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BAX Is Required for Neuronal Death after Trophic Factor Deprivation and during Development

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Summary

Members of the BCL2-related family of proteins either promote or repress programmed cell death. BAX, a death-promoting member, heterodimerizes with multiple death-repressing molecules, suggesting that it could prove critical to cell death. We tested whether *Bax* is required for neuronal death by trophic factor deprivation and during development. Neonatal sympathetic neurons and facial motor neurons from *Bax*-deficient mice survived nerve growth factor deprivation and disconnection from their targets by axotomy, respectively. These salvaged neurons displayed remarkable soma atrophy and reduced elaboration of neurites; yet they responded to readdition of trophic factor with soma hypertrophy and enhanced neurite outgrowth. *Bax*-deficient superior cervical ganglia and facial nuclei possessed increased numbers of neurons. Our observations demonstrate that trophic factor deprivation–induced death of sympathetic and motor neurons depends on *Bax*.

Introduction

Extensive programmed cell death occurs during the development and the maintenance of tissues of multicellular organisms (reviewed by Glücksmann, 1951). Early insights into the regulation of programmed cell death arose from the analysis of the molecular mechanisms underlying follicular B-cell lymphoma and genetic studies of the stereotypic cell deaths in nematode development. This led to the identification of homologous potent death repressors including the human *Bcl2* (Tsujimoto et al., 1985; Bakhshi et al., 1985; Cleary and Sklar, 1985) and the nematode *Ced9* gene (Hengartner and Horvitz, 1994). The *Bcl2* family of genes now consists of both apoptosis-promoting molecules (e.g., *Bax, Bad, Bak, Bclx*$_S$) and antagonists (e.g., *Bcl2, Bclx*$_L$*, Mcl1, Ced9*) whose gene products have been implicated as regulators of death in many cell types (Oltvai et al., 1993; Boise et al., 1993; Kozopas et al., 1993; Yang et al., 1995; Farrow et al., 1995; Chittenden et al., 1995a; Kiefer et al., 1995). These proteins function, at least in part, via competing protein interactions. Several conserved domains termed BH1, BH2, and BH3 are required for the formation of various heterodimers as well as for the regulation of cell death (Yin et al., 1994; Chittenden et al., 1995b). The apoptosis-promoting protein BAX displays a broad capability to form heterodimers with multiple members (BCL2, BCLx$_L$, MCL1, A1) (Sato et al., 1994; Sedlak et al., 1995), and all of these genes can be coexpressed in a single cell type (Merry et al., 1994; Krajewski et al., 1994; Gonzalez-Garcia et al., 1994; Greenlund et al., 1995b). Overexpression of BAX counters the protection afforded by antagonistic members such as BCL2 and BCLx$_L$ and promotes apoptosis. One model (the rheostat model) consistent with such data holds that a critical level of BAX homodimers activates downstream death effector molecules and that antagonists such as BCL2 prevent death by inactivating BAX through heterodimerization (Oltvai et al., 1993; Oltvai and Korsmeyer, 1994). In addition, there may exist a survival-promoting pathway that can be activated by BCLx$_L$ without requiring heterodimerization with BAX (Cheng et al., 1996). However, none of these studies established clearly whether the death agonist or antagonist molecules are dominant in determining cellular viability after a death-inducing stimulus. Thus, it was uncertain whether BAX could prove of singular importance in mediating an execution signal in a selected cell type.

Neuronal cell death plays a prominent role during the normal development of the vertebrate nervous system (reviewed by Oppenheim, 1991). At least half of the neurons that are generated in many populations die during specific developmental periods. This neuronal death is largely controlled by the availability of target-derived trophic factors to the distal nerve endings, once the neurons have become trophic factor–dependent and extend axons into their target areas. The mechanisms underlying neuronal death after trophic factor deprivation have been studied extensively in the sympathetic nervous system and in motor neurons. Neonatal sympathetic neurons depend on nerve growth factor (NGF) for survival (Levi-Montalcini and Booker, 1960; Gorin and Johnson, 1979; Crowley et al., 1994). In the absence of NGF, the death-inducing stimulus, sympathetic neurons die by apoptosis (Deckwerth and Johnson, 1993; Batistatou and Greene, 1993; Edwards and Tolkovsky, 1994). Neuronal death by trophic factor deprivation has been shown to be modulated by the levels of BCL2 family members. BCL2, BAX, and BCLx$_L$ are expressed in NGF-maintained neurons in vitro (Greenlund et al., 1995b) and in vivo (Krajewski et al., 1994; Miyashita et al., 1994). Overexpression of BCL2 (Garcia et al., 1992; Borner et al., 1994; Greenlund et al., 1995a) or BCLx$_L$ (Frankowski et al., 1995) by microinjection of expression vectors into sympathetic neurons retarded the kinetics of neuronal death after NGF withdrawal. Conversely, BCLx$_S$ reduced the neuroprotection afforded by BCL2 (Martinou et al., 1995) and BAK accelerated neuronal death (Farrow et al., 1995), consistent with their respective roles as death agonists and antagonists.

For motor neurons, deprivation of target-derived growth factors produced by peripheral nerve axotomy rapidly leads to death in the postnatal period unless exogenous growth factors are supplied (reviewed by

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Snider et al., 1992). In this experimental paradigm, as well as during the period of developmental cell death, motor neuron survival is modulated by BCL2. Murine facial motor neurons overexpressing BCL2 survived the period of naturally occurring neuronal death in larger numbers than control neurons (Martinou et al., 1994) and resisted degeneration after disconnection from their source of trophic support after axotomy (Dubois-Dauphin et al., 1994). Likewise, spinal motor neurons were partially protected from both insults by elevated levels of BCL2 (Farlie et al., 1995). These gain-of-function studies with *Bcl2* family members have documented that the common pathway of programmed cell death is involved in these neuronal deaths.

Because of the capacity for multiple interactions among BCL2 family members, overexpression studies cannot assure the singular importance of a particular member. The role of the death-repressing proteins $BCLx_L$ and BCL2 in neuronal development has been examined in mice in which these genes were inactivated by targeted deletion. These loss-of-function studies identified $BCLx_L$ as a critical positive regulator of neuronal survival at early developmental stages for sensory neurons and CNS neurons that were differentiating after having migrated out of the ventricular mitotic zone (Motoyama et al., 1995). Mice deficient in *Bcl2*, however, showed few overt developmental neuronal abnormalities, suggesting that BCL2 plays less of a role in the development of the nervous system (Veis et al., 1993; Nakayama et al., 1994; Kamada et al., 1995). No studies to date have addressed whether death-promoting BCL2 family members critically regulate survival of neurons.

In this study, we utilize *Bax*-deficient (*Bax* −/−) mice, the first knockout of a death-promoting family member (Knudson et al., 1995), to assess the role of BAX in neuronal cell death. *Bax* −/− mice display hyperplasia of selected non-neuronal lineages, including lymphocytes and ovarian granulosa cells, and demonstrate an unusual testicular degeneration. Yet, the *Bax* −/− lymphocytes had no definable altered resistance to several death stimuli (Knudson et al., 1995). In contrast, we find that *Bax* −/− sympathetic neurons are independent of NGF for survival and that neonatal motor neurons survive disconnection from their targets by axotomy. The trophic factor–independent neurons show reduced neurite outgrowth and have atrophic somas. Yet, they respond to addition of trophic factor with enhanced neurite outgrowth and soma hypertrophy. Furthermore, developmental sympathetic and motor neuronal death is reduced in *Bax* −/− mice. Thus, BAX is required for neuronal death after deprivation of neurotrophic factors.

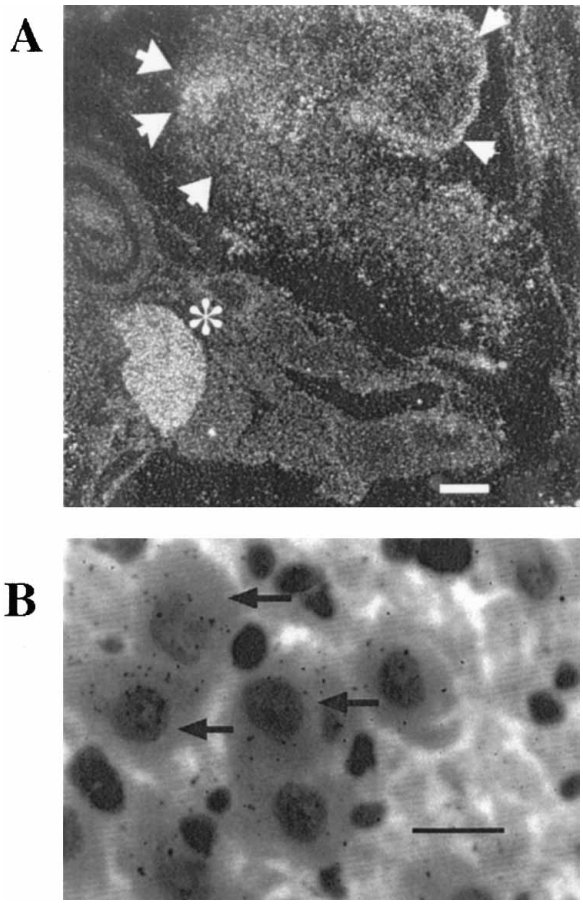

Figure 1. *Bax* mRNA Expression in Sympathetic and Facial Motor Neurons of Wild-Type Mice during the Period of Survival Dependence on Target-Derived Neurotrophic Factors

(A) Darkfield photomicrograph of a parasagittal section through brainstem and neck of a newborn wild-type mouse demonstrates *Bax* expression in the sympathetic SCG (asterisk), the ventral pons at the level of the trigeminal motor nucleus (large arrowheads), and the external germinal layer of the cerebellum (small arrowheads). Scale bar, 200 μm.

(B) Brightfield high power photomicrograph of *Bax* mRNA expression in motor neurons of the facial nucleus of P4 wild-type mice. The large neurons with abundant eosinophilic cytoplasm (arrows) are motor neurons. Silver grains are present over motor neurons but not over the surrounding glia. Scale bar, 25 μm.

Results

Bax mRNA Levels Are High in Sympathetic and Facial Motor Neurons

BAX is expressed in a temporal and spatial pattern compatible with it having a role in regulating neuronal survival during development. Wild-type (*Bax* +/+) neonatal sympathetic superior cervical ganglion (SCG) neurons (Figure 1A, asterisk) and facial motor neurons (Figure 1B, arrows) express *Bax* mRNA at a time when these neuronal populations are susceptible to growth factor deprivation. Besides being expressed in these two neuronal types, *Bax* expression is also prominent in the pons at the level of the trigeminal motor nucleus and in the external germinal layer of the developing cerebellum (Figure 1), among others, suggesting functions in the CNS beyond motor neurons.

Survival of *Bax* −/− Sympathetic Neurons Is Trophic Factor–Independent

The role of BAX in neuronal death by trophic factor deprivation was examined in a severe experimental model. Neonatal sympathetic neurons from SCGs of *Bax* −/−, *Bax* +/−, and *Bax* +/+ mice were enzymatically and mechanically dissociated into single cells, and plated on collagen in medium containing neutralizing

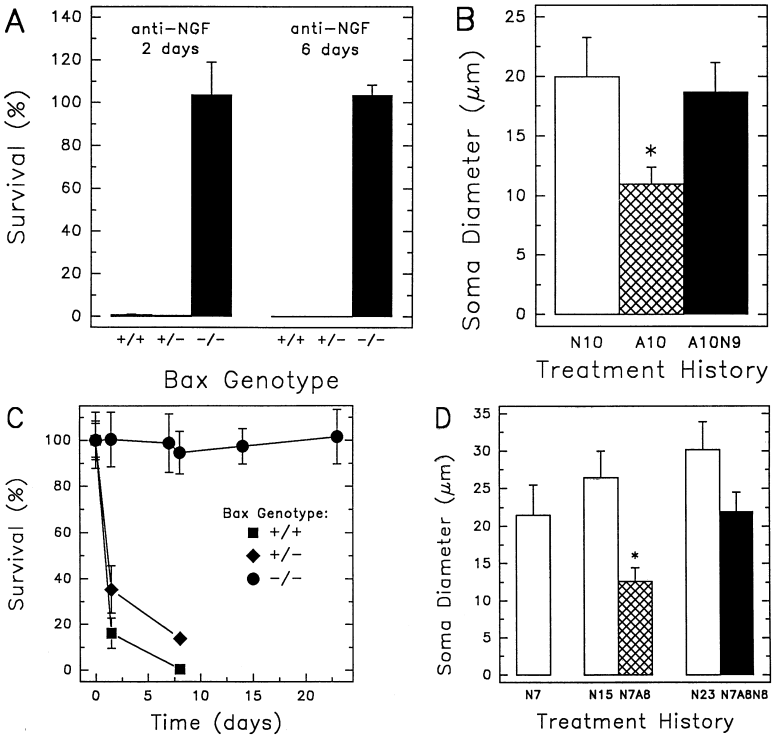

Figure 2. *Bax* −/− Sympathetic Neurons Are NGF-Independent for Survival

(A) Survival of freshly plated wild-type (+/+), heterozygous (+/−), and *Bax*-deficient (−/−) sympathetic neurons exposed to anti-NGF antiserum for 2 or 6 days followed by exposure to NGF for 9 days. The number of neurons was normalized to the mean number of living neurons in cultures from the same animal maintained with NGF for 13 days (1000–4600 neurons) and is indicated on the ordinate. Only *Bax* −/− neurons survive plating in the absence of NGF.

(B) Soma diameters of NGF-maintained and NGF-deprived *Bax* −/− sympathetic neurons. Sympathetic neurons plated in the absence of NGF for 10 days (A10, n = 41) have smaller somas than neurons maintained in NGF for 10 days (N10, n = 35), and somas enlarge when exposed to NGF for 9 days following the period of NGF deprivation (A10N9, n = 43). The difference in soma size between A10 and the other two conditions is statistically significant (* $p < 0.0001$, two-sided Mann Whitney U test).

(C) Survival of *Bax* −/−, *Bax* +/−, and wild-type sympathetic neurons cultured for 7 days in the presence of NGF and thereafter deprived of NGF for 35 hr to 23 days as indicated on the abscissa. Survival after 35 hr and 8 days is normalized to the survival of NGF-maintained 8-day-old cultures from the same animal. Survival after 7, 14, and 23 days is normalized to the survival in NGF-maintained 13-day-old cultures from the same animal. The NGF-maintained cultures contain between 1400 and 4700 neurons. *Bax* −/− neurons survive in the absence of NGF while *Bax*-expressing neurons die rapidly and show a distinct *Bax* gene dosage effect.

(D) Soma diameters of *Bax* −/− sympathetic neurons maintained by NGF for 7 (N7), 15 (N15), or 23 (N23) days, plated in the presence of NGF for 7 days and followed by 8 days of NGF deprivation (N7A8), or treated as N7A8 and then switched back to NGF for 8 days (N7A8N8). NGF deprivation (N7A8) induces soma atrophy, which is reversed upon readdition of NGF (N7A8N8) (mean ± SD, * $p < 0.0001$ compared with all other conditions, two-sided Mann Whitney U test). A total number of 16, 29, 25, 33, and 38 somas was measured for conditions N7, N15, N23, N7A8, and N7A8N8, respectively.

anti-NGF antibodies. Neonatal SCG neurons are normally exquisitely NGF-dependent for survival, and virtually all wild-type neurons die within 2 days in this paradigm. After 2 days, or an even more stringent 6 days, of NGF deprivation, the cultures were exposed to NGF-containing medium and living neurons counted 9 days later. This paradigm assesses whether the neurons remain alive during the period of NGF deprivation and whether they are capable of long-term survival after addition of NGF. Cultures from the same animal continuously exposed to NGF for 13 days from the time of plating were used as control for neuronal survival mediated by NGF. All neuronal numbers are presented normalized to the mean number of neurons in these control cultures. While all BAX-expressing neurons had died within 2 days in the absence of NGF, *Bax* −/− neurons survived NGF deprivation for 2 and 6 days and remained alive for 9 additional days after NGF was added (Figure 2A). Therefore, we conclude that *Bax* −/− neurons do not require NGF for survival. *Bax* +/− neurons showed no survival after 2 days of NGF deprivation, indicating that even a reduced gene dose of *Bax* can induce programmed cell death in neurons. However, the *Bax* gene dose does affect the kinetics of death (see below).

Although all neurons survived, the morphology of NGF-deprived *Bax* −/− neurons (Figure 3B) differed from that of NGF-maintained neurons (Figure 3A). NGF-deprived neurons had markedly smaller somas, which were only about half the diameter after 10 days of NGF deprivation compared with NGF-maintained neurons (see Figure 2B). Remarkably, however, the *Bax* −/− neurons grew neurites in the absence of NGF (Figure 3B). When examined by phase contrast microscopy or immunofluorescence with an anti-neurofilament M antibody, the extent of neurite outgrowth from NGF-deprived neurons was similar to that of NGF-maintained neurons after 1 day (data not shown). Thereafter, neurite outgrowth in the absence of NGF continued but was markedly less extensive than in the presence of NGF (Figures 3A and 3B). Most importantly, this atrophic appearance was fully reversible when NGF was added to NGF-deprived neurons. After exposure to NGF for 9 days, the soma diameter (see Figures 2B and 3D) and neurite outgrowth (Figure 3D) increased dramatically. These experiments demonstrate that NGF-deprived neurons prevented from dying by *Bax* deficiency retain a basic functional metabolism capable of producing NGF-independent neurite outgrowth and are able to respond to addition of NGF with typical trophic responses such as soma hypertrophy and enhanced neurite growth.

To examine whether there was a gene dosage effect of *Bax* on the kinetics of death, we examined sympathetic neuronal death in a different experimental paradigm, in which the kinetics of many molecular events underlying programmed cell death have been studied (Deckwerth and Johnson, 1993, 1994; Estus et al., 1994; Freeman

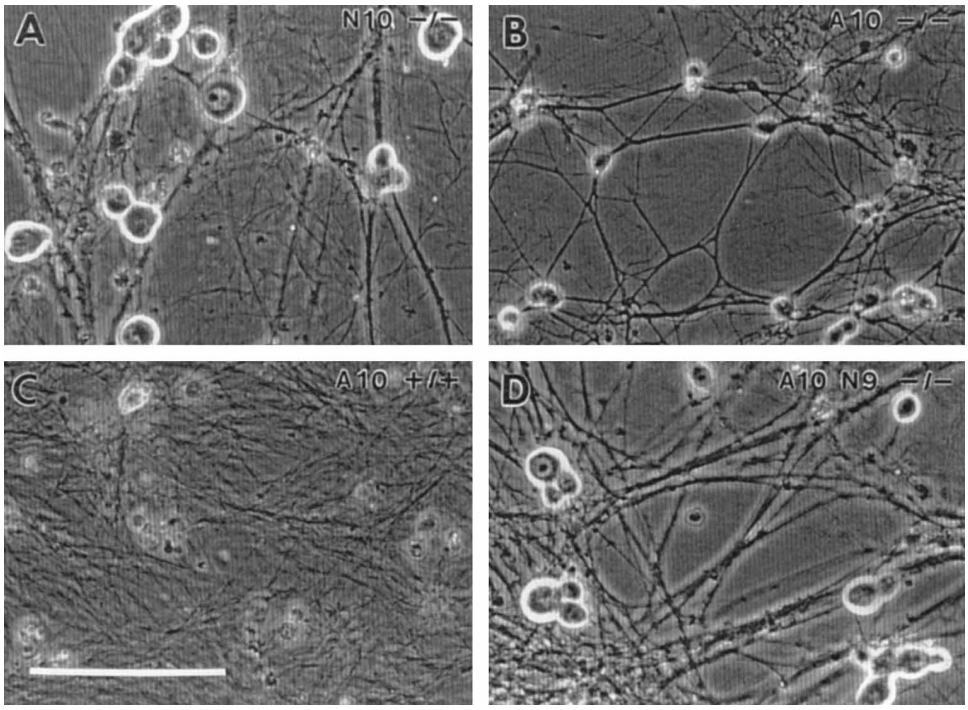

Figure 3. Dissociated *Bax* −/− Neonatal Sympathetic Neurons Survive In Vitro in the Absence of NGF and Show Classical Trophic Responses to Addition of NGF

(A) Primary cultures of dissociated *Bax* −/− murine sympathetic neurons maintained in NGF for 10 days.
(B) and (C) *Bax* −/− (B) and wild-type (C) neurons in the presence of anti-NGF antiserum for 10 days.
(D) Same field as (B), after an additional 9 days in the presence of NGF. *Bax* −/− sympathetic neurons survive in the absence of NGF (B) and extend neurites. Their cell bodies remain atrophic. Upon addition of NGF (D), the somas hypertrophy and neurite outgrowth increases greatly. Wild-type neurons die after withdrawal of NGF and their debris adheres to prominently visible collagen fibrils (C). Scale bar, 100 μm.

et al., 1994; Greenlund et al., 1995a, 1995b). Cultures of sympathetic neurons from wild-type, *Bax* +/−, and *Bax* −/− mice were first established in the presence of NGF for 7 days before being deprived of NGF. In this paradigm, more than 80% of all wild-type neurons have died after 35 hr and all by 8 days after NGF deprivation. In striking contrast, *Bax* −/− neurons survived in the absence of NGF for 23 days, the last time point examined (see Figure 2C). *Bax* +/− neurons died with slower kinetics than wild-type neurons, such that after 8 days 14% of the *Bax* +/− neurons remained viable (see Figure 2C). The retarded kinetics of death of *Bax* +/− neurons indicates that the gene dosage of *Bax* controls the kinetics of cell death after trophic factor withdrawal.

Besides assessing viability by phase contrast microscopy and crystal violet staining, viability of NGF-deprived *Bax* −/− neurons was also examined by staining with MTT, fluorescein diacetate, and Hoechst 33258 (Deckwerth and Johnson, 1994; data not shown). The cytoplasm of soma and neurites of NGF-deprived *Bax* −/− neurons stained dark blue with MTT, albeit weaker than NGF-maintained neurons, indicating a functional energy metabolism. Neurites and soma showed green fluorescence with fluorescein diacetate, indicating an intact plasma membrane and esterase activity. The nuclear chromatin stained homogeneously with Hoechst 33258 and showed the bright patches of heterochromatin characteristic of murine nuclei while lacking any sign of an apoptotic nuclear morphology.

As in the more stringent paradigm, NGF deprivation altered the morphology of SCG neurons. Their somas atrophied greatly such that the soma diameter was reduced by about 40% after 8 days of NGF withdrawal (see Figure 2D) and the extent of neurite outgrowth appeared diminished (data not shown). As for freshly plated neurons (see Figure 2B), the soma atrophy was reversed by readdition of NGF for 8 days (see Figure 2D).

A gene dosage effect of *Bax* on neuronal survival would be consistent with the inactivation of *Bax* being solely responsible for the observed phenotype. To further ensure that the massive reduction of trophic factor deprivation–induced death was not an artifact caused by a survival-promoting element introduced by the 129SV strain, NGF deprivation–induced death of sympathetic neurons was compared using both paradigms for 129SV mice, C57BL/6, and 129SV-C57BL/6 mice. Neuronal death was comparable for all strains as long as both copies of *Bax* were present (data not shown). Furthermore, we have not observed any *Bax*-deficient mouse whose neurons did not show a dramatic reduction of death after trophic factor deprivation.

BAX Is Required for Motor Neuron Death Following Neonatal Axotomy

To determine whether BAX was necessary for neuronal death by trophic factor deprivation in vivo, we used the paradigm of facial nerve axotomy in which the motor neurons in the facial nucleus are disconnected from

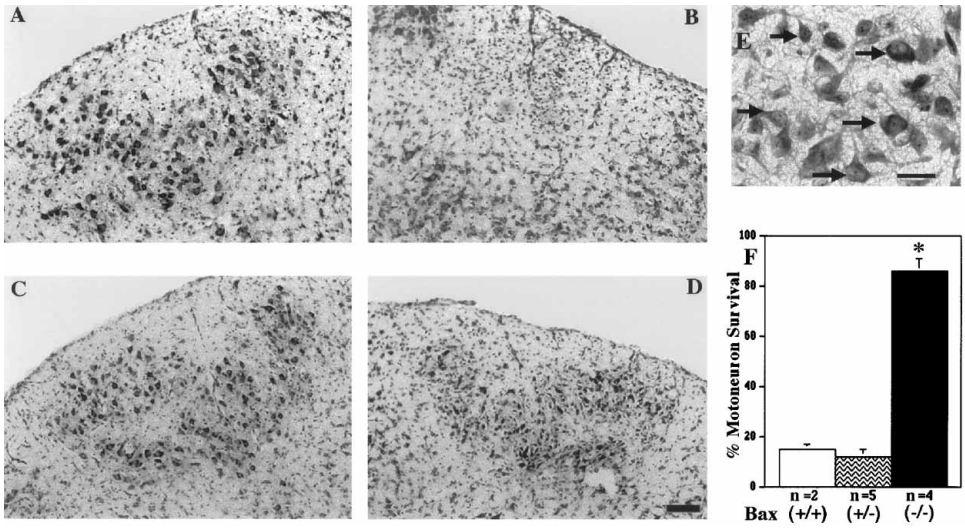

Figure 4. Motor Neuron Survival 1 Week after Facial Nerve Transection at P1 in *Bax* −/− and Wild-Type Mice

(A) and (B) Transverse sections of the pons at the level of the facial motor nucleus in wild-type mice showing the control, contralateral nonaxotomized (A), and ipsilateral axotomized facial nucleus (B). Almost all motor neurons degenerated after axotomy.

(C) and (D) Transverse section through the facial nucleus of a *Bax* −/− mouse showing the contralateral (C) and ipsilateral (D) facial nucleus. Virtually all facial motor neurons survived axotomy in *Bax* −/− animals, but many neurons became markedly atrophic.

(E) High power view of *Bax* −/− facial motor neurons that survived after axotomy (arrows).

(F) Quantitation of the number of facial motor neurons after a facial nerve transection in *Bax* +/+, *Bax* +/−, and *Bax* −/− mice. Motor neuron survival is presented as mean ± SEM of the percentage of viable neurons on the lesioned side relative to the nonlesioned side. The means for *Bax* +/− and *Bax* −/− animals were compared with the mean of *Bax* +/+ mice using the Student's *t* test (* $p < 0.001$; n, number of animals). Scale bar, 100 μm (A–D); 25 μm (E).

the trophic support provided by facial muscles. When performed on control *Bax* +/+ P1 mice, this procedure results in the loss of 90% of motor neurons by the seventh postoperative day. The axotomized wild-type mice show loss of motor neurons and subsequent gliosis (Figure 4B) relative to the contralateral unoperated side (Figure 4A). In striking contrast, in *Bax* −/− mice, 86% of the facial motor neurons survived nerve transection and there was only a limited glial reaction (Figures 4C–4F). The surviving neurons displayed considerable soma atrophy (Figures 4D and 4E). *Bax* +/− mice exhibited a similar loss of motor neurons as *Bax* +/+ mice, at the time points assessed.

Motor neurons in *Bax* −/− animals survived into adulthood after nerve transection at P1. The facial nucleus was still clearly discernible as a distinct cytoarchitectural unit 4 weeks after neonatal axotomy (Figure 5). Facial motor neurons remained viable at this time, although many of them were markedly atrophic. This pattern contrasts with that of *Bax* +/− and *Bax* +/+ mice, where facial motor neurons degenerated completely and were replaced with glia. These results demonstrate that BAX is obligate for the death of facial motor neurons following axotomy-induced trophic factor withdrawal.

Developmental Neuronal Death Is Reduced in *Bax* −/− Mice

Since BAX was required for death to occur after trophic factor deprivation, we sought to determine whether BAX levels would influence developmental neuronal death, a process in which trophic factor deprivation appears to play a prominent role. When ganglia were dissected from neonatal mice, it was noted that the largest ganglia would usually be later identified as being derived from *Bax* −/− animals. Furthermore, the number of trypan-blue excluding cells recovered after dissociation was larger for *Bax* −/− ganglia than for *Bax* expressing ganglia (data not shown). To determine the number of SCG neurons in animals with different *Bax* gene dosage, pairs of SCGs were dissected, dissociated, and allowed to adhere to collagen for 2 hr in the presence of NGF. The number of living neurons was determined after staining with an anti-tyrosine hydroxylase antibody and the nuclear dye Hoechst 33258. *Bax* −/− ganglia yielded 2.5 and 1.8 times more neurons than wild-type and heterozygous ganglia, respectively (Figure 6A). These data are consistent with a substantial reduction of naturally occurring death of sympathetic neurons during normal development.

We next asked whether *Bax* −/− mice had an increased number of facial motor neurons following the period of developmental death. The contralateral nonaxotomized facial nucleus was counted in 4-week-old mice, revealing a 51% increase in neuronal number in *Bax* −/− mice relative to control *Bax* +/+ mice (Figure 6B). The *Bax* −/− mice displayed a substantial increase in the number of small- to medium-sized motor neurons, while this population was less prominent in wild-type mice. *Bax* +/− mice also demonstrated a modest increase in the total number of facial motor neurons of ~13%, which just achieved statistical significance. Taken together, these results strongly suggest that normal developmental death is diminished in sympathetic and motor neurons from *Bax* −/− and *Bax* +/− mice.

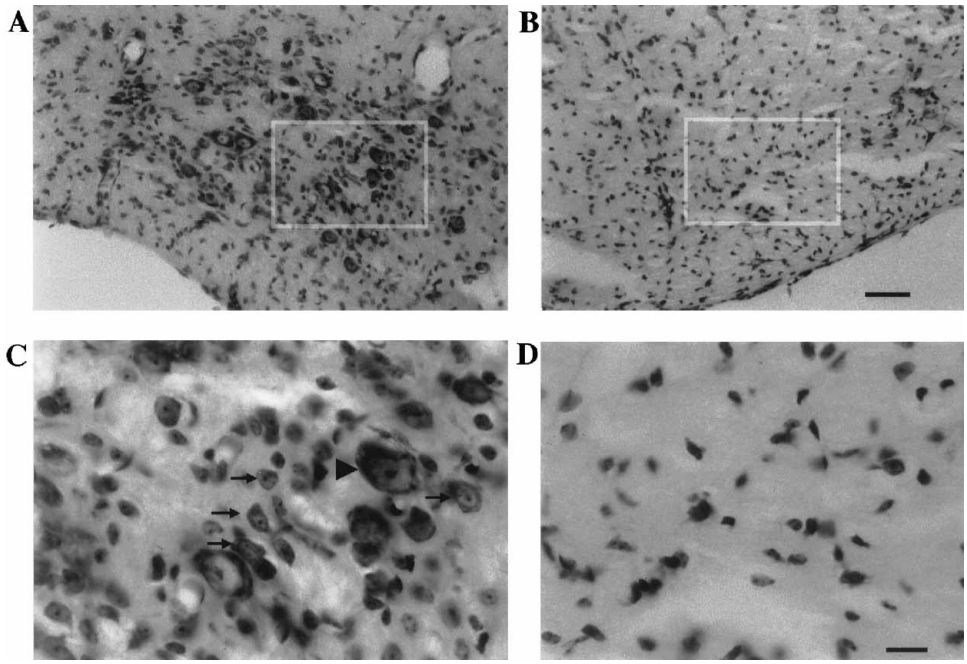

Figure 5. Motor Neurons in *Bax* −/− Mice Show Long-Term Survival Following Neonatal Axotomy

(A) and (B) Transverse sections of the facial nucleus 4 weeks after an ipsilateral axotomy performed at P1 in *Bax* −/− (A) and wild-type (B) mice. In *Bax* −/− animals, the motor neurons did not die after prolonged target deprivation. In contrast, the facial nucleus in wild-type animals was devoid of motor neurons and replaced with glial elements. Scale bar, 100 μm.

(C) and (D) High power magnification of images demarcated above in (A) and (B), respectively. The surviving facial motor neurons in *Bax* −/− mice are heterogeneous in size. Besides many very atrophic neurons (arrows) some neurons with large neuronal somas (arrowhead) are noted as well. Scale bar, 25 μm.

Discussion

Bax Is Required for Neuronal Death Induced by Trophic Factor Deprivation

The rheostat model hypothesizes that the balance between death-promoting and death-repressing members of the BCL2 family contributes a critical checkpoint that determines whether a cell will execute an apoptotic program after receiving a death stimulus (Oltvai et al., 1993; Oltvai and Korsmeyer, 1994). The results of this study demonstrate that in mice lacking BAX, sympathetic and motor neurons do not die in the experimental setting of trophic factor deprivation, but show long-term survival. Despite the coexpression of multiple *Bcl2* family members within these neurons (*Bcl2, Bclx$_L$, Mcl-1, Bax, Bak, and Bad*) (prior references and S. J. K. and W. D. S., unpublished data), the apoptotic pathway induced by the deprivation of neurotrophic factor is remarkably dependent upon a single member, *Bax*. The capacity of *Bax* −/− sympathetic neurons to establish in culture even in the absence of NGF is a rigorous test of their resistance to programmed cell death. Cell death induced by neonatal axotomy of motor neurons was also nearly completely prevented for at least 4 weeks by the

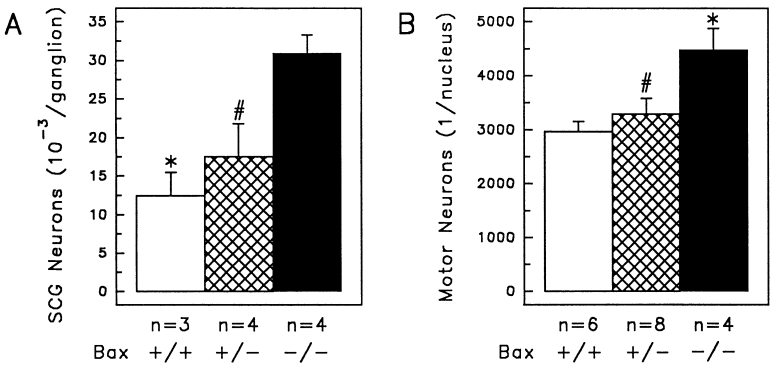

Figure 6. *Bax* Deficiency Suppresses Naturally Occurring Neuronal Death of Sympathetic and Facial Motor Neurons

(A) Number of viable sympathetic neurons per SCG after dissociation, plating in the presence of NGF for 2 hr, and identification of living neurons by staining for tyrosine hydroxylase and with Hoechst 33258. The number of viable neurons per wild-type (+/+), heterozygous (+/−), and *Bax*-deficient (−/−) ganglion is expressed as mean ± SD and was compared for *Bax* −/− ganglia with the number of neurons in wild-type or heterozygous ganglia using the two-sided Student's *t* test (* $p = 0.0003$; # $p = 0.0015$).

(B) Facial motor neuron number in the nucleus contralateral to axotomy of 4-week-old mice with different *Bax* genotypes. The number of neurons is presented as mean ± SD and was compared for *Bax* +/− and *Bax* −/− with the number of neurons in wild-type mice using the Student's *t* test (# $p < 0.04$; * $p < 0.001$; n, number of animals).

absence of *Bax*. This latter finding importantly generalizes our result, as it constitutes a second cell type dependent upon a different set of neurotrophic factors.

Our results seem to conflict with recent work that noted a weak survival-promoting effect when murine *Bax* was expressed in a number of trophic factor–deprived neurons of a nonmammalian species (chicken) (Middleton et al., 1996). Our loss-of-function experiments argue against a generalization of these results. Since single amino acid substitutions can alter the activity of BCL2 family proteins (Yin et al., 1994; Sedlak et al., 1995), the overexpression of *Bax* from another species might result in dominant negative effects or markedly alter stoichiometry (Middleton et al., 1996). Taken together, these results indicate that the consequences of altering BCL2 family members can depend upon the context in which they interact (Knudson et al., 1995).

Bax Deficiency Reduces Developmental Neuronal Death

Bax deficiency also leads to a reduction in the extent of naturally occurring developmental neuronal death. This finding demonstrates the developmental significance of the experimental resistance of *Bax* −/− neurons to neurotrophic factor deprivation. During development at E13-E18 in the mouse, 60% of the spinal motor neurons initially generated undergo programmed cell death (Lance-Jones, 1982; Oppenheim, 1991; Houenou et al., 1994). Motor neuron death during this period can be greatly ameliorated by the addition of specific motor neuron growth factors such as ciliary neurotrophic factor, brain-derived neurotrophic factor, and glial cell line–derived neurotrophic factor (GDNF), which also rescue motor neurons from axotomy-induced death (Sendtner et al., 1990; Oppenheim et al., 1992, 1995; Yan et al., 1992, 1995; Houenou et al., 1994). *Bax* −/− mice have a 51% increase in the total number of facial motor neurons, and it is likely that this increase is due to the inability of motor neurons to complete the normal programmed cell death pathway in the absence of BAX. Likewise, there are more sympathetic neurons in neonatal *Bax* −/− SCG than in *Bax*-expressing ganglia. The increased number of neurons is consistent with a decrease of naturally occurring cell death controlled by NGF, since mouse sympathetic neurons start to respond to NGF with increased survival at E16-E17 (Coughlin and Collins, 1985). It is conceivable that the increase in neuronal numbers may also reflect contributions of NGF-independent processes. For example, it is untested whether *Bax* deficiency would at an earlier developmental stage affect the survival of sympathetic neuroblasts dependent on NT-3 or other trophic factors (Birren et al., 1993; DiCicco-Bloom et al., 1993).

Neurons heterozygous for *Bax* ultimately die following experimental neurotrophic factor deprivation (Figures 2A and 4E). However, the kinetics of death of NGF-deprived *Bax* +/− sympathetic neurons was clearly retarded when neuronal cultures established for 7 days in the presence of NGF were studied (Figure 2C). *Bax* +/− mice also demonstrated slightly enhanced numbers of sympathetic and motor neurons when compared with wild-type mice (Figure 6). It is conceivable that the signals in some experimental paradigms may be stronger than in developmental deaths. For example, if trophic factor deprivation was accompanied by injury that was to induce BAX in *Bax* +/− mice, the altered setpoint between pro- and anti-apoptotic *Bcl2* family members could further diminish a heterozygous effect. Such an increase in BAX protein has been observed in CNS neurons following ischemic injury (Krajewski et al., 1995), providing support for this possibility.

While neurotrophic factor–dependent deaths were abrogated in *Bax* −/− mice, BAX does not appear to be requisite for all cell deaths. For example, the *p53*-dependent deaths of γ-irradiated thymocytes are not substantially altered in *Bax* −/− animals (Knudson et al., 1995). Moreover, when other lineages are quantified, not every cell type reveals hyperplasia in *Bax* −/− mice. The existence of many classes of neurons expressing differing subsets of BCL2 family members suggests that the control of cell death may differ with lineage and stage of development. Thus, while the *Bax* −/− mice provide the first testable model for the singular importance of a death-promoting family member, models examining other death-promoting members such as BAD, BAK, or $BCLx_S$ may prove their critical role in cell deaths not obviously influenced by BAX.

Bax-Deficient Neurons Demonstrate that Neuronal Survival Does Not Require Anabolic Responses Induced by Trophic Factors

In addition to promoting survival, neurotrophic factors exhibit anabolic effects on neuronal metabolism that ultimately result in morphological changes such as increases in soma size and neurite outgrowth. *Bax* is not involved in mediating these anabolic responses induced by atrophic factors, since NGF induces these changes in morphology in *Bax*-deficient sympathetic neurons as well as in wild-type neurons (Figures 2 and 3).

Conversely, the lack of anabolic stimulation upon withdrawal of trophic factors leads to atrophy. While this atrophy is apparent in NGF-deprived wild-type sympathetic neurons and its onset precedes the loss of viability (Deckwerth and Johnson, 1993, 1994), the extent of atrophy is much more prominent in neurons deficient in *Bax,* because atrophy is not terminated by the induction of apoptosis (Figures 2 and 3). Likewise, *Bax*-deficient motor neurons that survive neonatal axotomy exhibit a morphology distinct from axotomized motor neurons that survive following trophic factor administration. For example, GDNF administration prevents the considerable atrophy that normally accompanies axotomy and precedes death (Oppenheim et al., 1995; Yan et al., 1995). In contrast, *Bax* deficiency prevents only neuronal death but not the neuronal atrophy that is very apparent 1 week post-lesion and progresses during the following weeks (Figures 4 and 5). The atrophy of trophic factor–deprived neurons prevented from dying by *Bax* deficiency illustrates that anabolic responses exerted by trophic factor are not required for neuronal survival.

The atrophic morphology noted in the extra motor neurons surviving development illustrates the neurotrophic hypothesis of neuronal development. It states that the survival of trophic factor–dependent neurons is

regulated by limited amounts of target-derived trophic factors (reviewed by Oppenheim, 1991). With death abolished by *Bax* deficiency, the coupling between limited amounts of trophic factor leading to neuronal loss is disrupted. The limiting amount of trophic factors in the invariable target is no longer reflected by diminished neuronal survival but now manifests as atrophy of those supernumerary neurons. Despite the large number of atrophic motor neurons and probably other neuronal types, no abnormal behavior that might be related to the reduction of developmental neuronal death has been noted to date in the *Bax* −/− mice. It will be interesting to see whether *Bax* −/− mice manifest an observable phenotype when challenged in settings designed to test the limits or more subtle aspects of neuronal function.

Cells Protected by BCL2 Family Members from Death Retain Cell Type–Specific Characteristics

Inhibition by BCL2 family members of the progression of degeneration toward cell death preserves cells at a stage at which they display several seemingly normal responses, such as the expression of cell type–specific properties and the ability to mount classical anabolic responses to trophic factors. *Bax* −/− sympathetic neurons spontaneously extend neurites even in the absence of NGF, indicating that these salvaged neurons still express a hallmark of the neuronal phenotype, i.e., the elaboration of neurites. Along similar lines, it has been shown recently that facial motor neurons that survive axotomy when overexpressing BCL2 retain their electrophysiological properties (Alberi et al., 1996). These observations extend beyond the nervous system and are parallel to findings in the immune system in which overexpressed BCL2 not only prevented cell death, but also promoted differentiation of immature lymphocytes (Linette et al., 1994), and reminiscent of observations in nematode development in which cells rescued from developmental cell death by loss-of-function mutations in *Ced3* and *Ced4* acquire distinct phenotypes (Ellis and Horvitz, 1986). The ability of trophic factor–deprived *Bax* −/− neurons to extend neurites in the absence of NGF complements the finding by Martinou et al. (1994), who demonstrated that facial motor neurons overexpressing human BCL2 retained their axons in addition to surviving developmental neuronal death. The beneficial effects of BCL2 family members on neurites are particularly noteworthy, since axons and dendrites are neuronal compartments with a great degree of independence from the soma. The protection of neurites by BCL2 family members does not extend to any insult, however. In mice suffering from progressive motor neuronopathy, motor axons degenerate while motor neuronal somas remain protected by *Bcl2* overexpression (Sagot et al., 1995).

Bax-deficient mice constitute a genetic model with a defect in a distal common pathway of cell death. In addition to remaining alive, the salvaged trophic factor–deprived *Bax* −/− neurons express a neuronal phenotype, remain responsive to trophic factors, and display anabolic responses. This argues that in addition to the retention of at least some normal functional responses, the degenerative changes induced by trophic factor deprivation are arrested at a point where they are still reversible. Therefore, in a neurodegenerative disease or after an acute injury, in which BAX is critical in effecting neuronal death, a BAX-specific inhibitor may be neuroprotective by stabilizing degenerating neurons prior to the onset of irreversible damage. Since neurons retain the responsiveness to trophic factors and the ability to reverse incurred degenerative changes at this point, treatment with a BAX-specific inhibitor holds the promise of a functional recovery of such salvaged neurons upon exposure to trophic factors or upon elimination of the diverse proximal defects responsible for triggering neuronal degeneration.

Experimental Procedures

Breeding and Genotyping of Mice with Different Gene Dosages of *Bax*

Mice heterozygous for *Bax* (Knudson et al., 1995) were mated to yield F1 offspring with *Bax* −/−, *Bax* +/−, and wild-type genotypes. At the developmental age detailed below, tail DNA was prepared and screened for both the normal and the mutant allele using a single polymerase chain reaction. The normal allele was amplified using an exon 5 forward primer (0.64 μM: 5′-TGATCAGAACCATC ATG-3′) and an intron 5 reverse primer (0.64 μM: 5′-GTTGACCAGAG TGGCGTAGG-3′), which together generated a 304 bp product. The mutant allele was amplified with a neo/pgk primer (0.16 μM: 5′-CCGCTTCCATTGCTCAGCGG-3′) and the same intron 5 reverse primer, which together generated a 507 bp product. Cycling parameters were 1 min at 94°C, 55°C, and 72°C each for a total of 30–35 cycles. The primer ratio was adjusted to allow amplification of both products simultaneously with preferential amplification of the wild-type allele to assure correct genotyping of the *Bax*-deficient animals. The genotype of some of the litters was also examined by Southern hybridization (Knudson et al., 1995).

Sympathetic Neuronal Cultures

Primary cultures of sympathetic neurons were established from the two SCGs of every F1 offspring from *Bax* +/− × *Bax* +/− matings 1 day after birth using a modification of a previously published procedure (Deckwerth and Johnson, 1994). The genotype was determined from tail DNA after the cultures had been established. In brief, the two ganglia were dissected, digested with collagenase and dispase, dissociated by trituration, and plated at 3–5.5 × 10^3 trypan-blue excluding dissociated cells (neurons, fibroblasts, and Schwann cells) onto collagen-coated two-well chamber slides in 30 ml of MEM (Earle's salts) supplemented with 10% fetal calf serum. Between 4 and 30 cultures of the same plating density were established from each offspring, depending on the number of trypan-blue negative cells available. After allowing the neurons to adhere for 30–40 min, 1.5 ml of culture medium (MEM, 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 3.3 μg/ml aphidicolin to kill contaminating dividing non-neuronal cells) containing 50 ng/ml 2.5S mouse NGF or 0.025% goat anti-2.5S mouse NGF antiserum were added per well. The medium was changed every 3–4 days. While different cultures from the same offspring had similar numbers of neurons when examined by crystal violet staining (see below), after several days to weeks in the presence of NGF, the number of neurons in cultures from different animals was more variable (1–4.7 × 10^3/culture). To deprive established NGF-maintained neurons of NGF, the medium was replaced with medium lacking NGF and containing 0.025% goat anti-2.5S mouse NGF antiserum. To expose neurons to NGF after having been cultured with anti-NGF antiserum, the medium was exchanged twice with medium containing NGF.

Sympathetic Neuron Analysis

Living neurons in freshly dissociated ganglia were quantitated after plating the neurons for 2 hr, fixing the cultures with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (4% PFA), and

staining with 1:100 sheep anti-rat tyrosine hydroxylase (TH) antiserum (Chemicon, Temecula, CA), 1:400 Cy3-labeled donkey anti-sheep IgG (Jackson ImmunoResearch, West Grove, PA), and 1 μg/ml Hoechst 33258 (Molecular Probes, Eugene, OR). Cells with TH-positive cytoplasm and healthy nuclei were counted as living neurons. About 12–15 parallel stripes sampling representative 30% of the whole area of each culture were analyzed. Viability of sympathetic neurons in cultures older than 5 days was quantitated after fixation of the cultures with 4% PFA and staining with crystal violet (Deckwerth and Johnson, 1993). Living neurons have a dark blue cytoplasm, a lighter stained nucleoplasm, and dark nucleoli. Atrophy has little effect on the staining. Dead neurons and debris only stain faintly. The whole culture was counted. The number of neurons in each experimentally treated culture was normalized to the mean of the number of neurons from about two cultures from the same animal that were maintained in NGF for a time period comparable with that of the experimentally treated cultures. Neuronal death in the presence of NGF is less than 10%/week and does not affect the data presented. The normalized neuronal counts were averaged for cultures from different animals and litters but with the same genotype and treatment. Viability was also assessed qualitatively by staining of neuronal cultures with 0.4 mg/ml MTT (1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO) in culture medium for 10 min at 35°C, followed by fixation with 4% PFA or staining of living cultures with 5 μg/ml fluorescein diacetate (Sigma) in Locke's solution for 20 min at 35°C (Deckwerth and Johnson, 1994). The cytoplasm in soma and neurites of living neurons stains dark blue with MTT formazan crystals or shows green fluorescence with fluorescein diacetate.

Soma diameters were determined from phase contrast photomicrographs of living cultures. Neurons with nearly spherical soma, distinct cellular perimeter, and little contact with neighboring cells were included in the analysis. Neurite outgrowth was examined in 24-hr-old cultures after fixation with 4% PFA and by staining with 1:5000 rabbit anti-NF-M antiserum (Chemicon), 1:400 Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch), and Hoechst 33258. The statistical analysis of all quantitative data was performed using the two-sided Mann Whitney U test or Student's *t* test as indicated.

Facial Nerve Axotomy and Motor Neuron Analysis

Every F1 offspring from *Bax* +/− × *Bax* +/− matings was anesthetized on P1 by hypothermia, and its right facial nerve was transected as it exited the stylomastoid foramen. The contralateral side served as control for the axotomy and as source of the material for the analysis of developmental motor neuron death. Animals were sacrificed at 1 and 4 weeks after the axotomy with an overdose of sodium pentobarbital. Tail samples were taken for genotype analysis and the animals fixed by intracardiac perfusion with 4% PFA. The brains were removed, embedded in parafin, sectioned at 12 μm, and stained with cresyl violet. Facial motor neurons were readily identified by their appearance, and those with distinct nucleoli counted. About 12 sections were evaluated for each nucleus. The unpaired Student's *t* test was used to compare the mean of surviving motor neurons between wild-type control and *Bax* +/− and *Bax* −/− animals.

In Situ Hybridization for *Bax* mRNA

Newborn or P4 wild-type mice were anesthetized by hypothermia and sacrificed by decapitation. To examine *Bax* expression in the neonatal SCG, the heads were fixed in 4% PFA for 24 hr, transferred to 500 mM sucrose in PBS for 24 hr, and quickly frozen on dry ice in O. C. T. compound (Miles Inc., Elkhart, IN). Sagittal frozen sections (5 μm) were cut and mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). The remainder of the procedure was carried out essentially as described previously (Wanaka et al., 1990). To study the expression of *Bax* in P4 facial motoneurons, the brainstem was dissected and frozen on dry ice. Cryostat sections (20 μm) were cut in transverse planes, thawmounted onto SuperFrost slides, and stored at −20°C. On the day of hybridization slides were thawed, fixed for 10 min in 4% PFA, rinsed twice with PBS, and processed as described previously (Wright et al., 1995). *Bax* mRNA antisense riboprobes containing exons 4–6 were prepared from murine *Bax* cDNA (Oltvai et al., 1993) by digestion of the vector with Pst1 and synthesis of the cRNA using T7 RNA polymerase and [^{33}P]UTP as label. The specificity of the detection of *Bax* mRNA was controlled for by hybridization with a *Bax* mRNA sense probe.

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References

Alberi, S., Raggenbass, M., De Bilbao, F., and Dubois-Dauphin, M. (1996). Axotomized neonatal motoneurons overexpressing the *bcl2* proto-oncogene retain functional electrophysiological properties. Proc. Natl. Acad. Sci. USA *93,* 3978–3983.

Bakhshi, A., Jensen, J.P., Goldman, P., Wright, J.J., McBride, O.W., Epstein, A.L., Korsmeyer, S.J. (1985). Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around J_H on chromosome 14 and near a transcriptional unit on 18. Cell *41*, 889–906.

Batistatou, A., and Greene, L.A. (1993). Internucleosomal DNA cleavage and neuronal survival/death. J. Cell Biol. *122*, 523–532.

Birren, S.J., Lo, L., and Anderson, D.J. (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. Development *119*, 597–610.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. (1993). *Bcl-x*, a *Bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*, 597–608.

Borner, C., Martinou, I., Mattmann, C., Irmler, M., Schaerer, E., Martinou, J.-C., and Tschopp, J. (1994). The protein Bcl2α does not require membrane attachment, but two conserved domains to suppress apoptosis. J. Cell Biol. *126*, 1059–1068.

Cheng, E.H.-Y., Levine, B., Boise, L.H., Thompson, C.B., and Hardwick, J.M. (1996). Bax-independent inhibition of apoptosis by Bcl-x_L. Nature *379*, 554–556.

Chittenden, T., Harrington, E.A., O'Connor, R., Flemington, C., Lutz, R.J., Evan, G.I., and Guild, B.C. (1995a). Induction of apoptosis by the Bcl-2 homologue Bak. Nature *374*, 733–736.

Chittenden, T., Flemmington, S., Houghton, A.B., Ebb, R.G., Gallo, G.J., Elangovan, B., Chinnadurai, G., and Lutz, R.J. (1995b). A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. EMBO J. *14*, 5589–5596.

Cleary, M.L., and Sklar, J. (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint cluster region near a transcriptionally active locus on chromosome 18. Proc. Natl. Acad. Sci. USA *82*, 7439–7443.

Coughlin, M.D., and Collins, M.B. (1985). Nerve growth factor–independent development of embryonic mouse sympathetic neurons in dissociated cell culture. Dev. Biol. *110*, 392–401.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armanini, M.P., Ling, L.H., McMahon, S.B., Shelton, D.L., Levinson, A.D., and Phillips, H.S. (1994). Mice lacking nerve growth factor

display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell *76*, 1001–1011.

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

Deckwerth, T.L., and Johnson, E.M., Jr. (1994). Neurites remain viable after destruction of the neuronal soma by programmed cell death. Dev. Biol. *165*, 63–72.

DiCicco-Bloom, E., Friedman, W.J., and Black, I.B. (1993). NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival. Neuron *11*, 1101–1111.

Dubois-Dauphin, M., Frankowski, H., Tsujimoto, Y., Huarte, J., and Martinou, J.-C. (1994). Neonatal motoneurons overexpressing the *Bcl2* protooncogene in transgenic mice are protected from axotomy-induced cell death. Proc. Natl. Acad. Sci. USA *91*, 3309–3313.

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

Ellis, R.E., and Horvitz, H.R. (1986). Genetic control of programmed cell death in the nematode C. elegans. Cell *44,* 817–829.

Estus, S., Zaks, W.J., Freeman, R.S., Gruda, M., Bravo, R., and Johnson, E.M., Jr. (1994). Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J. Cell Biol. *127*, 1717–1727.

Farlie, P.G., Dringen, R., Rees, S.M., Kannourakis, G., and Bernard, O. (1995). *Bcl2* transgene expression can protect neurons against developmental and induced cell death. Proc. Natl. Acad. Sci. USA *92*, 4297–4401.

Farrow, S.N., White, J.H.M., Martinou, I., Raven, T., Pun, K.-T., Grinham, C.-J., Martinou, J.-C., and Brown, R. (1995). Cloning of a Bcl2 homologue by interaction with adenovirus E1B19K. Nature *374*, 731–733.

Frankowski, H., Misotten, M., Fernandez, P.-A., Martinou, I., Michel, P., Sadoul, R., and Martinou, J.-C. (1995). Function and expression of the *Bcl-x* gene in the developing and adult nervous system. NeuroReport *6*, 1917–1921.

Freeman, R.S., Estus, S., and Johnson, E.M., Jr. (1994). Analysis of cell cycle–related gene expression in postmitotic neurons: selective induction of cyclin D1 during programmed cell death. Neuron *12*, 343–355.

Garcia, I., Martinou, I., Tsujimoto, Y., and Martinou, J.-C. (1992). Prevention of programmed cell death of sympathetic neurons by the *Bcl2* proto-oncogene. Science *258*, 302–304.

Glücksmann, A. (1951). Cell deaths in normal vertebrate ontogeny. Biol. Rev. *26*, 59–86.

Gonzalez-Garcia, M., Perez-Ballestero, R., Ding, L., Duan, L., Boise, L. H., Thompson, C.B., and Nunez, G. (1994). Bcl-x_L is the major Bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. Development *120*, 3033–3042.

Gorin, P.D., and Johnson, E.M., Jr. (1979). Experimental autoimmune model of nerve growth factor deprivation: effect on developing peripheral sympathetic and sensory neurons. Proc. Natl. Acad. Sci. USA *76*, 5382–5386.

Greenlund, L.J.S., Deckwerth, T.L., and Johnson, E.M., Jr. (1995a). Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. Neuron *14*, 303–315.

Greenlund, L.J.S., Korsmeyer, S.J., and Johnson, E.M., Jr. (1995b). Role of Bcl2 in the survival and function of developing and mature sympathetic neurons. Neuron *15*, 649–661.

Hengartner, M.O., and Horvitz, H.R. (1994). C. elegans cell survival gene *Ced-9* encodes a functional homolog of the mammalian proto-oncogene *Bcl-2*. Cell *76*, 665–676.

Houenou, L.J., Li, L., Lo, A.C., Yan, Q., and Oppenheim, R.W. (1994). Naturally occurring and axotomy-induced motoneuron death and its prevention by neurotrophic agents: a comparison between chick and mouse. Prog. Brain Res. *102*, 217–225.

Kamada, S., Shimono, A., Shinto, Y., Tsujimura, T., Takahashi, T., Noda, T., Kitamura, Y., Kondoh, H., and Tsujimoto, Y. (1995). *Bcl-2* deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. Cancer Res. *55*, 354–359.

Kiefer, M.C., Brauer, M.J., Powers, V.C., Wu, J.J., Umansky, S.R., Tomei, L.D., and Barr, P.J. (1995). Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature *374*, 736–739.

Knudson, C.M., Tung, K.S.K., Tourtelotte, W.G., Brown, G.A.J., and Korsmeyer, S.J. (1995). *Bax*-deficient mice with lymphoid hyperplasia and male germ cell death. Science *270*, 96–99.

Kozopas, K.M., Yang, T., Buchan, H.L., Zhou, P., and Craig, R.W. (1993). *MCL1*, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to *BCL2*. Proc. Natl. Acad. Sci. USA *90*, 3516–3520.

Krajewski, S., Krajewska, M., Shabaik, A., Miyashita, T., Wang, H.G., and Reed, J.C. (1994). Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl2. Am. J. Pathol. *145*, 1323–1336.

Krajewski, S., Mai, J.K., Krajewska, M., Sikorska, M., Mossakowski, M.J., and Reed, J.C. (1995). Upregulation of Bax protein levels in neurons following cerebral ischemia. J. Neurosci. *15*, 6364–6376.

Lance-Jones, C. (1982). Motoneuron death in the developing lumbar spinal cord of the mouse. Dev. Brain Res. *4*, 473–479.

Levi-Montalcini, R., and Booker, B. (1960). Destruction of the sympathetic ganglia in mammals by an antiserum to the nerve growth–promoting factor. Proc. Natl. Acad. Sci. USA *46*, 384–391.

Linette, G.P., Grusby, M.J., Hedrick, S.M., Hansen, T.H., Glimcher, L.H., Korsmeyer, S.J. (1994). Bcl2 is upregulated at the CD4+8+ stage during positive selection and promotes thymocyte differentiation at several control points. Immunity *1*, 197–205.

Martinou, J.-C., Dubois-Dauphin, M., Staple, J.K., Rodriguez, I., Frankowski, H., Misotten, M., Albertini, P., Talabot, S., Catsicas, S., Pietra, C., and Huarte, J. (1994). Overexpression of bcl2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron *13*, 1017–1030.

Martinou, I., Fernandez, P.-A., Misotten, M., White, E., Allet, B., Sadoul, R., and Martinou, J.-C. (1995). Viral proteins E1B19K and p35 protect sympathetic neurons from cell death induced by NGF deprivation. J. Cell Biol. *128*, 201–208.

Merry, D.E., Veis, D.J., Hickey, W.F., and Korsmeyer, S.J. (1994). Bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. Development *120*, 301–311.

Middleton, G., Nunez, G., and Davies, A.M. (1996). *Bax* promotes neuronal survival and antagonizes the survival effects of neurotrophic factors. Development *122*, 695–701.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.-K., Liebermann, D., Hoffman, B., and Reed, J.C. (1994). Tumor suppressor p53 is a regulator of *Bcl2* and *Bax* gene expression in vitro and in vivo. Oncogene *9*, 1799–1805.

Motoyama, N., Wang, F., Roth, K.A., Sawa, H., Nakayama, K.-I., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S., and Loh, D.Y. (1995). Massive cell death of immature hematopoietic cells and neurons in *Bcl-x*-deficient mice. Science *267*, 1506–1510.

Nakayama, K., Nakayama, K.-I., Negishi, I., Kuida, K., Sawa, H., and Loh, D.Y. (1994). Targeted disruptions of *Bcl2*$\alpha\beta$ in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc. Natl. Acad. Sci. USA *91*, 3700–3704.

Oltvai, Z.N., and Korsmeyer, S.J. (1994). Checkpoints of dueling dimers foil death wishes. Cell *79*, 189–192.

Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell *74*, 609–619.

Oppenheim, R.W. (1991). Cell death during development of the nervous system. Annu. Rev. Neurosci. *14*, 453–501.

Oppenheim, R.W., Yin, Q.W., Prevette, D., and Yan, Q. (1992). Brain-derived neurotrophic factor rescues developing avian motoneurons from axotomy-induced death. Nature *360*, 755–757.

Oppenheim, R.W., Houenou, L.J., Johnson, J.E., Lin, L.F., Li, L., Lo, A.C., Newsome, A.L., Prevette, D.M., and Wang, S. (1995). Developing motoneurons rescued from programmed and axotomy-induced cell death by GDNF. Nature *373*, 344–346.

Sagot, Y., Dubois-Dauphin, M., Tan, S.A., de Bilbao, F., Aebischer, P., Martinou, J.-C., and Kato, A.C. (1995). Bcl2 overexpression prevents motoneuron cell body loss but not axonal degeneration in a mouse model of a neurodegenerative disease. J. Neurosci. *15*, 7727–7733.

Sato, T., Hanada, M., Bodrug, S., Irie, S., Iwama, N., Boise, L., Thompson, C.B., Golemis, E., Fong, L., Wang, H.-G., and Reed, J.C. (1994). Interactions among the members of the Bcl2 family analyzed with a yeast two-hybrid system. Proc. Natl. Acad. Sci. USA *91*, 9238–9242.

Sedlak, T.W., Oltvai, Z.N., Yang, E., Wang, K., Boise, L.H., Thompson, C.B., and Korsmeyer, S.J. (1995). Multiple Bcl2 family members demonstrate selective dimerizations with Bax. Proc. Natl. Acad. Sci. USA *92*, 7834–7838.

Sendtner, M., Kreutzberg, G.W., and Thoenen, H. (1990). Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature *345*, 440–441.

Snider, W.D., Elliot, J., and Yan, Q. (1992). Axotomy-induced neuronal death during development. J. Neurobiol. *23*, 1231–1246.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E., and Croce, C.M. (1985). The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science *229*, 1390–1393.

Veis, D.J., Sorenson, C.M., Shutter, J.R., and Korsmeyer, S.J. (1993). *BCL2*-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell *75*, 229–240.

Wanaka, A., Johnson, E.M., Jr., and Milbrandt, J. (1990). Localization of FGF receptor mRNA in the adult rat central nervous system by in situ hybridization. Neuron *5*, 267–281.

Wright, D.E., White, F.A., Gerfen, R.W., Silos-Santiago, I., and Snider, W.D. (1995). The guidance molecule semaphorin III is expressed in regions of spinal cord and periphery avoided by growing sensory axons. J Comp. Neurol. *361,* 321–333.

Yan, Q., Elliot, J., and Snider, W.D. (1992). Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. Nature *360*, 755–777.

Yan, Q., Matheson, C., and Lopez, O.T. (1995). In vivo neurotrophic factor effects of GDNF on neonatal and adult facial motoneurons. Nature *373*, 341–344.

Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B., and Korsmeyer, S.J. (1995). Bad, a heterodimeric partner for Bcl-x_L and Bcl-2, displaces Bax and promotes cell death. Cell *80*, 285–291.

Yin, X.-M., Oltvai, Z.N., and Korsmeyer, S.J. (1994). BH1 and BH2 domains of Bcl2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature *369*, 321–323.