Bcl-2 family regulation of neuronal development and neurodegeneration

Rizwan S. Akhtar a,b, Jayne M. Ness c, Kevin A. Roth a,*

a Division of Neuropathology, Department of Pathology, University of Alabama at Birmingham, 1530 Third Avenue South, SC 961, Birmingham, AL 35294-0017, USA
b Department of Neurobiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA
c Division of Pediatric Neurology, Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL 35294, USA

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Abstract

Neuronal cell death is a key feature of both normal nervous system development and neuropathological conditions. The Bcl-2 family, via its regulation of both caspase-dependent and caspase-independent cell death pathways, is uniquely positioned to critically control neuronal cell survival. Targeted gene disruptions of specific \textit{bcl-2} family members and the generation of transgenic mice overexpressing anti- or pro-apoptotic Bcl-2 family members have confirmed the importance of the Bcl-2 family in the nervous system. Data from studies of human brain tissue and experimental animal models of neuropathological conditions support the hypothesis that the Bcl-2 family regulates cell death in the mature nervous system and suggest that pharmacological manipulation of Bcl-2 family action could prove beneficial in the treatment of human neurological conditions such as stroke and neurodegenerative diseases.

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1. Introduction

Regulated cell death plays an important role in the development and survival of multi-cellular organisms. During embryogenesis, programmed cell death results in the regression of phylogenetically vestigial structures, the sculpting of organs, and establishment of appropriate cell numbers [1,2]. In the mature animal, apoptotic and non-apoptotic forms of regulated cell death are involved in both normal cellular turnover and in the biological response to pathological insults. The significance of cell death regulation is clearly evidenced in the mammalian nervous system where altered levels of programmed cell death may lead to a wide spectrum of neurodevelopmental anomalies. Similarly, increased cell death in the mature nervous system may lead to a variety of neurodegenerative pathologies. For these reasons, the Bcl-2 family has been the focus of intense neuroscientific interest.

The Bcl-2 family consists of three major subgroups [3]. Anti-death Bcl-2 family members, such as Bcl-2, Bcl-X L, Bcl-w and Mcl-1, typically possess four conserved motifs termed Bcl-2 homology (BH) domains. When overexpressed, these proteins inhibit cell death responsiveness. Multi-domain pro-death Bcl-2 family members, the second subgroup, typically possess at least BH1 and BH2 domains and include Bax, Bak, Bok, and Bcl-X S. The third, and most structurally diverse subgroup, is the BH3 domain-only pro-death subgroup. BH3-only molecules include Bim, Bid, Bcl-2-associated death protein (Bad), death protein 5/\textit{harakiri} (DP5/Hrk), Puma, and Noxa and induce cell death following a variety of stimuli. Bcl-2 family members can heterodimerize via their BH domains and execution of a cell death stimulus may depend on the intracellular balance between various Bcl-2 subfamily members [4]. The subcellular distribution of Bcl-2 family members varies from purely cytosolic to predominantly mitochondrial, to being present in multiple intracellular membranous compartments [5]. Bcl-2 family regulation of mitochondrial cytochrome \textit{c} release and mitochondrial function plays a large part in controlling cell death [3,6]. The death-promoting effects of Bcl-2 family action may occur secondary to downstream caspase activation, resulting in an apoptotic cellular phenotype, or be caspase-independent and display alternative degenerative morphological features. Determination of the significance of the Bcl-2 family in the regulation of neuronal
cell death and that of individual family members in particular has been greatly aided by the generation of transgenic mice. The overexpression of Bcl-2 and/or Bcl-XL in neurons has clearly demonstrated a significant neuroprotective function for these molecules in the mature nervous system. Targeted gene disruptions have similarly proved valuable in demonstrating critical roles for specific Bcl-2 family members in regulating neuronal programmed cell death. Genetically modified mice have also been useful in demonstrating a potential role for Bcl-2 family regulation of neuronal cell death in a variety of neuropathological conditions. Combined with direct studies of human tissues, these investigations strongly implicate the Bcl-2 family as a key regulator of neuronal cell fate.

2. Bcl-2 family regulation of neuronal programmed cell death

2.1. Bcl-2

Bcl-2 is the prototypical member of the Bcl-2 family [3]. Bcl-2 mRNA and protein are present at relatively high levels in the developing nervous system and decline significantly in the postnatal brain [7,8]. Bcl-2 expression is maintained at relatively high levels in sensory and sympathetic neurons in the adult peripheral nervous system [8,9]. The role of Bcl-2 in neuronal programmed cell death has been examined using several experimental approaches. Trophic factor withdrawal-induced death of a variety of neuronal cell lines and primary neuron populations, an in vitro model of target-dependent programmed cell death, is inhibited by Bcl-2 overexpression [10–13]. Similarly, neuronal overexpression of Bcl-2 in transgenic mice increases the number of neurons in many brain regions by inhibiting naturally occurring neuronal cell death [11,14]. These overexpression studies, however, only indicate a potential role for endogenous Bcl-2 in regulating programmed cell death since other anti-apoptotic family members may play a more significant role in vivo. Targeted disruption of the bcl-2 gene has indeed revealed selective effects of endogenous Bcl-2 expression on different neuronal populations.

Despite high levels of Bcl-2 mRNA and protein expression in both neural precursor cells and in postmitotic neurons in the embryonic brain, Bcl-2-deficient embryos show relatively normal nervous development and no significant increase in neuronal programmed cell death. Bcl-2-deficient mice do, however, exhibit a profound loss of motoneurons, sympathetic neurons, and sensory neurons during early postnatal life [15–17]. Unlike neurons in the brain, these peripheral neuronal populations normally exhibit significant baseline Bcl-2 expression during the neonatal period [8]. The death of these neurons in Bcl-2-deficient mice occurs after the normal peak of programmed cell death in these populations, suggesting that Bcl-2 may play a more important role in maintenance of their survival rather than in regulating their programmed cell death per se.

2.2. Bcl-XL

The bcl-x gene can be alternatively spliced to produce two major protein isoforms, Bcl-XL and Bcl-XS [18,19]. Bcl-XL is highly homologous to Bcl-2 and is expressed at relatively high levels in both the embryonic and mature nervous systems. In contrast, Bcl-XS is pro-apoptotic and is found only at low levels in the mammalian nervous system [19]. Bcl-XL expression is up-regulated in immature neurons as they migrate away from the mitotically active ventricular zone and remains highly expressed in mature neurons in the adult brain [20,21]. Targeted gene disruption of bcl-x has revealed a significant role for Bcl-XL in regulating neuronal programmed cell death [20]. Bcl-XL-deficiency is lethal around embryonic day (E) 13.5 secondary to extensive hematopoietic cell apoptosis; analysis of Bcl-XL-deficient embryos prior to this point reveals massive cell death of immature neurons throughout the developing nervous system (Fig. 1) [20]. Bcl-XL expression is low in neural precursor cells and Bcl-XL deficiency has no obvious effect on this cell population [20]. The increased susceptibility of Bcl-XL-deficient neurons to death stimuli is reca-

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Fig. 1. Bcl-XL deficiency causes extensive death of neurons in the developing nervous system. Hematoxylin and cosin stained sections of the E12 mouse spinal cord show occasional neurons with apoptotic histological features undergoing programmed cell death in the wild-type embryo (left panel; example indicated by an arrow) and massive numbers of apoptotic neurons in the Bcl-XL-deficient spinal cord (right panel; examples indicated by arrows). Scale bars equal 20 μm.
pitulated in vitro. Telencephalic neurons isolated from bcl-x<sup>-/-</sup> embryos were markedly sensitive to trophic factor deprivation and other cell death stimuli such as genotoxic injury and chloroquine administration [22–24]. Together, these results suggest that the survival of newly post-mitotic immature neurons depends on Bcl-X<sub>L</sub> function.

The increased programmed cell death observed in the Bcl-X<sub>L</sub>-deficient nervous system appears to involve activation of the intrinsic, mitochondrial-dependent, apoptotic death pathway. Bcl-X<sub>L</sub>-deficient embryos exhibit a marked increase in activated caspase-3-like activity in the developing nervous system [21]. The increased neuronal cell death observed in these embryos is completely blocked by concomitant deficiency in caspase-9 or caspase-3 [21,25]. Thus, for immature neurons in the developing brain, caspase-dependent apoptotic cell death is inhibited by endogenous expression of Bcl-X<sub>L</sub>. In addition to Bcl-X<sub>L</sub> and Bcl-2, recent studies also suggest a role for Bcl-w in neuronal cell death [26–29]. However, Bcl-w deficiency does not cause obvious neurodevelopmental abnormalities [30]. Further studies will need to address the functional redundancy of anti-apoptotic Bcl-2 family members.

2.3. Bax

The pro-apoptotic molecule Bax was originally identified as a binding partner for Bcl-2 [4]. Bax is expressed in both the embryonic and adult brain and can heterodimerize with Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, and A1 [4,31–33]. Current data suggest that Bax regulates cytochrome c release from mitochondria, perhaps via formation of the mitochondrial transition pore [6,34]. Anti-apoptotic members of the Bcl-2 family may heterodimerize or hetero-oligomerize with Bax to inhibit Bax function. Targeted gene disruption of bax has demonstrated an important role for Bax in triggering neuronal programmed cell death.

In the developing nervous system, programmed cell death of synapse-bearing neurons during a period of competition for target-derived neurotrophic support helps determine neuronal cell numbers. Bax-deficient mice exhibit markedly decreased neuronal programmed cell death in a variety of sites including brainstem, cerebellum, dorsal root ganglia, hippocampus, and spinal cord, with resultant increased neuron numbers in these areas [35,36]. This phenomenon is recapitulated in vitro in neonatal sympathetic neuron cultures. Following nerve growth factor (NGF) withdrawal, wild-type sympathetic neurons exhibit redistribution of cytochrome c from the mitochondria to the cytosol and undergo fairly rapid apoptotic death [35,37]. In contrast, Bax-deficient sympathetic neurons do not show increased cytosolic cytochrome c and survive for weeks after NGF withdrawal [37]. Bax-deficient neurons under these conditions exhibit reduced soma size and axon diameter. Neuronal atrophy is also observed in vivo in some populations of Bax-deficient neurons, indicating that neurons “rescued” from programmed cell death may not be completely normal [35,36]. Interestingly, in certain models of trophic factor withdrawal-induced neuronal death, Bax may not play a critical role. For example, in NGF-deprived trigeminal neurons, Bax-deficiency delays, but does not prevent, cell death [38]. Also, in cultured chick sensory and ciliary neurons, Bax overexpression may have a pro-survival action following trophic factor withdrawal [39]. Bax may also play a role in regulating activity dependent neuronal apoptosis. Cerebellar granule neurons (CGNs) undergo apoptosis when cultured in non-depolarizing, serum-free media, and Bax-deficient CGNs are protected from this apoptotic stimulus [40,41].

Targeted disruptions of both bax and bcl-x revealed important roles for these Bcl-2 family members in neuronal programmed cell death. To examine the potential interaction of Bax and Bcl-X<sub>L</sub> in neuronal cell death regulation, bax<sup>-/-</sup>/bcl-x<sup>-/-</sup> embryos were generated. Bax, Bcl-X<sub>L</sub> dual-deficient embryos showed complete protection from Bcl-X<sub>L</sub> deficiency-induced neuronal apoptosis [42]. The neuroprotective effect of Bax deficiency on Bcl-X<sub>L</sub>-deficient cells was also observed in vitro in primary telencephalic neuronal cell cultures [42]. Bax deficiency did not, however, rescue Bcl-X<sub>L</sub>-deficient mice from embryonic lethality, indicating that Bcl-X<sub>L</sub> has both Bax-dependent and Bax-independent actions on different cell types [42].

Although Bax deficiency inhibits programmed cell death in many neuronal populations, Bax-independent death pathways also contribute significantly to nervous system morphogenesis. Like Bax-deficient mice, mice with targeted gene disruptions of caspase-9, apaf-1, and caspase-3 exhibit markedly reduced programmed cell death. However, unlike Bax-deficient mice, a significant number of embryos with caspase-9, apaf-1, and caspase-3 gene disruptions also possess gross structural brain abnormalities, including neural precursor cell hyperplasia and forebrain exencephaly [43–46]. These observations suggest that programmed cell death in neural precursor cells may be caspase-dependent but Bax-independent. This conclusion requires caution, however, for several reasons. First, the neurodevelopmental effects of caspase-9, apaf-1, and caspase-3 gene disruption are incompletely penetrant and strain-dependent. For example, caspase-3 deficiency causes perinatal lethality and severe neurodevelopmental pathology in 129x1/SvJ mice. In contrast, the same gene disruption in C57BL/6J mice leads to minimal neuropathological abnormalities [47]. This result indicates that direct comparisons between gene disruptions on different or mixed genetic backgrounds are problematic. Second, Bcl-2 family members have overlapping actions, thereby raising the possibility that changes in the expression of other family members may compensate for Bax deficiency.

2.4. Bak

The pro-apoptotic molecule Bak was simultaneously identified in a yeast two-hybrid screen with an adenoviral
Bcl-2 functional homologue and by cloning [48–50]. Bak is widely expressed in most murine tissues, and recently a neuron-specific Bak splice product has been described [51]. Bak is structurally similar to Bax and, like Bax, may regulate mitochondrial cytochrome c release and apoptotic death in a variety of cell types [48,52]. Bak-deficient mice, like Bax-deficient mice, show no gross developmental pathology; however, overexpression of Bak can accelerate trophic factor withdrawal-induced death in sympathetic neurons [48,50]. Bak deficiency has no effect on cytochrome c release and cell death in sympathetic neurons following trophic factor withdrawal, whereas Bax deficiency prevents these changes [53].

Given the potential functional overlap between Bax and Bak, bax<sup>−/−</sup>/bak<sup>−/−</sup> mice were generated [54]. Bax, Bak dual-deficient mice show extensive perinatal lethality, persistent interdigital webs, imperforate vagina, and other developmental abnormalities, indicating that Bax and Bak may act in concert to affect cell death [54]. The potent pro-survival effect of combined Bax and Bak deficiency is well demonstrated in neural precursor cells. DNA damage to neural precursor cells, either in vivo or in vitro, induces p53-, Apaf-1-, and caspase-9-dependent apoptosis [55]. Alone, neither Bax nor Bak deficiency has any effect on DNA damage-induced neural precursor cell death [55,56]. However, neural precursor cells lacking both Bax and Bak are completely protected from γ-irradiation and chemotherapeutic-induced death, both in vivo and in vitro [56]. Bax, Bak dual-deficient neonatal and young adult mice exhibit increased numbers of periventricular neural precursor cells, indicating that Bax and Bak can combine to affect neural precursor cell survival in vivo (Fig. 2). Interestingly, dual Bax, Bak deficiency produces a less severe neurodevelopmental phenotype than that seen in affected caspase-9-, Apaf-1-, and caspase-3-deficient embryos. In total, these findings suggest that neural precursor cell death is regulated by developmental stage- and/or stimulus-specific molecular pathways which differentially involve Bax and Bak.

2.5. Bid

Bid is a member of the BH3-only pro-apoptotic subgroup of the Bcl-2 family, and was isolated as an interacting partner of Bcl-2 and Bax [57]. Bid regulates cell death by interacting with both pro- and anti-apoptotic multi-domain Bcl-2 family members such as Bcl-2, Bcl-X<sub>L</sub>, Bak and Bak [57–61]. This interaction requires an intact BH3 domain in Bid and triggers caspase-dependent apoptosis [57,59,60]. Bid plays an important role in linking extrinsic, death receptor signaling with intrinsic, mitochondrial cell death pathways. Activation of the cell surface death receptor Fas leads to the activation of caspase-8, which cleaves Bid [58]. Truncated Bid (tBid) is able to translocate to the mitochondria and can induce mitochondrial cytochrome c release [58]. This release may result from oligomerization or conformational changes in Bak or Bax, events promoted by tBid [61–63]. Bcl-X<sub>L</sub> and Bcl-2 are able to inhibit Bid-dependent cytochrome c release from isolated mitochondria [58,59,64]. Bid is expressed in neurons in both the embryonic and adult nervous systems [57,65], and tBid has been shown to interact with Bak in brain mitochondria and induce cytochrome c release [66]. However, Bid does not appear to play a major role in regulating neuronal programmed cell death during development. Bid-deficient mice exhibit no gross or microscopic neuropathology and there is no effect of Bid deficiency on naturally occurring neuronal cell death [65,67]. Bid deficiency also has no effect on neuronal precursor cell death either in vivo or in vitro [65]. Since the BH3-only subfamily is large, it remains possible that other BH3-only molecules subserve Bid function in Bid-deficient cells.

2.6. Bim

Bim was originally isolated as a pro-apoptotic, Bcl-2 interacting protein. Bim is a BH3-only subfamily member and can interact with Bcl-2, Bcl-X<sub>L</sub> and Bcl-w. Bim has three major splice variants, BimEL, BimL, and BimS, some of which can bind Bax directly [68,69]. Bim is primarily

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Fig. 2. Bax, Bak dual deficiency affects postnatal neural precursor cell survival. Hematoxylin and eosin stained sections of the 4-week-old mouse brain show neural precursor cells in the periventricular region of the wild-type forebrain (left panel: examples indicated by arrows) and a marked expansion of this cell population in the forebrain of a mouse lacking both Bax and Bak (right panel). Scale bars equal 200 μm.
expressed in neurons in the central nervous system [70]. BimEL expression is induced after apoptotic stimuli in several neuronal populations. In sympathetic neurons following NGF withdrawal, increased Bim expression is dependent on JNK signaling and occurs upstream of Bax-dependent caspase activation [71–73]. Similarly, cerebellar granule neurons exposed to non-depolarizing, serum-free conditions undergo Bax-dependent apoptosis that is accompanied by increased Bim expression [41,64,73]. The addition of insulin-like growth factor 1 (IGF-1) in these conditions can block Bim induction and death [74]. Although the precise mechanisms that regulate Bim function in neuronal cells are unclear, recently NGF has been shown to promote the phosphorylation of Bim and thereby suppress its pro-apoptotic activity [75].

Bim-deficient mice have been generated and show incompletely penetrant, strain-dependent embryonic lethality around E9.5 [76]. Programmed cell death of dorsal root ganglion neurons at E14.5 may be mildly reduced in Bim-deficient embryos but this effect appears transitory [72]. There are no obvious neuropathological abnormalities in bim−/− mice that survive to birth [76]. In vitro, Bim deficiency may delay, but does not prevent, both trophic factor withdrawal-induced sympathetic neuron death and activity-dependent cerebellar granule neuron death [72]. Another study of postnatal dorsal root ganglion sensory neurons has demonstrated that Bim deficiency provides partial protection from trophic factor withdrawal-induced death [71]. In total, these results suggest that Bim plays an important, but not essential, role in regulating neuronal programmed cell death.

2.7. DP5/Hrk

Death protein 5 (DP5) was identified through a total mRNA differential display analysis of NGF-deprived sympathetic neurons [77]. DP5 is highly homologous to the human gene harakiri (Hrk), cloned by a two-hybrid screen with Bel-2 and Bel-XL [78]. DP5 mRNA and protein are expressed in several tissues and are developmentally regulated in the nervous system [77,79]. Several in vitro systems have demonstrated a role for DP5/Hrk in regulation of neuronal cell death. Sympathetic neurons deprived of NGF induce DP5 mRNA [77]. DP5 is up-regulated in cerebellar granule neurons incubated in non-depolarizing, serum-free media [73]. This up-regulation is independent of Bax and incompletely blocked by inhibitors of JNK signaling [73]. Finally, DP5 mRNA is induced in primary rat cortical neurons exposed to toxic concentrations of amyloid-β protein [80], and this induction is attenuated by inhibition of JNK signaling [81]. These results suggest that several cellular pathways control DP5 expression. Overexpression of DP5 induces apoptosis in sympathetic neurons, and this effect can be attenuated by co-expression of Bel-2 [77]. Hrk mRNA expression is relatively high in lymphoid tissues, bone marrow, and spleen but is low in brain. Overexpression of Hrk induces a dramatic loss in cell viability that is prevented by co-expression of Bel-2 or Bel-XL. The interaction between Hrk and Bel-2 or Bel-XL is dependent on Hrk’s BH3 domain [78]. It is unclear whether DP5/Hrk acts by sequestering pro-survival Bel-2 family members or by direct interaction with pro-apoptotic molecules.

2.8. Bad

Bad, Bel-2-associated death protein, is a BH3-only subfamily member that was isolated as a binding partner for both Bel-XL and Bel-2 [82]. Bad is widely expressed in murine tissues, including the brain [83]. Bad is readily detected in the embryonic nervous system and is down-regulated postnatally [84]. The regulation of Bad function by posttranslational modification has been extensively investigated and Bad phosphorylation may critically regulate apoptosis in some cell types. At baseline, growth factor signaling activates kinases that phosphorylate Bad, which resides in an inactive state complexed with the molecular chaperone protein 14-3-3 [85]. Following an apoptotic stimulus, dephosphorylation of Bad may disrupt its complex with 14-3-3, permitting its interaction with Bel-XL and producing downstream Bax-dependent cytochrome c release and caspase activation [85,86]. This mechanism has been described in several in vitro neuronal systems. In cultured rat hippocampal neurons, addition of transforming growth factor-β 1 increases Bad phosphorylation and decreases the elevation of Bad protein following exposure to apoptotic stimuli [87]. Overexpression of Bad in cerebellar granule neurons causes death that is very similar to that seen following trophic factor withdrawal. Furthermore, Akt, a mediator of growth factor-induced survival in many cell types, can phosphorylate Bad, and expression of active Akt can prevent Bad-induced cell death in this system [88]. Similarly, overexpression of Bad in mature sympathetic neurons induces death [89]. Clearly, phosphorylation of Bad is an important regulatory mechanism in neuronal cell populations. It is important to note that Bad can be phosphorylated on several sites, and not all phosphorylation events prevent Bad’s pro-apoptotic function. For example, both growth factor signaling and stimulation of JNK signaling induce Bad phosphorylation in cerebellar granule neurons. JNK-induced phosphorylation of Bad may make it less susceptible to phosphorylation by growth factor signaling and may thereby promote Bad-mediated apoptosis [90].

Bad-deficient mice have been generated but show no gross developmental abnormalities, neurodevelopmental abnormalities, or alteration in neuronal programmed cell death [23,83]. Bad has been implicated in several neuronal cell death models both in vivo and in vitro but the lack of an effect on neuronal programmed cell death in vivo suggests that Bad does not critically regulate naturally occurring neuronal cell death. Transgenic knock-in mice have been generated that express non-phosphorylatable mutant Bad.
These animals demonstrate defective growth factor signaling in lymphocytes but no gross abnormalities in neural tissues [91]. As mentioned, in non-depolarizing, serum-free conditions, cerebellar granule neurons undergo apoptosis that is blocked by the addition of IGF-1 [40,74]. This effect of IGF-1 is absent in mice expressing non-phosphorylatable Bad, suggesting that pro-survival growth factor signaling in neuronal cells is directly linked to Bad phosphorylation [91].

2.9. Noxa

Noxa, a BH3-only member of the Bcl-2 family, was identified by mRNA differential display as a being expressed in X-ray-irradiated mouse embryonic fibroblasts (MEFs) [92]. Noxa is unique in that it harbors two BH3 domains. Noxa is expressed in several adult mouse tissues, including brain, and localizes to the mitochondria, where it induces cytochrome \( c \) release [92,93]. Noxa is inducible by p53, a tumor-suppressor protein that is critical for preventing the growth of abnormal or damaged cells. p53 inhibits growth by inducing both cell cycle arrest and cell death, although it is not entirely clear how p53-mediated signals are transmitted to the cell death machinery. The fact that Noxa is both a transcriptional target of p53 and a pro-apoptotic member of the Bcl-2 family strongly suggests that it may have a role in mediating p53-dependent signals that control entry into intrinsic cell death pathways. Although detailed investigations of Noxa function in neuronal cell death are currently lacking, the role of Noxa in non-neuronal systems has been studied. In MEFs, Noxa expression is p53-dependent and can be induced by concomitant p53 overexpression [94]. Furthermore, Noxa knockdown using interfering RNA oligonucleotides protects MEFs from a variety of apoptosis-inducing agents [94]. Overexpression of Noxa in several human cancer cell lines induces cytochrome \( c \) release, caspase-9 activation, and apoptosis [92,93]. These studies provide the groundwork for further investigations of Noxa function in neuronal cell types to determine this molecule’s contribution to neuronal apoptosis.

Noxa deficient mice have recently been generated, and display no obvious developmental abnormalities [95,96]. Embryonic fibroblasts isolated from Noxa-deficient mice show resistance to several apoptotic stimuli [95]. Furthermore, membrane insertion of Bak and Bak oligomerization is decreased in Noxa-deficient MEFs as compared to wild-type MEFs, suggesting that Noxa may promote the actions of Bak [95]. Recombinant Noxa can induce cytochrome \( c \) release without Bak-oligomerization, suggesting that Noxa and Bak may act synergistically [93].

2.10. Puma

Puma, a BH3-only member of the Bcl-2 family, was simultaneously identified by a microarray screen and a serial analysis of gene expression method [97,98]. Puma, like Noxa, is induced by p53 and may play a role in coupling death stimuli to effectors of apoptosis. Puma binds Bcl-2 and localizes to the mitochondria, where it induces cytochrome \( c \) release [97,98]. In addition, Puma has been shown to interact with Bcl-X \(_ L \) [98]. Puma also activates caspase-9 and caspase-3 in a number of cell lines in a BH3-domain-dependent manner, followed by rapid onset of morphological apoptotic features [97,98].

Currently, Puma’s role in regulating neuronal cell death is unclear. Puma mRNA and protein expression are increased in SH-SY5Y human neuroblastoma cells exposed to endoplasmic reticulum (ER) stress. This expression is followed by increased caspase-3 like protease activity and apoptotic nuclear features [99]. Interestingly, this study did not find an increase in mRNA levels of Bim, Bad, Noxa, Bak, Bak, Bcl-2 or Bcl-x in this model. ER stress in SH-SY5Y cells causes mitochondrial localization of Bax as well as release of cytochrome \( c \) [99]. Puma-deficient mice have recently been generated, and show no abnormalities in organogenesis or neurodevelopment. Puma deficiency provides marked resistance to several apoptosis-inducing agents in non-neuronal cell types [96]. Further experimentation is required to determine the role Puma deficiency may play in regulating neuronal cell death. Such studies are warranted considering that gene targeting of Puma in a colorectal cancer cell line has demonstrated a critical role for the molecule in p53-dependent cell death induced by DNA damage, hypoxia, or ER stress [99,100].

In total, studies of genetically-modified mice have revealed a significant role for the Bcl-2 family in regulating neuronal programmed cell death. Particularly significant roles for Bcl-X \(_ L \) and Bax are apparent and their action is complemented by Bcl-2 and Bak, respectively. Although disruption of individual BH3-only genes has not resulted in obvious changes in neuronal programmed cell death, it remains likely that this Bcl-2 subfamily acts in concert to regulate neuronal cell death.

3. Bcl-2 family regulation of neurodegeneration

Given the clear role for Bcl-2 family members in regulating cell death during nervous system development, much interest has been given to Bcl-2 family involvement in neuropathological conditions. The mammalian nervous system has several unique features that make it susceptible to injury and thus, regulation of neuronal cell death is of paramount significance. First, neural tissue is exquisitely sensitive to hypoxia and ischemia. Second, neurons are excitable and neural activity is imperative for neuron survival and function. Third, dead neurons, with rare exception, are not replaced in the mature nervous system, and the activation of a “cell suicide” death program may have significant and permanent impact on nervous system function. Fourth, since neurons are long-lived, sublethal injurious stimuli may produce changes in cell death-associated molecules that may attenuate or exacerbate the effects of...
additional death stimuli. Finally, sublethal cellular insults that interfere with synaptic transmission may render a neuron dysfunctional even in the absence of neuron death. Apoptosis-associated molecules may therefore be involved in neurodegenerative processes prior to, or in the absence of, neuron death.

3.1. Hypoxic-ischemic injury

Cerebrovascular disease is one of the leading causes of human morbidity and mortality worldwide. The brain is particularly vulnerable to hypoxic-ischemic injury that may occur focally or globally, as seen in stroke or asphyxiation, respectively. The role of Bcl-2 family members in regulating neuron death has been extensively investigated in animal models of hypoxic-ischemic neuronal injury. Neuronal expression of Bcl-2 family members appears to be altered by hypoxic-ischemic injury both in vivo and in vitro [27,101,102]. Exposure to hypoxia alone can increase Bcl-2 immunoreactivity in cultured neocortical cells, and several in vivo rodent models of hypoxic-ischemic injury have demonstrated increased Bcl-2 immunoreactivity [101–103]. However, elevated Bcl-2 expression has not been observed in all animal models of hypoxic-ischemic brain injury and it appears that Bcl-2 expression may be influenced by the degree of insult, species-specific factors, and gender-specific factors [101,104–106].

Further evidence for the potential importance of anti-apoptotic Bcl-2 family members has been demonstrated in experiments that manipulate protein expression of Bcl-2, Bcl-X\textsubscript{L} or Bcl-w. Overexpression of Bcl-2 in mice partially protects against stroke-induced injury [14]. Furthermore, intraventricular infusion of Bcl-2 antisense oligonucleotides accentuated stroke size following middle cerebral arterial occlusion in rat [107] and prevented tolerance to subsequent ischemia [108].

In many, but not all, hypoxic-ischemic models, direct CNS injection of viral vectors to drive overexpression of anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-X\textsubscript{L}, and Bcl-w has resulted in significant diminution in neuronal cell death and infarct size [109–118]. A novel alternative approach has fused Bcl-X\textsubscript{L} [117] or phosphorylation-resistant Bcl-X\textsubscript{L} [119] to the protein transduction domain (PTD) of the human immunodeficiency virus (HIV) TAT protein. These fusion proteins penetrate the blood–brain barrier following intraperitoneal injection, and significantly increase the efficacy of the anti-apoptotic vectors.

The analysis of mice with targeted gene disruptions has confirmed the significance of specific Bcl-2 family members in the regulation of neuronal responsiveness to hypoxic-ischemic injury. Bcl-2-deficient mice show increased infarct size and more severe neurological deficits following transient middle cerebral artery occlusion as compared to heterozygous and wild-type littermates [120]. In contrast, Bax-deficient mice exhibit significantly decreased caspase-3 activation and infarct volume as compared to wild types in a neonatal hypoxic-ischemic injury model [121]. Similarly, BID-deficient mice subjected to transient middle cerebral artery occlusion had decreased cytochrome c release, caspase-3 activation, and infarct size compared to wild-type animals [122,123]. In total, these studies suggest that the expression of pro- and anti-apoptotic Bcl-2 family members critically regulates responsiveness to hypoxic-ischemic injury and that inhibition of Bcl-2 family-dependent death pathways could offer significant protection from cerebrovascular insults.

3.2. Seizure-induced neurodegeneration

Abundant evidence exists for neuronal cell death following significantly intense seizure activity [124,125]. A complex pattern of apoptotic, excitotoxic, and necrotic death pathways may be activated following seizure activity [124,126–128]. Changes in Bcl-2 family member expression have been observed in several experimental models of seizures induced by kainic acid, domoic acid, or electrical stimulation. Most often reported has been increased neuronal expression of Bax mRNA and protein [129–132]. Changes in Bcl-2 expression have not been consistently observed and various seizure models have produced increased, decreased, or unaltered Bcl-2 expression [129,130,132]. Bid, Bad, caspase-8, caspase-9, and caspase-3 have all been reported to be activated in seizure-induced neuronal death [133–135]. Unfortunately, overexpression of Bcl-2 has not consistently led to neuroprotection in animal models of seizure-induced neurotoxicity [136,137]. Studies of mice with targeted gene disruptions in Bcl-2 family members have been sparse. One study of Bak-deficient mice treated with kainic acid demonstrated increased seizure activity and greater hippocampal cell loss in bak\textsuperscript{−/−} compared to bak\textsuperscript{+/−} animals, suggesting that Bak may modulate both neuronal excitability and cell death pathways [138]. Additional studies are required before any conclusions about the significance of Bcl-2 family involvement in seizure-induced neuron death can be drawn.

3.3. Motor neuron disease

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting upper motor neurons in the cerebral cortex and lower motor neurons in the spinal cord. In ALS, death of motor neurons leads to a progressive loss of voluntary muscle function and ultimately respiratory failure and death over a typical course of several years. Most cases of ALS are sporadic but approximately 10% of cases are familial. In familial ALS, the onset of disease typically occurs at a younger age and proceeds more rapidly than in sporadic ALS. One familial ALS subtype has been linked to mutations in the gene for copper/zinc superoxide dismutase 1 (SOD1), a free radical-scavenging enzyme [139,140]. Overexpression of mutated SOD1 in transgenic mouse and rat models leads to motor neuron loss through a proposed toxic gain-of-function effect [141–144].
Biochemical studies have implicated the Bcl-2 family in the regulation of motor neuron death in this disease. Decreased expression of Bcl-2 mRNA [145] and increased expression of harakiri mRNA [146] and Bax mRNA and protein [145,147] have been reported in ALS. This change in the balance of pro- and anti-apoptotic Bcl-2 family molecules, coupled with a reported increase in Bax and Bak mitochondrial localization and increased Bcl-2 cytosolic distribution, may contribute to neuron death in ALS [145,147–149]. Studies of mutant SOD1-overexpressing transgenic animals also support a role for the Bcl-2 family in motor neuron disease. As the disease progresses, mRNA and protein levels of Bcl-2 and Bcl-XL decrease, while Bax and Bad levels increase in motor neurons of mutant SOD1 transgenic mice [150–152]. Translocation of Bax and Bak from the cytosol to the mitochondria has also been shown to correlate with disease progression in SOD1-overexpressing mice [148,149]. In addition, increased Bid cleavage is detected in SOD1 mutant mice as compared to wild-type controls [153]. Studies of Bcl-2 transgenic and knock-out mice also implicate the Bcl-2 family in motor neuron disease pathogenesis. Overexpression of Bcl-2 in SOD1 transgenic mice results in delayed onset of symptoms and increased survival [154,155]. This neuron survival-promoting effect appears to result from a neutralization of Bax’s pro-apoptotic function and inhibition of caspase-1, -9, and -3 activity [150,156]. However, Bcl-2 overexpression fails to protect motoneurons from death in a second transgenic model of ALS [157], and morphologic studies of transgenic animal models and human ALS brain and spinal cord have provided limited evidence that neuron loss in ALS occurs via apoptotic death pathways [147,158]. Additional studies are required to determine the significance of the Bcl-2 family in the pathogenesis of motor neuron disease.

3.4. Nerve injury

Peripheral nerve injury may often lead to neuronal cell death. In early postnatal rodents, lesions of the facial or sciatic nerve cause rapid motoneuron degeneration in their respective nuclei [159–161], and transection of the optic nerve causes degeneration in retinal ganglion cells (RGCs) in both neonatal and adult mice [162,163]. Changes in neuronal expression of Bcl-2 family members following axotomy suggest a role for these proteins in regulating axotomy-induced neuron death. Bcl-2 mRNA and protein may either decrease or increase depending on the site of axotomy and type of injury [164–169]. Bax mRNA and protein have consistently been reported to be increased in neurons post-axotomy, and, combined with altered Bcl-XL and BimEL expression, may favor neuronal degeneration [72,164–167]. Overexpression of Bcl-2 or Bcl-XL in axotomized neurons is neuroprotective in many experimental models [11,114,162,163,170–173]. For example, Bcl-2-overexpressing transgenic mice have decreased RGC death following optic nerve transection. This effect is seen in both neonates and adults [163,165]. Based on electroretinography, RGC function in these animals is preserved [174,175]. Similarly, Bax-deficient mice show significant protection from facial nerve axotomy-induced neuron loss [35]. These studies indicate that Bcl-2 family members play an important role in regulating neuron loss following axonal injury.

3.5. Ethanol-induced neonatal brain injury

The neonatal rodent brain, which is roughly developmentally equivalent to the perinatal human brain, shows a marked sensitivity to a variety of insults. Olney and colleagues have reported extensive caspase-3 activation and apoptotic neuronal degeneration throughout the central nervous system of neonatal rats and/or mice exposed to antiepileptic drugs, anesthetic agents, or ethanol [176–179]. Prenatal or early postnatal ethanol exposure results in relative increases of Bax and Bcl-XL mRNA and protein levels as compared to Bcl-2 in the cerebellum and cortex [180–182]. Additionally, transgenic neonatal mice overexpressing Bcl-2 are protected against ethanol-induced neuronal cell death [180]. The role of pro-apoptotic Bcl-2 family members in ethanol-induced neuronal apoptosis has been examined in Bax-deficient neonatal mice [183]. Unlike wild-type mice, ethanol-exposed Bax-deficient animals show no increase in caspase-3 activity, TUNEL staining, or apoptotic neurons (Fig. 3). Quantitative determination of neuron density in ethanol-exposed wild-type and Bax-deficient neonatal mice showed a marked loss of neurons in wild-type mice, but no loss of neurons either 24 or 72 h after ethanol exposure in Bax-deficient mice, indicating that Bax deficiency provides prolonged protection from ethanol-induced neuronal degeneration. These studies suggest that Bax, and likely other Bcl-2 family members, play an important function during the perinatal period of human nervous system development.

3.6. Non-apoptotic neurodegeneration

Although some authors have suggested that caspase-dependent apoptosis underlies the pathogenesis of human neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease, this view has recently been questioned [2,184,185]. Specifically, there is relatively little morphological evidence for apoptosis in human neurodegenerative diseases and several alternative forms of cell death have been described. Increasing attention has focused on caspase-independent death pathways and, particularly, the occurrence and regulation of autophagic neuronal degeneration [186,187]. Evidence for Bcl-2 family involvement in autophagic cell death has been provided in several studies. Bid cleavage has been demonstrated following lysosomal damage [188,189]. Furthermore, an in vitro model of autophagic cell death revealed increased susceptibility of Bcl-XL-deficient neurons and decreased...
susceptibility of Bax-deficient neurons to lysosomotropic agents [22]. This degenerative process may involve Bcl-2 family-dependent mitochondrial injury and degradation [190]. Interestingly, autophagy has been reported in methamphetamine-induced dopaminergic neuritic degeneration, and steroid-induced neuronal cell death may also involve mitochondrial injury and autophagic pathways [191–193]. Regardless of the type of neuronal death induced, overexpression of anti-apoptotic Bcl-2 family members or bax gene disruption provides significant neuroprotection in several in vivo and in vitro disease degenerative models [194–196]. The protective effects of Bcl-2 overexpression or bax gene disruption can be cell-specific. Lurcher mice express a mutant, constitutively active glutamate receptor in cerebellar Purkinje cells [197]. The resultant increase in inward depolarizing current leads to the loss of Purkinje cells, followed by a secondary loss of cerebellar granule neurons. In Lurcher animals, apoptotic Purkinje cells express increased levels of Bax [198]. Recently, Bax-deficient, Lurcher double mutants were generated. In Bax-deficient/ Lurcher animals, the secondary loss of cerebellar granule cell neurons is blocked; however, Purkinje cell death is delayed but not prevented, suggesting the involvement of Bax-independent cell death pathways in this cell population [195,199].

In summary, both pro-survival and pro-death members of the Bcl-2 family play an essential role in normal nervous system development and are likely critical factors in certain neurological disease processes. In the nervous system, the expression of individual Bcl-2 family members and their contribution to survival or death are developmentally regulated. Both pro-survival and pro-death Bcl-2 family members play an essential role in normal nervous system development as illustrated by transgenic mouse strains with single or double gene disruptions of Bcl-2 family molecules. The very importance of this family is underscored by the functional redundancy of many of its members. Furthermore, the regulation of these molecules is complex, and may include mechanisms of transcriptional, translational, and posttranslational control. In many neurological disease models, altered Bcl-2 family member mRNA and protein expression has been observed. These findings provide hope that development of targeted pharmacological agents that enhance anti-apoptotic Bcl-2 family function or inhibit pro-apoptotic Bcl-2 family function will prove useful in the treatment of human neuropathological conditions. These interventions may block both apoptotic and non-apoptotic forms of neurodegeneration. In conclusion, the continued investigation of the basic biology of Bcl-2 family members in neuron populations will certainly make an impact on understanding neurodevelopment and neurodegeneration.

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