adenine nucleotide transport is altered in NGF-deprived, BAF-saved cells, we favor this model because it accounts for the similarity in protection against caspase-independent death of both oligomycin and CsA.

If reversal of F_0F_1 activity is an important event in caspase-independent death, why is the effect of oligomycin on $\Delta\Psi$m so small (Fig. 6)? At least two possible explanations exist. First, reverse operation of F_0F_1 may occur at a slow, but sustained, rate in NGF-deprived sympathetic neurons. In this scenario, oligomycin would have only a minor effect on $\Delta\Psi$m over the course of minutes, but a great effect on survival when applied for over a week. Consistent with this, the rate of reverse activity of F_0F_1 in anoxic skeletal muscle is lower than predicted, suggesting the existence of mechanisms to limit ATP hydrolysis by F_0F_1 (St. Pierre et al., 2000). Alternatively, F_0F_1 may reverse in only a small proportion of cells at any given time. F_0F_1 reversal may begin asynchronously throughout the population and followed by loss of $\Delta\Psi$m, because reverse activity of F_0F_1 contributes to caspase-independent death. Thus, the small effect of oligomycin on $\Delta\Psi$m, as measured on a population basis by this JC-1 assay, may be entirely accounted for by this small, transient subpopulation of cells. The techniques used in these studies cannot differentiate between these two possibilities.

CsA attenuates NGF deprivation–induced loss of mitochondrial proteins

Although CsA does not alter protein synthesis or prevent somal atrophy of NGF-deprived, BAF-saved cells (Chang and Johnson, 2002), CsA increases the amount of mitochondrial protein within these cells (Fig. 6 B), arguing that CsA directly or indirectly prevents the degradation of mitochondria. In other model systems, including a different paradigm of NGF deprivation–induced death of sympathetic neurons, which differs in some important respects with our system (Fletcher et al., 2000), selective mitochondrial elimination is mediated by autophagy (Tolkovsky et al., 2002). The striking difference in the appearance of cytoplasmic vesicles in NGF-deprived, BAF-saved cells treated with CsA further supports the conclusions that mitochondria within apoptotic cells saved with a caspase inhibitor are eliminated by autophagy. That CsA interferes with this process is not unprecedented because inhibition of PTP opening by CsA prevents autophagy of mitochondria in serum-deprived, glucagon-treated hepatocytes (Elmore et al., 2001). Thus, the PTP opening may be the trigger for the removal of mitochondria in NGF-deprived, BAF-saved neurons, marking them for degradation. Mitochondria are likely to be critical for the ability of a cell to survive after trophic factor readdition because oxidative phosphorylation is required for survival in the presence of NGF (Fig. 4 B). However, the loss of mitochondria in caspase inhibitor–saved cells may not cause caspase-independent death because mitochondria removed from these cells may be dysfunctional or damaged. Mitochondrial damage or dysfunction may have a role in regulating selective mitochondrial elimination (James et al., 1996; Elmore et al., 2001). Thus, the selective elimination of mitochondria may be a result, but not a cause, of impaired mitochondrial function. In support of this, CsA protects NGF-deprived, BAF-saved neurons from caspase-independent death (Chang and Johnson, 2002) and attenuated the loss of mitochondrial proteins (Fig. 7), but did not preserve oxidative phosphorylation within these neurons (Fig. 2).

Because degradation of intracellular organelles liberates free amino acids, mitochondrial elimination may represent an attempt to improve the energetic status of caspase inhibitor–saved cells. Although not producing ATP, these "damaged" mitochondria may be maintaining other critical func-

tions in the short term within these cells. The long-term effects of this effort, however, are deleterious because the potential short-term benefits jeopardize the ability of the cell to survive the ongoing, death-inducing stimulus and to recover after the stimulus is terminated.

Implications for postmitochondrial regulation of apoptosis

Much attention has been given to cytochrome *c* release as a critical point of regulation of apoptosis. However, clearly, mechanisms exist to regulate apoptotic machinery at points distal to cytochrome *c* release, such as inhibitors of apoptosis (Deveraux and Reed, 1999) and heat shock proteins (Beere et al., 2000; Bruey et al., 2000; Saleh et al., 2000). Regardless of the method by which caspase activation is prevented, caspase-independent sequelae of activating the cell death pathway, such as those examined in this work, will cause the eventual demise of these cells. Like caspase inhibitors, therapeutic use of inhibitors of apoptosis and heat shock proteins will be limited by the ability of the cell to survive these caspase-independent events. Thus, identifying the specific events that contribute to caspase-independent cell death may enhance our ability to exploit postmitochondrial strategies to prevent pathological cell death.

Materials and methods

Unless otherwise noted, all reagents were obtained from Sigma-Aldrich. Timed-pregnant Sprague-Dawley rats were obtained from Harlan Sprague-Dawley.

Cell culture

Sympathetic neurons from postnatal day 0–1 rats were maintained in a medium containing 50 ng/ml NGF (AM50) as described previously (Deshmukh et al., 1996). Cells were deprived of NGF by washing cells in a medium lacking NGF (AM0), followed by culture in AM0-containing neutralizing antibody to NGF. Commitment-to-die was measured by determining the proportion of NGF-deprived neurons that were rescued by NGF readdition. At the time of the rescue, cultures were washed extensively to remove residual anti-NGF and maintained in AM50. After 7 d, cells were washed and fixed in 4% PFA for at least 12 h at 4°C. Cultures were stained with 0.05% Toluidine blue in TBS (10 mM Tris and 0.9% NaCl, pH 7.6) and counted by using an inverted microscope (Eclipse TE300; Nikon) without knowledge of treatment group. All values are represented as a percentage of the mean number of cells in NGF-maintained sister cultures.

To determine the effect of altering glucose concentration on survival, glucose-free AM50 and AM0 were made with glucose-free MEM (Washington University Tissue Support Center) and dialyzed FBS. In some cases, glucose was added to bring the final concentration either to 5 mM, the concentration of glucose in standard MEM, or 25 mM. These alterations in formulation of the medium did not affect cell viability (Fig. 3 B, compare control vs. 5 mM glucose), suggesting that the glucose concentration was the only critical variable that was altered in this specially formulated medium.

Determination of ATP

ATP was measured by using a luciferase-based assay (Bioluminescent Somatic cell assay kit; Sigma-Aldrich) according to the manufacturer's instructions. All manipulations were performed on ice with ice-cold solutions. Sister cultures of 10,000 neurons per well in 24-well plates were washed once with PBS, lysed in a 1:1 dilution of the supplied releasing agent in water, and immediately frozen at −70°C after 0, 3, 6, or 9 d of treatment. After all samples from an individual experiment were collected, luciferase reagent was added to an aliquot of each lysate, and the amount of ATP was determined with a microplate luminometer (model TR717; Applied Biosystems). At each time point, the amount of ATP in sister cultures plated in anti-NGF was subtracted from all values to determine the amount of neuronal ATP. All values are expressed as a percentage of the average amount of ATP in cultures at time 0 for each experiment. By using known amounts of ATP, a standard curve was generated for each experiment to ensure that all values were in the linear range of the assay. In some experiments, an aliquot of each lysate was used for determination of total protein with the BCA method (Pierce Chemical Co.). The amount of neuronal protein in each sample was determined by subtracting the protein in sister cultures plated in anti-NGF.

To determine the source of intracellular ATP, cells were maintained in NGF or deprived of NGF in the presence of BAF for 3 d, and treated with a standard medium, glucose-free medium with 5 mM 2-deoxyglucose and 1 mM pyruvate, or a standard medium with 5 μg/ml oligomycin for 2 h.

Determination of $\Delta\Psi$m

Sympathetic neuronal cultures were grown in 96-well, opaque-walled, clear-bottom plates (Corning Costar). NGF-maintained cultures and those that had been deprived of NGF in the presence of BAF for 3 d were washed once and loaded with 3.3 μM JC-1 (Molecular Probes) in PBS with 1 g/liter glucose for 30 min at 37°C after which the cells were washed twice. JC-1 fluorescence was measured with a fluorescent plate reader (Fluoroskan II; Titertek) by using excitation/emission pairings of 485/538 nm, corresponding to the monomeric form of JC-1, and 544/590 nm, corresponding to the aggregated form of JC-1. The relative $\Delta\Psi$m was determined by calculating the ratio of the aggregated form of JC-1 to its monomeric form. Baseline readings were taken before vehicle or drug addition. The relative change in $\Delta\Psi$m in response to treatment was determined by normalizing the JC-1 ratio of each well 15 min after vehicle or drug addition to the baseline ratio for each well. Because JC-1 is a relatively slow equilibrating dye, changes in the JC-1 ratio were observed over the first 10–12 min after drug addition, but then remained stable over the next 30 min. Vehicle treatment in either NGF-maintained or -deprived, BAF-saved neurons slightly increased the JC-1 ratio over this period likely caused by diffusion of the monomeric form out of the cells. At the end of certain experiments, CCCP was added to each well and a reading was taken after 15 min. In all cases, the JC-1 ratio decreased to a value similar to that of cells initially treated with CCCP, suggesting that this reflects maximal mitochondrial depolarization. In some experiments, sister cultures that were deprived of NGF in the absence of BAF were rescued with NGF to monitor cytochrome *c* release (Putcha et al., 1999). In all cases, <5% of cells were rescued, demonstrating that at least 95% of the cells had released cytochrome *c*.

Western blotting

At the appropriate times, cultures were lysed in lysis buffer containing 100 mM Tris, pH 6.8, 4% SDS, 20% glycerol, and 5% β-mercaptoethanol. Proteins were resolved on Novex Tris-glycine gels (Invitrogen) and transferred to PVDF membranes (Millipore). Blots were blocked with 5% dry milk in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) before incubating with a mixture of mouse antitubulin, mouse anti-VDAC, and mouse anti–COX IV (Molecular Probes) in TBS-T with 5% milk overnight at 4°C. After washing, blots were incubated with HRP-conjugated anti–mouse secondary and visualized with SuperSignal Pico (Pierce Chemical Co.). The intensity of bands was determined with UnScan-It (Silk Scientific).

Electron microscopy

Cultures were grown on collagen-coated Permanox LabTek chamber slides (Nalge Nunc). After treatments, cells were fixed for 4 h in 3% glutaraldehyde in 100 mM phosphate buffer, pH 7.3, containing 0.45 mM Ca^{2+}. Cultures were fixed after in buffered OsO_4, dehydrated in graded alcohols, and embedded in Epon. Ultrathin sections were cut and examined with an electron microscope (model 1200; JEOL). For the purposes of quantification, photomicrographs taken at 10,000× were examined without knowledge of the treatment group. Cytoplasmic structures with or without limiting membranes that were at least half filled with electron-dense material were considered to be "electron-dense bodies." Membrane-limited, multilamellar structures in the cysosol that were less than half filled with electron-dense material were counted as "multilamellar whorls."

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References

Adams, J.M., and S. Cory. 1998. The Bcl-2 protein family: arbiters of cell survival. *Science.* 281:1322–1326.

Beere, H.M., B.B. Wolf, K. Cain, D.D. Mosser, A. Mahboubi, T. Kuwana, P. Tailor, R.I. Morimoto, G.M. Cohen, and D.R. Green. 2000. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat. Cell Biol.* 2:469–475.

Bruey, J.M., C. Ducasse, P. Bonniaud, L. Ravagnan, S.A. Susin, C. Diaz-Latoud, S. Gurbuxani, A.P. Arrigo, G. Kroemer, E. Solary, and C. Garrido. 2000. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat. Cell Biol.* 2:645–652.

Cai, J., and D.P. Jones. 1998. Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. *J. Biol. Chem.* 273:11401–11404.

Chang, L.K., and E.M. Johnson, Jr. 2002. Cyclosporin A inhibits caspase-independent death of NGF-deprived sympathetic neurons: a potential role for mitochondrial permeability transition. *J. Cell Biol.* 157:771–781.

Cregan, S.P., A. Fortin, J.G. MacLaurin, S.M. Callaghan, F. Cecconi, S.W. Yu, T.M. Dawson, V.L. Dawson, D.S. Park, G. Kroeomer, and R.S. Slack. 2002. Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J. Cell Biol.* 158:507–517.

Crompton, M. 1999. The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.* 341:233–249.

Crompton, M., S. Virji, and J.M. Ward. 1998. Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. *Eur. J. Biochem.* 258:729–735.

Cryns, V., and J.Y. Yuan. 1998. Proteases to die for. *Genes Dev.* 12:1551–1570.

Deckwerth, T.L., and E.M. Johnson, Jr. 1993. Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. *J. Cell Biol.* 123:1207–1222.

Deckwerth, T.L., J.L. Elliott, C.M. Knudson, E.M. Johnson, Jr., W.D. Snider, and S.J. Korsmeyer. 1996. BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron.* 17:401–411.

Deshmukh, M., J. Vasilakos, T.L. Deckwerth, P.A. Lampe, B.D. Shivers, and E.M. Johnson, Jr. 1996. Genetic and metabolic status of NGF-deprived sympathetic neurons saved by an inhibitor of ICE family proteases. *J. Cell Biol.* 135:1341–1354.

Deshmukh, M., K. Kuida, and E.M. Johnson, Jr. 2000. Caspase inhibition extends the commitment to neuronal death beyond cytochrome *c* release to the point of mitochondrial depolarization. *J. Cell Biol.* 150:131–143.

Deveraux, Q.L., and J.C. Reed. 1999. IAP family proteins—suppressors of apoptosis. *Genes Dev.* 13:239–252.

Du, C., M. Fang, Y. Li, L. Li, and X. Wang. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell.* 102:33–42.

Dunn, W.A. 1990. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J. Cell Biol.* 110:1923–1933.

Edwards, S.N., and A.M. Tolkovsky. 1994. Characterization of apoptosis in cultured rat sympathetic neurons after nerve growth factor withdrawal. *J. Cell Biol.* 124:537–546.

Elmore, S.P., T. Qian, S.F. Grissom, and J.J. Lemasters. 2001. The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *FASEB J.* 15: 2286–2287.

Ferrand-Drake, M., H. Friberg, and T. Wieloch. 1999. Mitochondrial permeability transition induced DNA-fragmentation in the rat hippocampus following hypoglycemia. *Neuroscience.* 90:1325–1338.

Fletcher, G.C., L. Xue, S.K. Passingham, and A.M. Tolkovsky. 2000. Death commitment point is advanced by axotomy in sympathetic neurons. *J. Cell Biol.* 150:741–754.

James, A.M., Y.H. Wei, C.Y. Pang, and M.P. Murphy. 1996. Altered mitochondrial function in fibroblasts containing MELAS or MERRF mitochondrial DNA mutations. *Biochem. J.* 318:401–407.

Kerr, J.F., A.H. Wyllie, and A.R. Currie. 1972. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer.* 26:239–257.

Li, L.Y., X. Luo, and X. Wang. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature.* 412:95–99.

Li, P., D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell.* 91: 479–489.

Martin, D.P., R.E. Schmidt, P.S. DiStefano, O.H. Lowry, J.G. Carter, and E.M. Johnson, Jr. 1988. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* 106:829–844.

Martinou, I., S. Desagher, R. Eskes, B. Antonsson, E. Andre, S. Fakan, and J.C. Martinou. 1999. The release of cytochrome *c* from mitochondria during apoptosis of NGF-deprived sympathetic neurons is a reversible event. *J. Cell Biol.* 144:883–889.

Matsumoto, S., H. Friberg, M. Ferrand-Drake, and T. Wieloch. 1999. Blockade of the mitochondrial permeability transition pore diminished infarct size in the rat after transient middle cerebral artery occlusion. *J. Cereb. Blood Flow Metab.* 19:736–741.

Miller, T.M., K.L. Moulder, C.M. Knudson, D.J. Creedon, M. Deshmukh, S.J. Korsmeyer, and E.M. Johnson, Jr. 1997. Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. *J. Cell Biol.* 139:205–217.

Mootha, V.K., M.C. Wei, K.F. Buttle, L. Scorrano, V. Panoutsakopoulou, C.A. Mannella, and S.J. Korsmeyer. 2001. A reversible component of mitochondrial respiratory dysfunction in apoptosis can be rescued by exogenous cytochrome c. *EMBO J.* 20:661–671.

Neame, S.J., L.L. Rubin, and K.L. Philpott. 1998. Blocking cytochrome *c* activity within intact neurons inhibits apoptosis. *J. Cell Biol.* 142:1583–1593.

Nicholls, D.G., and M.W. Ward. 2000. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci.* 23:166–174.

Oppenheim, R.W., R.A. Flavell, S. Vinsant, D. Prevette, C.Y. Kuan, and P. Rakic. 2001. Programmed cell death of developing mammalian neurons after genetic deletion of caspases. *J. Neurosci.* 21:4752–4760.

Putcha, G.V., M. Deshmukh, and E.M. Johnson, Jr. 1999. BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. *J. Neurosci.* 19:7476–7485.

Putcha, G.V., M. Deshmukh, and E.M. Johnson, Jr. 2000. Inhibition of apoptotic signaling cascades causes loss of trophic factor–dependence during neuronal maturation. *J. Cell Biol.* 149:1011–1018.

Rego, A.C., S. Vesce, and D.G. Nicholls. 2001. The mechanism of mitochondrial membrane potential retention following release of cytochrome c in apoptotic GT1-7 neural cells. *Cell Death Differ.* 8:995–1003.

Saleh, A., S.M. Srinivasula, L. Balkir, P.D. Robbins, and E.S. Alnemri. 2000. Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat. Cell Biol.* 2:476–483.

Scott, I.D., and D.G. Nicholls. 1980. Energy transduction in intact synaptosomes. Influence of plasma-membrane depolarization on the respiration and membrane potential of internal mitochondria determined in situ. *Biochem. J.* 186:21–33.

Smiley, S.T., M. Reers, C. Mottola-Hartshorn, M. Lin, A. Chen, T.W. Smith, G.D. Steele, Jr., and L.B. Chen. 1991. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc. Natl. Acad. Sci. USA.* 88:3671–3675.

Stefanis, L., D.S. Park, W.J. Friedman, and L.A. Greene. 1999. Caspase-dependent and -independent death of camptothecin-treated embryonic cortical neurons. *J. Neurosci.* 19:6235–6247.

St. Pierre, J., M.D. Brand, and R.G. Boutilier. 2000. Mitochondria as ATP consumers: cellular treason in anoxia. *Proc. Natl. Acad. Sci. USA.* 97:8670–8674.

Susin, S.A., H.K. Lorenzo, N. Zamzami, I. Marzo, B.E. Snow, G.M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, et al. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature.* 397:441–446.

Suzuki, Y., Y. Imai, H. Nakayama, K. Takahashi, K. Takio, and R. Takahashi. 2001. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol. Cell.* 8:613–621.

Tolkovsky, A.M., L. Xue, G.C. Fletcher, and V. Borutaite. 2002. Mitochondrial disappearance from cells: a clue to the role of autophagy in programmed cell death and disease? *Biochimie.* 84:233–240.

Troy, C.M., L. Stefanis, A. Prochiantz, L.A. Greene, and M.L. Shelanski. 1996. The contrasting roles of ICE family proteases and interleukin-1beta in apoptosis induced by trophic factor withdrawal and by copper/zinc superoxide dismutase down-regulation. *Proc. Natl. Acad. Sci. USA.* 93:5635–5640.

Vander Heiden, M.G., N.S. Chandel, P.T. Schumacker, and C.B. Thompson. 1999. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol. Cell.* 3:159–167.

Verhagen, A.M., P.G. Ekert, M. Pakusch, J. Silke, L.M. Connolly, G.E. Reid, R.L.

Moritz, R.J. Simpson, and D.L. Vaux. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell.* 102:43–53.

Von Ahsen, O., N.J. Waterhouse, T. Kuwana, D.D. Newmeyer, and D.R. Green. 2000. The "harmless" release of cytochrome c. *Cell Death Differ.* 7:1192–1199.

Waterhouse, N.J., J.C. Goldstein, O. von Ahsen, M. Schuler, D.D. Newmeyer, and D.R. Green. 2001. Cytochrome *c* maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. *J. Cell Biol.* 153:319–328.

Xue, L., G.C. Fletcher, and A.M. Tolkovsky. 1999. Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. *Mol. Cell. Neurosci.* 14:180–198.

Xue, L., G.C. Fletcher, and A.M. Tolkovsky. 2001. Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis. *Curr. Biol.* 11:361–365.