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BAK Alters Neuronal Excitability and Can Switch from Anti- to Pro-Death Function during Postnatal Development

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Summary

BAK is a pro-apoptotic BCL-2 family protein that localizes to mitochondria. Here we evaluate the function of BAK in several mouse models of neuronal injury including neuronotropic Sindbis virus infection, Parkinson's disease, ischemia/stroke, and seizure. BAK promotes or inhibits neuronal death depending on the specific death stimulus, neuron subtype, and stage of postnatal development. BAK protects neurons from excitotoxicity and virus infection in the hippocampus. As mice mature, BAK is converted from anti- to pro-death function in virus-infected spinal cord neurons. In addition to regulating cell death, BAK also protects mice from kainate-induced seizures, suggesting a possible role in regulating synaptic activity. BAK can alter neurotransmitter release in a direction consistent with its protective effects on neurons and mice. These findings suggest that BAK inhibits cell death by modifying neuronal excitability.

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Introduction

Inappropriate programmed neuronal death is implicated in the pathogenesis of many human acute and chronic neurological disorders, including stroke, Alzheimer's, Parkinson's, and Huntington's diseases, amyotrophic lateral sclerosis, and spinal muscular atrophy (Gervais et al., 2002; Zhang et al., 2002; Kerr et al., 2000). Despite the deleterious role of programmed cell death in pathological states, this process must be preserved because neuronal death is required for development and for protection against inappropriate propagation of neural precursor cells. The intracellular machinery that facilitates at least some neuronal death during development includes components of the cytoplasmic "apoptosome" complex, as knockout mice lacking the apoptosome components Apaf-1, caspase-9, or caspase-3 share a similar phenotype with significant numbers of excess neurons in the brain at birth (Kuan et al., 2000). Important modulators of developmental neuronal death include trophic factor- and activity-dependent signals, but the molecular mechanisms for activating the apoptosome or other cell death machinery during pathological neuronal death is less clear. Excitotoxicity is implicated as an underlying mechanism in many disorders, including ischemia, Sindbis virus infection, and amyotrophic lateral sclerosis (Le et al., 2002; Nargi-Aizenman and Griffin, 2001; Howland et al., 2002).

Upon receiving a death stimulus, pro-apoptotic BCL-2 proteins (e.g., BAX and BAK) facilitate the release of apoptosome-activating and other death-promoting factors from mitochondria by forming pores, inducing channels, or otherwise altering mitochondrial membrane permeability and the structural architecture of mitochondria (Martinou and Green, 2001; Scorrano et al., 2002). The anti-death BCL-2 family members (e.g., BCL-2 and BCL-xL) counteract the function of the pro-death family members, though the mechanisms are incompletely understood (Hengartner, 2000). Anti-death BCL-2 and BCL-xL can also be converted into killer proteins when they are cleaved by caspases or other proteases (Cheng et al., 1997; Clem et al., 1998). The resulting C-terminal fragments have "BAX-like" pro-death activity, induce cytochrome *c* release, and form pores in synthetic membranes (Kirsch et al., 1999; Basanez et al., 2001). The BCL-2 homolog of *C. elegans*, CED-9, can also exhibit pro-death as well as anti-death activity (Hengartner and Horvitz, 1994). Pro-death BCL-2 family members also require activation to become potent killer proteins. A variety of activation mechanisms may be involved, including dephosphorylation, relocalization to mitochondria, conformational changes, oligomerization, and proteolysis (Eskes et al., 2000; Wolter et al., 1997; Nechushtan et al., 1999).

BAX is widely expressed in the nervous system, and promotes developmental neuronal death in the peripheral and central nervous system of mice (White et al., 1998). However, the role of BAX in deleting neurons during development is specific to neuronal subsets and to developmental stage, as some neuron populations

seem unaffected by BAX deficiency (White et al., 1998; Gibson et al., 2001; Doughty et al., 2000). Furthermore, there is compelling evidence that BAX can function as a pro-survival factor in neurons. BAX promotes survival of trigeminal ganglia neurons during development in mice that are deficient for NGF or TrkA, even though BAX promotes death of superior cervical ganglia neurons in these same models (Middleton and Davies, 2001). BAX potently protects mice and cultured hippocampal neurons from Sindbis virus-induced apoptosis, but BAX promotes death of Sindbis virus-infected cultured dorsal root ganglia neurons (Lewis et al., 1999).

Less information is available for the BAX homolog, BAK, but BAK generally exhibits pro-apoptotic activity similar to that of BAX when assayed in most cell culture systems (Chittenden et al., 1995). However, BAK protects lymphoblastoid WI-L2 cells from serum deprivation or menadione treatment, suggesting the possibility that its function in regulating cell death may also be context dependent (Kiefer et al., 1995). Deficiency in BAK appears to be developmentally inconsequential except in mice that lack both BAX and BAK (Lindsten et al., 2000; Rathmell et al., 2002). Double BAX/BAK-deficient mice have increased numbers of neurons, larger brains, and excess neural stem cells compared to mice deficient in BAX alone. The double knockouts are also more prone to spontaneous and stress-induced seizures. However, the combined effects of deleting both BAX and BAK only partially mimic the neuronal defects observed with deletion of the apoptosome components. The large brain protrusions found in *Apaf-1*$^{-/-}$ or *caspase 9*$^{-/-}$ mice are not observed in BAX/BAK double-deficient mice, indicating that additional factors contribute to apoptosome activation or that BAX and BAK are not efficiently activated in these knockouts.

Like BAX, BAK is widely expressed in various organ systems, including neurons of the central nervous system (Kiefer et al., 1995; Krajewska et al., 2002), but the functional role of BAK in pathological neuronal death has not been explored. We investigated the function of BAK in neurons using a neuronotropic virus vector and BAK-deficient mice, and found that BAK can either promote or inhibit neuronal cell death depending on a variety of conditions. Several lines of evidence suggest that mitochondrial BAK inhibits neuronal cell death by influencing synaptic activity. BAK-deficient neurons are more excitable, consistent with the anti-death function of BAK in a seizure model. These findings implicate BCL-2 family proteins in a mechanism by which presynaptic mitochondria regulate neuronal activity.

Results

BAK Overexpression Protects Immature Mice and Neurons from Sindbis Virus-Induced Death

Sindbis virus induces apoptosis in a variety of cell lines, but because of its profound neuronal tropism in vivo, Sindbis virus-induced death is primarily limited to neurons of the central nervous system of infected mice (Jackson et al., 1988; Lewis et al., 1996). Therefore, Sindbis virus can be used as a vector to express and quantify the apoptotic function of cellular factors in vitro and in vivo by measuring the ability of these factors to enhance or suppress Sindbis virus-induced cell death and mouse mortality (Cheng et al., 1996; Levine et al., 1996).

To verify the pro-apoptotic function of BAK in cell lines using the Sindbis virus vector, BHK cells were infected with Sindbis virus encoding either HA-tagged human BAK (SV-BAK) or a control virus (SV-Control) with BAK in reverse orientation. BAK significantly enhanced cell death at 24 hr postinfection of BHK cells (Figure 1A), and 293 and COS-1 cells (data not shown). In contrast, overexpressed BAK protected mice from a fatal virus infection, as 80% of the mice infected at postnatal day 3 with SV-BAK survived compared to only 40% of those infected with SV-Control virus (Figure 1C). Results obtained with untagged BAK were indistinguishable from HA-tagged BAK; another control virus encoding GFP (SV-GFP) produced the same results as the reverse BAK SV-Control virus, indicating that there was no anti-sense effect of the reverse BAK construct (data not shown). Immunoblot analysis confirmed expression of HA-BAK in BHK cells and in infected mouse brains (Figures 1B and 1D). To eliminate the possibility that differences in virus replication could explain the differences in mouse mortality, titers of SV-BAK and SV-Control viruses in mouse brains were determined and found to be indistinguishable throughout the course of infection (Figure 1E). Thus, BAK protects mice not by killing virus-infected neurons to prevent the spread of virus.

To verify a direct effect of BAK in mouse neurons, hippocampal tissue slices were prepared from 3-day-old mice and infected with the same recombinant viruses. SV-Control virus induced intense propidium iodide (PI) staining with the recognizable pattern of hippocampal pyramidal and granule neurons, indicating severe neuronal loss that is consistent with the neuronotropic infection pattern of Sindbis virus in mice (Figures 1F and 1G, right). In contrast, cultures infected with SV-BAK showed little PI staining, similar to mock-infected cultures (Figure 1G, middle, left). Results from four independent experiments are shown in Figure 1H. A similar protective effect of overexpressed BAK was observed in spinal cord tissue slices (Figures 1I and 1J), indicating that BAK is a protective factor in these systems.

Endogenous BAK Protects Immature Mice and Neurons from Sindbis Virus and Excitotoxic Death

To determine whether endogenous BAK is also protective in newborn mice, the progeny from *BAK*$^{+/-}$ and *BAK*$^{-/-}$ crosses were infected with control Sindbis virus. In agreement with the overexpression system, ~30% fewer *BAK*$^{-/-}$ mice survived compared to their *BAK*$^{+/-}$ (Figure 2A) and *BAK*$^{+/+}$ littermates (67%, n = 20). If BAK deficiency is the only defect that renders *BAK*$^{-/-}$ mice more susceptible to a Sindbis virus infection, then reconstitution of these mice with exogenous BAK should rescue the fatal phenotype. Indeed, SV-BAK improved survival of *BAK*$^{-/-}$ mice, and further improved survival of *BAK*$^{+/-}$ mice (Figure 2B). Immunoblot analysis of mouse brain lysates verified the PCR genotype results, and revealed a decline in endogenous BAK expression between 1 week and 3 months of age (Figure 2C), consistent with the observations of others (Krajewska et al.,

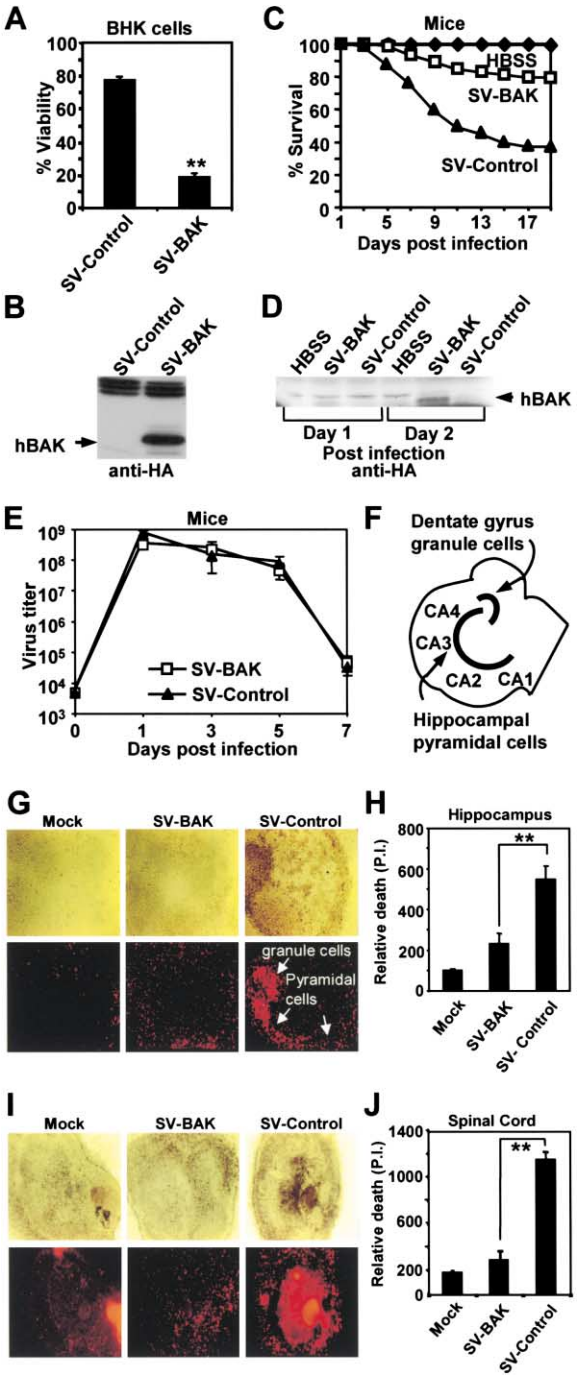

Figure 1. Overexpressed BAK Protects Young Mice/Neurons from Sindbis Virus-Induced Death

(A) Viability of BHK cells infected with recombinant Sindbis viruses was measured by trypan blue exclusion counting 200–400 cells/sample at 24–28 hr postinfection. Data are represented as mean ± SEM of four independent experiments (**, $p < 0.01$ by Student's t test).

(B) Immunoblot of SV-Control- and SV-BAK-infected BHK cells at 24 hr postinfection.

(C) Mouse survival following intracranial injection of 2- to 3-day-old CD-1 outbreed mice with the indicated recombinant viruses or buffer only ($n > 450$ mice per treatment group).

(D) Immunoblot of mouse brain lysates at the indicated times postinjection.

2002). Again, virus titers in infected brains of mice of different genotypes revealed no differences (data not shown).

Endogenous BAK was also protective in organotypic hippocampal (Figure 2D) and spinal cord (Figure 2E) cultures prepared from postnatal day 3 mice, as $BAK^{-/-}$ cultures were significantly more susceptible than $BAK^{+/-}$ cultures to virus-induced death detected by PI staining. Furthermore, exogenously expressed BAK (SV-BAK) partially rescued the BAK-deficient phenotype (Figures 2D and 2E). Results obtained with $BAK^{+/-}$ cultures were similar to $BAK^{+/+}$ cultures (data not shown). Again, no differences in virus titers were observed in these organotypic cultures (data not shown).

To determine whether BAK can protect neurons from cell death stimuli other than virus infection, organotypic hippocampal cultures prepared from postnatal day 3 mice were treated with kainate, NMDA, or glutamate to induce excitotoxic neuronal death. Endogenous BAK protected hippocampal cultures from excitotoxicity, as $BAK^{-/-}$ cultures exhibited intense focal PI staining whereas $BAK^{+/+}$ cultures survived significantly better (Figure 2F). Thus, endogenous BAK protects early postnatal neurons against cell death induced by both Sindbis virus infection and excitotoxicity.

Deletion of the Hydrophobic C Terminus Converts BAK from Anti-Death to Pro-Death Function

Unlike the BAX protein, which is cytosolic in healthy cells and translocates to mitochondria during cell death, BAK is reported to be constitutively localized on mitochondria in both healthy and dying cells (Griffiths et al., 1999; Wei et al., 2000). To determine whether BAK is cytosolic or mitochondrial in neurons where BAK is protective, organotypic hippocampal cultures were fractionated. Endogenous BAK was found predominantly in the heavy membrane fraction in both uninfected and Sindbis virus-infected cultures, consistent with mitochondrial localization in a paradigm where BAK inhibits cell death (Figure 3B, top).

To determine whether mitochondrial localization is required for the anti-death activity of BAK, the C-terminal hydrophobic membrane anchor domain was deleted (SV-BAKΔC) or replaced with the analogous domain of BCL-xL (SV-BAK-xL) (Figure 3A). Deletion or substitution of the BAK tail did not alter the pro-death

(E) Titers of infectious virus (pfu/100 mg brain tissue) were determined in duplicate plaque assays for five mice per data point (mean ± SEM).

(F) Diagram of mouse hippocampal organotypic cultures used in Figures 1G, 2D, and 2F. Orientation of cultures will vary.

(G) Light microscopy (top) and dead cell staining with propidium iodide (PI, bottom) of the same organotypic hippocampal slice cultures (prepared from CD-1 mice) infected with the indicated recombinant viruses.

(H) Relative PI intensity quantified by computer-based image analysis (setting mock-infected cultures to 100) in five independent experiments like that shown in (G); mean ± SEM (**, $p < 0.01$ by Student's t test).

(I) Spinal cord organotypic culture experiment as described for (G).

(J) Relative PI intensity for three independent experiments shown in (I) (described for [H]).

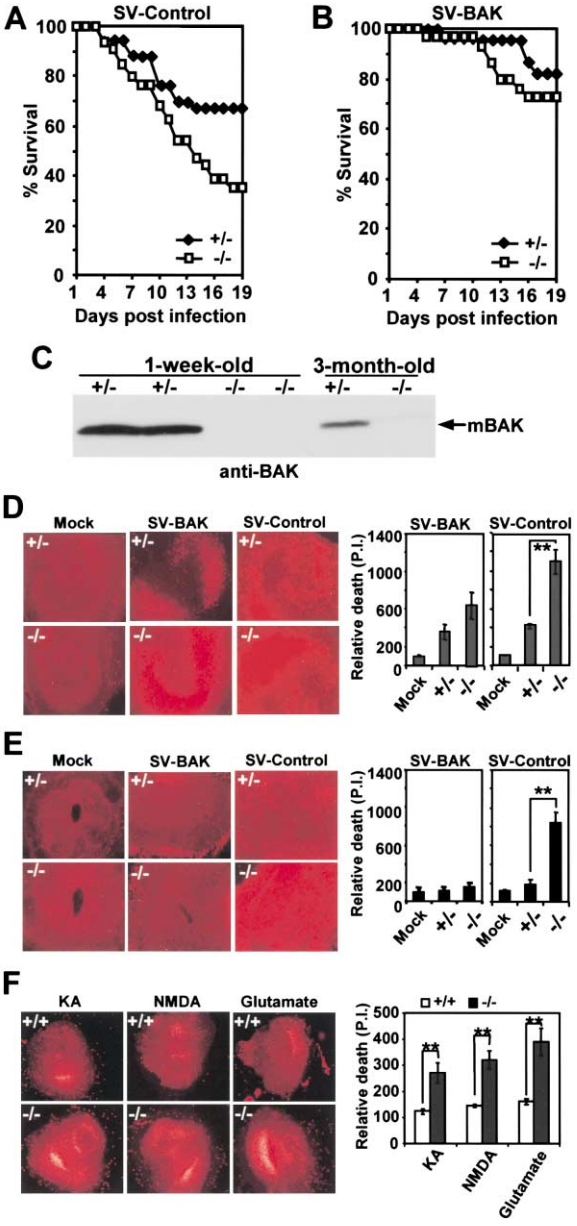

Figure 2. Endogenous BAK Protects Young Mice and Organotypic Neuron Cultures from Sindbis Virus- and Excitotoxicity-Induced Cell Death

(A) Survival of 4-day-old BAK littermates on a 129 x C57BL/6 background (33–40 mice/condition) following infection with control Sindbis virus (SV-Control).

(B) Survival of mice infected with Sindbis virus encoding HA-BAK (SV-BAK) as described for (A) (22–49 mice/condition).

(C) Immunoblot of endogenous BAK from brains of 1-week-old and 3-month-old littermates (each lane is a different mouse).

(D) Propidium iodide (PI) staining of dead cells in organotypic hippocampal cultures infected with recombinant viruses (left panels). Mean relative PI intensity for three independent experiments ± SEM (right panels; **, $p < 0.01$ by Student's t test).

(E) PI staining of organotypic spinal cord cultures as described for (D).

(F) PI staining of organotypic hippocampal cultures treated with kainate (KA), NMDA, or glutamate (left panels), for three independent experiments (right panels; mean ± SEM; **, $p < 0.01$ by Student's t test).

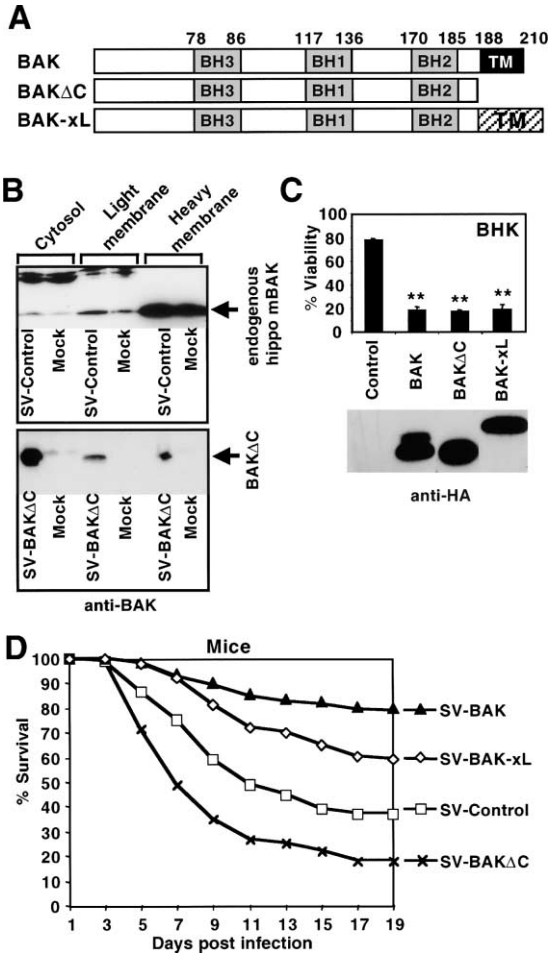

Figure 3. Hydrophobic C Terminus of BAK Is Required for Anti-Death Activity

(A) Map of wild-type and mutant BAK proteins. Residues 1–184 of BAK were fused to residues 197–233 of BCL-xL (BAK-xL).

(B) Immunoblot analysis of fractionated CD-1 organotypic hippocampal cultures infected with control virus (top), and *BAK*$^{-/-}$ organotypic hippocampal cultures infected with SV-BAK-ΔC (bottom).

(C) BHK cell viability by trypan blue exclusion at 24–28 hr postinfection with the indicated viruses expressing HA-tagged proteins (top); mean ± SEM for four independent experiments (**, $p < 0.01$ by Student's t test). Viability results are aligned with a corresponding immunoblot (bottom).

(D) Survival following infection of 2- to 3-day-old CD-1 outbreed mice with the indicated recombinant viruses (>200 mice per treatment group). Infection with another control virus (SV-GFP) generated survival data that were indistinguishable from BAK in the reverse orientation (data not shown).

function of BAK in BHK cells infected with Sindbis virus; immunoblot analysis verified the expected protein sizes (Figure 3C). In contrast to the effect in BHK cells, deletion of the BAK C terminus converted BAK from a potent protector into a killer protein in mice (Figure 3D); that is, newborn mice died faster and to a greater extent when infected with SV-BAKΔC compared to SV-Control. Replacing the BAK tail with the tail of BCL-xL (BAK-xL) partially restored the protective function of BAK in mice. The function of BAK mutants in mice was mimicked in organotypic hippocampal cultures (data not shown). To verify that BAKΔC is cytosolic in neurons, SV-BAKΔC

was used to infect organotypic hippocampal cultures prepared from BAK-deficient mice (to distinguish BAKΔC from endogenous protein) and cells were fractionated. As expected, BAK-ΔC was primarily found in the cytosol, though some BAK-ΔC protein was present in the membrane fractions (Figure 3B, bottom). The ability of BAKΔC to promote death in neurons argues that BAK retains the potential to kill cells even under circumstances where its killing activity is normally not activated.

BAK Is Anti- or Pro-Death in Older Mice Depending on the Neuron Subtype and the Neuronal Injury Model

To directly compare the function of BAK in newborn mice with the function of BAK in neurons of older mice, 4-week-old mice were infected with a neuroadapted Sindbis virus vector encoding BAK (NSV-BAK). NSV targets the same neuron populations as SV, including cortex, hippocampus, and spinal cord, but does so more efficiently in older mice (Jackson et al., 1988; Kerr et al., 2002). Similar to results obtained with hippocampal tissue slices from newborns, BAK protected hippocampal neurons (CA1-4) of older mice from Sindbis virus-induced death (Figure 4A). However, in contrast to newborn mice, older mice infected with NSV-BAK had higher mortality compared to NSV-GFP controls (Figure 4B). Thus, in those neurons that determine mortality rates in mice, BAK is also converted from an anti-death to a pro-death factor as mice age. The specific neuron population responsible for Sindbis virus-induced mouse mortality is not known but is postulated to be neurons of the brain stem that regulate vital functions. Again in contrast to young mice, BAK increased death of spinal cord neurons as determined by counting the number of morphologically normal (arrowheads) and pyknotic/deleted neurons (arrows) in spinal cord cross-sections of mice infected with NSV-BAK and NSV-GFP (Figures 4C and 4D, left).

Loss of motor neurons in the spinal cord correlated with the loss of axons in the ventral nerve roots that contain axons originating from motor neurons in the lumbar spinal cord. Mice infected with NSV-BAK had greater axonal degeneration in the lumbar ventral nerve roots (VR) compared to mice infected with NSV-GFP (Figures 4C and 4D, right). Consistent with the inefficient targeting of sensory neurons by Sindbis virus, the dorsal roots (DR) were less affected. Consistent with a pro-death function of BAK in the spinal cord, BAK enhanced hindlimb paralysis compared to GFP controls (Figure 4E). Almost all of the mice infected with NSV-BAK became paraplegic by 10 days postinfection. In contrast, most mice infected with NSV-GFP only developed kyphoscoliosis (thoracic muscle weakness) and mild hindlimb weakness. Interestingly, paralysis was occasionally observed in newborn BAK-deficient mice infected with Sindbis virus (data not shown), a phenomenon not previously observed in our prior experience with newborn mice, but consistent with the organotypic spinal cord studies shown in Figures 1 and 2, where BAK protects spinal cord neurons of newborns. Comparing the results from newborn and older mice infected with Sindbis virus, BAK appears to be converted from an anti-death to a pro-death factor in spinal cord neurons, but not in hippocampal neurons, during postnatal development.

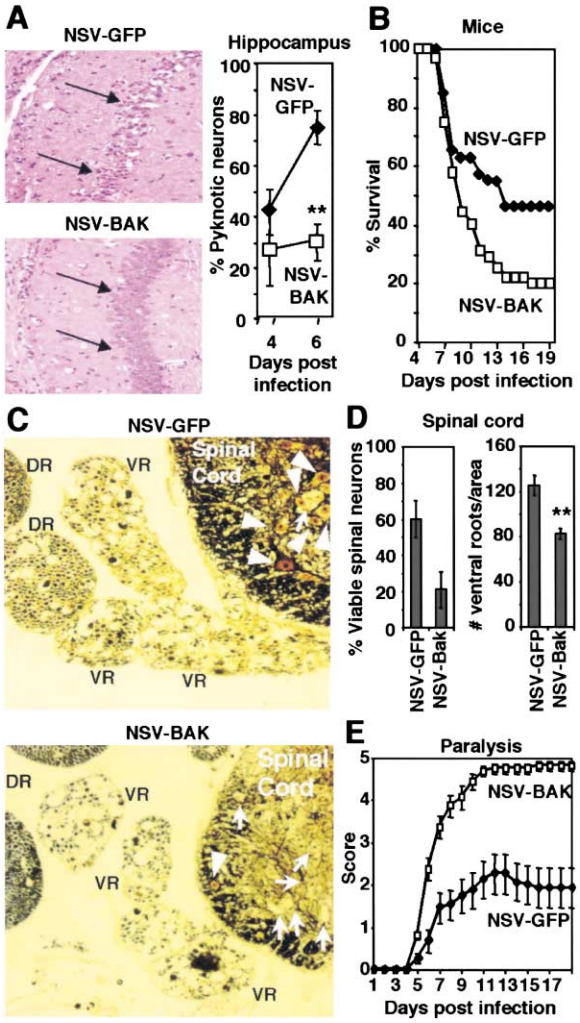

Figure 4. BAK Is Anti-Death or Pro-Death in Older Mice Depending on Neuronal Subtype

(A) Analysis of hippocampal neuronal death of NSV-infected mice ($n \geq 4$ mice per data point) by H&E staining (at 6 days postinfection, left) and percent pyknotic neurons (right). Black arrows indicate hippocampal neurons in CA2 and CA3.

(B) Survival of NSV-infected 4-week-old C57BL/6 mice ($n = 35$ per treatment group in two independent experiments).

(C) Silver staining of neurofilament in L4-L5 spinal column cross-sections from mice at 20 days postinfection with the indicated NSV virus vectors as described for (B). Large spinal cord neurons (arrowheads), pyknotic/deleted cells (arrows), ventral roots (VR), and dorsal roots (DR).

(D) Percent of viable spinal cord neurons (left) determined by counting all neuron cell bodies in spinal cord cross-sections like those shown in (C). Number of axons per area of ventral roots from the same sections (right). Data shown are for 12–18 areas from each of four mice per condition; mean ± SEM (**, $p < 0.01$).

(E) Severity of paralysis in C57BL/6 mice inoculated with recombinant NSV (20 mice per condition; mean ± SEM).

The function of BAK was also evaluated in older mice using models of stroke and Parkinson's disease (MPTP) even though these models do not allow direct comparisons with the same neuron subtypes in newborn mice. Interestingly, BAK promoted death in stroke/ischemic brain injury affecting the striatum and the cortex (Supplement 1, available at http://www.developmentalcell.

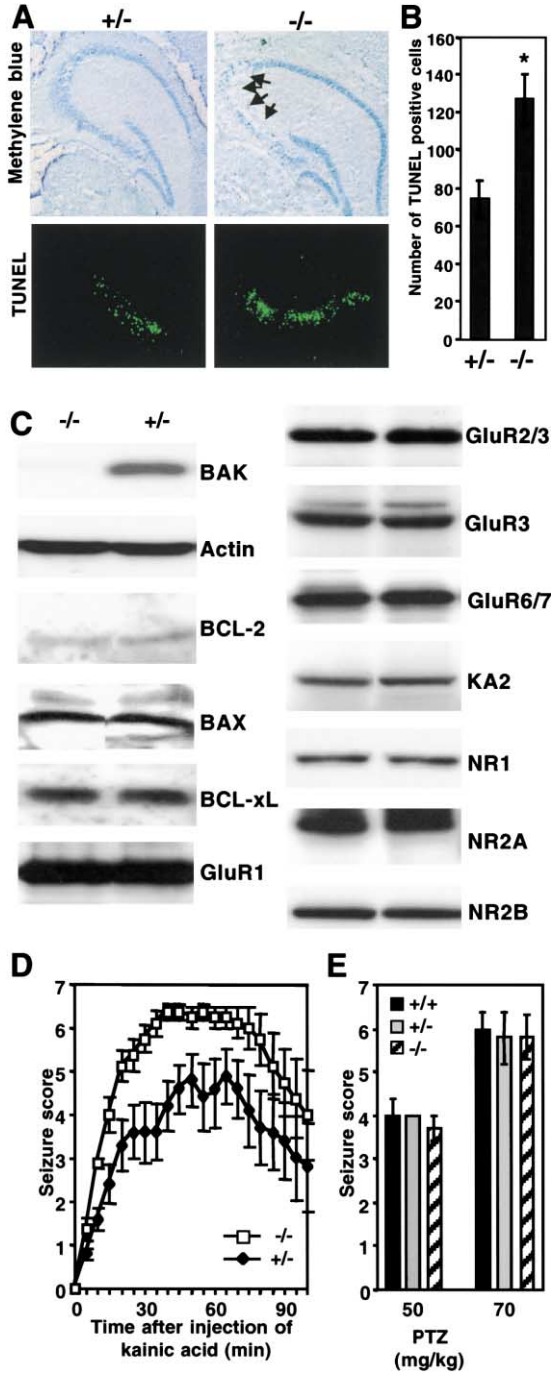

Figure 5. BAK Protects 4-Week-Old Mice from Kainate-Induced Seizures and Hippocampal Neuronal Cell Death

(A) Analysis of kainate (KA)-induced hippocampal neuronal death by histopathology (top) and TUNEL assay (bottom) on adjacent brain sections of $BAK^{+/-}$ (n = 8) and $BAK^{-/-}$ (n = 6) mice at 72 hr posttreatment (one $BAK^{+/-}$ and three $BAK^{-/-}$ mice died during treatment). Black arrows indicate regions with severe neuronal loss.

(B) Number of TUNEL-positive neurons in the hippocampus from mice described for (A) (mean ± SEM; *, $p < 0.05$, Student's t test).

(C) Immunoblot analysis of dissected hippocampal tissue from 4-week-old male $BAK^{-/-}$ and $BAK^{+/+}$ mice for the indicated BCL-2 family proteins and glutamate, kainate, and NMDA receptor subunits compared to the actin loading control.

(D) Seizure scores for BAK littermates (male) injected ip with kainate (mean ± SEM for 9 mice/genotype).

(E) Seizure scores for BAK male mice injected ip with PTZ (mean ± SEM; n ≥ 4 mice/group).

com/cgi/content/full/4/4/575/DC1), but was protective in mice treated with MPTP, which induces extensive injury of dopaminergic neurons in the substantia nigra pars compacta (Supplement 2).

BAK Protects Older Mice from Kainate-Induced Excitotoxic Neuronal Cell Death and Seizure

Treatment of rodents with the glutamate analog kainate induces limbic motor seizure followed by delayed cell death of neurons in the hippocampus (Ben-Ari, 1985). Therefore, to determine the function of BAK in excitotoxic death of mature hippocampal neurons, 4-week-old $BAK^{+/-}$ and $BAK^{-/-}$ mice were treated with kainate. Like the Sindbis virus model, BAK retained its protective function, as neuronal loss in CA3 and CA4 regions of the hippocampus was greater in $BAK^{-/-}$ mice than in control mice (Figure 5A, top). Similarly, neuronal death assessed by DNA fragmentation in a TUNEL assay was 40% higher in $BAK^{-/-}$ mice compared to their $BAK^{+/-}$ littermate controls (Figure 5A, bottom, and Figure 5B). This protective effect of BAK in the hippocampus cannot be attributed to altered protein expression levels of BCL-2, BCL-xL, BAX, or several glutamate, kainate, and NMDA receptor subunits, as immunoblot analysis of dissected hippocampi from 4-week-old mice failed to detect significant differences between $BAK^{+/+}$ and $BAK^{-/-}$ mice (Figure 5C). Assuming that the organotypic hippocampal cultures in Figure 2F reflect the function of BAK at early postnatal stages in mice, BAK appears to retain its pro-survival function in the hippocampus irrespective of age when subjected to an excitotoxic stimulus or to Sindbis virus infection.

The excitatory effect of kainate also contributes to the accompanying seizure that occurs within seconds to minutes after injection (Mulle et al., 1998). However, genes that regulate cell death occurring hours or days later would not be expected necessarily to alter the severity of seizures. For example, mice lacking p53 are protected from kainate-induced hippocampal neuron death but have the same degree of seizure activity as wild-type mice (Morrison et al., 1996). Analogously, knockout mice lacking the anti-apoptotic factor NAIP have increased kainate-induced neuronal death but NAIP has no affect on seizures (Holcik et al., 2000). Thus, it was initially surprising to find that $BAK^{-/-}$ mice had significantly greater susceptibility to kainate-induced seizures (Figure 5D). This implies that endogenous BAK suppresses the underlying mechanism of glutamate receptor-mediated seizure generation. Furthermore, this suppression mechanism by BAK must occur within seconds/minutes, prior to the subsequent cell death that takes hours/days to detect in a TUNEL assay. This raises the possibility that BAK suppresses cell death by regulating the excitability of neurons, either by suppressing excitatory transmission or enhancing inhibitory transmission. In contrast to kainate, there were no differences between $BAK^{-/-}$ mice and control littermates following

treatment with pentylenetrazol (PTZ), another epileptogenic agent (Figure 5E), suggesting that the BAK-specific effects of kainate are probably not caused by differential drug delivery across the blood-brain barrier.

BAK Modifies Synaptic Activity of Hippocampal Neurons

The protective effect of BAK against seizure activity and subsequent cell death is consistent with the hypothesis that BAK alters the intrinsic excitability of neurons. To investigate this possibility, we recorded synaptic responses in acutely isolated hippocampal tissue slices from 4-week-old $BAK^{+/+}$ and $BAK^{-/-}$ mice. We first investigated the characteristics of spontaneous miniature AMPA receptor excitatory currents (mEPSCs) using whole-cell voltage clamp recording in CA1 pyramidal neurons. Slices were pretreated with tetrodotoxin (TTX) to prevent action potential firing. Under these conditions, currents represent the spontaneous release of neurotransmitter from presynaptic terminals. The $BAK^{-/-}$ neurons showed a statistically significant increase in the frequency of excitatory mEPSCs compared to neurons from $BAK^{+/+}$ mice (Figure 6A). The increase in frequency was not accompanied by an increase in amplitude or a change in kinetics of the individual currents, indicating that BAK influences the probability of spontaneous release of neurotransmitter vesicles rather than influencing either the amount of transmitter in vesicles or the sensitivity of the postsynaptic cell. We also measured spontaneous release of neurotransmitter from inhibitory neurons. In contrast to mEPSCs, the frequency of miniature inhibitory postsynaptic currents (mIPSCs) was decreased in neurons from $BAK^{-/-}$ mice, again without change in amplitude or kinetics (Figure 6B). These findings indicate that either the intrinsic properties of neurotransmitter release or the density of synaptic endings are altered in $BAK^{-/-}$ animals.

To test for differences in excitatory transmission evoked by presynaptic stimulation, we recorded field potentials evoked by stimulation of the hippocampal slices. These recordings represent extracellular recordings of the postsynaptic response of pyramidal neurons. At low frequencies of stimulation ($<$1/30 s), we found no differences in the field potentials evoked in slices from $BAK^{+/+}$ or $BAK^{-/-}$ mice at any stimulus intensity (Figure 6C). When two stimuli are applied in rapid succession, the response to the second stimulus is typically enhanced. This paired-pulse facilitation, which is known to reflect enhanced transmitter release in response to the second stimulus, was also unchanged in $BAK^{-/-}$ mice (Figure 6D). With repetitive high-frequency stimulation, which evokes facilitation followed by a subsequent depression of synaptic transmission, however, we found a significant difference in the time course of postsynaptic responses. In particular, the excitatory synaptic responses from $BAK^{-/-}$ slices decremented significantly more rapidly during stimulation at 2.7 Hz (Figure 6E). Moreover, at the end of a 100-pulse 2.7 Hz stimulus train, the responses from $BAK^{-/-}$ slices were depressed relative to $BAK^{+/+}$, but recovered significantly more rapidly. These results are consistent with a change in the kinetics or the amount of neurotransmitter released from excitatory synapses. Taken together, these data indicate that both spontaneous and evoked neurotransmitter release is altered in $BAK^{-/-}$ animals. In particular, the reduction in spontaneous release from inhibitory GABAergic nerve terminals, and the increase from excitatory glutamatergic inputs are consistent with the enhanced severity of kainate-induced seizures in BAK-deficient mice.

Discussion

BAK is a versatile molecule that alters the fate of neurons by either promoting survival or inducing death depending on the stage of postnatal development and the specific cell type (summarized in Figure 7). Although the limitations of various models preclude a direct comparison of all neuronal subtypes at all ages, BAK was protective in all paradigms tested involving early postnatal neurons, but was converted to a pro-death protein in spinal cord neurons during postnatal development. This age-dependent conversion did not occur in hippocampal neurons, as BAK retained its protective function in models of excitotoxicity/seizure and virus infection. Proteolytic cleavage to sever the N terminus activates the cell killing activity of several BCL-2 family members. However, this type of proteolytic activation is apparently not sufficient to activate the pro-death function of BAK in immature neurons because ΔN-BAK (lacking amino acids 2–56), which potently induces death in BHK cells, failed to promote death in organotypic cultures and neonatal mice infected with Sindbis virus (unpublished data). Whereas this result does not exclude the possibility that proteolysis plays a role in situations where BAK is pro-death, additional mechanisms must regulate the anti- versus pro-death activity of BAK in hippocampal neurons.

Electrophysiological studies combined with the effects of excitatory stimuli on cultured neurons and mice indicate that endogenous BAK inhibits neuronal excitability. We suggest that the effect of BAK on synaptic transmission underlies its protective effect against excitotoxic agents and virus infection. Although BAK-deficient mice have no obvious anatomical defects, behavioral changes, or altered expression levels of surveyed BCL-2 family proteins and glutamate receptors, we cannot eliminate the possibility that neuronal connectivity and density of synaptic endings also contribute to the observed differences in synaptic activity. Nevertheless, our finding that synaptic transmission at low frequencies is not significantly altered by BAK suggests that there are no gross abnormalities in synaptic wiring.

BAK alters synaptic activity and neuron survival when localized to mitochondria. Mitochondria have a central role in regulating programmed cell death, but are also known to exert a profound influence on amplitude and time course of neurotransmitter release during repetitive synaptic stimulation (Friel and Tsien, 1994; Jonas et al., 1999). One key role for mitochondria within nerve terminals is the uptake and release of intracellular calcium during repetitive stimulation like those conditions used in this study. The enhanced rate of synaptic depression during high-frequency stimulation detected in $BAK^{-/-}$ neurons would be expected to occur if cytosolic calcium levels were elevated due to alterations in mitochondrial buffering (Zucker and Regehr, 2002). A similar

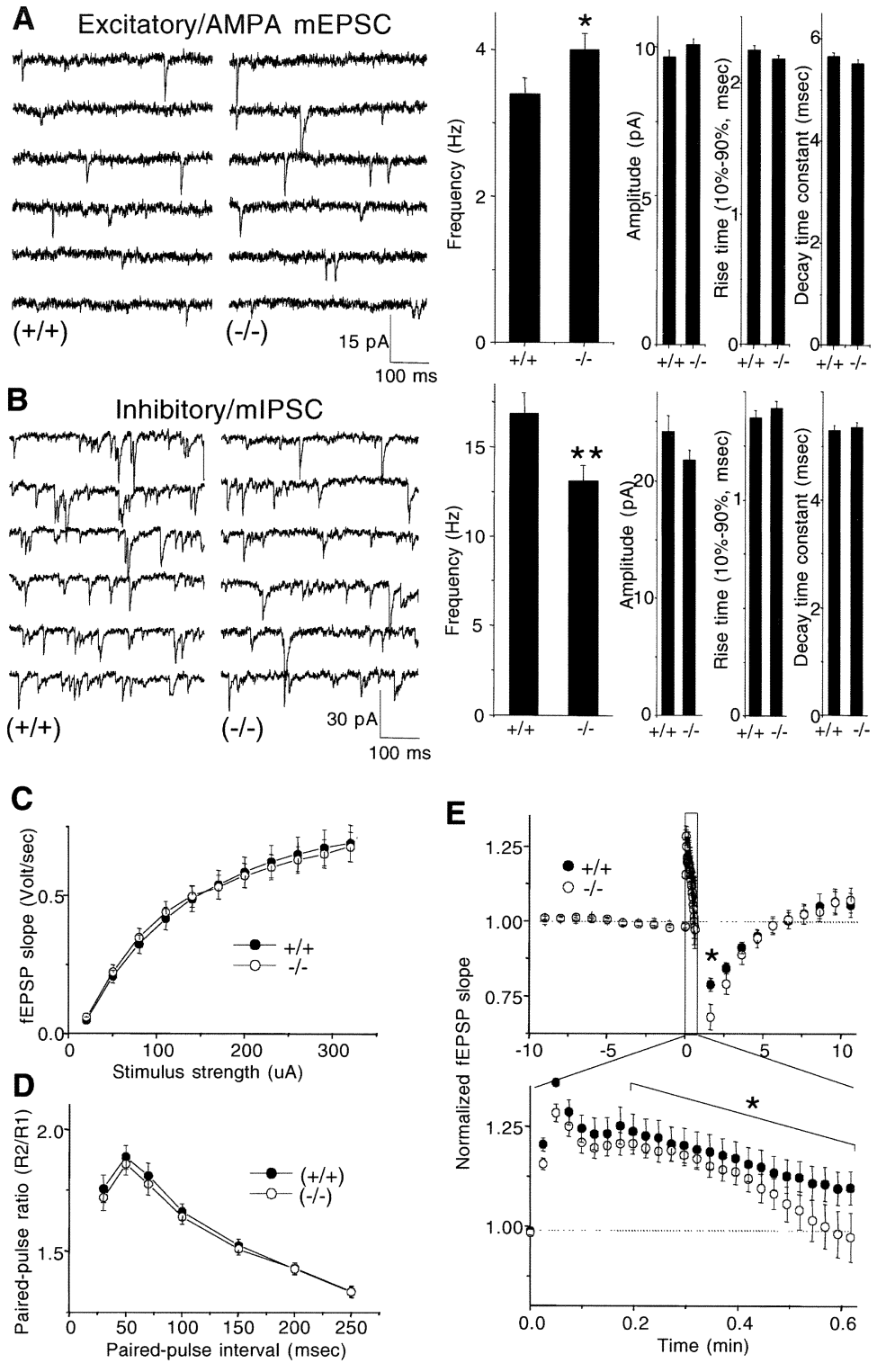

Figure 6. Characterization of Excitatory and Inhibitory Synaptic Responses from CA1 Hippocampal Pyramidal Neurons of *BAK*$^{-/-}$ Mice

(A) Measurements of miniature excitatory postsynaptic currents (mEPSCs). Traces show representative examples from wild-type and *BAK*$^{-/-}$ mice. Histograms (+/+, n = 25; −/−, n = 25) show the frequency of mEPSCs (+/+, 3.37 ± 0.23 (Hz); −/−, 3.96 ± 0.24 (Hz), *, p = 0.038). Also shown are the mEPSC amplitudes (+/+, 9.64 ± 0.23 pA; −/−, 10.07 ± 0.20 pA), rise times (10%–90% peak amplitude), and decay time constants.

(B) Measurements of miniature inhibitory postsynaptic currents (mIPSCs). Traces show representative examples from wild-type and *BAK*$^{-/-}$ mice. Histograms (+/+, n = 32; −/−, n = 32, from four pairs of male mice) show frequency of mIPSCs (+/+, 16.81 ± 1.20 [Hz]; −/−, 13.08 ± 0.89 [Hz], p = 0.0075), mIPSC amplitude (+/+, 24.16 ± 1.36 pA; −/−, 21.73 ± 0.89 pA), rise times, and decay time constants.

Anti, pro-death or neutral functions of endogenous BAK and BAX

Mouse model	Encephalomyelitis						Seizure		Parkinson disease	Stroke
Experimental method	Sindbis virus						Kainate/ glutamate		MPTP Toxicity	MCAO
Target neurons	Hippo-campal		Spinal cord		Mortality		Hippo-campal		Dopami-nergic	Cortical/ striatal
Age (weeks)	0.5	4	0.5	4	0.5	4	1	4	8-11	9-12
BAK	Anti	Anti	Anti	Pro	Anti	Pro	Anti	Anti	Anti	Pro
BAX	Anti	Anti	Anti	Neu	Anti	Anti	?	?	Pro	Pro

Figure 7. Summary of Anti- and Pro-Death Functions of BAK and BAX in Several Neuronal Injury Models Using Knockout Mice

Neuronal insults include middle cerebral artery occlusion and reperfusion (MCAO), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Results obtained with BAK-deficient mice are reported here. Results for BAX-deficient mice are reported elsewhere: Sindbis virus (Kerr et al., 2002), MPTP (Vila et al., 2001), and MCAO (Lee et al., 1999). Neu, neutral/no function observed.

elevation of presynaptic calcium is known to enhance the rate of recovery of transmission following repetitive stimulation (Wang and Kaczmarek, 1998; Sakaba and Neher, 2001). BCL-xL has been shown to regulate export of ATP from mitochondria (Vander Heiden et al., 1999). Thus, BAK could potentially affect synaptic activity if BAK alters the availability of ATP for the energy-requiring synaptic vesicle release mechanisms. BCL-xL may also influence neurotransmission. BCL-xL is known to promote neuron survival, is abundantly expressed in the adult brain, and is required for embryonic development of the nervous system (Motoyama et al., 1995; Krajewska et al., 2002). Injection of recombinant human BCL-xL protein into the giant presynaptic terminal of the squid stellate ganglion significantly increases the amplitude of synaptic potentials measured in the postsynaptic axon (unpublished data). Thus, BAK and BCL-xL may have different effects on synaptic function. Whether or not these are fixed or reversible functions of BAK and BCL-xL is not known. It is also reasonable to expect that altering synaptic activity could be beneficial and protective in one situation but detrimental and pro-death in another. Whereas synaptic activity is central in development and in strengthening synapses for long-term potentiation, excessive or perhaps context-inappropriate firing of action potentials can also be deadly, for example by promoting seizures (Gaiarsa et al., 2002; Selkoe, 2002). Thus, whereas BAK may protect some neurons by altering synaptic depression, BAK also could be potentially harmful to other neurons by influencing synaptic transmission in addition to its proposed role as an activated mitochondrial "death pore."

Separable Functions of BAK and BAX

Based on the analysis of knockout mice lacking BAK, BAX, or both, it appears that these proteins are redundant pro-death factors in several organ systems including neural progenitor cells of the central nervous system and in the lymphoid compartment (Lindsten et al., 2000; Rathmell et al., 2002). However, the mechanisms by which BAK and BAX regulate programmed cell death differ in at least some respects because they have opposite functions in certain neurons (Figure 7). In contrast to the results described here for BAK, both endogenous and overexpressed BAX protect 4-week-old mice from a fatal Sindbis virus infection and have no effect in the mature spinal cord (Kerr et al., 2002). Like BAK, the function of BAX may be dependent on the death stimulus. Although BAX protects neonatal hippocampal neurons from Sindbis virus (Lewis et al., 1999), BAX also promotes the few developmental hippocampal neuron deaths occurring at this same developmental stage (White et al., 1998).

The hydrophobic C-terminal tail of BAK and BAX may contribute to their differential regulation. Substitution of Ser184 in the hydrophobic C-terminal tail of BAX with different amino acids can have opposite effects on cell viability by either forcing or impairing mitochondrial localization of BAX in some cell types, perhaps depending on the affinity of the mutant tail for a binding pocket on the BAX molecule (Nechushtan et al., 1999). Interestingly, BAX mutants that remained cytosolic also improved cell viability compared to control, suggesting that forced cytosolic localization of BAX may be protective in some situations. Consistent with this idea, BAX remains cytosolic when it protects hippocampal neurons from Sindbis virus-induced cell death (our unpublished data).

Cell-Autonomous Function of BAK

We favor the idea that the reversible functions of BAK are due to neuron-specific regulatory events and do not require the indirect contribution of BAK in nonneuronal cells. A role for BAK in neurons is supported by the observation that BAK protein in the nervous system is predominantly neuronal (Krajewska et al., 2002). Of course, the role of nonneuronal BAK protein cannot be discounted in the study of BAK-deficient mice. However,

(C and D) Evoked excitatory postsynaptic responses as a function of stimulus strength (C) and paired-pulse facilitation ratio as a function of interval between the two stimulus pulses (D) (+/+, n = 24; −/−, n = 26 from four pairs of male mice).

(E) Synaptic responses during and after a 2.7 Hz stimulus train (100 pulses). The asterisk indicates a significant difference in fEPSP slope 1 min after the 2.7 Hz train (one-tailed t test, $p = 0.015$; at two-tailed t test, $p = 0.031$; +/+, n = 23; −/−, n = 25) that diminishes within 3 min, indicating an enhanced rate of recovery in the −/− slice; the slope from 1 to 4 min was significantly faster by t test in −/− mice (0.0883 ± 0.0071) compared to +/+ mice (0.054 ± 0.001), $p = 0.0379$. The lower graph shows an expanded time scale. The marked range (*) shows a significant enhancement of depression in the *BAK*$^{-/-}$ mice (two-factor ANOVA, $p = 0.0324$). Data presented are from four pairs of male mice; error bars indicate SEM. Electrophysiology methods are described in Supplement 3.

the use of Sindbis virus to deliver BAK specifically to neurons makes it likely that the anti- and pro-death functions of BAK observed in this model are due to the actions of BAK inside neurons. In addition, these and other results argue that BAK protects not by an indirect effect of inducing cell death in a subset of neurons or of other cell types.

Experimental Procedures

Viruses and Cell/Tissue Culture Assays

Recombinant Sindbis virus vectors (SV/dsTE12Q and NSV) were generated as described (Hardwick and Levine, 2000; Kerr et al., 2002). BHK (baby hamster kidney) cells were infected ~12 hr after plating with ten plaque-forming units (pfu)/cell.

Organotypic cultures were prepared as described (Vornov et al., 1995). One-week-old cultures were infected with 1×10^6 pfu per slice by slow drip of 100 μl inoculum. At 48 hr postinfection, cultures were stained for another 24 hr at 37°C with propidium iodide (Sigma), and fluorescence was measured by computer-based image analysis. Fluorescence intensity of mock-infected cultures was set to equal 100.

For excitotoxicity studies, ten day-old organotypic cultures prepared from 7-day-old mice were treated with 1 ml of culture media plus kainic acid (30 μM), NMDA (50 μM), or glutamate (500 μM) for 1 hr in a 37°C dry incubator. During a 24 hr recovery period, cultures were stained with propidium iodide as described above. In each experiment, three to four culture slices were used per virus construct or excitotoxin.

Virus-Infected Mice

For recombinant Sindbis virus (SV/dsTE12Q) studies, 2- to 3-day-old CD-1 mice (Charles River Laboratories) or 4- to 5-day-old progeny of BAK heterozygous crossings were inoculated by intracerebral injection of 5×10^3 pfu of virus diluted in 30 μl of HBSS. Mice were genotyped as described (Lindsten et al., 2000). Recombinant NSV viruses (5×10^3 pfu) were injected intracerebrally into 4-week-old male C57BL/6 mice (Charles River Laboratories). Animal paralysis severity was scored as follows: 0, no paralysis; 1, kyphoscoliosis; 2, kyphoscoliosis with mild hindlimb weakness; 3, paraparetic; 4, paraplegia; 5, death.

For histological analysis, NSV-infected mouse brains were harvested at 4 and 6 days postinfection and prepared as described (Kerr et al., 2002). To determine the extent of spinal cord neuron damage, the entire lumbar spinal column was processed, silver stained by modified Bielschowsky methods, and counted as described (Kerr et al., 2002).

Kainate Treatment

BAK$^{+/-}$ and *BAK*$^{-/-}$ male mice (4 weeks old) were injected intraperitoneally with a single dose of 45 mg/kg kainic acid (A.G. Scientific). Seizure activity was recorded every 5 min for 100 min following injection as described (Morrison et al., 1996); 1, arrest of motion; 2, rigid posture; 3, automatism; 4–6, increasing severity of bilateral tonic-clonic activity; 7, death. At 72 hr after kainate injection, brains were frozen in isopentane/dry ice and 20 μm hippocampal sections were stained with 0.1% methylene blue or analyzed for DNA fragmentation by TUNEL assay (Fliss and Gattinger, 1996).

Immunoblot and Fractionation Analysis

BHK cells or mouse brains were homogenized with 20 strokes in a type B dounce homogenizer and immunoblotted with anti-HA antibody (HA-12CA5, Roche) or with polyclonal rabbit antibody against human BAK (66026E, PharMingen). Infected mouse organotypic hippocampal cultures were harvested at 72 hr postinfection and fractionated as described (Chau et al., 2000).

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