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News and Commentary

BH3-only proteins: key regulators of neuronal apoptosis

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The molecular mechanisms of neuronal apoptosis have been intensively studied because a considerable amount of apoptosis occurs during the normal development of the mammalian nervous system. This death is important for establishing neuronal populations of the correct size and for ensuring that neurons that contact inappropriate targets are eliminated.[1,2] A second reason for the interest in this area is that there is increasing evidence that apoptosis is one of the mechanisms of neuronal death following acute injuries to the nervous system, such as stroke or traumatic brain injury, and neurons often die by apoptosis in cell culture and animal models of chronic human neurodegenerative disorders.[2] The aim of this article is to review recent work on the function and regulation of the BH3-only subfamily of Bcl-2 proteins in neurons, with an emphasis on studies with sympathetic neurons, cerebellar granule neurons (CGNs) and motoneurons, the best studied *in vitro* models of neuronal apoptosis, and work that has involved the analysis of neurons from mutant mice.

NGF Withdrawal-induced Death Requires Transcription and Involves the Mitochondrial Death Pathway

An important step forward in the neuronal cell death field was the discovery in the late 1980s that the death of developing nerve growth factor (NGF)-dependent sympathetic neurons following NGF withdrawal requires *de novo* transcription and protein synthesis.[3] This was one of the early observations that contributed to the idea of apoptosis as an active form of cell death and it proved to be true for other types of neuron deprived of survival signals, including CGNs and motoneurons. It stimulated a number of laboratories to search for genes that are transcriptionally induced in neurons undergoing apoptosis. One of the first regulated genes to be identified was the basic/leucine zipper transcription factor c-Jun, a member of the AP-1 family. The level of the *c-jun* mRNA and c-Jun protein increases rapidly in sympathetic neurons after NGF withdrawal, and microinjection of neutralizing antibodies against c-Jun or expression of a c-Jun dominant-negative mutant protects sympathetic neurons against NGF withdrawal-induced death,[4,5] as does conditional knockout of the *c-jun* gene in sympathetic neurons isolated from mice with a floxed *c-jun* gene.[6] These observations supported the idea that NGF withdrawal-induced death involves the transcriptional induction of genes that activate the cell death programme. In addition, the observation that c-Jun N-terminal phosphorylation increases after NGF withdrawal led to the demonstration that c-Jun N-terminal kinases (JNKs) are activated in sympathetic neurons deprived of NGF and that JNK activity is required for NGF withdrawal-induced death.[5,7–11] The level of c-Jun and of c-Jun N-terminal phosphorylation also increases in CGNs following serum and KCl withdrawal, and expression of dominant-negative c-Jun inhibits cell death induced by KCl/serum deprivation.[12]

How does the JNK/c-Jun pathway promote apoptosis in neurons? In the case of sympathetic neurons, the intrinsic mitochondrial death pathway is activated by NGF withdrawal. Cytochrome *c* is released from the mitochondria into the cytosol and is required for NGF withdrawal-induced death.[13,14] The JNK/c-Jun pathway has been shown to regulate the release of mitochondrial cytochrome *c* in sympathetic neurons. Thus, expression of dominant-negative c-Jun inhibits the release of cytochrome *c* after NGF withdrawal, whereas overexpression of MEKK1, an activator of the JNK pathway, can induce cytochrome *c* release and apoptosis in the presence of NGF.[15] These results suggested the hypothesis that the JNK/c-Jun pathway may promote neuronal apoptosis by activating the transcription of genes that increase mitochondrial outer membrane permeability, such as proapoptotic members of the Bcl-2 family.[15]

Transcriptional Induction of *dp5* and *bim* in Neurons

The Bcl-2 family of proteins can be divided into three subfamilies: (1) antiapoptotic proteins, such as Bcl-2, Bcl-x_L and Mcl-1, which inhibit mitochondrial cytochrome *c* release and apoptosis, and which share four Bcl-2 homology (BH) domains; (2) multidomain proapoptotic proteins, such as Bax and Bak, which have three BH domains, but which promote cytochrome *c* release and apoptosis; (3) BH3-only proteins, which only share the BH3 domain with other members of the family, and which are all proapoptotic. Both sympathetic neurons and CGNs express multiple proapoptotic Bcl-2 family proteins.[15–18] Of the multidomain proapoptotic proteins (Bax and Bak), Bax is essential for the release of mitochondrial cytochrome *c* in sympathetic neurons and for NGF withdrawal-induced death, and for the KCl/serum deprivation-induced death of CGNs, whereas Bak is not required.[18–20] This is an interesting observation because in other cell types

the expression of both Bax and Bak must be lost to prevent cell death induced by survival factor withdrawal.[21,22] This difference may be related to the fact that postnatal sympathetic neurons, hippocampal neurons and CGNs cultured *in vitro* exclusively express N-Bak, a neuron-specific splice variant of Bak, which is a BH3-only protein lacking the BH1 and BH2 domains of full-length Bak.[23,24] Thus, *in vitro* these cells only express one multidomain proapoptotic protein (Bax) in contrast to other cell types, which express both Bax and full-length Bak. It has been reported that overexpressed N-Bak inhibits the NGF withdrawal-induced death of sympathetic neurons *in vitro* but induces apoptosis in non-neuronal cells.[23] However, other workers have reported that overexpression of N-Bak can induce the death of cortical, hippocampal and CGNs in a Bax-dependent manner.[24]

In sympathetic neurons deprived of NGF, Bax translocates from the cytoplasm to the mitochondria and inserts into the mitochondrial outer membrane.[25] This process is transcription-dependent but the expression of Bax itself does not increase following NGF withdrawal.[15,17,25] Instead, BH3-only proteins that could directly or indirectly regulate Bax translocation and Bax-dependent mitochondrial outer membrane permeabilization are transcriptionally induced after NGF withdrawal. These include DP5, Bim and Puma. DP5 was the first BH3-only protein to be found to be induced by NGF withdrawal and was identified in NGF-deprived sympathetic neurons by the differential display technique.[16] DP5 is the rodent homologue of human Harakiri (Hrk)[26] and encodes a 92 amino-acid protein with a BH3 domain and carboxy-terminal hydrophobic membrane insertion sequence. In rodents, the expression of the *dp5* mRNA is largely restricted to the nervous system.[16] The *dp5* mRNA and DP5 protein increase in level in sympathetic neurons and neuronally-differentiated PC12 cells deprived of NGF, and reach a peak by 15 h after the removal of NGF.[16] The induction of the *dp5* mRNA by NGF withdrawal may require the JNK pathway because it is reduced by approximately 75% by the neuroprotective compound CEP-1347, a mixed lineage kinase (MLK) inhibitor that inhibits JNK activation in sympathetic neurons.[27] However, this hypothesis will need to be confirmed by other approaches because CEP-1347 and related MLK inhibitors can also activate the PI3-kinase pathway under certain conditions.[28,29] As well as being induced by NGF withdrawal, the *dp5* mRNA also increases in level in CGNs deprived of KCl and serum, cortical neurons treated with neurotoxic concentrations of amyloid β peptide, retinal ganglion cells of axotomized rat retinas, axotomized postnatal mouse motoneurons and in the spinal cords of human amyotrophic lateral sclerosis (ALS) patients compared to non-ALS controls.[27,30–33]

The function of the DP5 protein in neurons has been studied in gain-of-function and loss-of-function experiments. Overexpression of DP5 in microinjected sympathetic neurons induces apoptosis and this can be inhibited by coexpression of antiapoptotic Bcl-2.[16] Similarly, overexpression of DP5 can induce apoptosis in CGNs and this death requires the expression of Bax.[27] *Dp5* $-/-$ knockout mice have been generated, and these survive development, are viable and have no major anatomical defects in the nervous system.[33] Sympathetic neurons isolated from *dp5* $-/-$ knockout mice die marginally slower than wild-type neurons after NGF withdrawal.[33] In contrast, postnatal *dp5* $-/-$ motoneurons are relatively resistant to axotomy-induced death compared to wild-type neurons.[33] These results suggest that DP5 can promote neuronal apoptosis and that the importance of its contribution varies from one type of neuron to another. This variable requirement may depend on whether other BH3-only proteins that can substitute for the loss of DP5 are also expressed in the same cells and the relative concentrations of these proteins.

The idea that more than one BH3-only protein may be induced in neurons undergoing apoptosis is supported by the observation that the Bim_{EL} protein substantially increases in level in sympathetic neurons and CGNs deprived of survival factors.[15,17] Several different Bim isoforms have been described. Bim_{EL}, Bim_L and Bim_S are the major variants (see Figure 1a), generated as a result of alternate splicing of *bim* transcripts.[34] All three proteins are proapoptotic but differ in their potency such that $Bim_S > Bim_L > Bim_{EL}$. Bim_{EL} and Bim_L contain a region, not present in Bim_S (Figure 1a), that allows them to interact, in certain cell types, with LC8 (dynein light chain 1), a component of the dynein motor complex associated with the microtubule cytoskeleton.[35] When these cells undergo apoptosis, Bim_{EL} and Bim_L dissociate from the microtubule cytoskeleton and associate with the mitochondrial outer membrane by means of a C-terminal transmembrane domain. The Bim_{EL} protein is the major Bim isoform expressed in sympathetic neurons, CGNs and dorsal root ganglion (DRG) neurons.[15,17] The level of *bim* mRNA and Bim_{EL} protein increases rapidly in sympathetic neurons after NGF withdrawal, and at least part of this increase is due to increased transcription from the *bim* promoter since a reporter gene containing the *bim* promoter cloned upstream of the firefly luciferase gene (*bim*-LUC) is activated by NGF withdrawal in microinjected sympathetic neurons.[36] The JNK/c-Jun pathway contributes to this induction since expression of dominant-negative c-Jun in sympathetic neurons or treatment of the cells with CEP-1347 reduces the increase in *bim* mRNA by approximately 50%.[15,17,27] However, it is not yet known whether the *bim* gene is directly activated by c-Jun/AP-1 or whether the effect of the JNK/c-Jun pathway is indirect (Figure 1b). The promoter, first noncoding exon and first intron of the *bim* gene contain a number of potential c-Jun binding sites (AP-1 and ATF sites) that are conserved between the rat, mouse and human genes (Jonathan Gilley and Jonathan Ham, unpublished observations). Site-directed mutagenesis and DNA binding studies will need to be performed to establish the role of these sequences.

Overexpression studies and experiments with *bim* antisense oligonucleotides or neurons isolated from *bim* $-/-$ knockout mice have established that Bim plays an important role in neuronal death induced by survival factor withdrawal.[15,17,27] In the case of sympathetic neurons, microinjection of an expression vector for Bim_{EL} can induce the release of mitochondrial cytochrome *c* and apoptosis in the presence of NGF, and microinjection of *bim* antisense oligonucleotides that reduce the level of expression of Bim_{EL} protein can significantly, but not completely, protect sympathetic neurons against NGF withdrawal-induced death.[15,36]

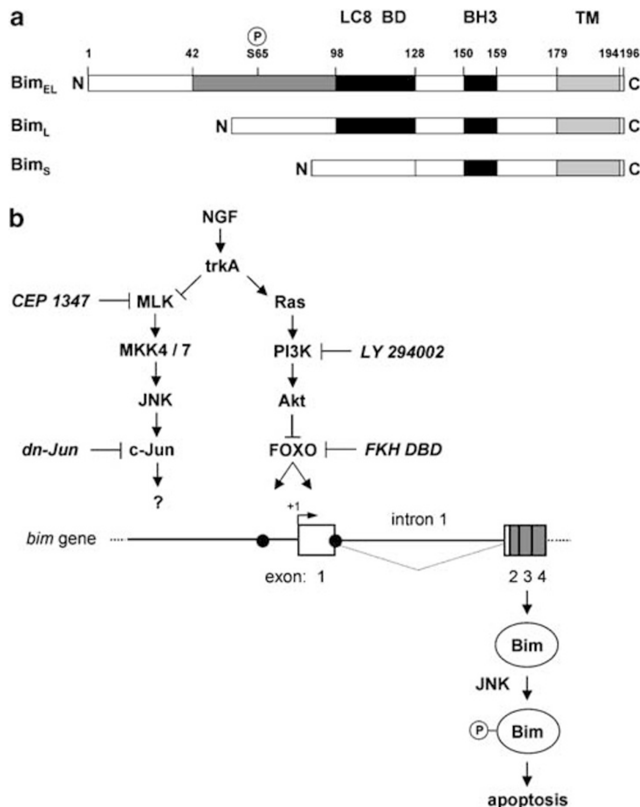

Figure 1 Structure of the major Bim isoforms and model of how Bim expression may be regulated in sympathetic neurons. (**a**) Structure of Bim_{EL}, Bim_{L} and Bim_{S}. The relationship between the major Bim isoforms is shown. Numbers refer to amino-acid residues in the mouse Bim protein.[34] The positions of the LC8 binding domain (LC8 BD), BH3 domain and hydrophobic transmembrane domain (TM) are indicated. The region unique to Bim_{EL} (shaded) is shown together with the ERK/JNK phosphorylation site at serine 65. (**b**) Hypothetical model of how *bim* transcription and Bim activity may be regulated in sympathetic neurons. The structure of the 5′ end of the rat *bim* gene is shown. Boxes represent exons. Exon 1 is noncoding. Exons 2, 3 and 4 are the first of several coding exons. NGF withdrawal leads to a decrease in PI3-kinase and Akt activity and the dephosphorylation and nuclear translocation of FOXO transcription factors, which can directly activate *bim* transcription by binding to the conserved FOXO binding sites in the *bim* gene (represented by filled circles). This can be inhibited by the FKH DBD, a dominant interfering mutant of FKHRL1.[36] NGF withdrawal also leads to activation of the MLK/JNK/c-Jun pathway, which contributes to induction of Bim expression by an unknown mechanism. The MLK inhibitor CEP-1347 or expression of dominant-negative c-Jun (dn-Jun) can reduce the increase in *bim* mRNA induced by NGF withdrawal.[15,27] Whether *bim* is a direct or indirect target of the JNK/c-Jun pathway is unknown. After NGF withdrawal, JNKs phosphorylate the Bim_{EL} protein at serine 65, which promotes the proapoptotic activity of Bim by an unknown mechanism[50]

Similarly, sympathetic neurons, DRG neurons or CGNs isolated from *bim* −/− mice are partially protected against cell death induced by survival factor withdrawal *in vitro* and the number of thoracic and lumbar DRG neurons undergoing programmed cell death *in vivo* during development is reduced in *bim* −/− mice.[15,17] For sympathetic neurons, when *bim* is knocked out, the level of protection against NGF withdrawal-induced death *in vitro* is not as great as that seen when the *bax* gene is inactivated.[19] Again, it is likely that other BH3-only proteins expressed in sympathetic neurons can partially compensate for the loss of Bim. DP5 is one candidate and another might be Puma, which has been reported to increase in level in sympathetic neurons after NGF withdrawal.[37] A full understanding of the role of these proteins in the death pathway in sympathetic neurons and other types of neuron will require the construction of double or triple knockout mice and the careful comparison of neurons isolated from these mice with single knockout or wild-type controls.

How might Bim contribute to the death of sympathetic neurons following NGF withdrawal? Bim_{EL} has been found to be associated with the mitochondrial outer membrane after NGF withdrawal,[17] where it may promote outer membrane permeabilization and cytochrome *c* release by interacting with other Bcl-2 family proteins. Recent interaction studies have shown that the Bim BH3 domain can bind with a relatively high affinity to several different antiapoptotic proteins, including Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1,[38,39] and could thereby prevent these proteins from binding to and inhibiting Bax dimerization and multimerization. Similarly, DP5 and Puma can bind to several different antiapoptotic Bcl-2 family members.[38] In addition, it has been suggested that, like tBid, the Bim BH3 domain can directly bind to and activate Bax *in vitro*,[39] although whether Bim_{EL} actually does this in neurons is not known.

The regulation of *bim* transcription by the PI-3 kinase/Akt survival signalling pathway has also been studied in sympathetic neurons. Work with the mouse pro-B cell line Ba/F3 showed that overexpression of an activated mutant of the FOXO transcription factor FOXO3a (FKHRL1) can increase the level of *bim* mRNA and protein and can induce apoptosis.[40] In the presence of survival factors, active Akt phosphorylates FOXO3a, which is then bound by the chaperone protein 14-3-3 and sequestered in the cytoplasm. In sympathetic neurons, NGF withdrawal leads to decreased PI3-kinase and Akt activity and reduced phosphorylation of FOXO3a, which is then released from 14-3-3 and translocates from the cytoplasm into the nucleus.[36] Examination of the DNA sequence of the 5′ end of the rat *bim* gene identified two FOXO binding sites in the region around the transcription initiation site, that are conserved between the rat, mouse and human *bim* genes (Figure 1b).[36] FOXO3a can bind to these sites *in vitro* and overexpression of a constitutively active mutant of FOXO3a can activate a *bim*-LUC reporter gene in sympathetic neurons. In addition, overexpression of FOXO3a in the presence of NGF induces apoptosis in a *bim*-dependent manner. More importantly, mutation of the two conserved FOXO binding sites present in the *bim*-LUC reporter construct greatly reduces the activation of the reporter gene by NGF withdrawal and expression of FKH DBD, a dominant interfering mutant of FOXO3a, in sympathetic neurons increases neuronal survival after NGF withdrawal (Figure 1b).[36] These results suggest that FOXO transcription factors contribute to the induction of *bim* transcription after NGF withdrawal by directly binding to the *bim* promoter. This might also occur in motoneurons deprived of neurotrophic factors.[41] In these cells, FOXO3a is activated after neurotrophic factor withdrawal and this correlates with an increase in the level of expression of Fas ligand and Bim. Like *bim*, the *Fas ligand* gene is a direct target of FOXO3a.[42] In CGNs, IGF-1, which activates PI3-kinase and Akt, blocks induction of *bim* following serum/KCl withdrawal and prevents activation of FOXO3a.[43] However, it is not yet known whether FOXO3a is necessary

for the induction of *bim* transcription in this system or in motoneurons following survival signal withdrawal.

Post-translational Regulation of Bim and Bad by Phosphorylation

Not only is Bim expression regulated at the transcriptional level in neurons and other cell types but intracellular signalling pathways can also regulate the stability and activity of the Bim protein by phosphorylation. Biswas and Greene showed that in PC12 cells, NGF can induce phosphorylation of Bim_{EL} and that this is mediated via the MEK/ERK pathway, but the sites in Bim phosphorylated by ERK were not identified in this study.[44] ERK-mediated phosphorylation of Bim_{EL} was also observed by Ley *et al*. in serum-treated fibroblasts.[45] These authors showed that this phosphorylation leads to the ubiquitylation and degradation of Bim via the proteasome, that is, ERK-mediated phosphorylation reduces the stability of the Bim protein. ERK1/2 directly binds and phosphorylates Bim_{EL}, but not Bim_L or Bim_S, *in vitro* and serine 65, which is only found in Bim_{EL} (Figure 1a), is a key phosphorylation site. Mutation of this residue to alanine blocks the phosphorylation of Bim_{EL} by ERK1/2 and prevents the degradation of the protein following activation of this pathway.[46] It has also been shown for osteoclasts that trophic factors, such as M-CSF, promote the phosphorylation, ubiquitylation and degradation of Bim, and that overexpression of a lysine-free Bim mutant that cannot be ubiquitylated in *bim −/−* cells abrogates the antiapoptotic effect of M-CSF.[47] In addition, it has been suggested that phosphorylation of Bim by ERK1/2 inhibits its interaction with Bax.[48] Whether ERKs phosphorylate Bim_{EL} at serine 65 in sympathetic neurons maintained in the presence of NGF is unknown.

In contrast to ERKs, the JNK pathway has been proposed to potentiate the proapoptotic activity of Bim. JNK has been reported to phosphorylate both Bim_{EL} and Bim_L in the LC8 binding region (Figure 1a) *in vitro* and it was suggested that this causes the release of Bim from the dynein motor complex in kidney 293T cells.[49] In sympathetic neurons, NGF withdrawal leads to increased phosphorylation of Bim_{EL} and this is blocked by CEP-1347, a MLK inhibitor, and SP600125, a JNK inhibitor, suggesting that MLK and JNK activity is required for this phosphorylation.[50] Interestingly, in this study it was suggested that serine 65 (the site phosphorylated by ERK1/2) was the key JNK phosphorylation site and that this potentiated the proapoptotic activity of Bim (Figure 1b). Phosphorylation of Bim_{EL} at serine 65 also occurs in CGNs following KCl/serum deprivation, and in PC12 cells infected with a recombinant adenovirus overexpressing the p75NTR.[51] The mechanism by which JNK phosphorylation of serine 65 increases the proapoptotic activity of Bim in neurons, rather than promotes its degradation, as in the case of ERK1/2 phosphorylation, remains to be determined.

Another BH3-only protein, whose regulation by phosphorylation has been extensively studied is Bad. Growth factors induce the phosphorylation of Bad at three sites (serine 112, serine 136 and serine 155), which allows the chaperone protein 14-3-3 to bind and sequester phosphorylated Bad in the cytoplasm.[52] Several protein kinases implicated in survival signalling have been proposed to mediate Bad phosphorylation, including Akt, Rsk, PAK, p70S6K and PKA.[52] Upon growth factor withdrawal, Bad is dephosphorylated and this active form of Bad binds to and inhibits prosurvival Bcl-2 family members. *Bad −/−* knockout mice have no gross abnormalities in the nervous system and *bad −/−* sympathetic neurons die at the same rate as wild-type neurons after NGF withdrawal.[18,53] However, Bad knockin mice have been constructed in which serines 112, 136 and 155 in Bad have been mutated to alanines so that the endogenous Bad protein cannot be phosphorylated (Bad^{3SA} mice).[54] Bad^{3SA} mice are viable and have no gross abnormalities although there are alterations in the normal development of pro-B and pro-T cells. Furthermore, studies with CGNs and other cell types isolated from Bad^{3SA} or wild-type mice and various apoptotic stimuli revealed that, in general, growth factor-dependent phosphorylation of Bad raises the threshold at which mitochondria release cytochrome *c* in response to apoptotic stimuli.[54]

A different mechanism by which protein kinases can regulate the activity of Bad involves phosphorylation of serine 128. This site can be phosphorylated by the cyclin-dependent kinase Cdc2 and by JNKs.[55,56] In developing rat CGNs cultured *in vitro* Cdc2 kinase activity increases after KCl deprivation and promotes apoptosis of these neurons.[55] Cdc2 phosphorylates Bad at serine 128 in cell free assays and in neurons in culture, and this phosphorylation inhibits the interaction of Bad phosphorylated by growth factor treatment with 14-3-3 proteins.[55] JNKs can also phosphorylate Bad at serine 128 *in vitro* and in cultured CGNs and again this promotes the apoptotic effect of Bad.[56] Furthermore, overexpression of the p75NTR in cultured cortical neurons, PC12 cells or glioma cells induces apoptosis associated with JNK-dependent phosphorylation of Bad at serine 128.[57] However, the significance of JNK-mediated phosphorylation of Bad at serine 128 was recently challenged by a study in which it was shown that JNK is required for the IL-3-mediated survival of pro-B cells.[58] These authors reported that JNK can phosphorylate Bad at threonine 201 and that this inhibits the interaction between Bad and $Bcl\text{-}x_L$. Replacement of threonine 201 by alanine generated a Bad mutant that promotes IL-3 withdrawal-induced apoptosis. Future studies will determine why JNKs phosphorylate different sites in Bad with different consequences in pro-B cells and neurons.

An Emerging Role for Other BH3-only Proteins in Neuronal Apoptosis

The p53 tumour suppressor protein has been shown to promote neuronal apoptosis in a variety of situations.[59] For example, overexpression of p53 can induce apoptosis in sympathetic neurons cultured *in vitro*, whereas expression of ΔNp73, a truncated form of the p53 family member p73 that decreases in level after NGF withdrawal, can protect sympathetic neurons against NGF withdrawal-induced death.[60,61] In neurons, in which the transcriptional activity of p53 family activator proteins increases during apoptosis, it is likely that Puma and Noxa, BH3-only protein genes that are direct targets of p53 transactivation, are induced. *Puma −/−*

and *Noxa* −/− knockout mice are viable and have no developmental defects so they can be used to study the role of these BH3-only proteins in the nervous system.[62–64] Thymocytes isolated from *Puma* −/− mice are resistant to apoptosis triggered by ionizing radiation (IR) and when P5 *Puma* −/− mice are irradiated, IR-induced apoptosis in the thymus and developing nervous system is greatly reduced.[62] In addition, *Puma* −/− fibroblasts and thymocytes are also protected against apoptosis induced by p53-independent insults, including cytokine deprivation and exposure to glucocorticoids, the protein kinase inhibitor staurosporine or phorbol ester.[64] Fibroblasts isolated from *Noxa* −/− mice are resistant to the p53-dependent apoptosis induced by DNA-damaging agents, such as etoposide, adriamycin and cisplatin.[63,64] More recently, the role of p53 and Noxa in the axotomy-induced death of motoneurons has been studied using *p53* −/− and *Noxa* −/− knockout mice.[65] In adult C57BL/6 mice, hypoglossal nerve injury leads to delayed but extensive motoneuron death. RT-PCR analysis demonstrated that *Noxa*, but not *Puma*, RNA levels increase in the hypoglossal nuclei of injured mice. This injury-induced increase in *Noxa* mRNA was partially reduced in *p53* −/− mice, and both *p53* −/− and *Noxa* −/− mice had increased numbers of motoneurons after axotomy. These results suggest that following axotomy, p53 promotes adult motoneuron apoptosis, at least in part by inducing the transcription of the BH3-only protein Noxa.

There is evidence that the extrinsic death receptor (Fas/TNF receptor) signalling pathway promotes caspase activation in some models of neuronal apoptosis. The BH3-only protein Bid is an important substrate of caspase-8 in the death receptor pathway. Cleavage of Bid by caspase-8 generates tBid, which can bind to and activate Bax and thereby promote mitochondrial outer membrane permeabilization.[66] *Bid* −/− knockout mice have no major abnormalities in the nervous system. In the case of sympathetic neurons cultured *in vitro*, Bid is not cleaved after NGF deprivation and *Bid* −/− neurons die at the same rate after NGF withdrawal as wild-type neurons.[15,18] However, there is some evidence that Bid does have a role in cortical neuron death induced by oxygen/glucose deprivation *in vitro* and following focal cerebral ischaemia *in vivo*.[67,68] In C57BL/6 mouse cortical neurons cultured *in vitro* oxygen/glucose deprivation leads to caspase-8 activation, Bid cleavage and apoptosis, and cortical neurons isolated from *Bid* −/− mouse brain are somewhat resistant to death induced by oxygen/glucose deprivation compared to wild-type neurons.[67] Furthermore, ischaemic damage *in vivo* is reduced in *Bid* −/− mice compared to wild-type mice following middle cerebral artery occlusion.[67,68] These results suggest that Bid makes some contribution to the induction of ischaemic neuronal death.

Conclusions and Future Directions

Mammalian neurons express multiple BH3-only proteins and the transcriptional induction or post-translational modification of more than one BH3-only protein occurs in a specific neuronal type in response to a specific death stimulus. For example, in sympathetic neurons, NGF withdrawal leads to an increase in the levels of DP5, Bim and Puma and the phosphorylation of Bim and Bad changes. Studies with knockout mice indicate that the loss of individual BH3-only proteins can delay but not completely inhibit apoptosis probably because the remaining family members can partially compensate for the loss of a single BH3-only protein. Why is more than one BH3-only protein induced/activated? Recent studies have shown that the BH3 domains of different BH3-only proteins have different binding specificities.[38,39] Some BH3-only proteins can bind to all antiapoptotic Bcl-2 family members, for example, Bim and Puma, whereas others have a more restricted binding specificity, for example, Bad and Noxa.[38] Thus, induction of more than one BH3-only protein will ensure that the intrinsic pathway is efficiently activated, that is, that all antiapoptotic family members present in the cell are inhibited and that Bax is activated.

Studies with sympathetic neurons and CGNs isolated from postnatal *bax* −/− knockout mice have demonstrated that Bax is essential for the survival signal withdrawal-induced death of these cells *in vitro*. Furthermore, the number of viable sympathetic neurons isolated from the superior cervical ganglia (SCG) of *bax* −/− knockout mice is increased by 2.5 fold compared to wild-type mice, suggesting that Bax is required for the naturally occurring developmental death of SCG neurons *in vivo*.[19,20] However, in contrast to *caspase-9* −/− and *caspase-3* −/− knockout mice,[69,70] *bax* −/− knockout mice do not have severe brain abnormalities. This suggests that either the caspase-9 and caspase-3-dependent apoptosis in the developing embryonic CNS does not require Bax, or that Bak can substitute for the loss of Bax at that stage of development but not in postnatal sympathetic neurons and CGNs. Clearly, this question requires further investigation. In the case of Bim and DP5, experiments with *bim* −/− and *dp5* −/− knockout mice suggest that Bim makes an important contribution, and DP5 a more minor contribution, to the NGF withdrawal-induced death of sympathetic neurons *in vitro*.[15,17,33] However, to formally conclude that BH3-only proteins are key regulators of developmental neuronal apoptosis, it would be necessary to characterize sympathetic neurons from double or even triple knockout mice to determine whether loss of more than one BH3-only protein has an effect similar to that of *bax* deletion. It will also be important to use knockout mice to investigate the role of individual BH3-only proteins in various mouse models of neurodegeneration. For example, experiments with *bim* −/− mice have demonstrated that Bim does not play an indispensable role in the cerebellar neurodegeneration that occurs in *Lurcher* mutant mice, whereas *bax* inactivation does reduce the death of CGNs, but not Purkinje neurons, in *Lurcher* mice.[71,72] In the case of *bim* −/− *Lurcher* neurons, other BH3-only proteins may substitute for the loss of Bim.

In addition to further studies with knockout mice, it is clear that relatively little is known about the mechanisms by which the transcription and activity of individual BH3-only proteins are regulated in neurons responding to cell death signals. The model for which we currently have the most information is the NGF withdrawal-induced death of sympathetic neurons. However, even in this case, there are many questions that need to be answered. For example, how does the JNK/c-Jun pathway contribute to the induction of Bim and DP5 following

NGF withdrawal; what other transcription factors activate or repress the transciption of the *bim* and *dp5* genes; how exactly do ERK and JNK regulate Bim at the post-translational level in the presence and absence of NGF, respectively; what is the role of Puma and how is Puma regulated by NGF? Furthermore, it is likely that there will be important differences in the mechanisms by which specific BH3-only proteins are regulated in other kinds of neurons. It is therefore safe to conclude that there will continue to be a considerable amount of interest and much research work in this area for the next few years.

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