Neurons Exclusively Express N-Bak, a BH3 Domain-only Bak Isoform That Promotes Neuronal Apoptosis*\[S\]

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Bak is generally recognized as a multidomain, proapoptotic member of the Bcl-2 family. Bak and Bax are functionally redundant in non-neuronal cells and represent a mitochondrial convergence point for cell death signaling pathways. This functional redundancy, however, may not exist in neurons in which the single deletion of Bak is sufficient to confer protection against a variety of cytotoxic insults. In the present study, we demonstrate that postnatal cortical and cerebellar granule neurons exclusively express an alternatively spliced, BH3 domain-only form of Bak (N-Bak), whereas astrocytes express only the full-length, multidomain form. Overexpression of N-Bak promotes Bax translocation in HeLa cells and induces neuronal cell death in cortical, hippocampal, and cerebellar granule neurons in a Bax-dependent manner. N-Bak interacts with Bcl-XL, but not BAX, suggesting an indirect mechanism for promoting Bax translocation to the mitochondria. N-Bak message and protein levels are elevated in cortical neurons in response to DNA damage, and subsequent induction of neuronal death is significantly delayed by expressing a full-length Bak antisense plasmid. These results demonstrate that postnatal neurons solely express a BH3 domain-only form of Bak, which contributes to DNA damage-induced neuronal apoptosis. The absence of full-length Bak expression explains the near exclusive requirement for Bak in neuronal apoptosis.

Intrinsic and extrinsic death cues commonly alter the balance between anti- and pro-apoptotic members of the Bcl-2 family of cell death regulators (1, 2). Bcl-2 and Bcl-X\(_L\) represent antiapoptotic members of the Bcl-2 family, and contain four characteristic Bcl-2 homology (BH)\(^2\) domains. Proapoptotic members of this family lack the BH4 domain and can be further subdivided into multidomain (i.e. Bak and Bax) and BH3 domain-only proteins (i.e. BID, BAD, Noxa, PUMA, etc.) (1, 2). Recent genetic and biochemical studies using non-neuronal cells have defined the functional interplay among various Bcl-2 family members; BH3-only proteins exert apoptotic activity only through direct or indirect activation of the multidomain proapoptotic members Bak and Bak (3). Several studies have further established that Bak and Bax are functionally redundant and represent obligate but alternative gateways to mitochondrial dysfunction and cell death (4–6). Antiapoptotic Bcl-2 family members antagonize the proapoptotic actions of these members through direct protein-protein interactions.

Bcl-2 family members contribute to the regulation of neuronal viability both in normal development and in the pathogenesis of brain injury and neurodegenerative disease. Mice deficient in the bcl-\(_{Xl}\) gene die at embryonic day 13 with excessive neuronal loss in the central and peripheral nervous system (7); deletion of the bax gene partially alters this outcome (8). Mice lacking the bcl-2 gene display substantial loss of motor, sensory, and sympathetic neurons in the postnatal period (9). In cultured neural progenitor cells, only the combined absence of Bak and Bax (\(bax^{-/-}\)\(\_\_\_\_\)) can provide complete resistance to cell death induced by growth factor deprivation (10) and treatment with DNA-damaging agents (11), whereas the majority of cells singly deficient in \(bax\_\_\_\_\_\_\) or \(bax\_\_\_\_\_\_\) as well as wild-type cells die by the same treatment. In differentiated neurons, however, single deletion of Bak is sufficient to confer significant protection from a variety of insults including neurotrophic factor deprivation (12–14), excitotoxicity (15), and DNA damage (15), whereas the absence of the Bcl-2 gene provides no protection (16). These results raise the possibility that the functions of Bak and Bax may not be redundant in neurons and suggest that the contribution of Bak to neuronal apoptosis is not significant relative to Bax, at least for the death stimuli and neuronal populations examined thus far.

Bak and Bax may not be interchangeable in neurons simply because Bak is not functionally coupled to apoptosis. For instance, Bax deletion is sufficient to protect cerebellar granule neurons from various insults although they reportedly express both the Bax and Bak protein (16). On the other hand, the lack of redundancy may be caused by neurons not expressing the multidomain, full-length Bak protein (designated FL-Bak) as recently reported in sympathetic and hippocampal neurons (17). These neurons instead express a novel message encoding a neuron-specific splice variant of Bak (designated N-Bak) that contains only the BH3 domain (17), leaving Bax as the only multi-domain proapoptotic protein expressed by these neurons. As a BH3-only protein, N-Bak would then be expected to act upstream of Bax to promote cell death. Unexpectedly, however, N-Bak overexpression showed no effect on the viability of sympathetic and hippocampal neurons (17). These results suggest that the expression and function of the Bak gene may vary among different populations of neurons and in relation to developmental age and the type of insult.

In the present study, we sought to clarify the neuronal expression profile of Bak as well as its functional relationship...
with Bak in neurons. Here, we demonstrate that postnatal cortical and cerebellar granule neurons exclusively express N-Bak, which is up-regulated in response to neuronal injury. Overexpression of N-Bak induces neuronal cell death in cortical, hippocampal, and cerebellar granule neurons in a Bak-dependent manner. Moreover, N-Bak binds to Bcl-X\textsubscript{L} and induces Bak translocation to the mitochondria. Thus, N-Bak serves as a critical element in a cell death pathway activated in response to DNA damage, in that cell death is delayed upon suppression of N-Bak expression.

**MATERIALS AND METHODS**

**Animals and Cell Culture**—The p53 knock-out and Bak knock-out mouse strains used were described previously (18–20), and the latter is now on a C57BL/6 background after 12 backcrosses. Cortical and hippocampal neuronal cultures were established from newborn mice as described previously (20) and maintained for 3 or 4 days before experimental manipulations. Cerebellar tissues from 7 day-old pups were trypsinized, dissociated, and plated in poly-D-lysine coated dishes in B27-supplemented Neurobasal medium (Invitrogen) in essentially the same way as for cortical/hippocampal neurons but at a higher density (4 × 10\textsuperscript{5} cells/100 μl). Cells were transfected and continuously maintained in the presence of zVAD-fmk (100 μM; caspase inhibitor I; EMD Biosciences). To monitor Bak translocation, HeLa cells were co-transfected with an appropriate experimental plasmid and a GFP-Bak plasmid (23) at a 1:1 ratio in the presence of zVAD-fmk (100 μM).

**Co-immunoprecipitation**—HeLa cells were transfected with an empty vector or pcDNA3-FLAG-N-Bak and lysed in 1 ml of EBC buffer (150 mM NaCl, 20 mM Tris at pH 7.8, and 0.5% Nonidet P-40) containing protease inhibitors (5 μg/ml leupeptin, 1 μg/ml phenylmethylsulfonyl fluoride, 7 μg/ml pepstatin, and 5 μg/ml aprotinin) and phosphatase inhibitors (1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, and 1 mM sodium fluoride). Lysates (250 μl) were cleared by centrifugation at 15,000 × g for 15 min at 4 °C and were incubated overnight with 2 μg of anti-Bax monoclonal (BD Biosciences Pharmingen) or anti-Bcl-X\textsubscript{L} polyclonal antibody (Ab-2; EMD Biosciences). Immune complexes were precipitated with Protein G-Sepharose (Pierce Biotechnology). Anti-FLAG M2 agarose (Sigma) was used for immunoprecipitation. Tagged proteins were washed four times with the same buffer, immune complexes were released from the beads by boiling in Laemmli buffer and then resolved by SDS-PAGE, followed by blotting and probing with an appropriate antibody as indicated in the text and figure legends.

**Western Blot Analysis**—Neurons or astrocytes were lysed in an extraction buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and the phosphatase inhibitors and protease inhibitors as described above. Protein extracts (50 μg of protein/lane) were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immun-Blot; Bio-Rad Laboratories). After blocking in phosphate buffered saline containing 0.1% Tween 20 and 2% casein, the membrane was incubated with primary antibodies in the same buffer. After processing with secondary antibodies (anti-mouse, anti-rabbit, or anti-goat, EMD Biosciences), the blots were developed with ECL reagents (Amersham, Arlington Heights, IL). Antibodies and their respective dilutions were as follows: Bak (Ab-2, 1:200; EMD Biosciences), Bax (Ab-2, 1:200; EMD Biosciences), Bcl-X\textsubscript{L} (Ab-2, 1:500; EMD Biosciences), FLAG (M2, 1:500; Sigma), β-actin (AC-15, 1:10,000; Sigma), and species-specific horseradish peroxidase-conjugated secondary antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA).

**RESULTS**

**Bax Mediates the Cytotoxic Actions of BH3-only Proteins**

*Bid* and *Bak* in Cortical and Hippocampal Neurons—We have demonstrated previously that Bax\textsuperscript{−/−} cortical and hippocampal neurons are resistant to different forms of apoptotic stress. The protection observed was nearly complete for DNA damage (camptothecin, ionizing radiation) but less complete for insults (glutamate, kainate, nitric oxide) that may involve more complicated apoptotic pathways (15, 24, 25). To determine whether Bax is the sole multidomain protein responsible for mediating apoptotic activity in neurons as suggested with DNA-damaging agents, we attempted to directly activate Bax or Bak by overexpressing *Bid* or *Bak* to over-repress *Bid* or *Bak* by overexpressing *Bid* or *Bak*. Components immediately upstream of the mitochondrial apoptotic pathway.

The *Bak*-only proteins *Bid* and *Bak* mediate mitochondrial apoptosis through distinct mechanisms that ultimately involve either Bid or Bak (26, 27). Cytosolic full-lengthBid (FL-Bid) is activated by caspase-8 cleavage to produce truncated Bid (tBid). This liberated proapoptotic fragment directly binds and activates Bax and Bak (28). In contrast, BAD does not directly bind to Bax or Bak; rather, it binds to the antiapoptotic proteins Bcl-2 and Bcl-X\textsubscript{L} at the mitochondrial membrane, which in turn causes Bax/Bak-dependent apoptosis by a mechanism that remains poorly defined (29, 30).

Viability of Bax\textsuperscript{−/−} and Bax\textsuperscript{−/−} neurons was examined after overexpression of FL-Bid, tBid, BAD, and Bak. Bax\textsuperscript{−/−} cortical and hippocampal neurons were remarkably sensitive to the cytotoxic actions of tBid and BAD overexpression, whereas...
data are presented as mean 

mid ( ) alone was used as a control for transfection with GFP-BAD, pCDNA3-FL-BID (tBid), or pCDNA3-tBID ( ). The pEGFP plasmid ( ) alone was used as a control for transfection with GFP-BAD and GFP-Bax. The viability of GFP-positive neurons was assessed 24 h after transfection. At least 500 cells were counted per condition. The data are presented as mean ± S.D. of three independent experiments. **, *p < 0.001.

Bax ( ) neurons were nearly completely protected from these insults (Fig. 1). Overexpression of FL-BID, which is inactive by itself, induced only a small decrease in viability in Bax ( ) neurons, suggesting limited processing to tBid, whereas no such cell death was observed in Bax ( ) neurons. The protection observed with Bax ( ) neurons was not caused by an inherent resistance of these cells to undergo apoptosis, because restoring Bax expression resulted in similar degrees of cell death in Bax ( ) and Bax ( ) neurons (Fig. 1). Thus, direct activation of mitochondrial apoptosis reveals that the ability of tBid and BAD to promote neuronal cell death is largely dependent on the presence of Bax. These results indicate that mitochondrial apoptosis does not proceed without Bax and, therefore, strongly suggest that Bak gene expression is not sufficient by itself to mediate apoptosis in these neurons.

Cortical and Cerebellar Granule Neurons Exclusively Express a Neuron-specific Bak Isoform, N-Bak—The predominant contribution of Bax to neuronal apoptosis observed in our studies could simply be caused by lower levels of the Bak protein being expressed in cortical and hippocampal neurons than in other cell types. On the other hand, these neurons may express a different isoform of Bak that cannot function as a multidomain proapoptotic protein, as observed in neonatal sympathetic and embryonic hippocampal neurons (17).

According to Sun et al. (17), alternative splicing produces at least two distinct species of mRNA (multidomain, full-length Bak, and N-Bak) from the Bak locus (Fig. 2A). The FL-Bak protein contains three BH domains (BH3, BH1, and BH2, in order of appearance) together with a transmembrane domain at the carboxyl terminus (Fig. 2B). N-Bak is generated by neuron-specific splicing that incorporates a novel 20-base pair exon (exon N) with a premature stop codon consequently created in exon 5, thereby producing a truncated protein lacking BH1 and BH2 domains (Fig. 2B). Thus, the two Bak isoforms fall into different groups of proapoptotic Bcl-2 family members: N-Bak represents a BH3-only protein, whereas FL-Bak is a multidomain protein.

We determined by RT-PCR and Western blot analysis which Bak isofrom is expressed in postnatal cortical and cerebellar granule neurons. For the identification of FL-Bak and N-Bak mRNA, we employed a two-step RT-PCR approach to ensure the specificity of amplification. The cDNAs amplified by RT-PCR using P1 and P2 primers were subsequently subjected to nested PCR with P3 and P4 primers (Fig. 2A). As shown in Fig. 2C, postnatal cortical neurons only express the mRNA for N-Bak, whereas astrocytes exclusively express FL-Bak mRNA. Western blotting confirmed the mutually exclusive expression of N-Bak in neurons and FL-Bak in astrocytes (Fig. 2C). The level of N-Bak mRNA expression was not dependent on Bax expression (Fig. 2C, Western blot, compare Bax ( ) versus Bax ( ) neurons: N-Bak/actin ratio, 0.21 versus 0.19, respectively). Consistent with the report by Sun et al. (17) showing the expression of N-Bak in addition to FL-Bak at the mRNA level in the cerebellum, we also detected mRNA expression of the N-Bak isoform in cerebellar granule neurons, whereas only FL-Bak was expressed in astrocytes (Fig. 2D). A minor amplification product corresponding to FL-Bak was also detected in the neuronal cultures at the mRNA level apparently because of the small number of contaminating astrocytes combined with the high sensitivity of detection by PCR amplification. At the protein level, however, only N-Bak protein was demonstrable (Fig. 2D), suggesting that cerebellar granule neurons represent another neuronal cell type that exclusively expresses N-Bak protein but not FL-Bak. To our knowledge, this is the first demonstration that the N-Bak protein is indeed expressed in neurons. This is important because previous studies on the expression and function of Bak in neurons either did not address the issue of which isoforms are expressed in neurons (31) or could not demonstrate N-Bak expression at the protein level (16, 17). From these results, we conclude that the apparent lack of any contribution by Bak to neuronal apoptosis observed in the present and previous studies is caused not simply by low expression levels of Bak, but rather by the absence of FL-Bak expression and, instead, the expression of a BH3-only form of Bak in cortical and cerebellar granule neurons. The lack of FL-Bak expression implies that Bax is the only Bcl-2 protein present in neurons capable of directly disrupting the mitochondrial membrane and explains why Bax deficiency provides complete protection against some forms of apoptotic insults in these neurons.

**N-Bak Induces Apoptosis in Cortical, Hippocampal, and Cerebellar Granule Neurons in a Bax-Dependent Manner**—Because BH3-only proteins are generally proapoptotic (26), it was unexpected that overexpression of N-Bak in sympathetic neurons reportedly did not induce cell death by itself and even protected these neurons from NGF deprivation-induced cell death (17). This prompted us to evaluate the influence of N-Bak overexpression on the viability of postnatal cortical, hippocampal, and cerebellar neurons. Control neurons expressing only enhanced green fluorescent protein (EGFP) elaborated well-developed dendritic and axonal processes typical of healthy cortical neurons cultured under these conditions (Fig. 3A). When FL-Bak protein was overexpressed, cortical neurons were efficiently killed, displaying extensive neurite fragmentation and degeneration (Fig. 3A). The killing occurred even in
the absence of Bax, indicating that FL-Bak can function as a multi-domain proapoptotic protein in these neurons when expressed (Fig. 3A). Overexpression of N-Bak also efficiently induced apoptosis but, in marked contrast to FL-Bak, did so only in the presence of Bax (Fig. 3A). Quantitative analysis in cortical as well as hippocampal and cerebellar granule neurons demonstrated that N-Bak is highly apoptogenic, and Bax deficiency provided complete protection in all three neuronal types (Fig. 3B). Thus, these findings indicate that increased levels of the N-Bak protein promote an acute decline in neuronal viability in these representative CNS neuronal populations, in sharp contrast to the antiapoptotic function of this protein reported for sympathetic neurons (17). Moreover, these results demonstrate that Bax is an essential downstream mediator of N-Bak-induced apoptosis in cortical, hippocampal, and cerebellar granule neurons.

Fig. 2. Cortical and cerebellar granule neurons exclusively express N-Bak, whereas astrocytes express only multidomain Bak. A, schematic representation of alternative splicing that generates two mouse Bak isoforms (FL-Bak and N-Bak) by selective incorporation of exon N, which creates a stop codon in the exon 5-derived sequence in N-Bak mRNA because of a frameshift. The sequences used for RT-PCR primers are shown as P1–P4. B, schematic domain structures of the FL-Bak and N-Bak proteins. The locations of the BH3, BH1, BH2, and transmembrane (TM) domains are illustrated as shaded boxes. C, Bak expression in cortical neurons and astrocytes. D, Bak expression in cerebellar granule neurons and astrocytes. N-Bak and FL-Bak are expressed exclusively in neurons and astrocytes, respectively, both at the mRNA and protein levels. For RT-PCR identification, the authenticity of amplified fragments was validated by size comparison with control FL-Bak and N-Bak fragments amplified from pCDNA3 FL-Bak and pCDNA3 N-Bak, respectively, by PCR using P3 and P4 primers. On Western blots N-Bak migrated at ~22 kDa (versus the predicted size of 16.4 kDa) and FL-Bak at ~29 kDa (versus the predicted size of 23.3 kDa). The slower migration of both N-Bak and FL-Bak relative to their predicted sizes was also observed when these proteins were transiently expressed in HeLa cells from the corresponding expression plasmids (data not shown). In addition, the identity of the protein bands was validated by running in parallel authentic N-Bak (IVT N-Bak) and FL-Bak (IVT FL-Bak) proteins that were in vitro translated and immunoprecipitated (D). RT-PCR and Western blotting experiments were repeated at least twice with essentially the same results.

Fig. 3. Elevated expression of N-Bak promotes neuronal cell death in a Bax-dependent manner. A, morphological assessment of neuronal cell death induced by overexpression of FL-Bak and N-Bak. Bax<sup>−/−</sup> or Bax<sup>+/−</sup> cortical neurons were co-transfected with a GFP expression vector and either pCDNA3 (vector), pCDNA3-FL-Bak (FL-Bak), or pCDNA3-N-Bak (N-Bak). The cultures were fixed and assessed for viability 24 h after transfection. Transfected neurons (green fluorescence) were judged as non-viable when they showed fragmented, degenerating neurites (arrowhead 1) as opposed to intact healthy neurites (arrowhead 2). Nuclear staining was done with Hoechst 33258 (blue). B, quantitative assessment of the cytotoxic action of FL-Bak and N-Bak expression on cortical, hippocampal and cerebellar granule neurons. At least 300 cells (200 cells for cerebellar granule neurons) were counted per condition. The data are presented as mean ± S.D. of three independent experiments. **, p < 0.001.
N-Bak Induces Cell Death by Caspase-dependent and -independent Pathways—

N-Bak-induced neuronal death in postnatal cortical neurons was only partially inhibited by the pan-caspase inhibitor, zVAD-fmk (Fig. 4A). This is consistent with the relative lack of sensitivity to caspase inhibition previously demonstrated for DNA damage-induced apoptosis of postnatal neurons (32, 33). Ectopic expression of N-Bak in HeLa cells induced apoptosis more robustly than FL-Bak and tBID (Fig. 4B). In marked contrast to neurons, however, N-Bak-induced apoptosis in HeLa cells was efficiently blocked by the pan-caspase inhibitor zVAD-fmk (Fig. 4B). The same apoptotic response, including nearly complete protection with caspase inhibition, was also observed in SH-SY5Y neuroblastoma cells (Supplemental Fig. 1). These results suggest that apoptotic signaling downstream of the mitochondria is not as dependent on caspase activity in neurons as in other cell types.

N-Bak Binds to Bcl-XL and Promotes Bax Translocation to the Mitochondria—
The results described above collectively demonstrate that N-Bak can act as a proapoptotic BH3 domain-only protein in postnatal cortical, hippocampal, and cerebellar granule neurons. We then sought to characterize the mechanism by which N-Bak promotes Bax-dependent cell death. We used HeLa cells for this study because N-Bak induces cell death in HeLa cells as efficiently as in neurons, but cell death can be effectively blocked with zVAD-fmk in HeLa cells, in marked contrast to postnatal neurons (Fig. 4). Inhibiting cell death at the caspase activation step facilitates characterization of N-Bak-mediated activities that contribute to the commitment phase of mitochondrial apoptosis without being obscured by postmitochondrial execution phases of apoptosis. The high transfection efficiency achieved with HeLa cells also facilitated biochemical analyses.

First, we determined whether N-Bak expression caused mitochondrial translocation of the Bax protein, a critical and requisite step for Bax to promote mitochondrial apoptotic changes (23, 34). A GFP-Bax fusion protein was co-expressed with N-Bak in the presence of zVAD-fmk to prevent cell death. Control cells (empty vector) displayed a diffuse, cytosolic distribution of Bax (Fig. 5A, vector). In marked contrast, overexpression of N-Bak produced a punctate distribution of Bax that overlapped with the mitochondrial marker HSP60, as seen with tBID overexpression, which served as a positive control (35) (Fig. 5A). Quantitative analysis clearly demonstrated that N-Bak induced a redistribution of the Bax protein in HeLa cells as efficiently as tBID (Fig. 5B).

Next, we examined the distribution of a myc-tagged N-Bak protein in the presence of zVAD-fmk. The overexpressed N-Bak protein did not show a diffuse cytoplasmic distribution; rather, it seemed to be principally associated with the plasma membrane and subcellular cytoplasmic structures, consistent with the presence of its C-terminal transmembrane domain. It was not concentrated at the mitochondria (Supplemental Fig. 2), in contrast to FL-Bak, which is known to constitutively reside in the mitochondria (36).

Finally, we performed co-immunoprecipitation experiments to determine whether N-Bak directly interacts with Bel-X<sub>L</sub> or Bax. We first confirmed that the N-Bak protein fused with the FLAG peptide (FLAG-N-Bak) at its amino terminus could induce apoptosis as efficiently as non-tagged N-Bak in HeLa cells and in cortical neurons as well (data not shown). From extracts of HeLa cells overexpressing FLAG-N-Bak in the presence of zVAD-fmk, anti-Bel-X<sub>L</sub> antibody co-immunoprecipitated the FLAG-N-Bak protein. Conversely, the anti-FLAG antibody co-immunoprecipitated the Bel-X<sub>L</sub> protein (Fig. 5C). Under the same conditions, however, we did not detect an immune-complex consisting of Bax and N-Bak (Fig. 5D). These results suggest that N-Bak promotes Bax activation not by directly interacting with Bax but by interacting with antiapoptotic members of the Bel-2 family.

N-Bak Is Up-regulated and Promotes Neuronal Cell Death after Injury—

Contrary to the observation made in sympathetic neurons (17), we found N-Bak to exhibit proapoptotic activity. To further elucidate the significance of this finding, we determined whether the endogenous N-Bak protein changes its expression level in response to apoptotic insults and consequently contributes to neuronal cell death. Exposure to the topoisomerase I inhibitor, camptothecin, generates DNA strand breaks and results in p53 and Bax-dependent cell death within 24 h of treatment (15, 37). N-Bak expression was elevated 12 h after exposure to camptothecin, as determined by semiquantitative
RT-PCR and Western blot (Fig. 6, A and B). The extent of up-regulation was similar in wild-type and p53-deficient neurons (Fig. 6, A and B), suggesting that N-Bak expression is regulated independently of p53 in the context of DNA damage. In contrast to N-Bak, the Bax protein was only modestly elevated in response to camptothecin-treatment (Fig. 6 B), in agreement with our previous report (15).

Next, the contribution of endogenous N-Bak to camptothecin-induced neuronal cell death was evaluated by suppressing N-Bak expression. Antisense Bak vectors have been used successfully to suppress expression of endogenous Bak proteins in a number of cell lines, including astrocyte-derived tumors (38, 39). We first validated the effectiveness of our antisense Bak vector, pCDNA3-AS-Bak, by demonstrating that it can significantly protect Bax−/− astrocytes from tBid-induced toxicity (Supplemental Fig. 3). We assumed that the antisense Bak construct could also effectively suppress the expression of N-Bak because FL-Bak and N-Bak share identical nucleotide sequences except for the inclusion of exon N in the N-Bak mRNA (see Fig. 2 A).
N-Bak Promotes Neuronal Cell Death

Recent studies have demonstrated in non-neuronal cells that the presence of both multidomain proapoptotic proteins, Bax and Bak, imparts functional redundancy; either protein provides a gateway to mitochondrial dysfunction and cell death (4–6). In neurons, however, despite the advances made in characterizing the role of Bax in apoptosis, the function of Bak remains poorly characterized. In the current study, we show that cortical and cerebellar granule neurons exclusively express a BH3-only isoform of Bak termed N-Bak, whereas astrocytes from the same brain regions exclusively express the multidomain FL-Bak. N-Bak, which is unable to induce mitochondrial apoptotic changes on its own as a BH3-only protein, instead binds to Bcl-X<sub>L</sub> and promotes Bax translocation to the mitochondria. Consistent with the absence of FL-Bak expression, apoptosis induced by overexpression of N-Bak, as well as tBID or BAD, is completely dependent on the presence of Bax in these N-Bak-expressing neuronal populations. Our results further demonstrate that the N-Bak protein is significantly elevated in response to DNA damage and promotes neuronal cell death. The action of N-Bak is thus proapoptotic and typical of BH3-only proteins. To execute apoptosis, N-Bak requires a multidomain proapoptotic protein, which is restricted to Bax for these neurons.

Neurons Express N-Bak—Our results demonstrate that postnatal mouse cortical and cerebellar granule neurons exclusively express the N-Bak isoform and therefore lack FL-Bak expression. Although a third multidomain proapoptotic Bcl-2 family member, Bok/Mtd, may be expressed in the brain (41) and is known to mediate DNA damage-induced apoptosis in SH-SY5Y neuroblastoma cells (42), there is no evidence that it is expressed in neurons (16) or that it contributes to neuronal cell death. Therefore, Bax seems to be the only multidomain proapoptotic protein available to mediate apoptosis at least in major neuronal populations such as cortical and cerebellar granule neurons (the present study) and sympathetic and hippocampal neurons (17). This is consistent with previous reports describing the complete protection conferred by Bax-deficiency against apoptotic cell death in these neuronal populations (12, 13, 15).

N-Bak mRNA is expressed only in the nervous system and in all brain regions examined (17). Furthermore, neonatal (P1-P2) sympathetic and embryonic (E17) hippocampal neurons in culture have been shown to express only N-Bak but not FL-Bak mRNA (17). At the protein level, however, N-Bak expression has not been previously demonstrated in these or any other types of neurons (16, 17). In addition, N-Bak protein expression was not detected in human temporal cortical tissue (43). In the present study, we successfully detected N-Bak protein expression in cortical and cerebellar granule neurons. Nonetheless, it remains to be determined whether expression of the N-Bak protein is restricted to certain neuronal populations or neurons at a specific stage of differentiation/maturation. It is also important to determine whether neurons can simultaneously express both Bak isoforms or are restricted to expressing a single isoform. FL-Bak protein expression was reported previously in cerebellar granule neurons in culture (16). Granule neurons in the cerebellum are actively generated when cultures are routinely prepared from 6–8 day-old postnatal brain (44); therefore, the cultures may contain neural/neuronal precursor cells. Neural precursor cells, including those from the cerebellum, are known to express FL-Bak (10, 45). Thus, the presence of FL-Bak expression in postnatal cerebellar granule neurons (16) might reflect the immature state of these neurons, which may

mined whether the inability of the antisense vector to promote long term survival is attributable to suboptimal suppression of N-Bak expression or to the redundancy of neuronal BH3-only proteins, including p53-inducible Noxa and PUMA that have recently been implicated in neuronal cell death (40), under the experimental conditions employed. However, it is unlikely that the FL-Bak protein becomes involved in cortical neuron cell death because of a shift in splicing from N-Bak to FL-Bak by skipping exon N during the course of apoptosis. FL-Bak expression was not detected either by RT-PCR (Fig. 6A) or Western blot analysis (not shown) at 12 h after camptothecin exposure, a time point at which mitochondrial apoptosis (caspase 3 activation) has already been initiated (data not shown). Together, these results demonstrate that endogenous N-Bak protein is not only up-regulated but contributes functionally to declining neuronal viability during the course of DNA damage induced cell death.

DISCUSSION

Recent studies have demonstrated in non-neuronal cells that the presence of both multidomain proapoptotic proteins, Bax and Bak, imparts functional redundancy; either protein provides a gateway to mitochondrial dysfunction and cell death (4–6). In neurons, however, despite the advances made in characterizing the role of Bax in apoptosis, the function of Bak remains poorly characterized. In the current study, we show that cortical and cerebellar granule neurons exclusively express a BH3-only isoform of Bak termed N-Bak, whereas astrocytes from the same brain regions exclusively express the multidomain FL-Bak. N-Bak, which is unable to induce mitochondrial apoptotic changes on its own as a BH3-only protein, instead binds to Bcl-X<sub>L</sub> and promotes Bax translocation to the mitochondria. Consistent with the absence of FL-Bak expression, apoptosis induced by overexpression of N-Bak, as well as tBID or BAD, is completely dependent on the presence of Bax in these N-Bak-expressing neuronal populations. Our results further demonstrate that the N-Bak protein is significantly elevated in response to DNA damage and promotes neuronal cell death. The action of N-Bak is thus proapoptotic and typical of BH3-only proteins. To execute apoptosis, N-Bak requires a multidomain proapoptotic protein, which is restricted to Bax for these neurons.

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N-Bak mRNA is expressed only in the nervous system and in all brain regions examined (17). Furthermore, neonatal (P1-P2) sympathetic and embryonic (E17) hippocampal neurons in culture have been shown to express only N-Bak but not FL-Bak mRNA (17). At the protein level, however, N-Bak expression has not been previously demonstrated in these or any other types of neurons (16, 17). In addition, N-Bak protein expression was not detected in human temporal cortical tissue (43). In the present study, we successfully detected N-Bak protein expression in cortical and cerebellar granule neurons. Nonetheless, it remains to be determined whether expression of the N-Bak protein is restricted to certain neuronal populations or neurons at a specific stage of differentiation/maturation. It is also important to determine whether neurons can simultaneously express both Bak isoforms or are restricted to expressing a single isoform. FL-Bak protein expression was reported previously in cerebellar granule neurons in culture (16). Granule neurons in the cerebellum are actively generated when cultures are routinely prepared from 6–8 day-old postnatal brain (44); therefore, the cultures may contain neural/neuronal precursor cells. Neural precursor cells, including those from the cerebellum, are known to express FL-Bak (10, 45). Thus, the presence of FL-Bak expression in postnatal cerebellar granule neurons (16) might reflect the immature state of these neurons, which may
continue expressing some degree of FL-Bak before completely switching to N-Bak. This could also account for the residual levels of FL-Bak mRNA expression observed in our cerebellar granule neuron cultures, although we did not detect FL-Bak protein. This residual level of FL-Bak mRNA, however, is more likely to be derived from contaminating astrocytes, which abundantly express FL-Bak (Fig. 5). It is interesting that neuroblastoma cells, which are thought to represent transformed neuronal precursor cells, express FL-Bak with no indication that N-Bak is co-expressed even after being induced to differentiate (46, 47). These results demonstrate that Bak gene expression is subject to developmentally regulated alternative splicing in the neuronal cell lineage, which may not be recapitulated in neuroblastoma cells. Together, individual neuronal populations and “neuron-like” cell lines may have to be separately evaluated for Bak isoform expression. Using customized protocols for protein extraction and immunoblotting analysis, as in the present study, and using an N-Bak-specific antibody may reveal more widespread expression of the N-Bak protein in neuronal cells.

Is N-Bak Function Context-dependent?—The present study has shown that N-Bak expression induces apoptosis in cortical, hippocampal, and cerebellar granule neurons, suggesting that the function of N-Bak in central nervous system neurons is to promote apoptosis in response to stress. Moreover, our results indicate that N-Bak protein expression is indeed up-regulated in response to DNA damage in cortical neurons and suppression of its expression delays the progression of apoptosis. Likewise, Fannjiang et al. (31) reported a proapoptotic function for the Bak gene in neuronal apoptosis in a stroke model using Bak knock-out mice. In this context, the antiapoptotic function observed when N-Bak was overexpressed in NGF-deprived sympathetic neurons is intriguing. How this BH3-only protein might enhance cell viability is not clear, however. Moreover, it is not known whether physiological levels of endogenous N-Bak can influence survival in NGF-deprived sympathetic neurons (17).

Nevertheless, there is a distinct possibility that proapoptotic Bcl-2 family members may confer protection from apoptosis, but only in neurons of the peripheral nervous system. On the other hand, it is conceivable that N-Bak is only neuroprotective under specific circumstances, such as when neurons are challenged by trophic factor withdrawal. Middleton et al. (48) previously reported that Bax overexpression conferred protection against neurotrophic factor deprivation-induced apoptosis in a multitude of sensory neuron populations, including those from the trigeminal mesencephalic nucleus (brain-derived neurotrophic factor), the dorsomedial part of the trigeminal ganglion (NGF) and dorsal root ganglia (NGF) and in parasympathetic neurons from the ciliary ganglion (ciliary neurotrophic factor). Thus, neurons of neural crest origin and the apoptotic paradigm involving survival factor deprivation may provide a cellular context in which normally proapoptotic Bcl-2 family members (Bax and N-Bak) can function as antiapoptotic proteins.

Viral-infected/transformed cells represent another condition in which Bak gene expression confers protection against apoptosis. This was demonstrated at the time of Bak’s discovery for serum deprivation-induced cell death in an Epstein-Barr virus-transformed lymphoblastoid cell line (49). A more recent example is reflected in the neuronal apoptosis induced by sindbis virus infection. Deletion of the Bak gene renders neurons more susceptible to neuronal cell death caused by this neurotropic virus (31). It is interesting that the effect of Bak gene deletion changes from promoting to suppressing spinal cord pathology and mortality as mice develop postnatally but remains proapoptotic in the hippocampus (31), again supporting the context-dependent nature of Bak gene function. Because the pathology caused by viral infection also involves the contribution of non-neuronal cells (50), the results obtained with whole animals and slice cultures will require further clarification using pure neuronal cultures. In addition, the fact that N-Bak is actually deleted in neurons in Bak-deficient mice, and not FL-Bak as previously assumed, may prove instrumental in elucidating the mechanism underlying the protective function of the N-Bak protein.

How Does N-Bak Function As a BH3-only Protein?—The mechanism by which N-Bak functions as a BH3-only protein to trigger mitochondrial translocation of Bax and enhance neuronal apoptosis remains to be investigated. Our data indicate that N-Bak interacts with Bcl-XL but not Bax, suggesting that its action is similar to that of Bad or Bik, which act as “enabler” BH3-only proteins (30). This is in contrast to the “activator” BH3-only protein Bid or Bim, which directly activates Bax and FL-Bak (26, 30). Although suppression of N-Bak expression provided only transient protection against DNA damage-induced apoptosis in our study, this is similar to the effect of Bim deletion in sympathetic neurons (51) and is believed to reflect a certain degree of functional redundancy among the various BH3-only proteins expressed in any given neuron. A recent report indicates that PUMA, a p53 inducible BH3-only protein, can mediate a significant fraction of p53-induced apoptotic activity in cerebellar granule neurons (40). PUMA is expected to function similarly to N-Bak in that it also interacts with Bcl-2/Bcl-XL (52). The fact that the absence of PUMA does not provide complete long-term protection from p53-induced neuronal death (40) supports the possibility that N-Bak and PUMA may work cooperatively as “enabler” BH3-only proteins to initiate mitochondrial apoptosis in neurons. It is notable that PUMA localizes at mitochondria in normal cells (52), whereas N-Bak does not (the present study), suggesting that these two BH3-only proteins in neurons may exert spatially segregated and therefore distinct functions in triggering mitochondrial apoptosis.

Another distinct feature of neuronal apoptosis suggested by the demonstrated expression of N-Bak is that the reciprocal lack of FL-Bak expression effectively eliminates one mechanism for apoptotic signaling in neurons. The interaction between p53 and FL-Bak at the mitochondria was recently shown to be critical for p53 to mediate its direct apoptogenic action at the mitochondria (53). The absence of FL-Bak in neurons eliminates this potential interaction, suggesting that the regulation of apoptosis in neurons differs from those pathways operating in non-neuronal cell types.

Concluding Remarks—The present study examined the expression and function of the Bak gene in central nervous system neurons. The results demonstrated that N-Bak is the sole Bak isoform expressed in neurons and it contributes significantly to DNA damage-induced apoptosis. To our knowledge, this is the first direct demonstration that the endogenous N-Bak protein is expressed and has a biological function in neurons. Despite the absence of the multi-domain proapoptotic FL-Bak, the corresponding addition of N-Bak to the list of available “enabler” BH3-only proteins may result in additional apoptotic signaling to Bax, facilitating Bax dependent apoptosis in these neurons. This may prove significant as the prototypic “enabler” BH3-only protein, BAD, primarily mediates cell death caused by survival factor deprivation in non-neuronal cells (54, 55), and may have a further limited role in neuronal apoptosis (16). N-Bak together with PUMA may thus function as the key enabler BH3-only proteins regulating mitochondrial apoptosis in neurons in response to DNA damage and other forms of stress. Finally, the identification of N-Bak provides an additional, neuron-specific target for pharmacological manipulation that could prove bene-
ficial in the treatment of human neurological conditions such as stroke and neurodegenerative diseases.

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REFERENCES

Supplemental Data

**Supplemental Figure 1.** Pan-caspase inhibitor, zVAD-fmk, fully protects SH-SY5Y cells from N-Bak-induced cell death.

SH-SY5Y cells were co-transfected with a pEGFP vector and either pCDNA3 (vector), pCDNA3-N-Bak (N-Bak), pCDNA3-FL-Bak (FL-Bak) or pCDNA3–tBID (tBid) in the presence or absence of 100 µM zVAD-fmk. DMSO was used as a vehicle control. Cells were fixed and processed for fluorescence and phase contrast imaging 24 hr after transfection. The absence of fluorescent cells in cultures transfected with the N-Bak plasmid in the absence of zVAD-fmk (DMSO) reflects the loss of transfected cells due to the induction of apoptosis, which occurred as efficiently as cell death induced by FL-Bak and tBid overexpression. N-Bak-induced cell death was almost fully inhibited in the presence of the pan-caspase inhibitor (zVAD-fmk).

**Supplemental Figure 2.** Subcellular localization of the N-Bak protein expressed in HeLa cells.

HeLa cells were transfected with either pCS2+MT N-Bak or pCS2+MT FL-Bak plasmids in the presence of zVAD-fmk (100 µM). 24 h after transfection, cells were fixed and processed for immunostaining for the myc tag (green) and the mitochondrial marker HSP60 (red). Hoechst 33258 (blue) was used for nuclear staining. The N-Bak protein fused with the myc tag at its amino terminus was confirmed to induce apoptosis as efficiently as non-tagged and FLAG-tagged N-Bak in cortical neurons and HeLa cells (data not shown). Note that myc-N-Bak is not associated with mitochondrial clusters in contrast to myc-FL-Bak, which displays nearly complete overlap with the mitochondrial marker. This suggests that N-Bak triggers apoptotic changes without involving its direct action at the mitochondria.

**Supplemental Figure 3.** Introduction of the full-length Bak antisense plasmid protects Bax−/− but not Bax+/− astrocytes from cell death induced by tBid overexpression.

Bax+/− and Bax−/− cortical astrocytes were triply transfected with pCDNA3-tBID (tBid), pCDNA3-AS-Bak (anti-sense) and a GFP expression plasmid at a 2:3:1 ratio. The empty vector pCDNA3 (vector) was used as a control for tBid and AS-Bak to normalize for the amount of plasmid used in the transfection. Viability of GFP-expressing cells was assessed 24 hr later on the basis of nuclear morphology. A minimum of 200 cells were counted per condition. Transfection with the anti-sense Bak vector significantly protected Bax−/− astrocytes from tBid-induced toxicity. Since the presence of either Bax or FL-Bak is sufficient for tBid to execute apoptosis (Wei et al., 2000; Wei et al., 2001) expression of the Bak antisense construct in Bax−/− astrocytes (Bax is still expressed) or the use of Bax−/− astrocytes (FL-Bak is still expressed) without the antisense vector failed to provide protection against tBid-induced toxicity. The data are presented as mean ± S.D. of three independent experiments. **, *p* < 0.01.

References

Supplemental Figure 2

myc/N-Bak
myc-DNA HSP60/DNA Merge

Supplemental Figure 3

Cell Survival (% of GFP-positive cells)

vector tBid Bax -/-
vector tBid Bax +/-
Neurons Exclusively Express N-Bak, a BH3 Domain-only Bak Isoform That Promotes Neuronal Apoptosis

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