Biochemistry and Biophysics Reports 4 (2015) 158–168

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Bax and caspases regulate increased production of mitochondria-derived reactive species in neuronal apoptosis: LACK of A role for depletion of cytochrome c from the mitochondrial electron transport chain

Rebecca A. Kirkland [a], James L. Franklin [b,*]

[a] College of Family and Consumer Sciences, University of Georgia, 1372 Dawson Hall, Athens, GA 30602, USA
[b] Department of Pharmaceutical and Biomedical Sciences, The University of Georgia College of Pharmacy, 357 Wilson Pharmacy, Athens, GA 30602, USA

ARTICLE INFO

Article history:
Received 5 June 2015
Received in revised form
20 August 2015
Accepted 9 September 2015
Available online 11 September 2015

Keywords:
Bax
Mitochondria
NGF
Apoptosis
Reactive Oxygen
Cytochrome *c*

ABSTRACT

A Bax-dependent increase of reactive oxygen species (ROS) and other reactive species (RS) occurs after withdrawing NGF from mouse sympathetic neurons in cell culture. Possible mechanisms underlying the increased ROS/RS are leakage of electrons from the mitochondrial electron transport chain secondary to caspase cleavage of respiratory complexes or leakage secondary to depletion of cytochrome *c* from the chain. We previously demonstrated that deletion of Bax or caspase 3 from these cells reduces ROS/RS production to near baseline levels indicating a central role for both Bax and caspase 3 in generating the ROS/RS. Here we depleted cytochrome *c* to a similar level in neurons from wild type and *bax* hemizygous or knockout mice by NGF withdrawal or treatment with H_2O_2. Death was prevented with a caspase inhibitor that caused a partial reduction of ROS/RS levels but did not completely prevent the ROS/RS increase. ROS/RS was highest in *bax* wild-type cells, lowest in *bax* knockout cells, and at an intermediate level in the *bax* hemizygous cells. These and our previous findings indicate that Bax and caspase 3 are necessary for the increased ROS/RS after withdrawing NGF from these cells and that little or none of the increased ROS/RS are secondary to a depletion of cytochrome *c* from the electron transport chain.

1. Introduction

At least half of the neurons generated during vertebrate neurogenesis die by apoptosis. Availability of a required neurotrophic factor is a primary determinant of which cells survive developmental death. Those neurons obtaining sufficient quantities of neurotrophin live while those that do not die. This process is involved in sculpting the developing nervous system [1]. The most extensively investigated model of developmental neuronal apoptosis consists of rat or mouse sympathetic neurons in cell culture deprived of nerve growth factor (NGF) [2–5]. As with many other types of neurons, the apoptotic death of these cells depends on the proapoptotic Bcl-2 family member, Bax [6]. Most Bax resides in the cytoplasm although some is associated with the outer mitochondrial membrane (OMM) of NGF-replete sympathetic neurons [7]. After NGF withdrawal, cytosolic Bax becomes tightly associated with the OMM where it permeabilizes the membrane and induces release of apoptogenic substances from the mitochondrial intermembrane space into the cytoplasm [8–10]. The most important of these factors for the death of sympathetic neurons and many other cell types is cytochrome *c*. Once in the cytoplasm, cytochrome *c* activates caspase-9 by inducing formation of the apoptosome [11]. Caspase-9 then activates downstream effector caspases that cleave many critical protein substrates, causing cell death.

The mechanism(s) by which Bax causes OMM permeabilization remains unclear [12,13]. Withdrawing NGF from rat or mouse sympathetic neurons causes increased levels of reactive oxygen species (ROS) and reactive species (RS) lying downstream of ROS in those cells [5,10,14–17]. These ROS are Bax-dependent and derive from the mitochondrial electron transport chain. A known mechanism by which Bax can increase cellular ROS is by caspase cleavage of respiratory complexes secondary to Bax-induced caspase activation and translocation to the mitochondrial

Abbreviations: NGF, nerve growth factor; ROS, reactive oxygen species; RS, reactive species; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; $\Delta\psi m$,, mitochondrial membrane potential; O_2.-, superoxide; CM-H_2 DCFDA, 5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate; $TMRM^+$, tetramethylrhodamine methyl ester; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; BAF, boc-aspartyl(OMe)-fluoromethylketone

* Corresponding author. Fax: +1 706 542 5358
E-mail addresses: rkirklan@uga.edu (R.A. Kirkland), jfrankli@rx.uga.edu (J.L. Franklin).

http://dx.doi.org/10.1016/j.bbrep.2015.09.004

intermembrane space [18,19]. Consistent with this mechanism in mouse sympathetic neurons, we reported that a broad-spectrum caspase inhibitor inhibits increased production of ROS/RS in NGF-deprived cells and that caspase 3 deletion blocks almost all of it. Another mechanism by which Bax might increase cellular mitochondrial ROS production is by depleting the chain of cytochrome *c* [20,21]. Such depletion could, ostensibly, increase leakage of electrons to molecular oxygen to produce the free radical ROS, superoxide ($O_2^{\cdot -}$). Other cellular ROS and RS are then produced downstream of $O_2^{\cdot -}$. Antioxidants block cytochrome *c* release and apoptotic death in NGF-deprived sympathetic neurons and other cells while pro-oxidants cause cytochrome *c* redistribution and death [5,16,17]. A recent finding demonstrates that Bax can increase mitochondrial-derived ROS in HepG2 and H9c2 cells and that these ROS then increase association of Bax with mitochondria in a positive feedback cycle that causes release of cytochrome *c* from all mitochondria in a cell over a short period [22]. Our published reports suggest that a similar mechanism exists in NGF-deprived sympathetic neurons [10,16,17]. Therefore, ROS/RS appear to lie both up- and downstream of cytochrome *c* redistribution in NGF-deprived sympathetic neurons and other cells and are a critical component of the mechanism by which Bax causes cytochrome *c* release. Our published findings suggest that most of the pro-oxidant effect of Bax is mediated via activation of caspase 3. Here we present evidence that depletion of cytochrome *c* from the electron transport chain makes little if any contribution to the increased Bax-dependent ROS/RS following NGF withdrawal. Our findings also suggest that, under some circumstances, Bax may have additional pro-oxidant effects that are independent of caspase activity.

2. Material and methods

2.1. Materials

5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate (CM–H_2DCFDA) and tetramethylrhodamine methyl ester ($TMRM^+$) were obtained from Molecular Probes (Eugene, OR). NGF 2.5S was from Harlan Bioproducts (Indianapolis, IN). Unless otherwise stated all other reagents were from Sigma (St. Louis, MO).

2.2. Breeding and genotyping of mice

Knockout ($bax^{-/-}$), hemizygous ($bax^{+/-}$), and wild-type ($bax^{+/+}$) mice were generated by mating mice hemizygous for the *bax* allele (Jackson Labs, Bar Harbor, ME) [23]. Genomic DNA was prepared from the tail of each pup using a Wizard Prep kit (Promega, Madison, WI) and genotype determined by PCR as previously described [17].

2.3. Cell culture

Superior cervical ganglia were dissected from newborn mice. Neurons were then enzymatically and mechanically dissociated from the ganglia, plated on a collagen substrate on #1 glass coverslips, and maintained in 35 mm culture dishes [6,17]. These coverslips were placed in an Attofluor cell chamber (Molecular Probes) for microscopy. Separate cultures were established for the ganglia from each pup of $bax^{+/-}$ X $bax^{+/-}$ matings. Cells to be used for immunoblotting experiments were plated on 35 mm culture dishes. Cells for survival experiments were plated on 35 mm or 24-well culture dishes. Neurons from ½ to 1 ganglion were plated per culture for most experiments. Cultures were maintained in medium containing Eagle's minimum essential medium with Earle's salts w/o L-glutamine (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, 20 μM uridine, 1.4 mM L-glutamine, and 50 ng/ml 2.5S NGF. Cultures were incubated at 35 °C in an incubator having a 5% CO_2 and 95% air atmosphere. Cultures were deprived of NGF by incubating them in culture medium containing a NGF-neutralizing antibody (Harlan Bioproducts or Abcam Inc., Cambridge, MA) and lacking NGF. All data presented are combined from experiments done with neurons from at least three separate platings. All experiments were begun when cultures were 6–9 days old.

2.4. Confocal microscopy

Confocal microscopy experiments were done with a Nikon C1 laser-scanning confocal microscope (Southern Micro Instruments, Marietta, GA) attached to a Nikon Eclipse TE300 inverted microscope. Capture of images was accomplished by EZC1 software. Neurons were scanned at 512 × 512 pixel resolution. Confocal pinhole size was always the same within an experiment.

2.5. ROS/RS and mitochondrial membrane potential ($\Delta\psi_m$) measurement

The ROS/RS-sensitive dye 5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate (CM-H_2 DCFDA) was used to determine neuronal ROS and other RS levels. This dye is readily membrane-permeant. Once in cells, it is effectively trapped by binding to cellular thiols. CM-H_2 DCFDA is only faintly fluorescent in reduced form but becomes intensely fluorescent when oxidized by several ROS and other RS [24]. We extensively characterized the use of this dye in sympathetic neurons and found it to be superior to other similar dyes for assessing changes in the ROS/RS levels in these cells [16,17]. Cultures were incubated for 20–25 min at 35°C in the appropriate experimental medium containing CM-H_2 DCFDA (10 μM). They were then washed 2 × with Leibovitz's L-15 medium containing the experimental treatments and were left in the last wash for microscopy. The dye was excited with the 488 nm line of the confocal lasers. Laser intensity was kept at ~10% of maximum to reduce photo-oxidation of the dye. Several passes of the laser at this power did not significantly increase CM–H_2 DCFDA intensity indicating that little photo-oxidation occurred in the cells at this power. Laser intensity and photomultiplier gain were always the same within an experiment. The green photomultiplier channel of the confocal microscopes was used for image acquisition.

We used $TMRM^+$ in non-quench mode to monitor $\Delta\psi_m$ [25–27]. Cultures were incubated for 20–25 min at 35 °C in the appropriate experimental medium containing $TMRM^+$ (10 nM). At the end of this time, cultures were washed 2 × with L15 media containing the appropriate experimental treatments and $TMRM^+$. They were left in the last wash for confocal microscopy. The dye was excited with the 543 nm line of the confocal lasers. The TRITC photomultiplier channel of the confocal microscope was used for image acquisition. That $TMRM^+$ was being used in non-quench mode was confirmed by the diminution of dye intensity when cells were acutely treated with the protonophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 200 nM).

Both CM–H_2 DCFDA and $TMRM^+$ fluorescence intensities in confocal micrographs were quantified by measuring the raw pixel intensity in a 60 μm^2 area within individual neuronal somas using the elliptical region tool of MetaMorph software (Universal Imaging, West Chester, PA). All microscopy was done at room temperature. The intensity of each neuron was normalized to that of NGF-maintained $bax^{+/+}$ neurons receiving the same concentration of dye for the same time as the experimental cells.

2.6. Immunocytochemistry and immunoblotting

Immunocytochemical staining of cytochrome *c* was accomplished as described [16,17]. Western blotting for cytochrome *c* and anti-β tubulin III was also done essentially as described [17]. Cytochrome *c* concentration in cultures, as determined by immunoblot, was normalized to tubulin levels in the same culture unless otherwise indicated. Anti-mouse cytochrome *c* antibody (Pharmingen) was used at 1.5 μg/ml. Anti-β tubulin III antibody was used at a 1:1000 dilution. ECL blot analyses was done with a Fotodyne Foto/Analyst Dual-Light Luminary Workstation and TotalLab Software (Fotodyne, Hartland, WI).

2.7. Statistical analysis

Statistical analysis was done with SigmaPlot 6.0 (SPSS, Chicago, IL). Statistical comparisons were made either with *t* tests or Kruskal–Wallis one-way ANOVA on ranks with Dunn's multiple comparisons. The appropriate statistical method was determined for each data set. Means are shown as ± SEM.

3. Results

3.1. Increased ROS/RS after NGF withdrawal was inhibited by a broad-spectrum caspase inhibitor

We used the ROS/RS-sensitive dye, CM–H_2DCFDA, to determine ROS and other RS levels in mouse sympathetic neurons. CM–H_2DCFDA is relatively insensitive to oxidation by $O_2^{\cdot -}$ [24]. It also cannot distinguish between ROS and several other RS (*e.g.* hydroxyl radicals and peroxynitrite) [28]. It is oxidized by several ROS/RS downstream of $O_2^{\cdot -}$ dismutation and can, therefore, serve as an indirect measure of $O_2^{\cdot -}$ levels. Cells were loaded with the dye and confocal microscopic analysis was used to measure changes in dye intensity after NGF-withdrawal. Fig. 1a shows that, as previously reported [17], the average fluorescence intensity of CM–H_2DCFDA increased after NGF deprivation and remained elevated throughout the course of the apoptotic process. The time-course of the increase in CM–H_2DCFDA intensity is similar to that reported in NGF-deprived sympathetic neurons when other ROS/RS-sensitive dyes were utilized [5,14,15]. Pretreatment of NGF-deprived cultures for three hours with either cell-permeant PEG-catalase or PEG-superoxide dismutase completely blocked the increased ROS/RS at 24 h after withdrawal further indicating that CM–H_2DCFDA was detecting the dismutation products of $O_2^{\cdot -}$ (the CM–H_2DCFDA intensity in the PEG catalase treated cells was 0.23 ± 0.1 fold that of neurons maintained in NGF and the PEG-SOD intensity was 0.89 ± 0.04 fold that in NGF-supported cells; $p > 0.6$ by ANOVA on ranks; $n = 67$–112 neurons). These data indicate that the cells had entered a sustained pro-oxidant state after NGF withdrawal and that elevated $O_2^{\cdot -}$ was its source.

Production of $O_2^{\cdot -}$ by mitochondria is dependent on mitochondrial membrane potential ($\Delta\psi_m$). Decreasing (depolarizing) $\Delta\psi_m$ can reduce electron leakage from the electron transport chain and cause decreased production of $O_2^{\cdot -}$ [27]. We used the mitochondrial uncoupler, FCCP, to dissipate the proton gradient across the inner mitochondrial membrane (IMM) and assessed $\Delta\psi_m$ with the $\Delta\psi_m$-sensitive dye, $TMRM^+$ [26]. FCCP, at a concentration (1.6 μM) that nearly abrogated $\Delta\psi_m$ (7.7 ± 0.3% of control $TMRM^+$ intensity in NGF-replete cells; $n = 59$ neurons), blocked most of the increased ROS/RS after NGF withdrawal (0.22 ± 0.09-fold increase of average CM–H_2DCFDA intensity at 24 h after NGF withdrawal as compared to 1.53 ± 0.17 fold increase without the FCCP; $p < 0.001$; $n = 33$ neurons). Because $\Delta\psi_m$ is primarily generated by the electrochemical proton gradient, this

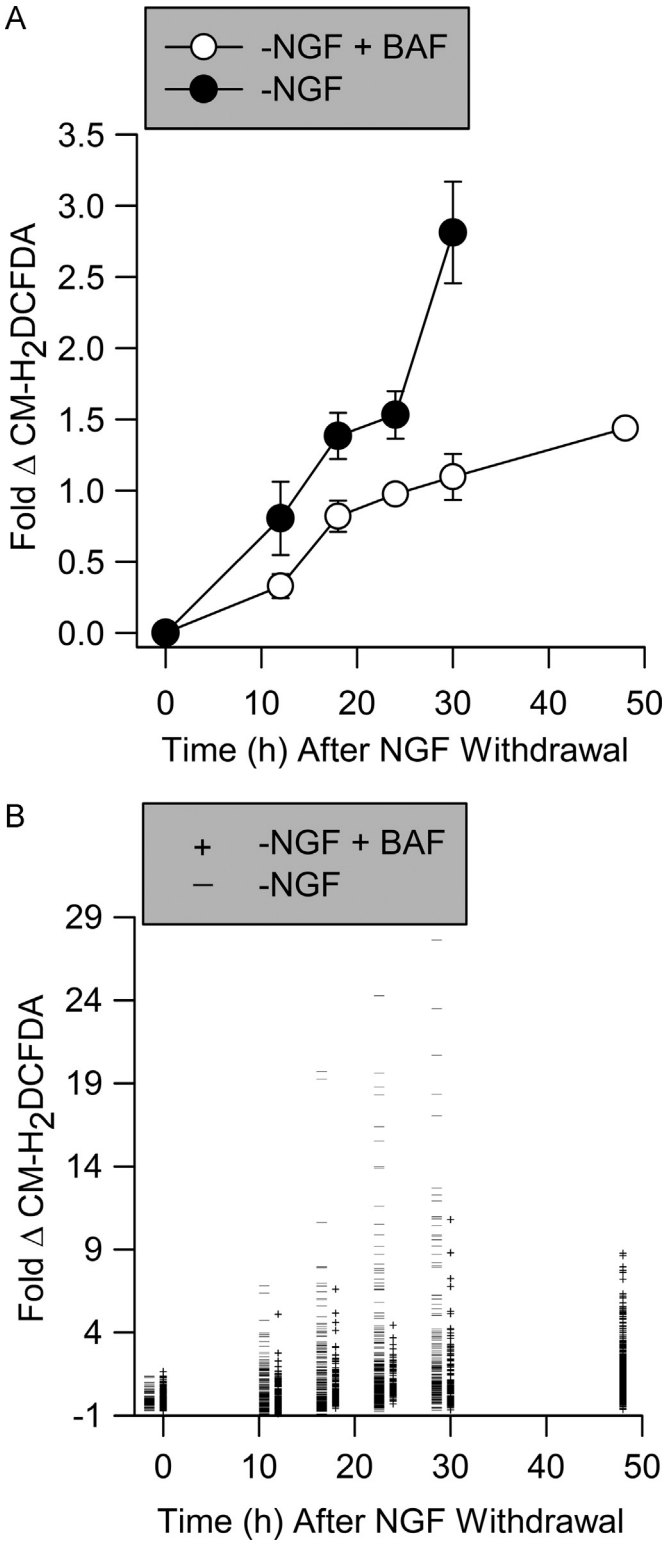

Fig. 1. A caspase inhibitor suppressed elevation of cellular ROS/RS after NGF withdrawal. (a) The broad-spectrum caspase inhibitor, BAF (Sigma or Enzyme Systems Products, Livermore, CA; 50 μM), attenuated but did not prevent increased ROS/RS levels caused by NGF deprivation. Over the period shown, this concentration of BAF prevents the death of > 80% of NGF-deprived mouse sympathetic neurons [3]. BAF was included in the culture media from the time of deprivation. $n = 112$–356 cells for each treatment and time after withdrawal. (b) BAF decreased the number of NGF-deprived neurons having modestly elevated cellular ROS/RS (CM-H_2DCFDA intensity) and also the number of neurons with greatly elevated ROS/RS. The single cell data shown is the same as that used for the averages in part a. The CM–H_2DCFDA intensity of each neuron was normalized to the average intensity of NGF-maintained $bax^{+/+}$ neurons receiving the same concentration of dye for the same time as the experimental cells. The fold changes are from these average values.

Table 1
Equal CM–H_2DCFDA loading occurred in all. experimental conditions.

Condition	Fold change in dye intensity after 30 min of H_2O_2 (2 mM)
$bax^{+/+}$-NGF+BAF	$+1.95 \pm 0.15$
$bax^{+/-}$-NGF+BAF	$+2.00 \pm 0.14$
$bax^{+/+}$ H_2O_2-NGF+BAF	$+2.17 \pm 0.19$
$bax^{+/-}$ H_2O_2-NGF+BAF	$+1.80 \pm 0.12$
$bax^{-/-}$ H_2O_2-NGF+BAF	$+2.00 \pm 0.23$
$bax^{+/+}$-NGF+BAF+Rot	$+2.06 \pm 0.13$
$bax^{+/+}$-NGF+BAF+Oligo	$+1.90 \pm 0.17$
$bax^{+/-}$-NGF+BAF+Oligo	$+1.97 \pm 0.15$

CM–H_2DCFDA loading and response to oxidation were not altered by any of the experimental conditions tested in this manuscript. Cells were loaded for 20 min with CM-H_2DCFDA in medium containing the experimental treatments. They were then transferred to L15 medium containing the experimental treatments for confocal microscopy. The CM–H_2DCFDA intensities in NGF-maintained $bax^{+/+}$ neurons from the same plating as the experimental cells were determined at the end of the loading period. Cultures were then exposed to 2 mM H_2O_2 for 30 min. CM–H_2DCFDA intensity is shown as average fold change from the average intensity measured in the NGF-maintained $bax^{+/+}$ neurons. n=29-93 cells. There were no significant differences in the average dye intensities of cells receiving any of the eight treatments ($p > 0.1$). These data show that none of the differences in CM–H_2DCFDA intensities reported in this manuscript can be explained by artifacts caused by differential dye loading or in the response of the dye to oxidation. All BAF-exposed cultures were deprived of NGF and exposed to BAF (50 μM) for 48 h. Rotenone (Rot, 10 μM) and oligomycin (Oligo, 5 μg/ml) were added in the CM–H_2DCFDA loading medium and were also in the L15 medium. H_2O_2 refers to cells treated with 2 mM H_2O_2 in the presence of NGF followed by NGF deprivation and maintenance in BAF for 48 h.

finding indicates a mitochondrial origin for the ROS/RS.

Permeabilization of the OMM during the apoptotic death of some cell types has been shown to allow activated cytosolic caspases access to the mitochondrial intermembrane space where they increase mitochondrial ROS production by cleaving respiratory chain subunits [18,19]. The ROS/RS increase occurring after withdrawing NGF from rat sympathetic neurons and during the apoptotic death of some other cell types is greatly attenuated by caspase inhibitors, also suggesting that some of the elevated ROS/RS is caused by action of caspases on the mitochondrial electron transport chain [5,16,29]. NGF-deprived mouse sympathetic neurons remain alive in culture for several days when exposed to the broad-spectrum caspase inhibitor, boc-aspartyl(OMe)-fluoromethylketone (BAF; 50 μM) [3]. Fig. 1a shows that BAF treatment attenuated the average ROS/RS increase occurring after NGF withdrawal from these cells although not to as great as extent as we found in NGF-deprived rat sympathetic neurons [16]. The effect of BAF on ROS/RS cannot be attributed to artifacts such as differential dye loading or dye response as neither this treatment, nor any others in this manuscript, affected these parameters (Table 1). The ROS/RS detected at 48 h after NGF deprivation was almost completely suppressed by treatment with 1.6 μM FCCP (0.16 ± 0.07 fold change from baseline CM–H_2DCFDA values in NGF-replete control cells; $p > 0.1$; n=24 neurons) indicating their derivation from the mitochondrial electron transport chain. Inspection of the effects of BAF on ROS/RS in single cells (Fig. 1b) revealed that this inhibitor attenuated modest increases in ROS/RS caused by NGF deprivation and blocked most of the very high ROS/RS levels found in many cells (cells with > 4 fold increase of CM–H_2DCFDA intensity; Fig. 1b). These and our previous data suggest that caspases are involved in generating the elevated ROS/RS caused by withdrawing NGF from mouse sympathetic neurons, perhaps by attacking components of the mitochondrial respiratory chain [5,16,17].

3.2. Bax regulated ROS/RS levels in cytochrome c-depleted neurons

Release of cytochrome *c* from the mitochondrial intermembrane space into the cytoplasm is critical for the apoptotic death of many cell types. Bax, a proapoptotic member of the Bcl-2 family of proteins is central to this release. Genetic deletion of *bax*, the gene coding for Bax, prevents cytochrome *c* redistribution and apoptotic death of many types of neurons, including NGF-deprived mouse sympathetic neurons [6,17,30]. We reported that Bax lies upstream from all of the increased ROS/RS following withdrawal of NGF from these neurons and presented evidence that these ROS/RS are a critical part of the mechanism by which Bax induces release of cytochrome *c* from their mitochondria [10,17,22]. The data shown in Fig. 1 suggests that some of the ROS/RS increase after NGF withdrawal is caused by caspases activated downstream of Bax-induced cytochrome *c* release. However, caspase inhibition did not suppress all of the elevated ROS/RS while caspase 3 or *bax* deletion almost abrogates it [5,10,17]. These findings suggest that Bax increases ROS/RS primarily via activation of caspases 3 in these cells. They also suggest that the inhibitor did not completely block caspase activity or that it has non-specific pro-oxidant activity independent of caspase inhibition.

One possible mechanism that could have explained the Bax-dependent ROS/RS remaining in NGF-deprived neurons exposed to BAF was depletion of cytochrome *c* from the electron transport chain of their mitochondria [20]. Such depletion could, ostensibly, enhance leakage of electrons from mitochondrial respiratory complexes and augment formation of $O_2^{\cdot -}$. To clarify the role of cytochrome *c* redistribution in generating Bax-induced ROS/RS, we depleted cytochrome *c* from NGF-deprived *bax* wild-type ($bax^{+/+}$) neurons and neurons hemizygous for *bax* ($bax^{+/-}$). The latter cells have half of the wild-type concentration of Bax protein and also exhibit greatly diminished ROS/RS levels after NGF withdrawal [17]. Depletion was effected by depriving cultures of NGF to induce cytochrome *c* redistribution and maintaining them in BAF-containing medium to prevent death. Cytochrome *c* rapidly degrades once it redistributes from mitochondria into the cytoplasm of sympathetic neurons and BAF does not prevent release or degradation [9,10,16,17]. The amount of cytochrome *c* remaining in NGF-deprived, BAF-maintained neurons was determined by immunocytochemistry and immunoblotting. Most or all mitochondria in these and other cell types [31] appear to release cytochrome *c* over a short period. Rapid release followed by rapid degradation generates two immunocytochemically distinguishable populations of sympathetic neurons: 1, cells that retain cytochrome *c* in their mitochondria and that stain intensely for it and 2, neurons that have released cytochrome *c* from mitochondria and show greatly reduced cytochrome *c* staining. Fig. 2a demonstrates the dissimilarity in the appearance of these cells and how we used this difference to score NGF-deprived neurons as having retained cytochrome *c* in mitochondria or having released it. The data in Fig. 2b, generated using this technique, shows that both $bax^{+/+}$ and $bax^{+/-}$ neurons maintained in BAF released cytochrome *c* over a similar period. These time-courses are also similar to those observed in the absence of BAF [17]. Therefore, as determined by immunocytochemistry, neither caspase inhibition with BAF nor reduction of Bax levels to about half of normal concentration affected the rate of cytochrome *c* redistribution. By 48 h after NGF withdrawal, immunocytochemical staining for cytochrome *c* indicated that~90% of neurons of both genotypes had released cytochrome *c*. Therefore, this method for determining cellular cytochrome *c* status suggests that both BAF-maintained $bax^{+/+}$ and $bax^{+/-}$ neurons were depleted of cytochrome *c* by the same amount at all times after NGF withdrawal.

Immunostaining can give a qualitative answer as to whether cytochrome *c* redistribution has occurred after NGF withdrawal. However, it does not provide clear quantitative information about the amount of cytochrome *c* released from or remaining in mitochondria. To more quantitatively determine the amounts of cytochrome *c* in NGF-deprived neurons, we withdrew NGF from cultures

having $bax^{+/+}$ and $bax^{+/-}$ genotypes, maintained them alive with BAF-containing medium, and determined total cellular cytochrome *c* levels by immunoblotting. Because of the rapid degradation of cytochrome *c* in the cytoplasm of NGF-deprived mouse sympathetic neurons, immunoblotting cannot detect cytoplasmic cytochrome *c* in cytosolic fractions from them at any time after its release from mitochondria [4]. The only immunoblot-detectable cytochrome *c* remaining in these cells is mitochondrial. Therefore, the total cellular levels of cytochrome *c* detected by immunoblotting in NGF-deprived cells reflect only the cytochrome *c* left in mitochondria [32]. The immunoblots in Fig. 2c show that the amounts of cytochrome *c* in $bax^{+/+}$ and $bax^{+/-}$ cultures deprived of NGF for 48 h and maintained in BAF- containing medium were similar. Fig. 2d quantifies cytochrome *c* data from a number of immunoblots. These data show that NGF-replete $bax^{+/+}$, $bax^{+/-}$, and *bax* knockout ($bax^{-/-}$) neurons all had identical amounts of cytochrome *c*. Withdrawing NGF for 24–48 h caused an equivalent decrease ($p > 0.1$ for both) in the total amount of cytochrome *c* in cultures of BAF-maintained $bax^{+/+}$ and $bax^{+/-}$ neurons. As expected [17], NGF-deprived, BAF-exposed $bax^{-/-}$ neurons did not lose cytochrome *c* after NGF withdrawal ($p > 0.1$ compared to NGF-replete control). The reduction in the amount of cytochrome *c* after 24 h of withdrawal was similar to that suggested by the immunocytochemical method used in Fig. 2b (~60% for both $bax^{+/+}$ and $bax^{+/-}$ cultures). However, the amount of cytochrome *c* remaining in cultures 48 h after withdrawal was somewhat higher than that suggested by the immuncytochemical experiments (~40% rather than the ~10% suggested by immunocytochemistry). One possible explanation for the discrepancy between the values generated by the two techniques is that the immunocytochemical method detected cytochrome *c* only in neurons while immunoblotting detected cytochrome *c* both in neurons and in nonneuronal cells in the cultures [16,17]. To ascertain whether this was the case, we determined cytochrome *c* levels in cultures made by plating cells without NGF. This technique establishes cultures having no neurons but containing the same number of nonneuronal cells as those that are replete with NGF [16,17]. These nonneuronal cultures had undetectable levels of cytochrome *c* (not shown). Therefore, the discrepancy between the 48 h immunocytochemical data and immunoblot data cannot be explained by the cytochrome *c* expressed in nonneuronal cells. These findings indicate that a substantial pool

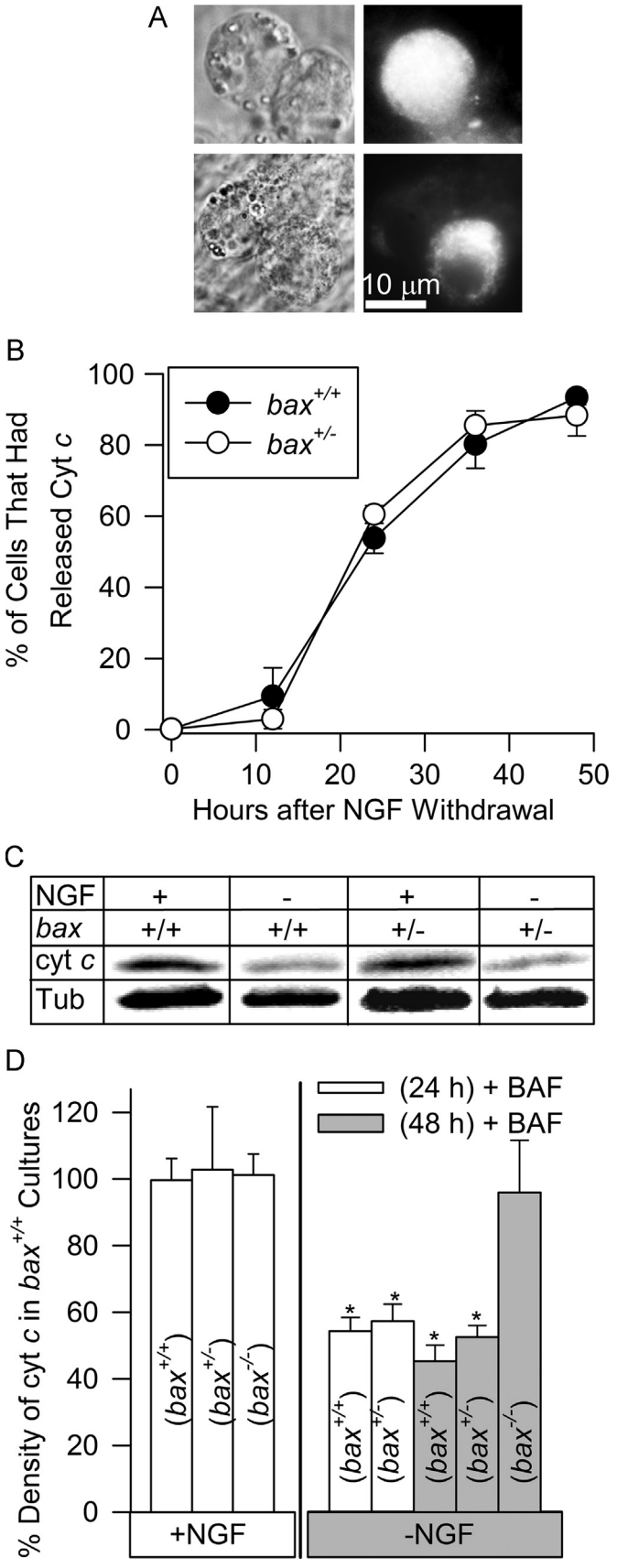

Fig. 2. The concentration of cytochrome *c* in mitochondria was similar in $bax^{+/+}$ and $bax^{+/-}$ neurons after NGF withdrawal. (a) Micrographs illustrating the criteria used to score cytochrome *c* localization by immunocytochemistry. The left panels show differential interference contrast images of neurons that had been deprived of NGF for 48 h and maintained in BAF (50 μM)-containing medium. The right panels are fluorescent micrograph of the same cells showing immunostaining for cytochrome *c*. Note the intense punctate staining in the top neuron in the top panel and the faint staining in the bottom one. The top neuron is representative of neurons that were scored as having retained cytochrome *c* in mitochondria. The bottom neuron and two of the three neurons shown in the lower panels are representative of cells scored as having released cytochrome *c* into the cytoplasm where it was rapidly degraded. (b) Time-courses of loss of cytochrome *c* (Cyt *c*) immunostaining in NGF-deprived $bax^{+/+}$ and $bax^{+/-}$ neurons. Cultures were maintained in medium containing BAF (50 μM) to prevent death. Scoring for cytochrome *c* status was done as in part a with fluorescence microscopy. Neurons were scored as having punctate (no cytochrome *c* release) or faint staining (released cytochrome *c*) by a naive observer. $n = 3$–4 cultures for each time point. c Western blots showing cytochrome *c* levels in cultures of neurons having the indicated genotypes. Loading control is β tubulin III (Tub) from the same cultures. Note the decrease in cytochrome *c* after NGF withdrawal. NGF-deprived cultures were maintained in BAF (50 μM)-containing medium for 48 h before immunoblot analysis. d Quantification of cytochrome *c* levels in cultures of neurons from mice having the indicated genotypes and receiving the indicated treatments. The left bars show that neurons with $bax^{+/+}$ $bax^{+/-}$, and $bax^{-/-}$ genotypes all expressed the same amount of cytochrome *c* when maintained in medium containing NGF. Cytochrome *c* concentration (density of immunoblot bands) was determined by immunoblotting and was normalized to amount of *β* tubulin III in the same cultures. The right bars show that cytochrome *c* had decreased by the same amounts in $bax^{+/+}$ and $bax^{+/-}$ neurons at 24 or 48 h after NGF withdrawal ($p > 0.1$ within the time-point). There was no decrease in $bax^{-/-}$ cultures. Deprived cultures were maintained in BAF (50 μM)-containing medium. Stars indicate significantly different ($p < 0.001$) from NGF-maintained controls. $n = 3$-6 cultures for each bar.

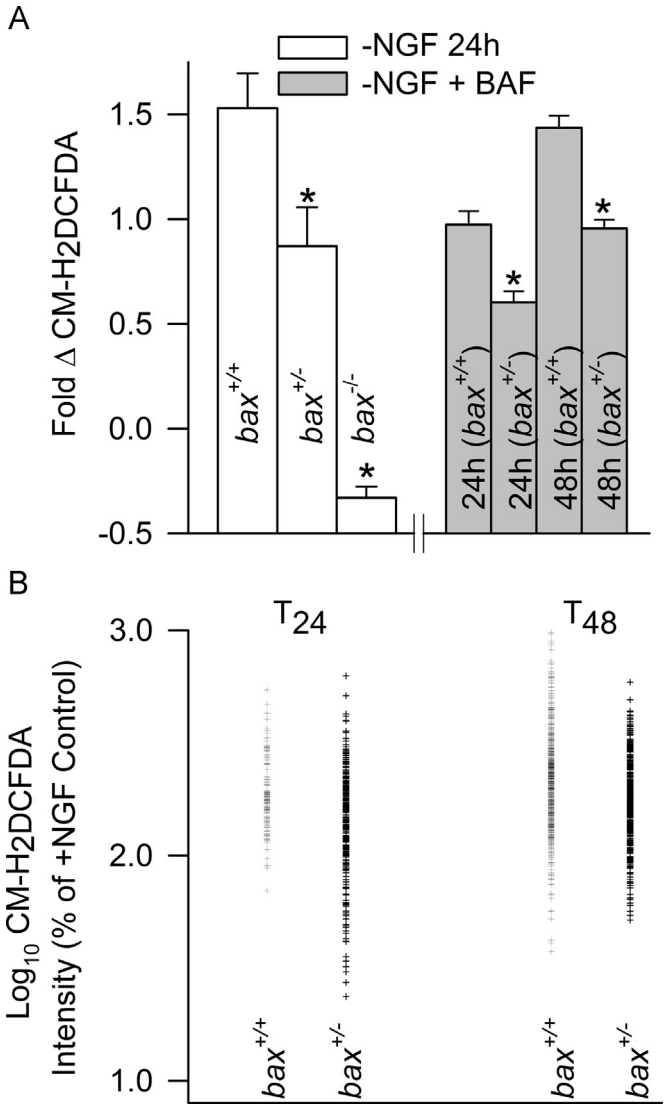

Fig. 3. Bax concentration determined ROS/RS levels in NGF-deprived neurons having similar cytochrome *c* content. (a) Decreasing *bax* gene dosage and, therefore amount of Bax protein expressed in neurons, decreased ROS/RS levels. Left, CM–H_2DCFDA intensities in neurons deprived of NGF-for 24 h and having the indicated *bax* genotypes. Right, CM–H_2DCFDA intensities in $bax^{+/+}$ and $bax^{+/-}$ neurons deprived of NGF for 24-48 h and maintained alive in BAF (50 μM)-containing medium. Stars indicate significantly different ($p < 0.001$) from $bax^{+/+}$ neurons at the same time-point. $n = 141–541$ neurons. (b) Point plot of the single-cell CM–H_2DCFDA data (BAF-treated) averaged in part a. Data is plotted on a semi-log scale to more clearly demonstrate the wide range of CM-H_2DCFDA intensities.

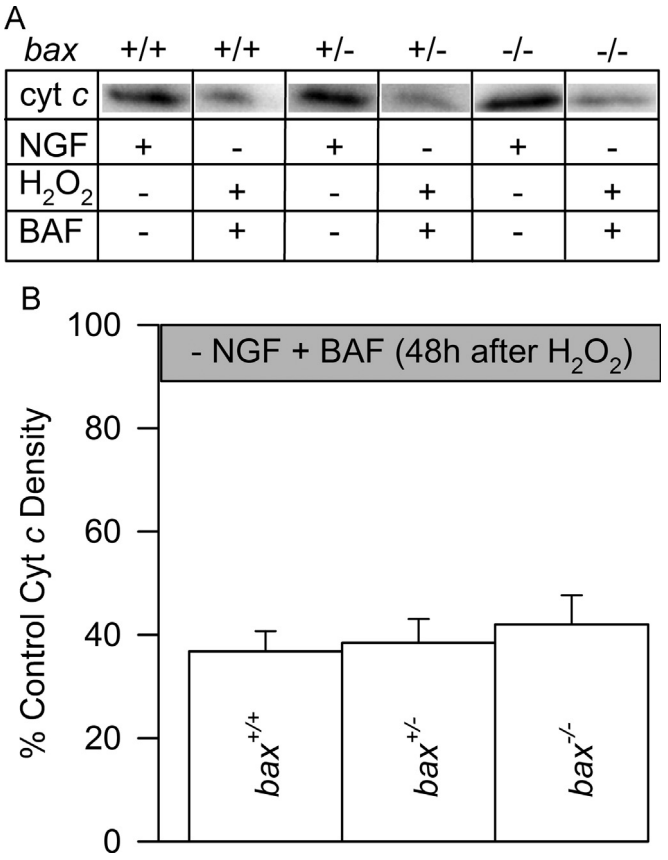

Fig. 4. Depletion of cytochrome *c* in $bax^{+/+}$, $bax^{+/-}$, and $bax^{-/-}$ neurons treated with H_2O_2. (a) Western blots showing cytochrome *c* levels in cultures of neurons that had been exposed to H_2O_2 48 h previously. Cultures were exposed for 30 min to medium containing NGF and 2 mM H_2O_2. They were then maintained for 48 h in medium containing no NGF and BAF (50 μM) to prevent death. Cultures with the same *bax* genotypes are siblings having the same plating densities. Note the decrease in cytochrome *c* to equivalent concentrations in H_2O_2-treated cultures of all *bax* genotypes. To assure that H_2O_2 treatment did not alter levels of protein loaded onto the gels, a non-specific protein band detected by the primary cytochrome *c* antibody was monitored (not shown) [32]. Similar amounts of this protein confirmed that comparable amounts of protein were loaded onto gels. (b) Quantification of cytochrome *c* levels in cultures of H_2O_2 -treated neurons having the indicated *bax* genotypes. Cytochrome *c* concentration was determined by immunoblotting. Cytochrome *c* had decreased by the same amounts in neurons with each *bax* genotype by 48 h after H_2O_2 (2 mM)-exposure and NGF withdrawal. Treatment protocol was the same as in part A. Cytochrome *c* was normalized to the amount of cytochrome *c* found in sibling cultures maintained since the time of plating in medium containing NGF. Equal protein loading in most cases was confirmed by monitoring the non-specific protein band detected by the primary antibody. In some cases equal protein loading was also determined by density of β tubulin III bands. Only those cultures where equal loading was confirmed are included. $n = 3–6$ cultures.

of cytochrome *c* remains in BAF-maintained neurons that have been deprived of NGF for 48 h and that this pool is not readily detectable by the immunocytochemical method. The salient data for the purposes of this report is that, as determined by two separate techniques, both $bax^{+/+}$ and $bax^{+/-}$ neurons had the same amount of cytochrome *c* at 24 and 48 h after NGF withdrawal. Therefore, ROS/RS levels in NGF-deprived neurons expressing different concentrations of Bax but the same concentration of cytochrome *c* could be compared to determine the effects of Bax levels on ROS/RS independent of its effects on mitochondrial cytochrome *c* levels.

As previously reported [17], 24 h of NGF withdrawal induced higher ROS/RS levels in neurons having a $bax^{+/+}$ genotype than in ones with a $bax^{+/-}$ genotype (Fig. 3a). $bax^{-/-}$ neurons exhibited no increased ROS/RS after NGF withdrawal. The latter neurons have no detectable Bax protein while those with the $bax^{+/-}$ – genotype have about half the Bax concentration found in $bax^{+/+}$ cells. These findings cannot be explained by artifacts such as differential dye loading but are due to effects of Bax protein concentration on actual cellular ROS/RS levels (Table 1) [16,17]. This effect also does not appear to be secondary to differences in the ability of cells having the different genotypes to detoxify ROS/RS but, rather, in the amount of ROS/RS produced by mitochondria [5,17]. The death and dissolution of many cells by 24 h after NGF withdrawal creates difficulties in interpretation of data. Therefore, we deprived cultures of $bax^{+/+}$ and $bax^{+/-}$ neurons of NGF and maintained them in BAF to prevent death (Fig. 1). Fig. 3a and b shows that $bax^{+/+}$ neurons maintained in this fashion had small but significantly higher ROS/RS levels ($p < 0.001$) at both 24 h and 48 h after NGF withdrawal than did $bax^{+/-}$ neurons. Because these cells had identical amounts of cytochrome *c* in their mitochondria at these times (Fig. 2), these data suggest that Bax concentration can affect ROS/RS levels

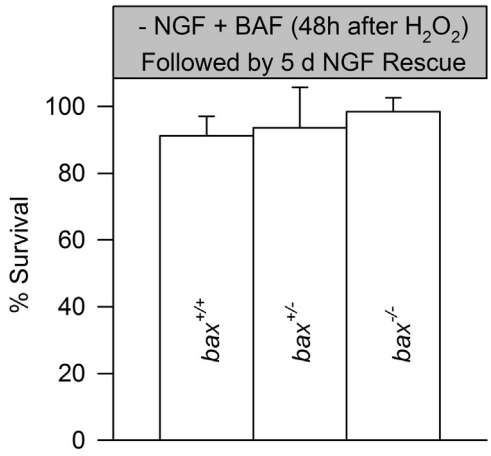

Fig. 5. H_2O_2 treatment did not affect neuronal survival. Neurons of all *bax* genotypes survived treatment with H_2O_2 followed by NGF withdrawal and maintenance in BAF (50 μM)-containing medium. Cultures were exposed for 30 min to medium containing NGF and 2 mM H_2O_2. They were then maintained for 48 h in medium containing no NGF and BAF to prevent death. At the end of this time this medium was replaced with standard culture medium containing NGF. All surviving neurons treated by this rescue paradigm hypertrophy when the experimental medium is replaced with standard culture medium containing NGF [17]. Five days after beginning this treatment, cells were counted by a naïve observer. Percentage of surviving neurons is normalized to the number of neurons counted in sibling cultures maintained continuously in NGF-containing culture medium for the same period. $n=5$–7 cultures.

independent of its effect on depleting cytochrome *c* from the mitochondrial electron transport chain.

Withdrawal of NGF from $bax^{-/-}$ neurons does not cause loss of cytochrome *c* from their mitochondria (Fig. 2d). However, it is possible to induce release of cytochrome *c* from the mitochondria of these cells by exposing them to H_2O_2 [17]. The mechanism underlying this redistribution is not understood. Neurons from $bax^{-/-}$ mice were exposed to 2 mM H_2O_2 for 30 min in culture medium containing NGF. At the end of this time, cultures were washed 2X with similar medium containing an anti-NGF antibody, no NGF, and 50 μM BAF. They were left in the last wash for 48 h. Neurons from $bax^{+/+}$ and $bax^{+/-}$ animals were treated in the same way so that cytochrome *c* and ROS/RS levels could be compared between the three genotypes in cells that had received exactly the same treatments. We next used immunoblotting to determine the amount of cytochrome *c* in these cultures. Neurons from $bax^{+/+}$, $bax^{+/-}$, and $bax^{-/-}$ animals all had the same cytochrome *c* concentration 48 h after this treatment began (~40% of control values; $p > 0.1$; Fig. 4a and b). Therefore, H_2O_2 treatment and NGF deprivation depleted cytochrome *c* to similar levels in neurons of all *bax* genotypes. This treatment did not kill any cells (Fig. 5).

The equivalent depletion of cytochrome *c* in H_2O_2-treated

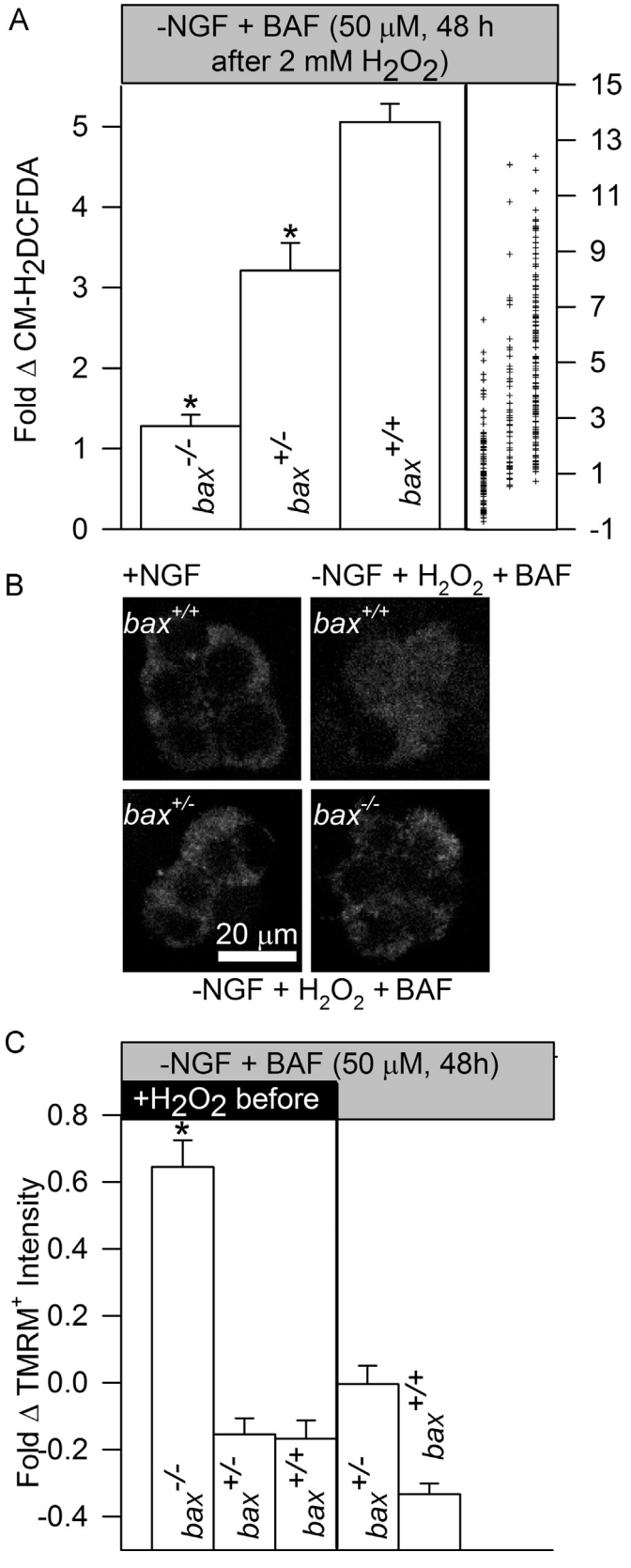

Fig. 6. Bax concentration regulated ROS/RS levels in neurons depleted of cytochrome *c* by H_2O_2 treatment. (a) Left, average CM-H_2DCFDA intensities of H_2O_2-treated neurons. Right, point plot of the single cell data averaged on the left. Cultures were exposed to H_2O_2 (2 mM) for 30 min in medium containing NGF then deprived of NGF and maintained in BAF (50 μM)-containing medium for 48 h. They were then loaded with CM-H_2DCFDA, and confocal microscopic imaging was done at this time. Neurons of each genotype treated in this manner loaded similar amounts of dye as determined by exposing CM–H_2DCFDA-loaded cells to 2 mM H_2O_2 for 30 min (Table 1). Stars mean significantly different from $bax^{+/+}$ cells. $n=54$-143 neurons. (b) Neurons treated with H_2O_2 retained $\Delta\psi_m$. Confocal micrographs showing $TMRM^+$ staining in neurons maintained in NGF and ones treated with 2 mM H_2O_2 for 30 min in NGF-containing medium followed by NGF deprivation and maintenance for 48 h in BAF (50 μM)-containing medium. Note intense punctuate $TMRM^+$ staining in cells with all three genotypes indicating retention of $\Delta\psi_m$ by the mitochondria of each. (c) Average $TMRM^+$ staining increased above baseline values in $bax^{-/-}$ neurons that had been depleted of cytochrome *c* by H_2O_2-treatment. $TMRM^+$ staining decreased in NGF-deprived, BAF-saved $bax^{+/+}$ and $bax^{+/-}$ cells by about the same amount regardless of whether they had been treated with H_2O_2 ($p > 0.1$). Therefore, Bax concentration was positively related to ROS/RS levels but negatively related to $\Delta\psi_m$. Culture conditions were the same as in part a. Star means significantly different from $bax^{+/+}$ cells. $n=75$–233 neurons.

neurons of each of the three *bax* genotypes made it possible for us to compare ROS/RS levels in neurons having the same reduced concentrations of cytochrome *c* along with the full complement of Bax protein, half that amount, or no Bax at all [17]. Fig. 6a shows that Bax concentration had a striking effect on cellular ROS/RS levels in these cells. Neurons with a $bax^{+/+}$ genotype that had been treated for 30 min with 2 mM H_2O_2, then deprived of NGF, and maintained in the presence of BAF (50 μM) for 48 h had about a 5-fold increase in average CM–H_2DCFDA intensity compared to NGF-replete controls. This was about three times higher than the average CM–H_2DCFDA intensity of $bax^{+/+}$ neurons that had not been exposed to H_2O_2 but had been deprived of NGF and maintained in BAF-containing medium for 48 h (Fig. 3; $p < 0.001$). Neurons with reduced *bax* gene dosages exhibited lower ROS levels than did $bax^{+/+}$ neurons after H_2O_2-treatment. Cells from mice with a $bax^{+/-}$ genotype that had been exposed to H_2O_2 and then maintained for 48 h in BAF-containing medium showed about a 3-fold increase of average CM–H_2DCFDA intensity while neurons from $bax^{-/-}$ mice treated in a similar manner exhibited only slightly more than a 1-fold increase ($p < 0.001$ for each *bax* genotype compared to each other). Because cells of each *bax* genotype had identical amounts of cytochrome *c*, these data again suggest that Bax can affect ROS/RS levels independent of Bax-induced cytochrome *c* redistribution.

Production of $O_2^{\cdot -}$ by mitochondria is influenced by $\Delta\psi_m$. Increasing (hyperpolarizing) $\Delta\psi_m$ can increase electron leakage from the electron transport chain and elevate production of $O_2^{\cdot -}$ [27]. Most, or all, of the increased ROS/RS in NGF-deprived sympathetic neurons appear to be mitochondrial-derived and, therefore, are likely downstream products of $O_2^{\cdot -}$ [16,17]. To determine whether Bax might have regulated ROS/RS levels in the H_2O_2-treated neurons by affecting $\Delta\psi_m$, neurons of each of the three *bax* genotypes were exposed to H_2O_2 (2 mM for 30 min) followed by NGF-deprivation and incubation for 48 h in medium containing BAF (50 μM). Relative $\Delta\psi_m$ was determined by exposing cells to $TMRM^+$ (10 nM; Fig. 6b, c). The mitochondria of all three genotypes retained $\Delta\psi_m$ at 48 h after H_2O_2 treatment and NGF removal (Fig. 6b). Punctate $TMRM^+$ staining was observed in 100 ± 0% of $bax^{+/+}$ cells, 98 ± 2% of $bax^{+/-}$ cells, and 100 ± 0% of $bax^{-/-}$ cells (n=73–182 neurons). These data indicate that mitochondria were intact and could generate a proton gradient in the H_2O_2-treated, NGF-deprived neurons.

While higher ROS/RS levels were positively related to Bax concentration in cells treated in this manner, the opposite was true for $\Delta\psi_m$ (Fig. 6c). $TMRM^+$ intensity was highest in the H_2O_2-

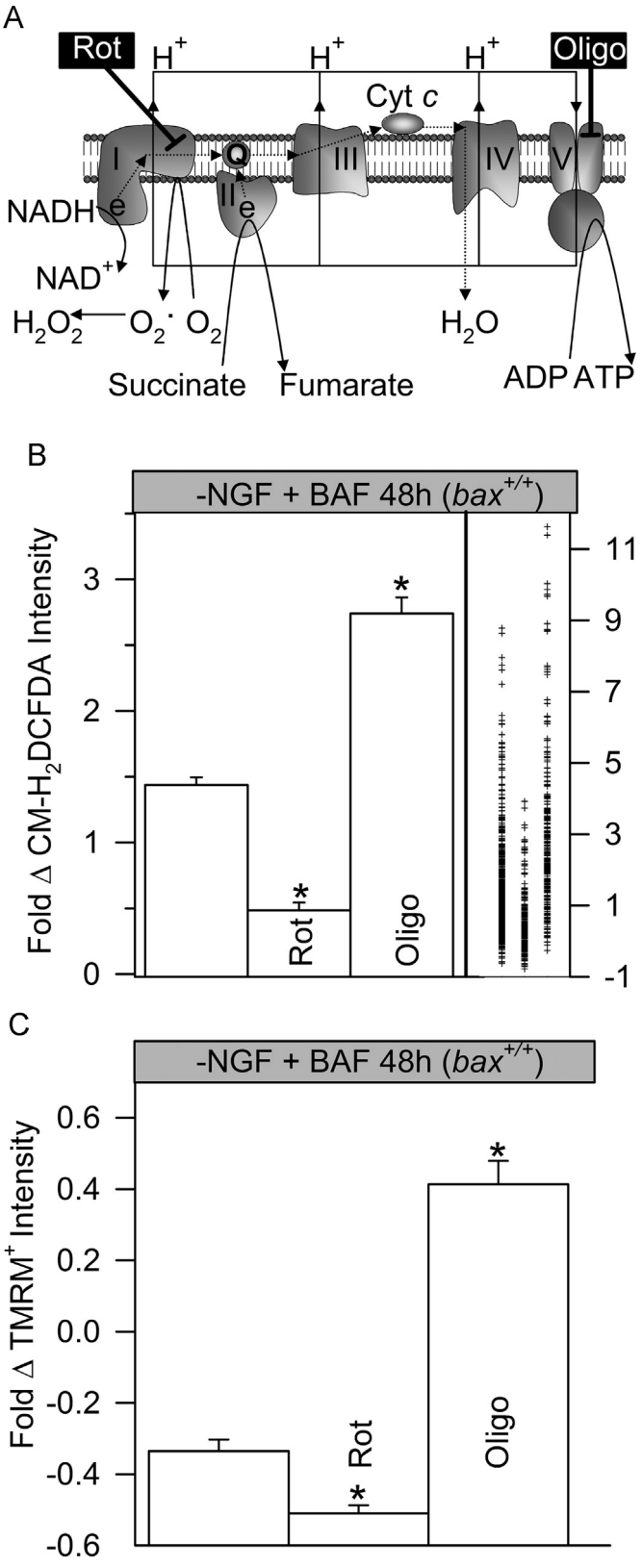

Fig. 7. Forward electron transfer was retained in cytochrome *c*-depleted neurons. (a) The electron transport chain. The mitochondrial matrix is toward the bottom of the page and the intermembrane space toward the top. Solid arrows indicate direction of proton flow when electron transfer is forward. The path of forward electron transfer is indicated by the broken arrows. Rotenone (Rot) blocks electron transfer through complex I to ubiquinone (coenzyme Q). Oligomycin (Oligo) blocks proton flow through complex V ($F_1.F_O$-ATP synthase). Under certain conditions (*e.g.*, loss of most cytochrome *c*) consumption of ATP by complex V produces ADP and reverses the direction of proton movement and, thus, electron transfer. Superoxide is shown being produced at complex I. It can also be produced at complex III (not shown) [26]. (b) The respiratory complex I inhibitor, rotenone (10 μM), decreased ROS/RS in NGF-deprived neurons maintained alive by BAF. Oligomycin (5 μg/ml), increased average CM-H_2DCFDA intensity in NGF-deprived neurons maintained alive for 48 h in BAF-containing medium, indicating that blocking the $F_1.F_O$ -ATP synthase increased ROS/RS levels. Left, averaged data. Right, point plot of raw data from single cells, in the same order. Neurons from $bax^{+/+}$ mice were deprived of NGF for 48 h and maintained alive in BAF (50 μM)-containing medium. They then received the indicated treatments for 20 min during the time of CM-H_2DCFDA loading. Treatments were included in the L15 medium to which cells were exposed during confocal microscopy. Neither rotenone nor oligomycin affected CM-H_2DCFDA loading (Table 1). Stars mean significantly different from cells without rotenone or oligomycin treatment ($p < 0.001$). n=216-580 neurons. (c) Oligomycin (5 μg/ml) increased $\Delta\psi_m$ while rotenone (10 μM) greatly decreased it. Neurons were deprived of NGF and maintained in BAF (50 μM)-containing medium for 48 h. Treatment with oligomycin or rotenone was done during the last 20 min of incubation and also in the L15 medium the cells were exposed to for microscopy. $TMRM^+$ (10 nM) was included during the last 20 min of incubation and was also included in the L15 medium. These, and the above data, indicate that electron transfer remained in a forward direction in cytochrome *c*-depleted neurons. Stars mean significantly different from cells without rotenone or oligomycin treatment ($p < 0.001$). n=116–233 neurons.

treated $bax^{-/-}$ neurons and lowest in the two other genotypes. Indeed, while $TMRM^+$ intensity was below that of control cells in neurons containing Bax, the intensity in the $bax^{-/-}$ neurons was well above the control level. ROS/RS levels in all three cell types were greatly decreased by treating cultures with FCCP (1.6 μM; $p < 0.1$ for each compared to NGF-replete control cells). Thus, ROS/RS production did depend on mitochondria having a $\Delta\psi_m$.

3.3. Forward electron transfer was maintained in cytochrome c-depleted neurons

Electrons derived from the TCA cycle in the mitochondrial matrix normally transfer from mitochondrial respiratory complexes I and II to ubiquinone which then transfers them to complex III. They are then carried to complex IV by cytochrome *c* (Fig. 7a). Under certain conditions, reversal of electron transfer in the mitochondrial electron transport chain can occur [21,27]. Such reversed flow is thermodynamically unfavorable and typically involves consumption rather than production of ATP by the $F_1.F_O$ -ATP synthase. When operating in reverse, the synthase pumps protons out of the mitochondrial matrix driving the electron transport chain in reverse. Reversed flow of protons through the synthase increases $\Delta\psi_m$ rather than decreasing it as does forward flow. Such reversal can be caused by depletion of cytochrome *c* from the chain and can lead to increased ROS/RS by augmenting leakage of electrons near the beginning of the chain, primarily from respiratory complex I [21]. Rotenone blocks electron flow through complex I (Fig. 7a). When forward electron transfer is occurring, rotenone typically increases mitochondrial ROS/RS production by augmenting leakage of NADH-derived electrons upstream from the point of block. Consistent with forward flow in NGF-supported cells, rotenone increased ROS/RS levels (not shown). When reversed flow is taking place, rotenone usually decreases ROS/RS production because the site of leakage is now downstream of the point of rotenone block. NGF-deprived neurons from $bax^{+/+}$ animals that had been maintained alive for 48 h by BAF had elevated ROS/RS levels (Figs. 1a and b; 3a and b). Rotenone greatly decreased ROS/RS in these cells (Fig. 7b; $p < 0.001$ compared to NGF-deprived control), suggesting that reversed electron transfer could be responsible for much of the increased ROS/RS.

To further test the possibility that reversed electron transfer was responsible for elevated ROS /RS in NGF-deprived neurons, we utilized the macrolide antibiotic, oligomycin. Oligomycin blocks proton flow in either direction through the $F_1.F_O$ -ATP synthase. This block increases $\Delta\psi_m$ when electron transfer is forward because the proton gradient cannot be dissipated by the synthase and, as a result, ATP production is blocked. If forward transfer is occurring, oligomycin should increase $\Delta\psi_m$ and, as a result, ROS/RS production. The opposite will happen if electron transfer is reversed as the proton gradient across the IMM is maintained by the synthase and is dissipated when it is blocked. Oligomycin used at a concentration (5 μg/ml) known to block most, or all, mitochondrial ATP production in these cells [33] greatly increased CM–H_2DCFDA intensity in NGF-deprived $bax^{+/+}$ neurons that had been incubated in BAF-containing medium for 48 h (Fig. 7b). Utilizing a bioluminescent ATP assay (Sigma) we found that this concentration of oligomycin decreased ATP levels in these cells by $26 \pm 14\%$ ($p < 0.009$). The non-oligomycin-sensitive ATP derives from glycolysis, the primary ATP source in NGF-deprived sympathetic neurons [33]. These findings suggest that forward transfer of electrons was sustained in these cells.

To further clarify the issue of whether forward or reversed electron transfer occurs in these neurons we determined the effects of oligomycin and rotenone on $\Delta\psi_m$. Fig. 7c shows that oligomycin increased $\Delta\psi_m$ in $bax^{+/+}$ cells deprived of NGF and maintained in BAF-containing medium for 48 h. Rotenone decreased $TMRM^+$ fluorescence intensity to very low levels in these cells, indicating decreased $\Delta\psi_m$. The increase of $\Delta\psi_m$ by oligomycin treatment and the decrease by rotenone treatment cannot be explained by reversed electron transfer. In light of these findings, the most parsimonious interpretation of the rotenone block is that most electrons contributing to the ROS/RS enter the chain at respiratory complex I and leak from the chain downstream of the

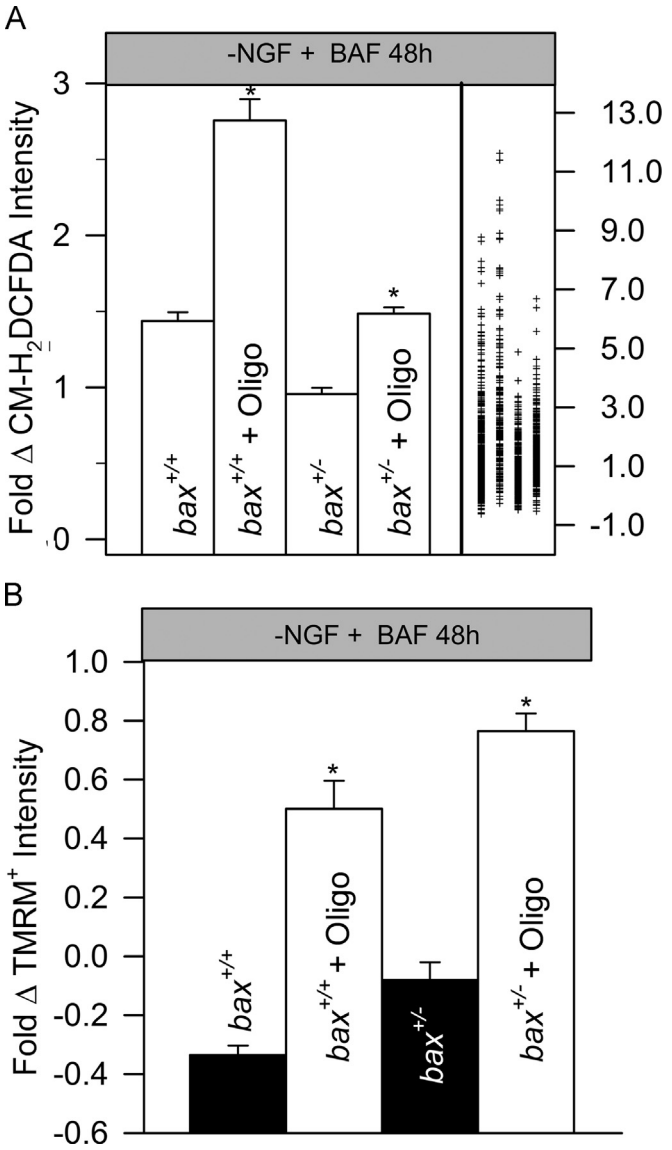

Fig. 8. Higher Bax concentration caused higher ROS/RS levels but lower $\Delta\psi_m$ in oligomycin-treated, cytochrome *c*-depleted neurons. a Oligomycin (5 μg/ml) increased CM-H_2DCFDA intensity more in cytochrome *c*-depleted $bax^{+/+}$ neurons than in $bax^{+/-}$ neurons ($p < 0.001$). Neurons were deprived of NGF and maintained alive for 48 h in BAF (50 μM)-containing medium. They then were treated with oligomycin (5 μg/ml) for 20 min during the time of CM-H_2DCFDA loading. Oligomycin was included in the L15 medium that cells were exposed to during confocal microscopy. Right is a point-plot of the data from individual cells averaged in the bar graphs. The order is the same. Stars mean significantly different from the same cell type without oligomycin treatment ($p < 0.001$). $n = 283–587$ neurons. b Oligomycin (5 μg/ml) increased $\Delta\psi_m$ more in NGF-deprived, BAF-saved $bax^{+/-}$ neurons than in neurons with a $bax^{+/+}$ genotype. NGF-deprived neurons were maintained in BAF (50 μM)-containing medium for 48 h. Treatment with oligomycin was done during the last 20 min of incubation and also in the L15 medium the cells were exposed to for microscopy. $TMRM^+$ (10 nM) was included during the last 20 min of incubation and was also included in the L15 medium. Stars mean significantly different from the same cell type without oligomycin treatment ($p < 0.001$). $n = 75–233$ neurons.

point of rotenone block.

3.4. Bax positively regulated ROS/RS but negatively regulated $\Delta\psi_m$ in oligomycin-treated neurons

To determine whether Bax regulates ROS/RS levels when the F_1. F_O -ATP synthase is blocked, we withdrew NGF from neurons taken from $bax^{+/+}$ and $bax^{+/-}$ animals and maintained them alive for 48 h with BAF (50 μM) before treating them with oligomycin (5 μg/ml). This treatment increased CM–H_2DCFDA intensity in both the $bax^{+/+}$ and $bax^{+/-}$ neurons compared to cells not treated with oligomycin (Fig. 8a). The intensity in the $bax^{+/+}$ neurons was about twice that in the $bax^{+/-}$ cells indicating that, under conditions of synthase block, higher Bax concentration caused higher ROS/RS levels.

Fig. 8b shows that oligomycin increased $\Delta\psi_m$ in both $bax^{+/+}$ and $bax^{+/-}$ neurons that were deprived of NGF and maintained for 48 h in BAF (50 μM)-containing medium. $\Delta\psi_m$ was greater in the oligomycin-treated $bax^{+/-}$ neurons than in oligomycin-treated $bax^{+/+}$ neurons.

4. Discussion

We conducted a study of the effects Bax on ROS/RS levels in NGF-deprived mouse sympathetic neurons in cell culture. As previously reported, ROS/RS levels were elevated in wild-type cells by 12 h after NGF withdrawal and continued to increase with time after deprivation [17]. Almost all ROS/RS were blocked by a protonophore at a concentration that eliminated $\Delta\psi_m$, indicating that the ROS/RS derived from electrons that leaked from the mitochondrial electron transport chain.

Elimination of Bax almost completely blocks elevation of ROS/RS in mouse sympathetic neurons after NGF deprivation [5,17]. A substantial portion of the increased ROS/RS after withdrawal is likely due to Bax/cytochrome *c*-dependent activation of caspases that then cleave mitochondrial respiratory complexes and cause increased leakage of electrons from the mitochondrial electron transport chain to molecular oxygen. Consistent with this mechanism, our previous work demonstrates that most of the ROS/RS increase in NGF-deprived cells lies downstream of caspase 3 activity [5]. Another possible source of the increased ROS/RS is Bax-induced depletion of cytochrome *c* from the electron transport chain. This depletion could increase ROS/RS by augmenting leakage of electrons from the chain [20]. To determine whether any of the increased ROS/RS was caused by release of cytochrome *c* from mitochondria, we depleted $bax^{+/+}$ and $bax^{+/-}$ neurons of cytochrome *c* by depriving them of NGF and maintaining them in medium containing the broad-spectrum caspase inhibitor BAF at a concentration that prevents cell death and suppresses ROS/RS levels [5]. By 48 h after withdrawal, the cytochrome *c* stores of neurons of both genotypes were reduced by equivalent amounts (~60%). $bax^{+/+}$ neurons, which have about twice the Bax protein concentration of $bax^{+/-}$ cells [17], had higher ROS/RS levels than did the latter cells. Cytochrome *c* was depleted in $bax^{-/-}$ neurons by treatment with H_2O_2, followed by NGF deprivation and maintenance in media containing BAF. Cells with the other two genotypes received similar treatment. All three *bax* genotypes retained~40% of their cytochrome *c* stores 48 h after treatment began. The H_2O_2 did not kill any cells, nor did it eliminate $\Delta\psi_m$. Both $bax^{+/+}$ and $bax^{+/-}$ neurons treated with H_2O_2, followed by maintenance in media lacking NGF and containing caspase inhibitor had higher ROS/RS levels than similar cells that had been deprived of NGF and exposed to caspase inhibitor for 48 h without prior H_2O_2 treatment. Possible explanations for this finding are that the H_2O_2 increased leakage of electrons to form ROS/RS by damaging respiratory complexes or that cellular antioxidant defenses were compromised. While ROS/RS levels were positively affected by Bax concentration, the opposite was true for $\Delta\psi_m$. Higher Bax concentrations were associated with lower (depolarized) $\Delta\psi_m$ in NGF-deprived neurons. This is a counterintuitive finding as higher (hyperpolarized) $\Delta\psi_m$ can provide more driving force for ROS/RS production. It is possible that the elevated ROS/RS levels in the neurons with higher Bax concentrations damaged mitochondria and resulted in fewer protons being pumped out. Regardless of the explanation, the data suggest that Bax concentration positively affects ROS/RS levels in NGF-deprived mouse sympathetic neurons largely independent of depletion of cytochrome *c* from the mitochondrial electron transport chain. They also demonstrate that the effect of Bax is not mediated through elevation of $\Delta\psi_m$.

The data presented here cannot completely exclude a role for depletion of cytochrome *c* from the electron transport chain in causing some of the increased ROS/RS in NGF-deprived sympathetic neurons. The cytochrome *c* stores in these cells can be separated into two pools based on mobility. One pool appears to be easily released when Bax associates with the OMM. The other pool (~40% of control) is not easily released, perhaps because it is bound to the inner mitochondrial membrane (IMM) or sequestered in cristae [34,35]. It is possible that this pool is competent to carry all electrons from respiratory complexes III to IV and that little increased electron leakage and ROS/RS occurs in NGF-deprived cells due to reduced cytochrome *c* levels. Consistent with cytochrome *c* depletion having little influence on increased ROS/RS in NGF-deprived cells our previous work shows a dose-dependent effect of caspase 3 on ROS/RS production [5]. Deletion of caspase 3 almost, but not completely (~92% reduction), prevented increased ROS/RS after NGF deprivation while caspase $3^{+/-}$ cells had reduced ROS/RS levels compared to wild-type neurons. All three cell types release similar amounts of cytochrome *c* from their mitochondria. The small remaining ROS/RS in the caspase $3^{-/-}$ cells could, ostensibly, be caused by depletion of cytochrome c from mitochondria. However, it could just as easily be caused by a caspase-independent effect of Bax on the mitochondria. Our present and previous findings cannot distinguish between these two possibilities.

The increased ROS/RS and $\Delta\psi_m$ caused by oligomycin treatment of NGF-deprived, cytochrome *c*-depleted neurons indicates that electron transfer remained in a forward direction (*i.e*, from complexes I and II to III and then IV) in their mitochondria. Preservation of forward flow was likely due to the substantial pool of cytochrome *c* remaining in these cells. Exact location of the site of electron leakage cannot be determined from the data presented here. However, the complex I inhibitor, rotenone, greatly decreased ROS/RS in these cells. Therefore, the site of leakage must be downstream of this location. The likely explanation is that most of the electrons that generated the ROS/RS derived from NADH at complex I and rotenone prevented them from transferring to the downstream leakage site. Moreover, rotenone depolarized $\Delta\psi_m$ in these cells to about the same degree as uncoupling mitochondrial respiration with a protonophore, suggesting that most electrons in the transport chain entered at complex I (Fig. 7a).

Other possible mechanisms by which Bax might regulate ROS/RS production include restriction of diffusion of compounds across the OMM that are required for respiration [36], effects of Bax on leakage of electrons from respiratory complexes, or effects of Bax on cellular antioxidant status. However, in light of the findings reported here and elsewhere [5], these mechanisms seem unlikely. Early work with the Bcl-2 family suggested that these proteins alter cellular antioxidant status [37,38]. Later work revealed that the antioxidant effect is likely due to cellular compensation for alterations in mitochondrial ROS/RS production caused by these

proteins [39]. There are no obvious effects of *bax* gene dosage on the ability of mouse sympathetic neurons to detoxify ROS/RS in either NGF-replete or -depleted cells [5,10,17]. Therefore, it is unlikely that any of the results reported here can be explained by Bax-mediated alterations in cellular antioxidant capabilities. Rather, the effect of Bax appears to be on mitochondrial ROS/RS production.

In conclusion, the findings reported here along with our previous findings [5] suggest that Bax regulates ROS/RS levels in NGF-deprived sympathetic neurons by augmenting mitochondrial ROS/RS production almost entirely via caspase activation and that depletion of cytochrome *c* from the electron transport chain contributes little or nothing to the increased ROS/RS.

Conflicts of interest

None.

Acknowledgments

This work was supported by grant from the National Institutes of Health (NS37110).

References

[1] R.W. Oppenheim, Cell death during development of the nervous system, Ann. Rev. Neurosci. 14 (1991) 453–501.
[2] D.O. Martin, R.E. Schmidt, P.S. DiStefano, O.H. Lowry, J.G. Carter, E.M. Johnson Jr., Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation, J. Cell Biol. 106 (1988) 829–844.
[3] M. Deshmukh, K. Kuida, E.M. Johnson Jr., Caspase inhibition extends the commitment to neuronal death beyond cytochrome *c* release to the point of mitochondrial depolarization, J. Cell Biol. 150 (2000) 131–144.
[4] G.V. Putcha, M. Deshmukh, E.M. Johnson Jr., Inhibition of apoptotic signaling cascades causes loss of trophic factor dependence during neuronal maturation, J. Cell Biol. 149 (2000) 1011–1018.
[5] R.A. Kirkland, G.M. Saavedra, B.S. Cummings, J.L. Franklin, Bax Regulates Production of superoxide in both apoptotic and nonapoptotic neurons: role of caspases, J. Neurosci. 30 (2010) 16114–16127.
[6] T.L. Deckwerth, J.L. Elliot, C.M. Knudson, E.M. Johnson Jr., W.D. Snider, S. J. Korsmeyer, Bax is required for neuronal death after trophic factor deprivation and during development, Neuron 17 (1996) 401–411.
[7] G.V. Putcha, C.A. Harris, K.L. Moulder, R.M. Easton, C.B. Thompson, E. M. Johnson Jr., Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice, J. Cell Biol., 157, (2002) 441–453.
[8] K.G. Wolter, Y.T. Hsu, C.L. Smith, A. Nechushtan, X.G. Xi, R.J. Youle, Movement of Bax from the cytosol to mitochondria during apoptosis, J. Cell Biol. 139 (1997) 1281–1292.
[9] G.V. Putcha, M. Deshmukh, E.M. Johnson Jr., Bax translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, Bcl-2, and caspases, J. Neurosci. 19 (1999) 7476–7485.
[10] R.A. Kirkland, G.M. Saavedra, J.L. Franklin, Rapid activation of antioxidant defenses by nerve growth factor suppresses reactive oxygen species during neuronal apoptosis: evidence for a role in cytochrome *c* redistribution, J. Neurosci. 27 (2007) 11315–11326.
[11] P. Li, D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, X. Wang, Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade, Cell 91 (1997) 479–489.
[12] N.N. Danial, S.J. Korsmeyer, Cell Death: Critical Control Points, Cell 116 (2004) 205–219.
[13] S.W.G. Tait, D.R. Green, Mitochondria and cell death: outer membrane permeabilization and beyond, Nat. Rev. Mol. Cell Biol. 11 (2010) 621–632.
[14] L.J.S. Greenlund, T.L. Deckwerth, E.M. Johnson Jr., Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death, Neuron 14 (1995) 303–315.
[15] L.L. Dugan, D.J. Creedon, E.M. Johnson Jr., D.M. Holtzman, Rapid suppression of free radical formation by nerve growth factor involves the mitogen-activated protein kinase pathway, Proc. Natl. Acad. Sci. 94 (1997) 4086–4091 , USA.
[16] R.A. Kirkland, J.L. Franklin, Evidence for redox regulation of cytochrome c release during programmed neuronal death: antioxidant effects of protein synthesis and caspase inhibition, J. Neurosci. 21 (2001) 1949–1963.
[17] R.A. Kirkland, J.A. Windelborn, J.M. Kasprzak, J.L. Franklin, A Bax-induced pro-oxidant state is critical for cytochrome c release during programmed neuronal death, J. Neurosci. 22 (2002) 6480–6490.
[18] J.E. Ricci, C. Munoz-Pinedo, P. Fitzgerald, B. Bailly-Maitre, G.A. Perkins, N. Yadava, I.E. Scheffler, M.H. Ellisman, D.R. Green, Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain, Cell 117 (2004) 773–786.
[19] J.E. Ricci, R.A. Gottlieb, D.R. Green, Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis, J. Cell Biol. 160 (2003) 65–75.
[20] J. Cai, D.P. Jones, Superoxide in apoptosis: mitochondrial generation triggered by cytochrome c loss, J. Biol. Chem. 273 (1998) 11401–11404.
[21] A.A. Starkov, B.M. Polster, G. Fiskum, Regulation of hydrogen peroxide production by brain mitochondria by calcium and Bax, J. Neurochem. 83 (2002) 220–228.
[22] C. Garcia-Perez, S.S. Roy, S. Naghdi, X. Lin, E. Davies, G. Hajnóczky, , Bid-induced mitochondrial membrane permeabilization waves propagated by local reactive oxygen species (ROS) signaling, Proc. Natl. Acad. Sci. USA 10 (2011) 4497–4502.
[23] C.M. Knudson, K.S.K. Tung, W.G. Tourtelotte, G.A.J. Brown, S.J. Korsmeyer, Bax-deficient mice with lymphoid hyperplasia and male germ cell death, Science 270 (1995) 96–99.
[24] J.A. Royall, H. Ischiropoulos, Evaluation of 2′, 7′-dichlorofluorescin and dihydrorhodamine123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells, Arch. Biochem. Biophys. 302 (1993) 348–355.
[25] D.G. Nicholls, S.L. Budd, Mitochondria and neuronal survival, Physiol. Rev. 80 (2000) 315–360.
[26] D.G. Nicholls, M.W. Ward, Mitochondrial membrane potential and cell death: mortality and millivolts, Trends Neurosci. 23 (2000) 166–174.
[27] D.G. Nicholls, S.J. Ferguson, Bioenergetics 3, Academic Press, London, 2002.
[28] B. Halliwell, J.M.C. Gutteridge, Free radicals in biology and medicine, fourth ed., Clarendon Press, Oxford, 2007.
[29] S. Tan, Y. Sagara, Y. Liu, P. Maher, D. Schubert, The regulation of reactive oxygen species production during programmed cell death, J. Cell Biol. 141 (1998) 1423–1432.
[30] F.A. White, C.R. Keller-Peck, C.M. Knudson, S.J. Korsmeyer, W.D. Snider, Widespread elimination of naturally occurring neuronal death in Bax-deficient mice, J. Neurosci. 18 (1998) 1428–1439.
[31] J.C. Goldstein, C. Muñoz-Pinedo, J.E. Ricci, S.R. Adams, A. Kelekar, M. Schuler, R. Y. Tsien, D.R. Green, Cytochrome *c* is released in a single step during apoptosis, Cell Death Differ. 12 (2005) 453–462.
[32] L. Xie, R.S. Johnson, R.S. Freeman, Inhibition of NGF deprivation-induced death by low oxygen involves suppression of BIM_{EL} and activation of HIF-1, J. Cell Biol. 168 (2005) 911–920.
[33] L.K. Chang, R.E. Schmidt, E.M. Johnson Jr., Alternating metabolic pathways in NGF deprived sympathetic neurons affect caspase-independent death, J. Cell Biol. 162 (2003) 245–256.
[34] M. Ott, J.D. Robertson, V. Gogvadze, B. Zhivotovsky, S. Orrenius, Cytochrome *c* release from mitochondria proceeds by a two-step process, Proc. Natl. Acad. Sci. USA 99 (2002) 1259–1263.
[35] E. Gottlieb, S.M. Armour, M.H. Harris, C.B. Thompson, Mitochondrial membrane potential regulates matrix configuration and cytochrome *c* release during apoptosis, Cell Death. Differ. 10 (2003) 709–717.
[36] M.H. Harris, C.B. Thompson, The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability, Cell Death. Differ. 7 (2000) 1182–1191.
[37] D.M. Hockenbery, Z.N. Oltvai, X.M. Yin, C.L. Milliman, S.J. Korsmeyer, Bcl-2 functions in an antioxidant pathway to prevent apoptosis, Cell 75 (1993) 241–251.
[38] L.M. Ellerby, H.M. Ellerby, S.M. Park, A.L. Holleran, A.N. Murphy, G. Fiskum, D. J. Kane, M.P. Testa, C. Kayalar, D.E. Bredesen, Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2, J. Neurochem. 67 (1996) 1259–1267.
[39] A.J. Kowaltowski, R.G. Fenton, G. Fiskum, Bcl-2 family proteins regulate mitochondrial reactive oxygen production and protect against oxidative stress, Free Radic. Biol. Med. 37 (2004) 1845–1853.