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Review

'Men are but worms:' ☆ neuronal cell death in *C. elegans* and vertebrates

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Abstract

Awarding the 2002 Nobel Prize in Physiology or Medicine to Sydney Brenner, H Robert Horvitz, and John E Sulston for 'their discoveries concerning the genetic regulation of organ development and programmed cell death (PCD)' highlights the significant contribution that the study of experimental organisms, such as the nematode *Caenorhabditis elegans*, has made to our understanding of human physiology and pathophysiology. Their studies of lineage determination in worms established the 'central dogma' of apoptosis: The BH3-only protein EGL-1 is induced in cells destined to die, interacts with the BCL-2-like inhibitor CED-9, displacing the adaptor CED-4, which then promotes activation of the caspase CED-3. The vast majority of cells undergoing PCD during development in *C. elegans*, as in vertebrates, are neurons. Accordingly, the genetic regulation of apoptosis is strikingly similar in nematode and vertebrate neurons. This review summarizes these similarities – and the important differences – in the molecular mechanisms responsible for neuronal PCD in *C. elegans* and vertebrates, and examines the implications that our understanding of physiological neuronal apoptosis may have for the diagnosis and treatment of acute and chronic human neurodegenerative disorders.
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Abbreviations: BH, BCL-2 homology (domain); CG, cerebellar granule (neuron); CREB, cAMP response element binding protein; Cyt *c*, cytochrome *c*; DRE, downstream regulatory element; ERK, extracellular-regulated kinase; FOXO, forkhead box; HSN, hermaphrodite-specific neuron; JAK, janus kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MLK, mixed lineage kinase; NSM, neurosecretory motoneuron; NGF, nerve growth factor; PI3 K, phosphatidylinositol-3-kinase; PCD, programmed cell death; SCG, superior cervical ganglion; STAT, signal transducer and activator of transcription; TFD, trophic factor deprivation; TMD, transmembrane domain; UTR, untranslated region; VDAC, voltage-dependent anion channel.

Introduction

Programmed cell death (PCD) is critical to the development and maintenance of many tissues. Deficiencies in PCD underlie some forms of oncogenesis, while excessive cell death may contribute to several pathological conditions, including stroke, autoimmune disorders, and certain neurodegenerative diseases. Cells undergoing PCD exhibit morphological and biochemical changes characteristic of apoptosis, including cytoplasmic shrinkage, plasma membrane blebbing, chromatin condensation, and DNA fragmentation.[1] Phagocytes, both professional and nonprofessional, eventually engulf the dying cells, thereby preventing an inflammatory response.

Much of what we know today about the evolutionary conservation and genetic regulation of PCD arises from the pioneering work of Sydney Brenner, John Sulston, and Robert Horvitz, for which they were jointly awarded the 2002 Nobel Prize in Physiology or Medicine. From their studies of cell-lineage determination in the nematode *Caenorhabditis elegans*, Brenner, Sulston, and Horvitz found that of the 1090 somatic cells generated during development of the *C. elegans* hermaphrodite, only 959 remain in the adult. Moreover, 105 of the 131 cells that undergo PCD are neurons, a finding that Horvitz noted 'particularly caught my attention' in his Nobel address. In this manner, neuronal development in nematodes resembles that in their vertebrate counterparts, characterized by overproduction and then cell death, ostensibly (according to the neurotrophic hypothesis) to match the number of innervating neurons with the size of their target tissue. Perhaps it should come as no surprise then that some of the most striking similarities between the signal transduction pathways responsible for PCD in worms and vertebrates occur in neurons.

In this review, we shall discuss some of these similarities, as well as some important differences, between PCD in *C. elegans* and in vertebrate neurons, focusing on one of the most extensively studied models of neuronal PCD, nerve growth factor (NGF) deprivation in sympathetic neurons from superior cervical ganglia (SCG). Then, we will examine the

☆ From *Punch Magazine*, 1882 (and H.R. Horvitz's Nobel lecture, 8 December 2002)

potential contribution of neuronal cell death to the pathogenesis of both acute and chronic neurodegenerative diseases and discuss the role of PCD-targeted therapies in the treatment of these disorders.

The Genetics and Biochemistry of Programmed Cell Death

In a series of elegant genetic experiments that span three decades, Horvitz and his colleagues defined the 'central dogma' of PCD, depicted schematically in Figure 1. According to this model, in at least two types of neurons in *C. elegans* (i.e., the hermaphrodite-specific neurons (HSNs) in males and the neurosecretory motoneuron (NSM) sister cells), the 'thanatin' EGL-1 is induced in cells destined to die, interacts with the cell death inhibitor CED-9, thereby displacing the adaptor CED-4, which can then promote the activation of the executioner protease CED-3. Parallel studies in vertebrates conducted by numerous laboratories have identified the vertebrate counterparts of these nematode proteins as BH3-only BCL-2 proteins (e.g., BIM and HRK), antiapoptotic BCL-2 proteins (e.g., BCL-2 and BCL-X_L), Apaf-1, and the caspase family of cysteine proteases, respectively.

Despite this remarkable evolutionary conservation, several fundamental differences appear to exist: First, caspase activation through this 'intrinsic' pathway during development

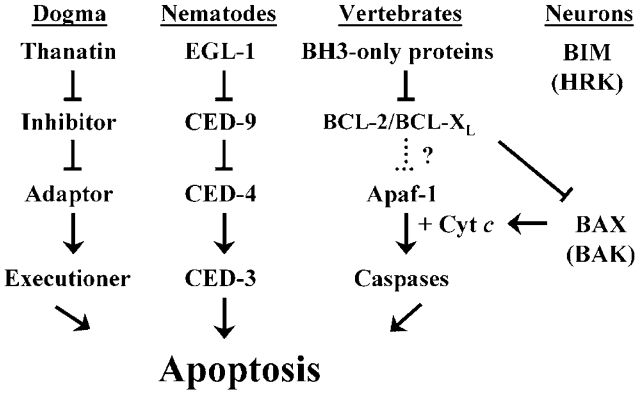

Figure 1 The 'central dogma' of PCD. Based on the work of Horvitz and his colleagues in HSNs and NSM sister cells in nematodes, the thanatin EGL-1 is induced in cells that are destined to die, interacts with inhibitor CED-9, displaces the adaptor CED-4, which then activates executioner protease CED-3. This basic scheme is conserved in vertebrates in the form of BH3-only proteins (e.g., BIM and HRK), antiapoptotic BCL-2 proteins (e.g., BCL-2 and BCL-X_L), Apaf-1, and the caspases. However, assuming that Apaf-1 is the one and only CED-4 ortholog in vertebrates*, two fundamental differences exist: First, caspase activation through this pathway requires cyt *c* release in vertebrates, but not in worms. And in sympathetic and CG neurons (and probably other neuronal subpopulations), this release requires BAX, whose 'activation' (see Figure 3) requires the BH3-only protein BIM, and probably others (e.g., HRK). Second, caspase activation in SCG (and probably other) neurons, requires BAX-dependent release of not only cyt *c* but also Smac/DIABLO, an IAP inhibitor. *(We equivocate here because the answers to certain questions may reveal even greater conservation than that described above: For example, is the mitochondrial localization of CED-9 reported by Chen *et al.*[130] purely serendipitous or does it belie a BCL-2-like function in regulating mitochondrial permeability in the nematode? Furthermore, is Apaf-1 the one and only true vertebrate CED-4 ortholog as suggested by preliminary sequence analysis of the human genome[131] or does another CED-4-like protein(s) exist whose activation of CED-3-like caspases truly can be inhibited at the level of the apoptosome by direct interaction with a CED-9-like antiapoptotic BCL-2 family member?)

requires cytochrome *c* (cyt *c*) in vertebrate, but not in nematode, neurons. (The structural basis for this difference seems apparent: CED–4 lacks the WD40 repeats required in Apaf-1 for interaction with cyt *c*, a potential caveat being that activation of the *Drosophila* Apaf-1-related killer (ARK), which also has WD40 repeats, may not require interaction with cyt *c*.[2]) Second, such cyt *c* release in multiple neuronal populations requires expression of at least one multidomain proapoptotic BCL-2 protein, usually BAX. And finally, caspase activation in sympathetic (and probably other) neurons in physiological settings requires not only BAX-dependent cyt *c* release but also inactivation of an inhibitor of apoptosis (IAP)-like activity by Smac/DIABLO, the phenomenon initially described as 'competence-to-die.'[3] Because this review, in highlighting the similarities between PCD in nematode and vertebrate neurons, will focus on the premitochondrial regulation of apoptosis, particularly by BCL-2 proteins, the interested reader is referred elsewhere for further discussion of the role of cyt *c* and IAPs in the postmitochondrial regulation of neuronal apoptosis.[4–9]

Premitochondrial Regulation of Intrinsic Pathway Apoptosis: The CED-9/BCL-2 Family

The CED-9/BCL-2 family of proteins consists of both antiapoptotic (e.g., BCL-2, BCL-X_L, BCL-w, and MCL-1) and proapoptotic members; the latter are further divided into two subfamilies, 'multidomain' (e.g., BAX, BAK, and BOK) and 'BH3-only' (e.g., BAD, BID, and BIM) proteins. Four regions of homology known as BCL-2 homology (BH) domains 1–4 define membership in the BCL-2 family.[10] Although certain exceptions exist, antiapoptotic BCL-2 proteins generally possess all four BH domains and a transmembrane domain (TMD), while multidomain proapoptotic members typically lack only the BH4 domain; and, true to their name, BH3-only proapoptotic proteins usually possess only a BH3 domain and (sometimes) a TMD. This conserved domain structure among the three subfamilies has functional significance because BH1–3 may mediate interactions between family members.

BCL-2 proteins can form certain homo- and heterodimers and multimers. Whether they do so *in vivo* or even *in vitro* in the absence of conformation-altering detergents that promote interactions (and may mimic a membrane environment) remains controversial. However, as a general rule, antiapoptotic BCL-2 proteins require BH1 and BH2 to prevent cell death and to heterodimerize with their proapoptotic counterparts.[11–14] Conversely, proapoptotic proteins generally require BH3 to promote cell death and, accordingly, to heterodimerize with BH1 and BH2 of their antiapoptotic cousins.[15–18] These *in vitro* findings from mutagenesis studies are consistent with the tertiary structure of BAK,[19] BAD,[20] and BIM[21] heterodimers with BCL-X_L showing that the BH3 domain of the proapoptotic members forms an amphipathic α-helix that associates through hydrophobic and electrostatic interactions with a 'binding pocket' formed by the BH1 and BH2 domains of BCL-X_L. (One minor caveat to these conclusions may be that none of these structures includes the entire C-terminus or TMD (where present) of the respective proteins, and the tertiary structures of the proteins

examined may differ in soluble *versus* membrane-bound forms.)

Consistent with studies such as these, Korsmeyer and coworkers[22] proposed the 'rheostat model', according to which the ratio of proapoptotic to antiapoptotic BCL–2 family members within a cell determines whether that cell will die in response to a death signal. Although certain findings (e.g., the ability of both pro- and antiapoptotic family members to function independently to regulate cell death[23–25]) have cast doubt on certain elements of the original hypothesis, the basic premise of this model still holds true: the net balance of proapoptotic versus antiapoptotic BCL-2 proteins determines a cell's fate.

Nonetheless, the biochemical mechanisms by which BCL-2 family proteins regulate survival remain unclear. The three-dimensional structure of BCL-X_L shows similarities to the pore-forming domains of bacterial toxins such as diphtheria toxin and the colicins.[26] Accordingly, BCL-2, BCL-X_L, BID, and BAX can form ion channels in synthetic lipid membranes.[27–30] These channels are characterized by multiconductance states, pH sensitivity, voltage gating, and poor ion selectivity. Whether BCL-2 family members form pores in mitochondria in intact cells and what regulates this activity are unclear; however, these findings suggest that these proteins may either form or regulate the formation of channels in mitochondrial membranes. Alternatively, BCL-2 proteins may regulate the integrity of intracellular (especially mitochondrial) membranes, either alone or in concert with other proteins, such as VDAC.[31–33] In any case, the molecular and biochemical basis for the regulation of apoptosis by BCL-2 proteins remains uncertain.

Programmed Cell Death in the Nervous System: The Sympathetic Neuron Model

From approximately embryonic-day-16 (E16) to postnatal-day-7 (P7), sympathetic neurons require NGF for survival. [34] *In vivo* administration of NGF antiserum during this period induces extensive loss of SCG neurons.[35] This cell death can be recapitulated *in vitro*: Neonatal sympathetic neurons, maintained in culture for 4–6 days in the presence of NGF, undergo an apoptotic cell death within 48 h of trophic factor withdrawal.[36,37] This death, which requires *de novo* protein synthesis,[38] expression of the proapoptotic BCL-2 family member *Bax*,[39] and development of competence-to-die,[3] is inhibited by caspase inhibitors[40–42] and neuroprotective agents, such as KCl and cAMP.[36,37,43,44]

Transcriptional Regulation of BH3-only Proteins, the Long-Sought-after Thanatins

Nearly two decades ago, we reported that inhibitors of RNA and protein synthesis protect against trophic factor deprivation (TFD)-induced apoptosis in sympathetic neurons. [38] This observation suggests that, true to its name, 'programmed' cell death is a differentiation program like any other requiring selective new gene expression to direct a cell to its physiologically appropriate fate, in this case death. However, the identities of these genes, which we called 'thanatins'[45] and defined as genes induced during apoptosis whose principal, or sole, function is to mediate cell death, remained elusive. However, here again genetic studies of PCD in *C. elegans* proved illuminating: Conradt and Horvitz[46] reported in 1998 that the BH3-only protein EGL-1, whose expression is induced in HSNs destined to die in male nematodes, physically interacts with CED-9, displacing the adaptor CED-4, which can then activate the caspase CED-3 (Figure 1). Subsequently, studies in sympathetic neurons identified a BH3-only protein – BIM – induced during NGF deprivation and required for TFD-induced apoptosis.[47,48] However, *Bim* deletion,[47] unlike *Bax* deficiency,[39] delays, but does not prevent, cyt *c* release, caspase activation, and cell death in this paradigm. These findings suggest the existence of at least one other functionally redundant, BH3-only protein in these neurons. Accordingly, in 1997, two groups reported the cloning of HRK/DP5, a BH3-only protein induced by NGF deprivation in SCG neurons[49] with kinetics strikingly similar to those for BIM.[50] Moreover, we recently have observed similar induction of another BH3-only protein, PUMA, during NGF deprivation in sympathetic neurons (C Besirli and EMJ, unpublished observations).

Cell death induced by overexpression of either *Bim* or *Hrk/Dp5* (or any other BH3-only protein examined) requires BAX alone despite coexpression of BAK in cerebellar granule (CG) neurons,[50–52] indicating that BH3-only proteins function genetically upstream of (or in parallel with) BAX-dependent cyt *c* release and its downstream sequelae in these neurons. Furthermore, although the physiological function of HRK and PUMA in neuronal cell death remains unknown, these findings suggest that recapitulation of the $Bax^{-/-}$ phenotype, in which cyt *c* release, caspase activation, and cell death are completely prevented long-term, will require inactivation of BIM, and almost certainly other BH3-only proteins (e.g., HRK and PUMA). However, two critical questions remain unanswered by these studies: What regulates the transactivation of BH3-only proteins and how (assuming Apaf-1 is the true vertebrate CED-4 ortholog), in the absence of an apoptosome complex resembling that in nematodes, do these BH3-only proteins function?

In *C. elegans*, in addition to the HSNs in males, the death of at least one other neuronal population, the NSM sister cells, requires transactivation of the BH3-only protein EGL-1. More recent studies in both cell types demonstrate that two zinc-finger transcription factors CES-1 and TRA-1 repress the transcription of *egl-1*.[53–55] Of interest, CES-1 and TRA-1 have human orthologs, SLUG and GLI proteins, respectively. Although the potential role of SLUG and GLI proteins in regulating BH3-only protein expression in vertebrates is unknown, both have been implicated in the pathogenesis of certain human cancers.[56] Nonetheless, recent findings shed some light on the transcriptional regulation of BH3-only proteins in vertebrate neurons, and (not surprisingly) the situation may be even more complex than that in worms.

Multiple signaling pathways modulate the NGF-dependent survival of sympathetic neurons (Figure 2), including (but not necessarily limited to) those involving cAMP response element binding protein (CREB), NF-κB, phosphatidylinositol-3-kinase (PI3 K), and mitogen-activated protein kinases (MAPKs), including various MAPKs (e.g., p42/44 extracellular-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), and p38 MAPKs), MAPK kinases (MAPKKs) (e.g.,

MKK3, 4, 6, and 7), and MAPKK kinase kinases (MAPKKKs) (e.g., mixed lineage kinases (MLKs)), as well as putative MAPKKK activators, such as small G-proteins (e.g., Cdc42/Rac). Some of these pathways, such as CREB,[57] NF-κB,[58,59] MEK/ERK,[60,61] and PI3K/Akt,[62–64] promote survival and/or differentiation in these neurons, whereas others, especially JNKs, promote cell death.[65–67]

Recent findings indicate that BCL-2 proteins such as BIM, HRK, and other BH3-only proteins are molecular targets for

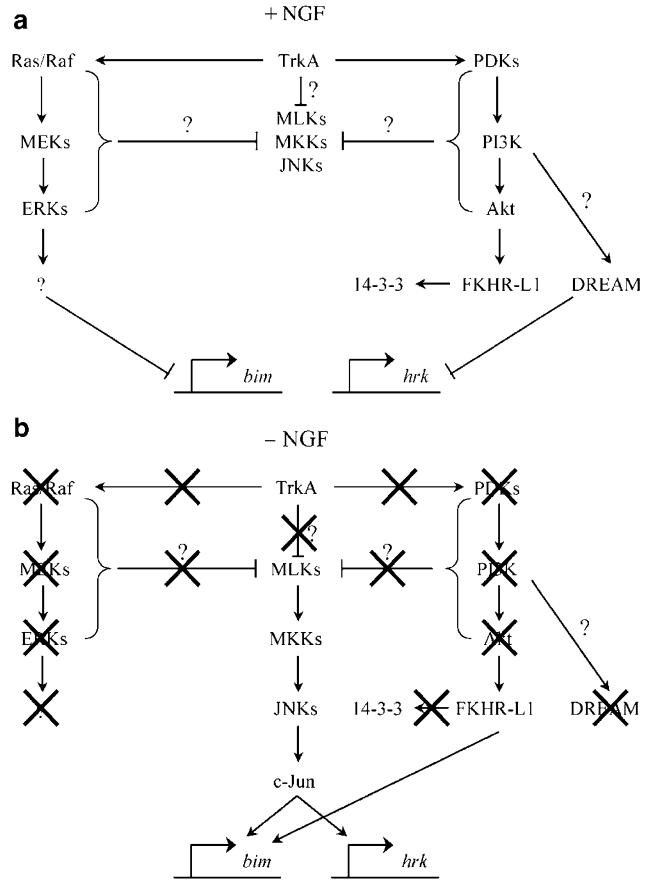

Figure 2 Multiple signal transduction pathways coordinately regulate the expression of BH3-only proteins, such as BIM and HRK, in sympathetic neurons. **(a)** In NGF-maintained sympathetic neurons, BIM and HRK expression is suppressed by multiple mechanisms, including (but not necessarily limited to) those described below. First, NGF-dependent activation of TrkA causes activation of the Ras/MEK pathway, which (somehow) represses BIM expression. In parallel, PI3K-dependent activation of Akt causes phosphorylation of Forkhead transcription factors, such as FKHR-L1, which then can be sequestered in the cytosol by 14-3-3 proteins, thereby preventing BIM expression.[132] Moreover, the transcriptional repressor DREAM, which can be activated by PI3 K signaling and/or elevated intracellular Ca^{2+}, represses HRK expression. Finally, TrkA activation by NGF suppresses activation of the MLK/JNK pathway, although the precise molecular mechanisms are unknown (e.g., mutual negative regulation by the Ras/MEK and PI3K/Akt signaling modules may contribute). **(b)** In contrast, during NGF deprivation, ERK and PI3K activation decays, derepressing BIM and HRK expression. In parallel, as PI3K-dependent Akt activity decreases, Forkhead transcription factors, such as FKHR-L1, become dephosphorylated, dissociate from 14-3-3 proteins and translocate to the nucleus, where they transactivate BIM expression. And finally, NGF deprivation causes activation of the JNK pathway, culminating in AP-1-mediated BIM and HRK induction. Moreover, activation of the JNK pathway also causes phosphorylation of BIM, perhaps regulating the proapoptotic activity of BIM post-translationally by modulating proteasome-dependent degradation

these kinase cascades that mediate at least some of their effects.[47,48,50,68,69] For example, maximal transactivation of BIM requires activation of the JNK/c-Jun pathway,[47,48,50,52] consistent with the identification of AP-1 sites in the Bim promoter.[70] Also striking is the extent to which the transcripts for *Bim* and *Hrk* are coordinately regulated by the JNK/c-Jun pathway,[50] and perhaps others, such as the PI3K/Akt module,[71,72] consistent with a TFD-induced transcriptional 'program' culminating in BAX-dependent neuronal apoptosis. Accordingly, overexpression of known or hypothesized components of the JNK/c-Jun signaling module, including MLK1–3, MKK4, MKK7, and JNK1, requires BAX alone even when BAK is coexpressed.[52] Similarly, in sympathetic neurons[72] as in hematopoietic cells,[69] in which TFD causes induction of both BIM and HRK, attenuation of signaling through the PI3K/Akt pathway during TFD causes dephosphorylation and nuclear translocation of FOXO transcription factors and FOXO-mediated *Bim* transcription. Consistent with this, ectopic expression of constitutively active FOXO transcription factors induces cell death that requires BIM and BAX in neurons[72] (GVP and EMJ, unpublished observations). In addition to FOXO transcription factors, another potential target of PI3K signaling in this context may be DREAM, a Ca^{2+}-dependent repressor that regulates *hrk* transcription.[71] In this case, trophic factors such as NGF activate PI3K and increase intracellular Ca^{2+}, both of which promote repression of *hrk* transcription by DREAM, which binds a downstream regulatory element (DRE) sequence in the 3′ untranslated region (UTR) of the *hrk* gene. In this manner, both the MLK/JNK and PI3K/Akt signaling modules may coordinately regulate the expression of the BH3-only proteins BIM and HRK during TFD-induced neuronal apoptosis. Finally, recent data suggest that activation of the Ras/MEK pathway, which may itself antagonize JNK signaling and promote CREB-mediated pro-survival signaling (see below), represses transcription of BIM. For example, inhibitors of MEK such as PD98059 and U0126 induce BIM expression in nonneuronal cell lines[73–76] as well as in neurons (GVP and EMJ, unpublished observations). However, interpretation of these findings is complicated by crosstalk between the Ras/MEK, PI3K/Akt, and MLK/JNK modules, as well as recent reports on the post-translational regulation of BIM's proapoptotic activity by two of these pathways (i.e., ERK and JNK) via phosphorylation-dependent modulation of protein stability, probably involving the proteasome.[52,73,77,78]

However, conclusions based on these reports that activation of MLK/JNK signaling and/or suppression of PI3K/Akt and Ras/MEK signaling are the only pathways controlling BIM transcription likely under-represent the complexity of BIM transcriptional control. First, Liu and Greene[79] report that Rb-dependent, E2F-mediated gene repression attenuates TFD-induced apoptosis in sympathetic neurons and that depression of certain E2F target genes, such as B- and C-myb, which are induced by NGF deprivation in SCG neurons,[65] can induce cell death in these neurons. Since the BIM promoter contains potential binding sites for c-Myb,[70] BIM may be one of the E2F target genes derepressed during TFD. Second, the transcriptional control of BH3-only proteins like BIM may vary in a cell type- and stimulus-specific manner. For example, NGF-deprived sympathetic neurons rely primarily on *activa-*

tion of the MLK/JNK pathway culminating in AP-1-dependent transactivation of BIM,[47,48,50] with some modest contribution from loss of PI3K/Akt and Ras/MEK signaling.[47] In contrast, in IL-3-deprived hematopoietic cells, *suppression* of the PI3K/Akt pathway culminating in FKHRL1-mediated BIM transcription predominates,[68,69] with some contribution again from loss of Ras/MEK signaling.[76] Even within a single cell type, control of BIM transcription may vary according to cellular context. For example, in the case of mass cultures of NGF-maintained sympathetic neurons, inhibition of the Ras/MEK or PI3K/Akt pathway by using U0126 or LY294002, respectively, causes some MLK/JNK-*dependent* induction of BIM; however, in the context of depolarization-induced survival of both SCG and CG neurons, which depends almost exclusively on PI3K/Akt signaling,[80–83] LY294002 alone causes significant MLK/JNK-*independent* BIM induction (GVP and EMJ, unpublished observations) and BAX-dependent cell death,[84] consistent with physiological crosstalk between the Ras/MEK, PI3K/Akt, and MLK/JNK pathways. Thus, coordinate transcriptional regulation of BH3-only proteins, such as that seen with BIM and HRK during neuronal apoptosis, will likely involve complex signaling networks that may be context dependent (i.e., cell- and stimulus-specific).

Finally, transcriptional targets other than BH3-only proteins may also contribute to survival responses mediated by some of the aforementioned signaling modules (e.g., Ras/MEK and PI3K/Akt). For example, the CREB family of transcription factors contributes to the trophic factor-mediated survival of both sympathetic and CG neurons (reviewed in Lonze and Ginty[57]) via a BAX-dependent pathway[85] (GVP and EMJ, unpublished observations). A previous report suggests that this pro-survival activity involves CREB-mediated upregulation of *bcl-2*.[86] However, as noted more recently,[85] this is unlikely because both *in vivo* and *in vitro*, the trophic factor-dependent survival of both PNS and CNS neurons is only modestly affected by *Bcl-2* deletion.[87,88] Although we clearly lack a definitive compendium of CREB family-regulated genes, among the known or hypothesized targets, a more likely candidate is BCL-X_L, which is critical for neuronal survival,[89,90] and is a transcriptional target of the JAK-STAT signaling module,[91] which includes at least one transcriptional target of CREB (i.e., STAT3).[92] In addition, notwithstanding the added complexity of phosphorylation-independent regulatory events, the phosphorylation-dependent activation of CREB may involve multiple kinase cascades, including the Ras/MEK, PI3K/Akt, and p38 MAPK pathways mentioned above.[57] Second, NGF-dependent activation of NF-κB contributes to the survival of sympathetic neurons.[58,59] Such regulation may be both premitochondrial, involving transactivation of anti-apoptotic BCL-2 proteins, such as BCL-X_L and/or A1/BFL-1,[93,94] and postmitochondrial, involving upregulation of IAPs.[95,96] However, one important caveat exists regarding the importance of transcriptional regulation of antiapoptotic BCL-2 proteins during TFD in SCG and CG neurons: The expression of BCL-X_L, BCL-2, and BCL-w remains essentially unchanged until very late in the cell death process (i.e., after the release of cyt *c*).[51,87] Therefore, although NF-κB and CREB signaling clearly contributes to survival in these neurons, whether antiapoptotic BCL-2 proteins represent physiologically significant transcriptional targets of these pathways is unclear.

Enter the Interloper: Regulation of BAX during Neuronal Apoptosis

As mentioned above, one fundamental difference between developmental neuronal PCD in *C. elegans* and vertebrates is that the latter involves cyt *c* release for caspase activation, and at least in sympathetic and CG neurons (and probably many others), such release absolutely requires expression of multidomain proapoptotic BCL-2 protein BAX.[3,97,98] Despite widespread expression of *Bax in vivo*, *Bax*-deficient mice exhibit relatively modest defects in non-neuronal lineages,[99] likely secondary to functional redundancy with other multidomain proapoptotic BCL-2 family members.[100,101] In contrast, their phenotype in neuronal lineages, whether central or peripheral, is dramatic.[39,84,90,102] For example, despite coexpression of BAK, TFD-induced cell death in CG neurons depends solely on BAX.[51] However, contrary to one report,[103] neither the mRNA nor protein level of BAX increases during NGF deprivation-induced apoptosis in sympathetic neurons[87] or K^+-withdrawal-induced death in CG neurons.[47,51,84,104] Therefore, BAX must be regulated post-translationally in these and other neuronal TFD paradigms,[98,104–107] not via transactivation by p53, which only has a nominal role in TFD-induced neuronal apoptosis[108–111] in contrast to its significant role in DNA damage-induced neuronal cell death.[108,112–115] With regard to the contribution of p53 (or other family members) to TFD-induced neuronal PCD, we favor the hypothesis that reduced *basal* BAX expression underlies the modest delay in cell death observed in NGF-deprived SCG neurons deficient in p53 for two principal reasons: (1) *p53* deletion is associated with reduced *basal* BAX expression in multiple neuronal populations including sympathetic neurons;[116] and (2) reducing the gene dosage of *Bax* (e.g., in $Bax^{+/-}$ neurons), like *p53* deficiency, *delays* TFD-induced apoptosis in SCG neurons.[39,108]

To our knowledge, TFD in sympathetic and CG neurons remains the only primary cell culture paradigms shown to be entirely dependent on the expression and translocation of BAX alone for cyt *c* release, caspase activation, and apoptosis,[3,39,84,98,105,106] although this is probably true for other neuronal populations.[39,107] Therefore, these models are excellent for studying the physiological mechanisms responsible for endogenous BAX translocation and BAX-dependent cyt *c* release. Moreover, macromolecular synthesis is required for BAX translocation, cyt *c* release, and apoptosis during TFD in SCG neurons;[3,39,105,117] and, the BH3-only proteins BIM and HRK are induced in cells destined to die and require BAX to mediate cell death.[47,48,50] Taken together, these findings clearly suggest that BH3-only proteins may trigger BAX translocation and its downstream sequelae. However, these results are consistent with several possibilities, which need not be mutually exclusive, for how this may occur (Figure 3a). (Please note that these arguments, which are similar to those previously presented by Putcha *et al.*,[51] have only been strengthened by more recent findings, such as Liu *et al.*[21])

First (Model 1), BH3-only proteins may interact *directly* with BAX in the cytosol to trigger its translocation to mitochondria, where it integrates and mediates the release of apoptogenic intermembrane-space proteins, such as cyt *c*. Alternatively

(Model 2), BH3-only proteins may interact directly with BAX at mitochondria to form a multiprotein complex that mediates cyt *c* release. We do not favor these models for several reasons. First, the tertiary structure of soluble, cytosolic BAX is inconsistent with BAX interacting with BH3-only proteins in the cytosol, arguing against Model 1. Specifically, the BH1 and BH2 domains, which presumably would mediate interactions with a 'BH3 donor,' are not accessible for such interactions.[118] (Whether the 'BH3-binding pocket' formed by BH1 and BH2 becomes accessible upon integration into the outer mitochondrial membrane by BAX or BAK is unclear.) Second, to date, only three known or putative BH3-only proteins may be cytosolic: BID, BAD, and MAP-1. Although the physiological role of MAP-1 is unknown, deletion of neither BID nor BAD altered the time course or extent of TFD-induced cyt *c* release or apoptosis in sympathetic or CG neurons,[51] arguing against Model 1 for these proteins. Therefore, neither tBID nor BID nor BAD appears to be the cytosolic trigger for BAX translocation and its downstream sequelae in SCG and CG neurons. Third, with the potential exception of BID (for which only overexpression data exist), no BH3-only proteins interact directly with BAX at endogenous levels in primary cells or tissues undergoing cell death, nor have any been co-precipitated with BAX multimers, although the possibility of a 'hit-and-run' model for BAX activation remains.[119] Finally, deletion of *Bim* (and presumably *Hrk*) attenuates BAX-dependent cyt *c* release.[47] However, neither protein appears to be cytosolic,[17,47] which argues against Model 1, nor can interact directly with BAX,[17,120] which argues against Models 1 and 2.

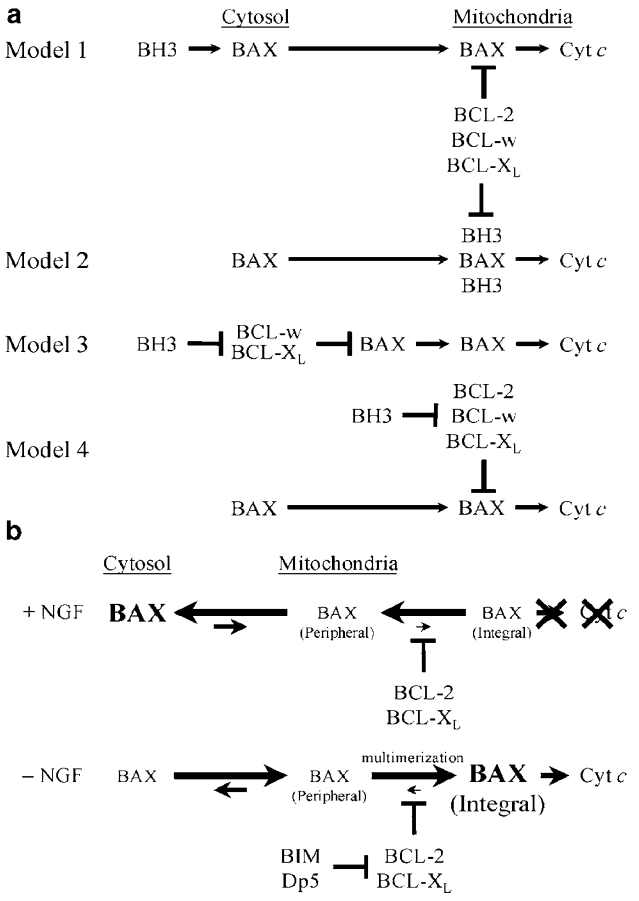

Figure 3 BH3-only proteins indirectly trigger BAX translocation, cyt *c* release, caspase activation, and apoptosis during TFD-induced neuronal cell death. **(a)** Four proposed models depicting how BH3-only proteins, such as BIM and HRK, may promote BAX translocation and its downstream sequelae during TFD in sympathetic and CG neurons. Models 1 and 2 invoke direct interaction of BH3 donors with BAX, while Models 3 and 4 involve BH3-only proteins indirectly triggering BAX translocation via inactivating interactions with antiapoptotic BCL-2 proteins. We favor Model 4 for reasons discussed in the text. **(b)** A dynamic equilibrium model for BAX translocation. During TFD in sympathetic and CG neurons, BIM and HRK are induced and inactivate antiapoptotic BCL-2 proteins, thereby altering the equilibrium between cytosolic and mitochondrial pools of BAX, culminating in apparent BAX translocation, multimerization, and integration, followed by cyt *c* release, caspase activation, and apoptotic cell death. See text for more details. Because this hypothesis implicitly suggests that all BH3-only proteins function similarly (at least in neurons), one potential caveat is that structural and/or functional subfamilies may exist within the BH3-only subfamily of proapoptotic proteins[32,133]

Next, BH3-only proteins may *indirectly* promote BAX-dependent cyt *c* release by interacting with and inactivating antiapoptotic BCL-2 proteins, either in the cytosol (Model 3) or at mitochondria (Model 4), thereby freeing BAX to multimerize and integrate, forming structures that release cyt *c*. Both models are generally consistent with the genetics and biochemistry of PCD in *C. elegans* in which the BH3-only protein EGL-1 is induced in cells destined to die and interacts with and inactivates an antiapoptotic BCL-2 protein, leading to apoptosome formation, caspase activation, and apoptosis. (As discussed above, an important caveat is the requirement for cyt *c* release in vertebrates, but not in worms.) However, we favor Model 4 for several reasons. First, antiapoptotic BCL-2 proteins may not interact directly with BAX in the cytosol, except in the presence of conformation-altering nonionic detergents,[121,122] which may mimic a membrane environment, arguing against Model 3, but for Model 4. Second, the tertiary structure of BAX is inconsistent with interactions with antiapoptotic BCL-2 proteins in the cytosol,[118] which argues against Model 3, but not Model 4. Third, BIM and HRK, two BH3-only proteins that we propose are critical for TFD-induced apoptosis in neurons, do not interact directly with BAX and appear to localize to mitochondria. Moreover, the proapoptotic activity of these proteins generally correlates with their ability to interact with, and inactivate, their antiapoptotic cousins.[16,17,120] Finally, this hypothesis may help explain a 'threshold effect' observed with both BIM and HRK. Specifically, neurons can tolerate a certain level of BIM and HRK induction without releasing cyt *c* or undergoing cell death.[47,50] For example, during TFD in *Bcl-2*-overexpressing sympathetic and CG neurons, BIM levels increase with time (to a point greater than that seen in wild type littermates) and then decline precipitously as cells begin to die; in contrast, in $Bax^{-/-}$ neurons, BIM levels continue to increase (to levels exceeding even those seen in *Bcl-2*-overexpressing cells) and only begin to decline days later when significant decreases in global macromolecular synthesis begin to dismantle the synthetic machinery needed to sustain the induction. Accordingly, overexpression of *Bcl-2* only attenuates TFD-induced cyt *c* release and apoptosis in SCG and CG neurons,[105,123] whereas *Bax* deletion completely prevents both.[3,39,84,98] The same is true for CGN death caused by ectopic overexpression of BH3-only proteins.[50–52]

Therefore, we propose that cellular BAX exists in equilibrium between two states (Figure 3b), soluble in the cytosol or peripherally associated with mitochondria, in NGF-maintained sympathetic neurons. With TFD, BH3-only proteins are induced and localize to intracellular membranes, including the outer mitochondrial membrane, where they interact with and inactivate antiapoptotic BCL-2 proteins, thereby allowing BAX peripherally associated with mitochondria or translocated from the cytosol to multimerize and integrate. This creates a thermodynamic 'sink' that shifts the BAX equilibrium strongly (and perhaps irreversibly) in favor of mitochondrial localization, resulting in further translocation and integration into the mitochondrial outer membrane. In this scenario, inhibitors of *de novo* protein synthesis should prevent BAX translocation (consistent with our earlier findings[105]) by preventing induction of BH3-only proteins.[47] Therefore, we suggest that BH3-only proteins are triggers, albeit indirect, for BAX translocation and its downstream sequelae during TFD-induced neuronal apoptosis. In this manner, vertebrate neurons resemble their nematode counterparts insofar as caspase activation requires transactivation of BH3-only proteins, which in turn inactivate antiapoptotic BCL-2 proteins, the principal differences being the addition of an intervening step involving BAX-dependent cyt *c* release, which is required for apoptosome formation, and BAX-dependent release of Smac/DIABLO, which inactivates an IAP-like activity.[7,124]

The Next Step: Neuronal Cell Death and Neurodegeneration

Over 30 years have passed since the term 'apoptosis' was coined by Kerr, Wyllie, and Currie[1] to describe cell death in response to physiological or pathological stimuli that has a particular morphology first described by Glucksman in 1951. However, only in the past decade or so has the study of apoptosis literally exploded, with over 50 000 articles published between 1990 and 2000. The reason for this tremendous interest is obvious: Dysregulated cell death may at the very least contribute to, and at most be responsible for, the pathogenesis of many of our most intractable disorders, ranging from heart disease and stroke to autoimmunity and cancer. Few, if any, areas in modern medicine, at least in theory, are not touched by apoptosis research. Similarly, the promise of apoptosis research is equally clear: By manipulating the cell-suicide program, we can prevent unwanted cell death (e.g., in stroke) or, conversely, induce death in unwanted cells (e.g., in cancer). However, even though the last decade witnessed amazing progress in the identification and, in some cases, our understanding of the regulation of the molecular machinery of cell death, many questions remain at both the theoretical and practical levels.

What is 'apoptosis?'

Kerr *et al.* [1] initially defined apoptosis purely in morphological terms. Today, with our armamentarium of biochemical and molecular assays, a cynic might suggest that the word means everything and nothing. Since the field truly came in vogue in the early 1990s, it sometimes seems that every cell death is 'apoptotic.' Accordingly, one long-time cell death researcher recently lamented that everything in the Sigma catalog has been shown to cause apoptosis. Even to a purist, what was once the 'gold standard' assay for apoptosis – ultrastructural analysis – has been diminished by results-driven, not hypothesis-driven, experimentation. Although some may dismiss such concerns as semantics, we respectfully disagree. Describing a particular cell death as 'apoptotic' is helpful if, and only if, such classification provides information about the mechanisms contributing to that death and (presumably) how to manipulate it. Accordingly, an operational definition of apoptosis that we favor is as follows: cell death with the morphological hallmarks described by Kerr *et al.*[1] that requires caspase activity for those morphological features. (Note that this definition allows for caspase-independent cell death, as well as 'apoptotic' morphology without caspase activation, but caspase-independent apoptosis would be an oxymoron.) However, any definition of apoptosis (including ours) will likely be criticized by some as either too narrow or too broad; therefore, future work in cell death may be best served by carefully describing and characterizing the death caused by a particular stimulus by using multiple morphological and biochemical criteria.

Does the cell death in a particular degenerative disorder, such as Alzheimer's disease or stroke, contribute to the clinical manifestations of the disease and, if so, is this death apoptotic?

Unfortunately, the best answer we can obtain for this question may be a correlative one. Such a correlation could be, but has rarely been, established by appropriate answers to the following questions. First, does cell death precede the onset of clinical signs and symptoms? Second, does the induction of comparable cell death in a spatial and temporal pattern similar to that seen in the disease recapitulate these signs and symptoms? And third, does preventing cell death – necrotic, apoptotic, or otherwise – ameliorate these signs and symptoms? The answer to this last question leads directly into the next question…

Regardless of whether excessive apoptosis *per se* is the proximate cause of the disease, does preventing this cell death alone without altering the primary pathogenic mechanisms provide sufficient therapeutic benefit to outweigh potential adverse effects? For example, how will antiapoptotic agents be administered to prevent the death of certain cells without pathologically disrupting homeostasis in other cell populations? Conversely, in situations (e.g., cancer) characterized by insufficient apoptosis, how will proapoptotic agents be delivered to ensure the death of intended, but not unintended, targets?

An early and reasonable premise of cell death research was that issues related to the pathogenesis of various degenerative

conditions could be largely circumvented because execution of the cell must proceed through a 'final, common pathway' mediated by BCL-2 proteins and caspases. Accordingly, in a murine model of amyotrophic lateral sclerosis (ALS), *Bcl-2* overexpression[125] or caspase inhibition[126] delays disease onset, prolongs the 'symptomatic' period, and delays mortality. However, neither of these interventions prevents dysfunction or death; and, the delay in disease progression, while real ($\sim$10–15%), may represent a modest extension of lifespan, quality of life concerns notwithstanding. At a cellular level, these observations probably reflect several realities: First, neuronal cell death *per se*, vis-à-vis neuronal dysfunction, may not be the primary, let alone the sole, pathogenic mechanism responsible for the clinical signs and symptoms of the disease. Second, cell death that contributes to the pathophysiology of such degenerative conditions may not be purely apoptotic. Third, modulation of the BCL-2 and caspase checkpoints alone will probably only provide temporary protection from cell death, especially since cells may progress from apoptotic to nonapoptotic (e.g., autophagic, necrotic, etc.) modes of cell death, particularly if the pathogenic insult remains unabated. By no stretch of the imagination is caspase inhibition as a monotherapy likely to be the 'magic bullet' for chronic neurodegenerative disorders because neurons 'saved' by caspase inhibitors alone suffer (and ultimately will die from) a significant mitochondrial 'hit' that lies genetically downstream of the BAX/BCL-2 checkpoint. This 'hit,' which includes not only loss of mitochondrial cyt *c* but also may involve activation of the cyclosporin A-inhibitable permeability transition pore,[127] defines a 'window of opportunity' during which trophic (and/or stimulus-abating) agents may be used in combination with antiapoptotic strategies to rescue cells from the brink of the abyss. And finally, degenerating cells, even when saved 'in perpetuity' (e.g., NGF-deprived $Bax^{-/-}$ neurons) are likely to be functionally compromised,[128] a particularly vexing problem with neurons since a live, but dysfunctional (e.g., 'disconnected' or inexcitable), neuron may be no better functionally to the organism than a dead one.

Furthermore, few studies have examined the adverse effects of chronic antiapoptotic treatments, which could, if not selectively targeted to specific tissues, cause multiple significant problems (e.g., oncogenesis and autoimmunity) since naturally occurring PCD is a critical homeostatic mechanism in all organisms. In any case, findings such as those described above for ALS, while encouraging, suggest that antiapoptotic strategies alone are unlikely to provide dramatic functional benefits to patients in the context of chronic degenerative disorders in the absence of therapies designed to address the underlying pathogenic mechanisms. In contrast, antiapoptotic treatments may provide considerable benefit in certain situations (e.g., stroke and myocardial infarction) in which the insult is acute, transient, and spatially well defined. In such situations, preventing cell death for a critical window of time may allow cells to 'ride out' the insult and, probably in combination with other approaches, re-establish a normal milieu. In other words, even in acute degenerative conditions in which antiapoptotic therapies may have their greatest clinical utility, optimal treatment will probably require combination therapies with trophic (and/or stimulus-abating) agents. Therein lies the promise of therapies, such as the MLK inhibitor CEP-1347, which not only prevents TFD-induced apoptosis but also promotes neuronal growth and differentiation, at least in model systems.[129]

In summary, the study of apoptosis has progressed remarkably in the past decade, thanks in large part to the pioneering efforts of Brenner, Sulston, and Horvitz in nematodes, and promises to mature further still in the post-genomics era. The next few decades should demonstrate whether the reality of therapies designed to modulate cell death for therapeutic benefit will achieve the promise articulated by their most ardent advocates, including ourselves.

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