

Inhibition of Apoptotic Signaling Cascades Causes Loss of Trophic Factor Dependence during Neuronal Maturation

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Abstract. During development, neurons are acutely dependent on target-derived trophic factors for survival. This dependence on trophic support decreases dramatically with maturation in several neuronal populations, including sympathetic neurons. Analyses of nerve growth factor deprivation in immature and mature sympathetic neurons indicate that maturation aborts the cell death pathway at a point that is mechanistically indistinguishable from *Bax* deletion. However, neither the mRNA nor protein level of BAX changes with neuronal maturation. Therefore, BAX must be regulated posttranslationally in mature neurons.

Nerve growth factor deprivation in immature sympathetic neurons induces two parallel processes: (a) a protein synthesis-dependent, caspase-independent translocation of BAX from the cytosol to mitochondria, followed by mitochondrial membrane integration and

loss of cytochrome *c*; and (b) the development of competence-to-die, which requires neither macromolecular synthesis nor BAX expression. Activation of both signaling pathways is required for caspase activation and apoptosis in immature sympathetic neurons. In contrast, nerve growth factor withdrawal in mature sympathetic neurons did not induce the translocation of either BAX or cytochrome *c*. Moreover, mature neurons did not develop competence-to-die with cytoplasmic accumulation of cytochrome *c*. Therefore, inhibition of both BAX-dependent cytochrome *c* release and the development of competence-to-die contributed to the loss of trophic factor dependence associated with neuronal maturation.

Key words: BAX • caspase • cytochrome *c* • mitochondria • neurotrophin

Introduction

Programmed cell death resulting in apoptosis is a prominent feature of the developing nervous system and may contribute to several neuropathological conditions, such as stroke, trauma, and neurodegenerative disorders (e.g., amyotrophic lateral sclerosis). During development, neurons are acutely dependent on trophic factors for survival. For example, sympathetic neurons require NGF for survival during late embryogenesis and the early postnatal period. However, this dependence on target-derived trophic support decreases dramatically with maturation (Angeletti et al., 1971; Bjerre et al., 1975; Goedert et al.,

1978; Otten et al., 1979), a phenomenon recapitulated in vitro. Neonatal sympathetic neurons maintained in culture for 3–4 wk are resistant to apoptosis induced by NGF withdrawal (Lazarus et al., 1976; Chun and Patterson, 1977). Several neuronal populations exhibit similar resistance to trophic factor deprivation (Snider et al., 1992) that probably serves an important role in the maintenance of the adult nervous system, which if compromised, may contribute to neurodegenerative diseases. The mechanisms responsible for this decreased trophic factor dependence are poorly understood.

Genetic and biochemical studies have identified several critical regulators of apoptosis in mammals: caspases, Apaf-1, and the BCL-2 family of proteins. In the mammalian nervous system, most naturally occurring programmed cell death requires the expression of BAX, a

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proapoptotic member of the BCL-2 family of proteins (Deckwerth et al., 1996; Shindler et al., 1997; White et al., 1998).

Analyses of the biochemical and molecular events induced by trophic factor deprivation in immature and mature sympathetic neurons indicate that the latter are functionally indistinguishable from *Bax*-deficient cells (Easton et al., 1997). That is, mature sympathetic neurons phenotype *Bax*^{-/-} immature neurons: all known biochemical and molecular events induced by trophic factor withdrawal that occur upstream of the BAX/BCL-2 checkpoint do so with similar kinetics in both *Bax*^{-/-} immature and *Bax*^{+/+} mature neurons. However, neither the mRNA nor protein level of BAX increases during NGF deprivation-induced apoptosis in neonatal neurons (Greenlund et al., 1995) or decreases with neuronal maturation (Easton et al., 1997), suggesting that BAX is regulated posttranslationally in both immature and mature sympathetic neurons.

In immature sympathetic neurons, NGF deprivation induces two parallel processes: (a) a protein synthesis-dependent, caspase-independent translocation of BAX (Putcha et al., 1999) from the cytosol to mitochondria, followed by loss of mitochondrial cytochrome *c* (Deshmukh and Johnson, 1998; Neame et al., 1998; Martinou et al., 1999; Putcha et al., 1999); and (b) the development of competence-to-die with cytoplasmic accumulation of cytochrome *c*, which requires neither macromolecular synthesis nor BAX expression (Deshmukh and Johnson, 1998). Activation of both pathways is required for caspase activation and apoptotic cell death in immature sympathetic neurons. In this study, we examined whether decreased trophic factor dependence in mature sympathetic neurons results from inhibition of one or both of these pathways. We found that NGF deprivation in mature sympathetic neurons, which causes Jun NH₂-terminal kinase (JNK) activation and some immediate-early gene transcription (Easton et al., 1997), did not induce the translocation of either BAX or cytochrome *c*. Moreover, mature neurons did not develop competence-to-die with cytoplasmic accumulation of cytochrome *c*, even after prolonged periods of NGF deprivation. Therefore, failure of both BAX-dependent cytochrome *c* release and the development of competence-to-die caused the loss of trophic factor dependence in mature sympathetic neurons.

Materials and Methods

Reagents

All reagents were purchased from Sigma Chemical Co. unless otherwise stated. Collagenase and trypsin were purchased from Worthington Biochemical. The caspase inhibitor boc-aspartyl(OMe)-fluoromethylketone (BAF)¹ was purchased from Enzyme Systems Products. Medium lacking NGF (AM0) consisted of MEM with Earl's salts (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, 20 μM uridine, and 3.3 μg/ml aphidicolin. AM50 medium consisted of AM0 medium plus 50 ng/ml mouse 2.5S NGF (Harlan Bioproducts).

¹Abbreviations used in this paper: BAF, boc-aspartyl(OMe)-fluoromethylketone; DIV, days in vitro; HM, heavy membrane.

Sympathetic Neuronal Cultures

Primary cultures of sympathetic neurons were established from the superior cervical ganglia of each postnatal day-1 (P1) mouse by using previously described methods (Johnson and Argiro, 1983; Deckwerth et al., 1996; Easton et al., 1997). Neurons were grown in AM50 for ~5 d (immature; 5 d in vitro [DIV 5]) or ~25 d (mature; DIV 25) and then either maintained in AM50 or treated as follows: for NGF deprivation, cultures were rinsed with AM0, followed by the addition of AM0 containing goat anti-mouse 2.5S NGF neutralizing antiserum (anti-NGF) (Ruit et al., 1990); for NGF deprivation in the presence of various reagents, 1 μg/ml cycloheximide or 50 μM BAF, respectively, was added to AM0 containing anti-NGF.

Immunocytochemistry

Neuronal cultures were immunostained as described previously (Easton et al., 1997). Cytochrome *c* was detected with mouse monoclonal primary antibodies (1:1,000) (PharMingen) and FITC-conjugated donkey anti-mouse secondary antibody (1:300) (Jackson ImmunoResearch). BAX was detected with a rat monoclonal primary antibody (1:250) (catalog number 13401A, lot M024742; PharMingen) and a Cy3-conjugated anti-rat secondary antibody (1:400) (Jackson ImmunoResearch).

Cell Counts: BAX and Cytochrome *c* Immunocytochemistry

Sympathetic neurons that had been maintained in NGF for 5 or 25 DIV in AM50 were deprived of NGF in the presence of the caspase inhibitor BAF (as described above). At various times after deprivation, cultures were fixed and immunostained with anti-BAX or anti-cytochrome *c* antibodies. For each timepoint, the number of cells that had acquired a punctate staining pattern for BAX or had lost the punctate staining pattern for cytochrome *c* was determined by a blinded observer from a random sampling of 200–250 cells. All experiments were conducted in the presence of BAF to prevent any cell loss that would otherwise affect the counts.

Subcellular Fractionation

Sympathetic neurons (~10⁶) were harvested in 10 ml of isotonic fractionation buffer (250 mM sucrose, 0.5 mM EDTA, 20 mM Hepes, 500 μM Na₃VO₄, pH 7.2) supplemented with protease inhibitor cocktail Complete (Roche Molecular Biochemicals) and centrifuged at 900 *g* for 5 min. All steps were conducted at 4°C. The pellet was then resuspended in 200 μl fractionation buffer, homogenized by using a ball-bearing homogenizer, and centrifuged at 900 *g* for 5 min to remove nuclei and intact cells. The postnuclear supernatant was transferred to a microfuge tube (Beckman) and centrifuged at 25,000 *g* for 10 min to collect the heavy membrane (HM) fraction enriched in mitochondria, followed by centrifugation of the post-HM supernatant at 100,000 *g* for 10–20 min to obtain the microsomal and cytosolic fractions. All pellets were resuspended in a volume of fractionation buffer equivalent to the cytosolic volume. All fractions were then resuspended to equivalent volumes with lysis buffer and evaluated by Western blotting.

We were unable to detect cytochrome *c* in the cytosolic fractions of both nonneuronal and neuronal primary cells and cell lines undergoing apoptosis induced by a variety of stimuli, even at multiple timepoints. The lack of detectable cytochrome *c* in the cytosol may indicate its rapid degradation once it has been released into the cytosol (Deshmukh and Johnson, 1998; Neame et al., 1998; Martinou et al., 1999; Putcha et al., 1999).

Alkali Extraction

The postnuclear supernatant from sympathetic neurons (~2–4 × 10⁶) was isolated as described above and aliquoted equally into three microfuge tubes. Samples were centrifuged at ~25,000 *g* for 10 min to collect the HM fractions. The post-HM supernatants were pooled, centrifuged at 100,000 *g* for 10–20 min to obtain the microsomal and cytosolic fractions, and prepared for immunoblot analysis as described above. The HM pellets were then resuspended in 800 μl fractionation buffer, 0.2 M Na₂CO₃, pH 11.5, or 1% (wt/vol) SDS, incubated at 4°C for 30 min while rotating, and centrifuged at 25,000 *g* for 10–20 min to retrieve the HM pellets. These pellets were resuspended in a volume of fractionation buffer equivalent to one-third the cytosolic volume and prepared for Western blot analysis as described above. Mitochondrial integral membrane proteins are resistant to

extraction by 0.2 M Na₂CO₃, yet sensitive to extraction by 1% SDS, whereas peripheral membrane proteins are extracted by both treatments.

Microinjections and Quantitation of Cell Death

These experiments were performed as described previously (Deshmukh and Johnson, 1998). In brief, sympathetic neurons were maintained in NGF for either 5 or 25 DIV and treated for ~36–48 h with NGF, anti-NGF, or anti-NGF plus 1 μg/ml cycloheximide. Cells were then microinjected with 25 mg/ml bovine cytochrome *c* and rhodamine dextran dye. At each timepoint after the injections, the number of microinjected cells that remained viable was determined by using morphological criteria and expressed as a percentage of the total number of microinjected cells.

Results and Discussion

Trophic Factor Withdrawal in Immature Sympathetic Neurons Triggers the Translocation of BAX from the Cytosol to Mitochondria, Integration into Mitochondrial Membranes, and Cytochrome *c* Release

Neonatal sympathetic neurons were maintained in the presence of NGF (50 ng/ml) for either ~5 (immature) or 25 d (mature). Cells were then deprived of NGF for various periods, and the subcellular localization of BAX and

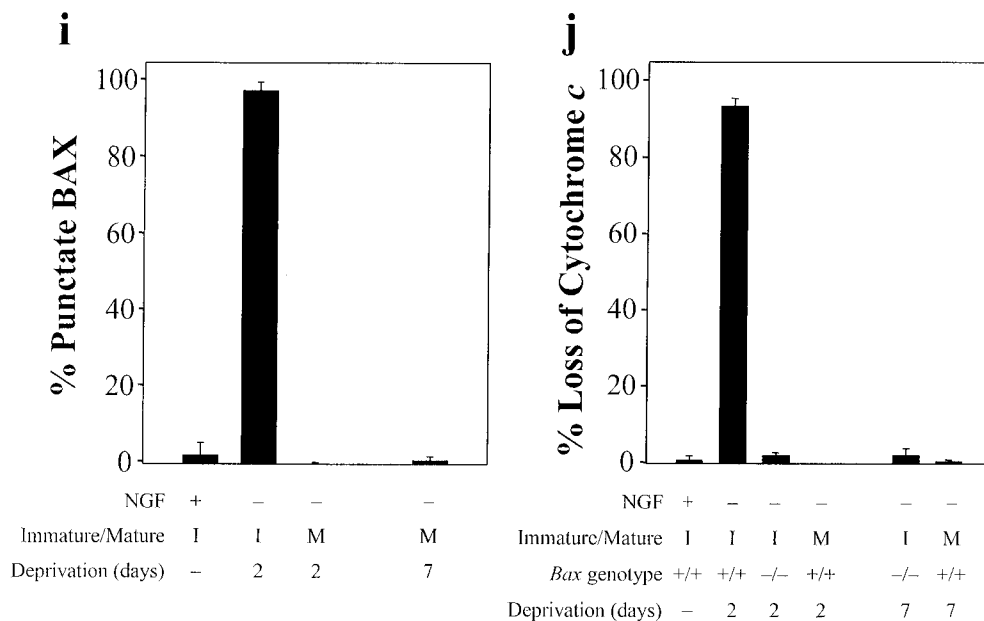
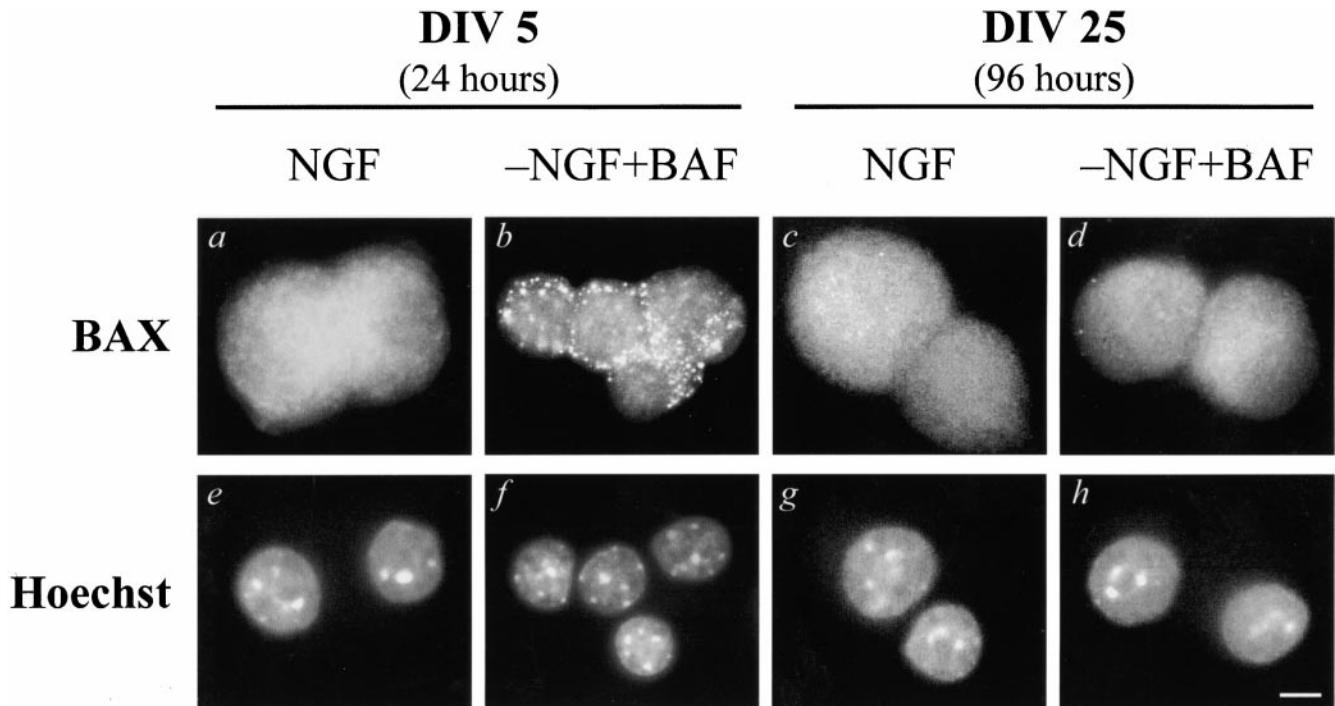


Figure 1. BAX and cytochrome *c* undergo subcellular redistribution during trophic factor withdrawal in immature, but not mature, sympathetic neurons. Neurons were maintained in NGF for ~5 (immature; DIV 5) or 25 d (mature; DIV 25) and then deprived of NGF in the presence of 50 μM BAF. At the indicated times, neurons were fixed and immunostained for BAX (a–d, i) or cytochrome *c* (j). All cells were costained with Hoechst 33258 (e–j). The number of cells exhibiting punctate BAX staining or diffuse cytochrome *c* staining was determined, with 200–250 cells counted for each condition. Mean ± SEM, *n* ≥ 3. Bar, 5 μm. Please note that a, b, e, and f have been reported previously in Putcha et al. (1999) and are included only for comparison.

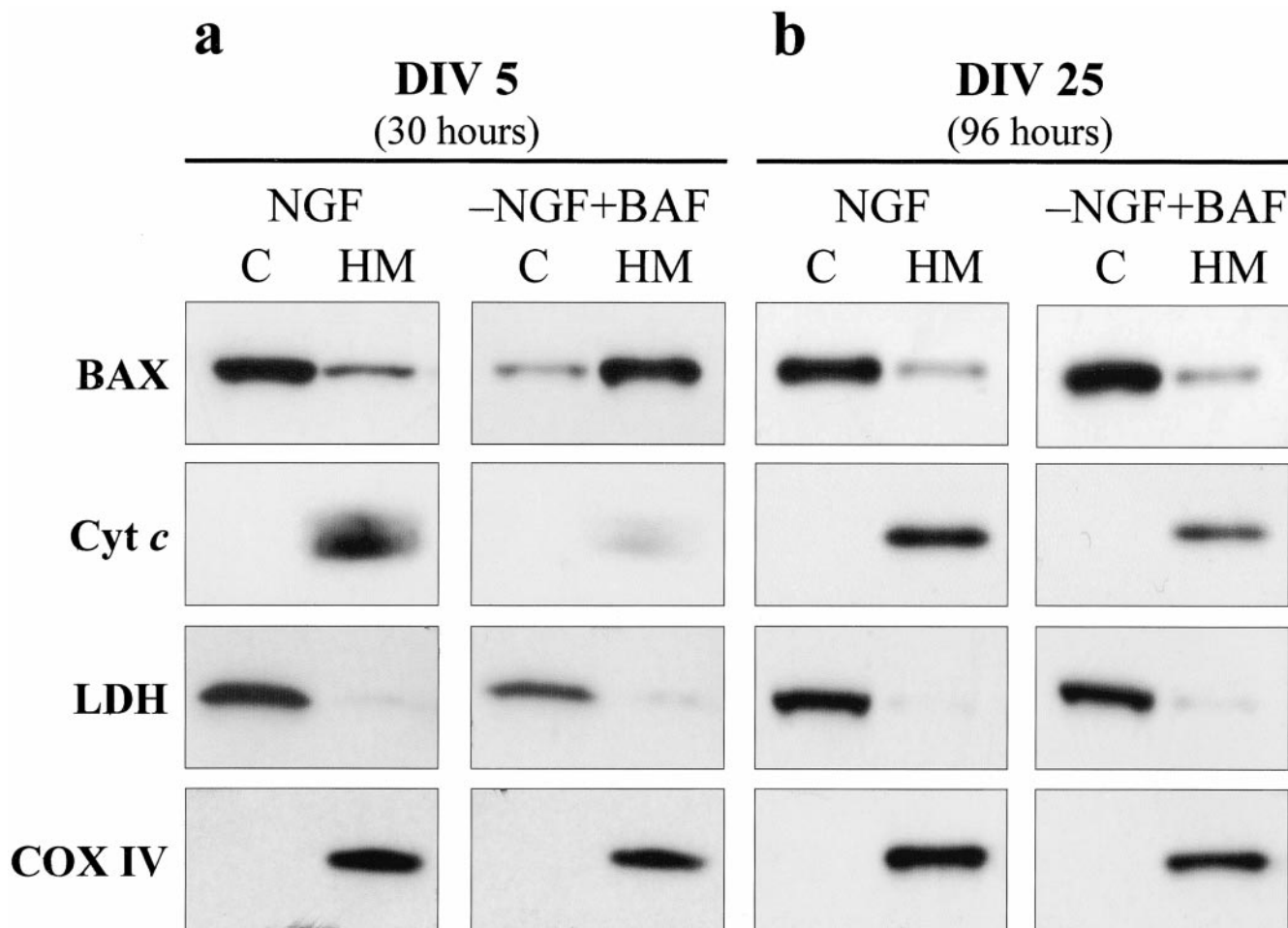


Figure 2. NGF deprivation induces the translocation of BAX and cytochrome *c* in immature, but not mature, sympathetic neurons. DIV 5 or DIV 25 neurons were deprived of NGF for (a) 30 h (DIV 5) or (b) 96 h (DIV 25), and the subcellular localization of BAX and cytochrome *c* was examined by subcellular fractionation. Lactate dehydrogenase (LDH) and cytochrome oxidase subunit IV (COX IV) served as markers for the purity of the cytosolic (C) and HM fractions, respectively, and as markers for equal protein loading.

cytochrome *c* was determined by using both immunocytochemistry (Fig. 1) and subcellular fractionation (Fig. 2). The pan-caspase inhibitor BAF was added to the cultures at the time of deprivation to prevent any cell death that might otherwise have complicated cell counts.

NGF-maintained, immature sympathetic neurons exhibit diffuse BAX staining (Putcha et al., 1999) and punctate cytochrome *c* staining (Deshmukh and Johnson, 1998; Neame et al., 1998; Martinou et al., 1999; Putcha et al., 1999), consistent with cytosolic and mitochondrial localizations, respectively (Fig. 1, a, i, and j). With NGF deprivation, BAX staining changed to a punctate pattern that colocalized extensively with mitochondrial markers (e.g., cytochrome oxidase subunit VIc), consistent with the redistribution of BAX to mitochondria (Fig. 1, b and i). Accordingly, we observed loss of punctate, mitochondrial cytochrome *c* staining, consistent with the release of cytochrome *c* from mitochondria and its subsequent degradation in the cytosol (Fig. 1 j). This loss of mitochondrial cytochrome *c* requires BAX, since sympathetic neurons from *Bax*^{-/-} mice maintain punctate cytochrome *c* stain-

ing, even after 1 wk of NGF deprivation (Fig. 1 j; Deshmukh and Johnson, 1998).

Recently, two groups reported that BAX did not translocate from the cytosol to mitochondria during cell death in nonneuronal cell lines. Instead, they suggested that BAX already at the mitochondria undergoes a conformational change that exposes the NH₂ terminus (Goping et al., 1998; Desagher et al., 1999). To distinguish between these two possibilities, we also examined the localization of BAX and cytochrome *c* by using subcellular fractionation. In NGF-maintained immature sympathetic neurons, BAX primarily localized to the cytosol, whereas cytochrome *c* was found exclusively in mitochondria (Fig. 2 a). NGF deprivation induced the translocation of BAX from the cytosol to mitochondria, leading to the loss of mitochondrial cytochrome *c* (Fig. 2 a). Taken together with our previous findings (Putcha et al., 1999), the results shown in Fig. 1 and Fig. 2 demonstrate that trophic factor withdrawal in immature sympathetic neurons induced the translocation of BAX from the cytosol to mitochondria, followed by the loss of mitochondrial cytochrome *c*.

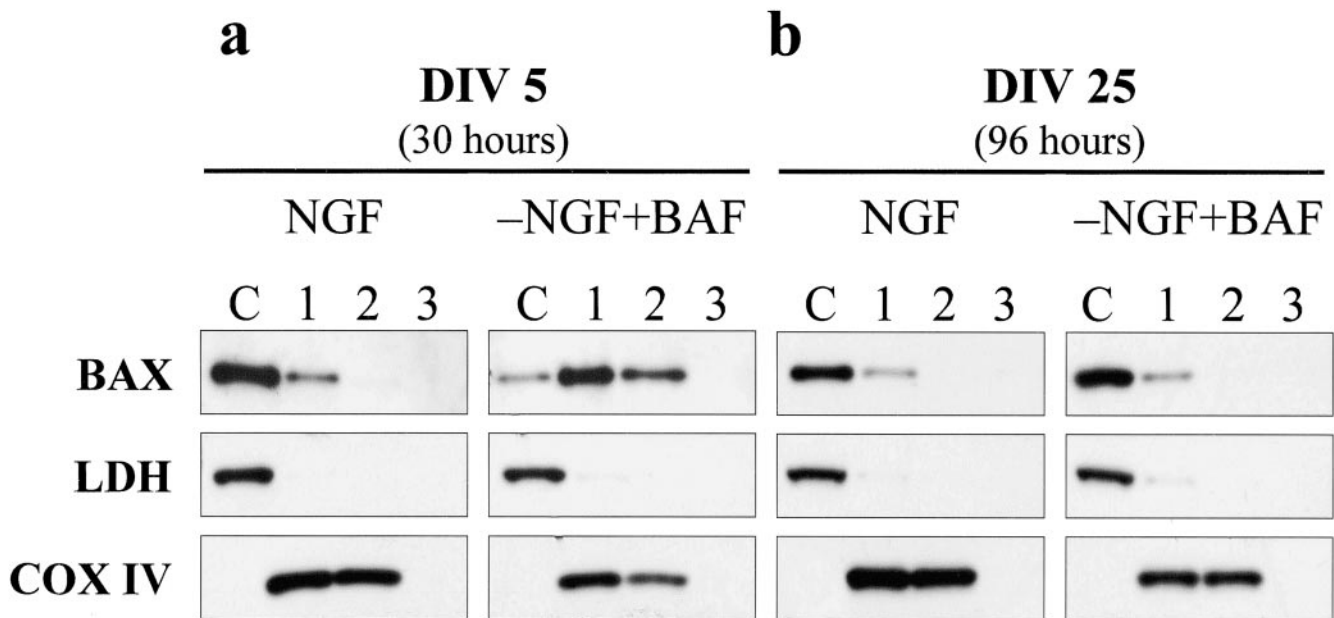


Figure 3. NGF deprivation induces the integration of BAX into mitochondrial membranes in immature, but not mature, sympathetic neurons. DIV 5 or DIV 25 neurons were deprived of NGF for (a) 30 h (DIV 5) or (b) 96 h (DIV 25), and the subcellular localization of BAX was examined by subcellular fractionation with alkali extraction by using 0.2 M Na₂CO₃, pH 11.5. SDS (1% wt/vol) served as a positive control for the extraction of integral membrane proteins. C, cytosol; 1, HM fraction extracted with isotonic buffer; 2, HM fraction extracted with 0.2 M Na₂CO₃; 3, HM fraction extracted with 1% SDS. In a, the apparent extraction of some mitochondrial BAX by alkali treatment in NGF-deprived immature neurons may indicate that not all mitochondrial BAX was integrated into mitochondrial membranes at this particular timepoint.

BAX is found in both the cytosolic and mitochondrial fractions of various tissues and cells, including sympathetic neurons, both in vivo and in vitro (Fig. 2 a and Fig. 3 a; data not shown) (Hsu et al., 1997; Eskes et al., 2000). To determine whether BAX associated with mitochondria is integrated into mitochondrial membranes, we performed alkali extraction of the mitochondrial fractions from NGF-maintained and -deprived immature sympathetic neurons (Fig. 3 a). Mitochondrial integral membrane proteins are resistant to extraction by 0.2 M Na₂CO₃, yet sensitive to extraction by 1% SDS, whereas peripheral membrane proteins are extracted by both treatments. In NGF-maintained cells, mitochondrial BAX was extracted by alkali treatment, indicating that under normal conditions, a small amount of cellular BAX is loosely associated with mitochondrial membranes. Conversely, mitochondrial BAX from NGF-deprived sympathetic neurons was resistant to alkali extraction, consistent with its insertion into mitochondrial membranes. Thus, trophic factor withdrawal in immature sympathetic neurons induced the translocation of BAX from the cytosol to mitochondria and integration of BAX into mitochondrial membranes.

NGF Deprivation in Mature Sympathetic Neurons Does Not Induce BAX Translocation, Cytochrome c Release, or the Development of Competence-to-Die

In contrast to immature sympathetic neurons, which show complete loss of viability 48–72 h after NGF deprivation, trophic factor withdrawal in mature neurons does not induce significant cell death, even after weeks of deprivation

(Easton et al., 1997). Functionally, these mature neurons behave like *Bax*-deficient cells. That is, all known biochemical and molecular events induced by NGF deprivation upstream of the BAX/BCL-2 checkpoint (e.g., *c-Jun* phosphorylation), but not those downstream (e.g., *c-fos* induction), occur with similar kinetics in both *Bax*^{-/-} immature and *Bax*^{+/+} mature neurons (Easton et al., 1997). Accordingly, like *Bax*^{-/-} sympathetic neurons (Deshmukh and Johnson, 1998), trophic factor withdrawal in mature neurons did not induce loss of mitochondrial cytochrome *c*, even after 1 wk of deprivation (Fig. 1 j and Fig. 2 b). Since neither the mRNA (Greenlund et al., 1995) nor protein (Easton et al., 1997) level of BAX decreases with maturation and since the subcellular compartmentalization of BAX contributes to the regulation of its proapoptotic activity, we examined whether inhibition of BAX translocation contributes to the inability of mature sympathetic neurons to release cytochrome *c* and their consequent resistance to cell death induced by trophic factor withdrawal. Like their immature counterparts, NGF-maintained mature neurons primarily exhibited a diffuse, cytosolic distribution of BAX by both immunocytochemistry (Fig. 1, c and i) and subcellular fractionation (Fig. 2 b and Fig. 3 b). However, in contrast to immature neurons, NGF deprivation in mature neurons did not induce the translocation of BAX from the cytosol to mitochondria (Fig. 1, d and i, Fig. 2 b, and Fig. 3 b). Moreover, mitochondrial BAX was sensitive to alkali extraction in both NGF-maintained and deprived mature sympathetic neurons (Fig. 3 b). Thus, NGF deprivation in mature sympathetic neurons did not induce the translocation of either BAX or cytochrome *c*. Moreover,

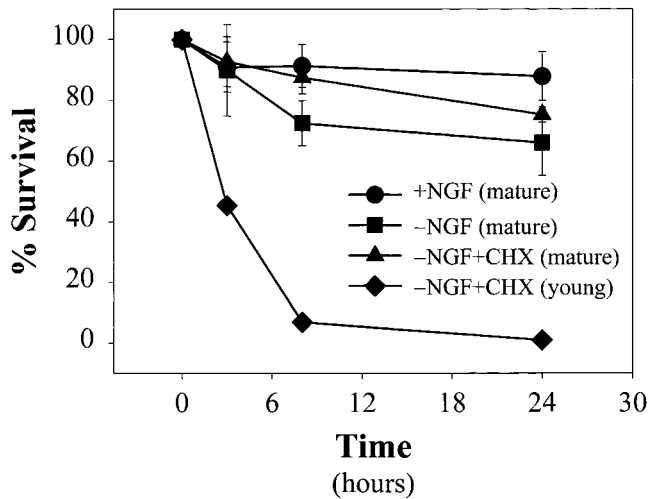


Figure 4. Mature sympathetic neurons do not develop competence-to-die with cytoplasmic microinjection of cytochrome *c*. Cells were maintained in NGF for ~5 (immature; diamonds) or ~25 d (mature) and then treated for 36–48 h with NGF (circles), anti-NGF (squares), or anti-NGF plus 1 μ g/ml cycloheximide (CHX; triangles and diamonds). Cells were then microinjected with 25 mg/ml bovine cytochrome *c* and rhodamine dextran dye. At each timepoint after the injections, the number of microinjected cells that remained viable was determined and expressed as a percentage of the total number of microinjected cells. Mean \pm range, $n = 2$.

since BAX is required for cytochrome *c* release (Fig. 1 j; Deshmukh and Johnson, 1998) and since the expression of BAX is essentially unchanged in immature and mature sympathetic neurons (Greenlund et al., 1995; Easton et al., 1997), our observations further indicate that BAX translocation *per se* was necessary for cytochrome *c* release, caspase activation, and apoptosis induced by trophic factor withdrawal in these cells.

Although BAX-dependent cytochrome *c* release is nec-

essary for NGF deprivation-induced cell death in immature sympathetic neurons, it is not sufficient, since cytoplasmic accumulation of cytochrome *c* does not induce apoptosis in NGF-maintained cells. Sympathetic neurons must also develop competence-to-die, a process that requires neither macromolecular synthesis nor BAX expression (Deshmukh and Johnson, 1998). Only activation of both signaling pathways, BAX-dependent cytochrome *c* release, and the development of competence-to-die, is sufficient to ensure rapid and efficient neuronal apoptosis after trophic factor withdrawal. To determine whether mature neurons develop competence-to-die after NGF withdrawal, we examined whether cytoplasmic accumulation of cytochrome *c* was sufficient to induce cell death in NGF-deprived, mature sympathetic neurons. Immature and mature sympathetic neurons were deprived of NGF for 36–48 h in the presence or absence of cycloheximide and then microinjected with holocytochrome *c*. In immature neurons, cycloheximide prevents cell death yet allows the development of competence induced by NGF deprivation (Deshmukh and Johnson, 1998). At various times after microinjection, cell viability was assessed. As shown in Fig. 4, cytoplasmic microinjection of cytochrome *c* did not induce cell death in NGF-deprived, mature sympathetic neurons, even with 1 wk of deprivation (data not shown) or with a fivefold higher concentration of cytochrome *c* than that required for immature cells (i.e., 25 vs. 5 mg/ml). Therefore, these neurons did not develop competence-to-die. Thus, the results shown in Fig. 1–4 demonstrate that inhibition of both BAX-dependent cytochrome *c* release and the development of competence-to-die was responsible for the loss of trophic factor dependence associated with neuronal maturation.

Taken together with other reports (Deshmukh and Johnson, 1998; Eskes et al., 1998, 2000; Goping et al., 1998; Gross et al., 1998; Neame et al., 1998; Nechushtan et al., 1999; Putcha et al., 1999; Antonsson et al., 2000), our results suggest the following model. Trophic factor withdrawal in immature sympathetic neurons induces two par-

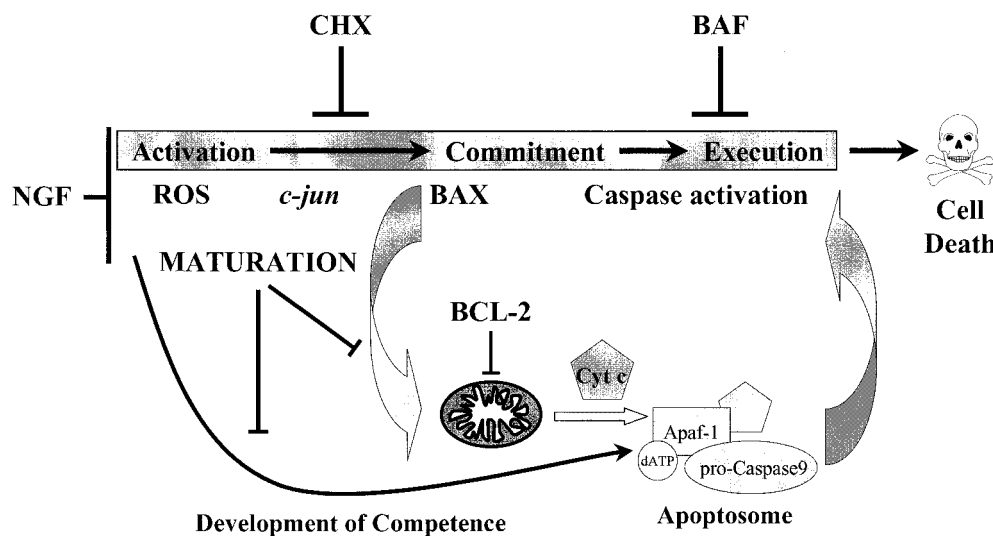


Figure 5. Schematic diagram of the sequence of critical events that occurs during sympathetic neuronal death induced by NGF deprivation. ROS, reactive oxygen species; CHX, cycloheximide; cyt *c*, cytochrome *c*. CHX appears to inhibit the induction of genes required for the multimerization and translocation of BAX to mitochondria (and its subsequent integration into mitochondrial membranes). The identities of these specific gene products, the expression of which may be regulated by *c-Jun*, are unknown. During development, neurons become progressively less dependent on tar-

get-derived trophic support. Inhibition of both BAX-mediated cytochrome *c* release and the development of competence-to-die is responsible for this loss of trophic factor dependence.

allele processes: (a) the development of competence-to-die with cytoplasmic accumulation of cytochrome *c*, which requires neither macromolecular synthesis nor *Bax* expression; and (b) the protein synthesis-dependent, caspase-independent translocation of BAX from the cytosol to mitochondria, followed by the integration of BAX into mitochondrial membranes and the loss of mitochondrial cytochrome *c*. This latter process requires the induction of some unidentified gene products, which are critical to the multimerization and translocation of BAX to the mitochondrial outer membrane, where it inserts and promotes the release of apoptogenic factors, such as cytochrome *c*, into the cytosol. This leads to the formation of a functional apoptosome, caspase activation, substrate cleavage, and cell death. With maturation, sympathetic neurons become markedly less dependent on target-derived trophic support, both in vivo (Angeletti et al., 1971; Bjerre et al., 1975; Goedert et al., 1978; Otten et al., 1979) and in vitro (Lazarus et al., 1976; Chun and Patterson, 1977; Easton et al., 1997). Failure of both arms of the cell death cascade induced by NGF deprivation in immature cells, BAX-dependent cytochrome *c* release, and the development of competence-to-die, contributed to this loss of trophic factor dependence in mature sympathetic neurons (Fig. 5).

Age-related decreases in target-derived trophic factor dependence, which have been reported for several neuronal populations (Snider et al., 1992), serve as an important homeostatic mechanism for maintenance of the adult nervous system. A defect in the process whereby neurons become progressively less dependent on trophic support with maturation may sensitize adult neurons to a variety of pathological insults, thereby contributing to neuronal cell dysfunction and death in neurodegenerative disorders.

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