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Programmed Cell Death

Kevin A. Roth

INTRODUCTION

Programmed cell death is critical for normal nervous system development. From the initial sculpting of the size and shape of the developing brain through establishment of the number of cells in specific neuronal populations, programmed cell death ensures that nervous system development proceeds in an orderly and regulated fashion. Although neuronal programmed cell death research has historically focused on synapse-bearing neurons and their competition for limited supplies of target-derived neurotrophic molecules, recent studies have revealed a significant role for programmed cell death in neural precursor cells and immature neurons, prior to the establishment of synaptic contacts. Rapid advances in molecular biology and the use of gene-targeting approaches have led to tremendous progress in our understanding of the molecular regulation of programmed cell death. Recent studies have also revealed unexpected complexity in neuronal cell-specific death pathways and raised questions about the intrinsic and extrinsic triggers of programmed cell death.

HISTORICAL PERSPECTIVE

Programmed cell death refers to the reproducible, spatially- and temporally-restricted death of cells during organismal development (Burek and Oppenheim, 1996). As such, the term is synonymous with "physiological," "naturally-occurring," or "developmental" cell death. The original discovery of programmed cell death in the vertebrate nervous system has been attributed to Beard who described the degeneration of neurons in the skate nervous system over 100 years ago (Beard, 1896; Jacobson, 1991). In 1926, Ernst described three types of developmental cell death involving the regression of vestigial organs, the cavitation, folding or fusion of organ anlage, and the elimination of cells during tissue remodeling (Ernst, 1926). These three functional types of programmed cell death were termed phylogenetic, morphogenetic, and histogenetic cell death, respectively, by Glücksmann approximately 50 years ago (Glücksmann, 1951). Examples of phylogenetic death in the mammalian nervous system include degeneration of the paraphysis, vomeronasal nerve, and nervus terminalis. Morphogenetic cell death occurs during formation of the mammalian optic and otic vesicles and during maturation of the neural tube and neural plate. Histogenetic cell death in the mammalian nervous system is fairly widespread and numerous neuronal cell populations throughout the central and peripheral nervous systems have been reported to undergo this type of degeneration (Jacobson, 1991). The extent of histogenetic death varies between neuronal populations but has been estimated to range between 20% and 80% of neurons in some populations (Oppenheim, 1991). Prominent among these are motor neurons in the spinal cord and neurons in the sensory and sympathetic nervous systems.

Histogenetic cell death was the focus of many studies of neuronal programmed cell death during the second half of the 20th century (Hamburger, 1992). Histogenetic neuron death is typically triggered by insufficient trophic factor support. Following initial neurogenesis, immature neuron migration, and synaptogenesis, many neuronal populations enter a period of competition for target-derived trophic factors. Neurons obtaining inadequate trophic support during this period are eliminated through activation of a cell autonomous death program. This competitive process has been thought to ensure the proper matching of the size of each newly generated neuronal population with that of its target field. Nerve growth factor (NGF) was the first neurotrophic factor to be isolated and characterized and much of the recent progress in defining the molecular regulation of neuronal cell death can be attributed to investigations of NGF and related molecules. Despite the historical significance of NGF-related research, the concept that neuronal programmed cell death serves largely to match neuron numbers with post-synaptic target size is overly simplistic (Kuan *et al*., 2000). Many neuronal populations exhibit no obvious requirement for target-derived neurotrophic molecules and a significant degree of cell death may occur in neural precursor cells and immature neurons prior to the elaboration of neuritic processes and formation of synaptic contacts. Ongoing research on neural precursor cells and immature neurons is extending our understanding of the role of programmed cell death in developmental neurobiology.

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MORPHOLOGICAL TYPES OF PROGRAMMED CELL DEATH

Cells undergoing programmed cell death in the developing nervous system may exhibit various morphological appearances (Clarke, 1990). The most common morphological type of programmed cell death is type 1 or apoptotic cell death and many authors have erroneously equated apoptosis with programmed cell death (Häcker, 2000). The term "apoptosis" was originally used to describe a unique type of "non-necrotic" cell death in which the degenerating cells displayed a specific set of morphological features (Kerr *et al*., 1972). Apoptotic cells exhibit chromatin condensation and margination, nuclear fragmentation, cytoplasmic membrane blebbing and convolution, and cell shrinkage (Fig. 1). These features are best appreciated by ultrastructural examination but can also be observed at the light microscopic level (Roth, 2002). The vast majority of cells that die during nervous system development, including neurons lost during competition for target-derived neurotrophic factor support, show apoptotic features.

The second major type of programmed cell death is type 2 or autophagic degeneration (Clarke, 1990). Autophagic cell death is characterized by the presence of numerous autophagic vacuoles and degradation of cytoplasmic elements in the degenerating cell (Fig. 2). The nucleus may become pyknotic and, in some cases, may exhibit typical "apoptotic-like" nuclear features. Autophagic vacuoles originate as double membrane sheets derived from the endoplasmic reticulum which engulf intracellular organelles and cytoplasmic materials followed by delivery of the vacuolar contents to lysosomes (Seglen and Bohley, 1992; Dunn, 1994). Unlike apoptotic cell death which typically involves single or scattered cells within normal parenchyma, autophagic cell death typically involves contiguous groups of degenerating cells (Lee and Baehrecke, 2001). Autophagic cell death has been observed at several sites in the vertebrate nervous system including the isthmo-optic nucleus where neurons undergo programmed cell death secondary to insufficient target-derived trophic factor support.

Less common morphological forms of programmed cell death have been described including type 3A, "non-lysosomal disintegration" and type 3B, "cytoplasmic type" (Clarke, 1990). These forms of death bear some resemblance to necrotic cell death in that swelling of intracellular organelles and fragmentation of the cell membrane are prominent. Type 3A and 3B programmed cell death are rarely observed in the mammalian nervous system. However, distinguishing between forms of cell death may be difficult, and in some cases, degenerating cells may exhibit features of multiple morphological death types.

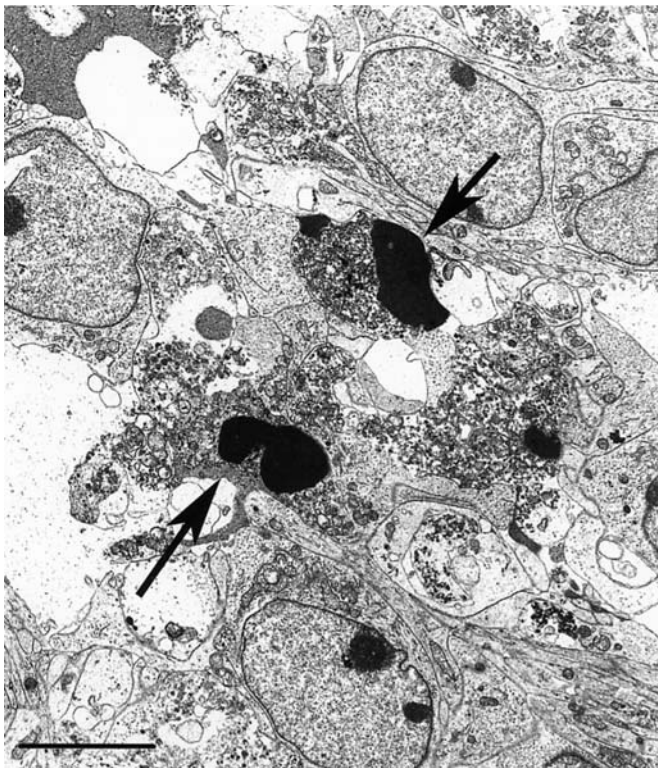

FIGURE 1. Ultrastructural examination of the embryonic day 12 mouse spinal cord shows several degenerating neurons (indicated by arrows) with apoptotic features including chromatin condensation and margination and cell shrinkage. Scale bar equals 5 μm.

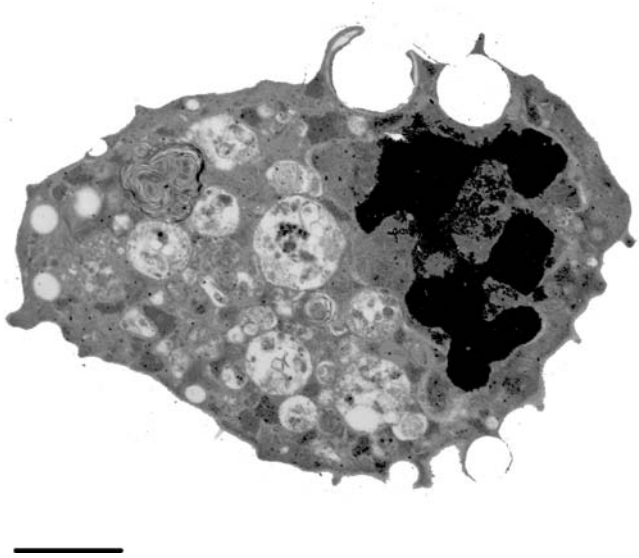

FIGURE 2. The morphological features of autophagic cell death are illustrated in this electron micrograph of a telencephalic neuron that was exposed to 20 μM chloroquine, a lysosomotropic agent, for 18 hr *in vitro*. The cell contains numerous membrane delimited autophagic vacuoles of various sizes, some containing osmophilic debris, a decreased number of cytoplasmic organelles, and degenerative nuclear features (clumped and fragmented chromatin) similar to those observed in cells undergoing apoptotic death. Scale bar equals 2 μm.

MOLECULAR REGULATION OF PROGRAMMED CELL DEATH

Apoptotic Cell Death

Apoptosis is the most common type of programmed cell death in the developing nervous system. Much of our understanding of the molecular pathways regulating mammalian cell apoptosis was anticipated by investigations of programmed cell death in the nematode *Caenorhabditis elegans* (Horvitz, 1999). In *C. elegans*, approximately 10% of the organism's cells undergo programmed cell death in a highly stereotyped, cell autonomous fashion. Four genes, *egl-1* (egg-laying defective), *ced-9* (ced, cell death abnormal), *ced-4*, and *ced-3*, act in a coordinated fashion to cause *C. elegans* cell death. Studies suggest that EGL-1 binds to CED-9, releasing CED-4 from a CED-9/CED-4 complex, and CED-4 in turn activates CED-3 which represents the commitment point to *C. elegans* cell death.

This basic pattern of apoptotic death regulation is recapitulated in mammals. Structural homologs of EGL-1, CED-9, CED-4, and CED-3 exist in mammals and consist of several multigene families. Mammalian EGL-1-like molecules are members of the BH3 domain-only Bcl-2 subfamily and include Bid, Bim, Bad, and Noxa (Korsmeyer, 1999). These molecules are thought to interact with multidomain, CED-9-like, Bcl-2 family members to regulate mitochondrial cytochrome *c* release and function. Multidomain Bcl-2 family members are divided into anti- (e.g., Bcl-2 and Bcl-X_L) and pro-apoptotic (e.g., Bax and Bak) subgroups. Bcl-2 and Bcl-X_L can block apoptotic stimulus-induced cytochrome *c* redistribution and Bax and Bak promote mitochondrial cytochrome *c* release. Apaf-1, the best-defined mammalian homolog of CED-4, binds cytosolic cytochrome *c*, and in the presence of dATP or ATP, assists in the conversion of caspase-9 into an active enzyme (Zou *et al*., 1997, 1999). Caspases are the mammalian homologs of CED-3 and consist of approximately 15 cysteine-containing, aspartate-specific proteases (Nicholson, 1999). Caspases exist at baseline as inactive zymogens and are converted into active enzymes via cleavage of the proenzyme form into large and small subunits which together form the active caspase. This processing occurs at specific aspartic residues which are themselves caspase cleavage sites. Caspase-9 is an initiator caspase and upon its activation cleaves caspase-3, one of three effector caspases (caspase-3, caspase-6, and caspase-7). In most cell types, including neurons, caspase-3 is the predominant caspase effector and its activity is responsible for producing many of the morphological features that define apoptotic cell death (Zheng *et al*., 1998; D'Mello *et al*., 2000). Targeted gene disruptions of *apaf-1*, *bcl-2*, and *caspase* family members have revealed an important role for apoptotic cell death regulators in neuronal programmed cell death (see below).

Despite the many parallels between programmed cell death in *C. elegans* and mammals, recent studies have revealed increased complexity in mammalian cell death regulation (Joza *et al*., 2002). For example, cytochrome *c* release from mitochondria plays an import role in mammalian apoptosome formation and caspase activation, yet cytochrome *c* is uninvolved in *C. elegans* cell apoptosis. An intriguing family of endogenously expressed mammalian caspase inhibitors has emerged as possible key regulators of mammalian programmed cell death. The inhibitors of apoptosis protein (IAP) family consists of multiple molecules including XIAP, cIAP-1, cIAP-2, and a subfamily of neuronal apoptosis inhibitory proteins (NAIPs), which in mice consist of multiple members (Deveraux and Reed, 1999). IAPs are characterized by the presence of one or more baculovirus inhibitory repeat (BIR) homologous domains and although IAPs may have other functions, they appear to affect apoptosis by potently inhibiting caspase enzymatic activity (Deveraux and Reed, 1999). IAP family members exhibit selective caspase inhibitory activity, and endogenous inhibition of activated caspases 2, 3, 7, and 9 has been reported (Chai *et al*., 2001; Huang *et al*., 2001; Riedl *et al*., 2001). NAIP was originally reported to lack caspase inhibitory activity; however, more recent studies have shown that NAIP is a potent group II caspase (caspases 2, 3, and 7) inhibitor (Robertson *et al*., 2000). Several IAPs have been reported to be expressed in the nervous system and a variety of studies suggest that IAPs may regulate neuronal apoptosis (Robertson *et al*., 2000). Overexpression of XIAP, cIAP-1, or cIAP2 can prevent or delay cell death in both *in vitro* and *in vivo* neuronal apoptosis paradigms (Götz *et al*., 2000; Kügler *et al*., 2000; Mercer *et al*., 2000; Perrelet *et al*., 2000). XIAP-deficient mice have been generated but showed no obvious nervous system abnormalities (Harlin *et al*., 2001). The potential role of NAIPs in regulating neuronal programmed cell death is particularly intriguing since partial deletions in the human *NAIP* gene have been found in patients with spinal muscular atrophy (Roy *et al*., 1995) and targeted gene disruption of *naip-1* in mice resulted in increased susceptibility to kainic-acid-induced neuronal apoptosis (Holcik *et al*., 2000). Recently, two molecules have been identified that can bind IAPs and block their caspase inhibitory effects. The first of these molecules, Smac/Diablo is released from mitochondria following an apoptotic stimulus and can promote apoptosis by displacing IAPs from caspase-9 (Verhagen *et al*., 2000; Zheng *et al*., 2000; Srinivasula *et al*., 2001). The second molecule, XAF1, has been reported to bind to XIAP and antagonize its caspase inhibitory activity (Liston *et al*., 2001). This multilevel regulation of caspase activity underscores the importance of caspases in apoptosis and suggests a possible role for IAPs in the developing nervous system.

Recent studies also suggest that apoptotic cell death may occur, in at least some cell types, independently of caspase activation (Nicotera, 2000; Cheng *et al.*, 2001). Apoptosis-inducing factor (AIF) is a mitochondrial localized flavoprotein that undergoes nuclear translocation in response to certain death stimuli (Susin *et al*., 1999). AIF can produce cell death in the absence of caspase activation and it may mediate the death-promoting effects of poly(ADP-ribose)polymerase-1 in several models of neuronal cell death (Yu *et al*., 2002). The significance of AIF in neuronal programmed cell death regulation remains to be determined.

Autophagic Cell Death

In contrast to apoptotic cell death, autophagic cell death has received relatively scant attention. However, recent studies

are beginning to provide insights into the molecules involved in autophagic cell death (Bursch, 2001; Tolkovsky *et al*., 2002). Several genes, including *beclin 1* and oncogenic *ras*, have been demonstrated to play a role in caspase-independent autophagic death and APG5, a molecule involved in the targeting of proteins for autophagic destruction, is upregulated in degenerating cells (Chi *et al*., 1999; Liang *et al*., 1999; Saeki *et al*., 2000). Similarly, mRNA and protein for the lysosomal protease cathepsin D are upregulated in apoptotic cells indicating possible crosstalk between lysosomal-dependent autophagic death and caspase-dependent apoptotic death (Deiss *et al*., 1996; Wu *et al*., 1998). This concept is further supported by the finding that Bax deficiency significantly inhibited cell death in an *in vitro* model of neuronal autophagic cell death and the observation that lysosomal extracts were capable of cleaving the pro-apoptotic Bcl-2 family member Bid; providing a possible pathway for lysosomal mediated caspase activation (Stoka *et al*., 2001; Zaidi *et al*., 2001). Interestingly, trophic factor withdrawal induced neuronal death, which is typically considered a trigger of apoptotic death, may produce extensive autophagic vacuole formation and be attenuated by 3-methyladenine, an inhibitor of autophagic vacuole formation (Shibata *et al*., 1998; Xue *et al*., 1999; Uchiyama, 2001). Together, these observations indicate extensive crosstalk in the molecular pathways regulating these two major morphological types of programmed cell death (Fig. 3).

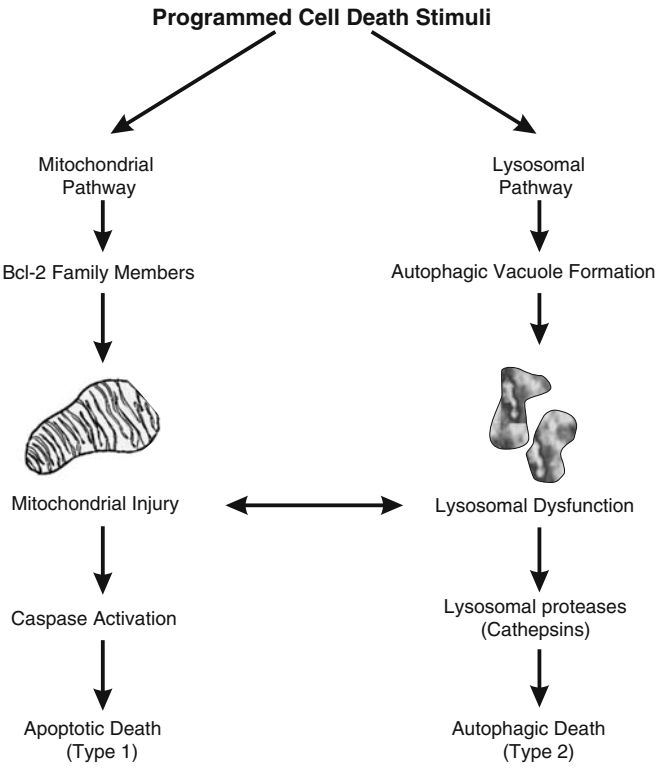

FIGURE 3. Programmed cell death stimuli trigger degeneration by activation of either mitochondrial or lysosomal-dependent pathways. The molecules involved in the apoptotic death pathway are fairly well defined and include Bcl-2 and caspase family members. Less is known about the autophagic death pathway, but lysosomal proteases, including cathepsins, are likely to play an important role in cellular destruction.

TARGETED GENE DISRUPTIONS

The generation of mice deficient in one or more cell death-associated molecule(s) has proven a powerful tool for investigating mammalian neuronal programmed cell death (Snider, 1994; Zheng, 2000). These "knockout" mice provide both an unambiguous assessment of the role of specific genes in neuronal development and an *in vivo* test of the epigenetic relationship between apoptosis-associated molecules (Kuan *et al*., 2000; Roth *et al*., 2000). Two caveats to the interpretation of the results obtained in such transgenic mouse studies deserve mention. First, a negative result, that is, the targeted gene disruption fails to affect neuronal programmed cell death, does not exclude a role for the disrupted gene in programmed cell death regulation. Compensatory changes in other genes or alternative cell death pathways may minimize the effects of single gene disruptions (Zheng, 2000). A positive result, however, implies that the disrupted gene has a noncompensatable, nonredundant function in programmed cell death regulation. Second, the results obtained with targeted gene disruptions may be incompletely penetrant and/or dramatically affected by mouse strain-specific genetic factors. For example, we have observed markedly different neurodevelopmental abnormalities in C57BL/6J and 129X1/SvJ caspase-3-deficient mice, and mouse strain-specific effects of other targeted gene disruptions have been reported (Lomaga *et al*., 2000; Leonard *et al*., 2002). Similarly, species-specific effects of gene disruption cannot be easily excluded and may limit extrapolation of results from mouse studies to human nervous system development. Despite these caveats, significant insights into the molecular regulation of neuronal programmed cell death have been obtained from transgenic mouse studies.

Bcl-2

Bcl-2 is the prototypical anti-apoptotic Bcl-2 family member (Korsmeyer, 1999). Bcl-2 is a 26 kDa protein that is localized to the outer mitochondrial membrane, nuclear envelope, and portions of the endoplasmic reticulum (Krajewski *et al*., 1993). Bcl-2 immunoreactivity is present in the developing nervous system in relatively high amounts in neural precursor cells in the ventricular and subventricular zones and in neurons in the developing cortical plate (Merry *et al*., 1994). Bcl-2 immunoreactivity decreases significantly in the postnatal central nervous system. High levels of Bcl-2 expression are retained, however, in sensory and sympathetic ganglion neurons in the adult (Merry and Korsmeyer, 1997).

The function of *bcl-2* in the nervous system has been explored in a variety of experimental paradigms. Overexpression of *bcl-2* in cultured sympathetic neurons prevents apoptosis in NGF-deprived cells (Garcia *et al*., 1992; Allsopp *et al*., 1993). *In vivo* neuronal overexpression of *bcl-2* in transgenic mice indicates a potential role for Bcl-2 in programmed cell death in the nervous system. Compared to nontransgenic littermates,

transgenic mice have a 12% increase in brain size, and some brain regions show up to a 50% increase in neuron number (Martinou *et al*., 1994). The targeted disruption of *bcl-2* indicates a particularly important role for Bcl-2 in the maintenance, during postnatal life, of motoneurons, sympathetic neurons, and sensory neurons, populations of neurons normally expressing high levels of Bcl-2 (Veis *et al*., 1993; Nakayama *et al*., 1994; Michaelidis *et al*., 1996). Surprisingly, the pro-apoptotic effect of Bcl-2 deficiency occurs largely after the peak period of naturally occurring cell death in these neuronal populations. This finding does not imply that *bcl-2* is functionless in the embryonic nervous system since Bcl-2 may be involved in other developmental processes besides apoptosis (Chen *et al*., 1997); rather, it may suggest functional redundancy for *bcl-2* and other anti-apoptotic *bcl-2* gene family members in regulating neuronal programmed cell death.

Bcl-X_L

Bcl-X_L is an anti-apoptotic member of the Bcl-2 family (Boise *et al*., 1993). The *bcl-x* gene (*Bcl2l1*) can be alternatively spliced to produce two major protein isoforms Bcl-X_L and Bcl-X_S, a pro-apoptotic molecule (González-García *et al*., 1994). Bcl-X_L is the predominant isoform in mammals and Bcl-X_S is present at only low levels, if at all, in the nervous system (González-García *et al*., 1995). Bcl-X_L immunoreactivity is present at relatively high levels in neurons in the adult nervous system. In the embryonic brain, Bcl-X_L immunoreactivity is not detected in neural precursor cells in the ventricular zone but is abundant in immature neurons in the intermediate and marginal zones and in more mature neurons in the developing cortical plate (Motoyama *et al*., 1995; Roth *et al*., 2000). The targeted gene disruption of *bcl-x* failed to affect apoptosis of neural precursor cells but produced a dramatic increase in apoptotic neurons throughout the developing nervous system (Motoyama *et al*., 1995). Degenerating cells with apoptotic nuclear features and TUNEL positivity were numerous in the embryonic day (E)11–12 mouse spinal cord, brainstem, and dorsal root ganglia (Fig. 4). These degenerating neurons were abundant in the intermediate zone of the developing brain suggesting that Bcl-X_L plays a particularly important role in promoting the survival of newly post-mitotic immature neurons. Since Bcl-X_L deficiency is lethal at approximately E13.5, secondary to hematopoietic apoptosis, the effects of Bcl-X_L deficiency on telencephalic neurons, which are only sparsely present prior to E13, was examined in primary E12 telencephalic cells that were cultured for several days in medium promoting neuronal differentiation. Bcl-X_L-deficient telencephalic neurons were found to be markedly susceptible to a variety of *in vitro* apoptotic insults including trophic factor deprivation and genotoxic injury indicating that Bcl-X_L is a key regulator of neuron survival throughout the nervous system (Roth *et al*., 1996; Shindler *et al*., 1998; Zaidi *et al*., 2001).

The findings cited above indicate that Bcl-X_L is the most important Bcl-2 anti-apoptotic protein during the period of neuronal programmed cell death since its deficiency cannot be compensated for by other endogenously expressed family members. Bcl-2 does, however, complement Bcl-X_L's action in immature neurons as mice lacking both Bcl-X_L and Bcl-2 show even more substantial neuron apoptosis than mice lacking Bcl-X_L alone (Shindler *et al*., 1998). It is also possible that other anti-apoptotic Bcl-2 family members such as Bcl-W (Bcl2l2) and Bfl1/A1 (Bcl2a1) will be found to play a role in neuronal programmed cell death; however, such data are currently lacking.

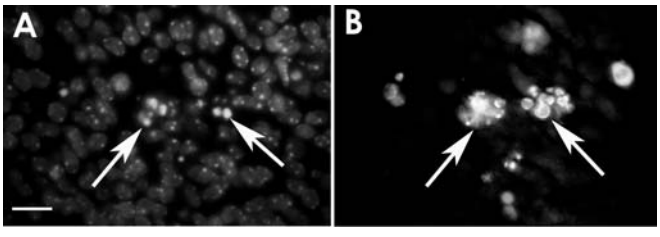

FIGURE 4. Apoptotic nuclei (examples indicated by arrows) are frequent in the Bcl-X_L-deficient embryonic spinal cord and can be detected by condensed nuclear labeling with (A) Hoechst 33,258 and/or (B) TUNEL staining. Scale bar equals 10 μm.

Bax

The pro-apoptotic Bcl-2 subfamily consists of approximately 15 molecules divided into two subgroups (Korsmeyer, 1999). The EGL-1-like subgroup consists of molecules with a single Bcl-2 homology domain, the so called "BH3 domain-only" group, and the multidomain subgroup whose members contain two or more Bcl-2 homology domains. Targeted gene disruption studies have revealed a particularly important role for the pro-apoptotic multidomain family member Bax in neuronal programmed cell death (Knudson *et al*., 1995; Deckwerth *et al*., 1996). Bax is capable of heterodimerizing with Bcl-X_L or Bcl-2 and it is expressed at relatively high levels in the embryonic and adult nervous system (Oltvai *et al*., 1993; Krajewski *et al*., 1994; Sedlak *et al*., 1995). Bax-deficient mice exhibited markedly decreased neuronal programmed cell death and increased neuron numbers in neurotrophic factor dependent neuronal subpopulations (Deckwerth *et al*., 1996). Bax-deficient neurons were resistant to trophic factor withdrawal induced apoptosis both *in vivo* and *in vitro*. Regionally dependent decreases in TUNEL positive cells were also observed in Bax-deficient embryos as early as gestational day 11.5, suggesting that Bax may also regulate immature neuron apoptosis (White *et al*., 1998). No significant expansion in the embryonic neural precursor cell population or gross brain abnormalities were observed in Bax-deficient mice suggesting that Bax may not play a significant role, by itself, in regulating morphogenetic cell death in the developing brain.

A critical interaction between Bax and Bcl-X_L in regulating immature neuron death was demonstrated in $bax^{-/-}/bcl\text{-}x^{-/-}$ embryos (Shindler *et al*., 1997). Bax/Bcl-X_L dual deficient embryos were completely protected from the increased neuronal apoptosis observed in the Bcl-X_L-deficient embryonic nervous system (Fig. 5). This finding indicates that the mechanism of Bcl-X_L's anti-apoptotic action in immature neurons is through its

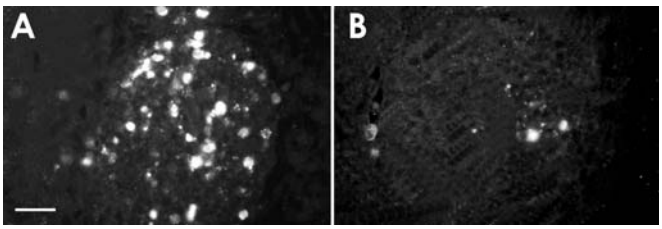

FIGURE 5. (A) Numerous apoptotic neurons with activated caspase-3 immunoreactivity are detected in the dorsal root ganglia of a Bcl-X_L-deficient embryo. (B) In contrast, an embryo lacking both Bcl-X_L and Bax shows only rare caspase-3 immunoreactive dorsal root ganglion neurons. Scale bar equals 20 μm.

ability to inhibit the pro-apoptotic effects of *bax* expression. In many cell types and apoptotic paradigms, Bax and Bcl-X_L exhibit additional interactions with BH3 domain-only molecules and the multidomain pro-apoptotic molecule Bak. For example, the anti-apoptotic effects of Bcl-2 and Bcl-X_L are modulated by BH3-domain molecules such as Bad, Bim, and Noxa, and Bak interacts with Bax to regulate mitochondrial cytochrome *c* release and function following many different death-promoting stimuli (Bouillet *et al*., 2001; Wei *et al*., 2001).

BH3 domain-only proteins may affect neuronal apoptosis as evidenced by the fact that sympathetic neurons derived from Bim-deficient mice showed significantly reduced apoptosis in response to NGF deprivation *in vitro* (Putcha *et al*., 2001). However, Bim deficiency had little effect on neuronal programmed cell death *in vivo* and targeted disruption of other BH3 domain-only genes, for example, *bid* and *bad*, have not resulted in significant neurodevelopmental abnormalities (Shindler *et al*., 1998; Leonard *et al*., 2001). Since several BH3 domain-only genes are expressed in the developing nervous system, the targeted disruption of a single BH3 domain-only gene may be compensated for by other family members resulting in an underestimation of the importance of this Bcl-2 subfamily in programmed cell death regulation.

The complementary effects of multidomain pro-apoptotic Bcl-2 family members is strikingly demonstrated in $bax^{-/-}$/$bak^{-/-}$ mice. Bax-deficient mice showed decreased neuronal programmed cell death, male infertility, and reduced female fertility, but were otherwise healthy, while Bak-deficient mice showed no obvious pathology in either the nervous system or other organs. In contrast, mice lacking both Bax and Bak typically died during the perinatal period and exhibited multiple developmental defects that were not observed in mice deficient in either Bax or Bak alone (Lindsten *et al*., 2000). In the nervous system, $bax^{-/-}/bak^{-/-}$ mice showed decreased programmed cell death of neurons, similar to that observed in $bax^{-/-}/bak^{+/+}$ animals. In addition, dual-deficient mice exhibited a significantly increased periventricular neural precursor cell population which was more striking than that observed in either Bax- or Bak-deficient mice. The $bax^{-/-}/bak^{-/-}$ embryos did not, however, exhibit the massive expansion of neural precursor cells or display exencephaly and cranial bone defects that have been

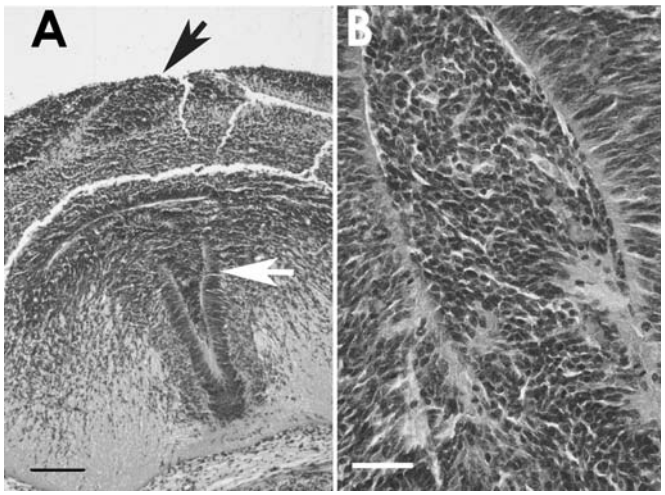

FIGURE 6. (A) An embryonic day 12.5 caspase-3-deficient mouse shows an expanded neural precursor cell population in the developing cerebellum (black arrow) and supernumerary cells in the intraventricular space (white arrow). Higher magnification of (B) the intraventricular infiltrate shows densely packed neural precursor cells within the ependymal lined ventricle. Scale bars in (A) and (B) equal 100 and 25 μm, respectively.

observed in *caspase-*$9^{-/-}$ or *apaf-*$1^{-/-}$ mice (see below). In total, these results suggest that Bax is the predominant pro-apoptotic multidomain Bcl-2 family member in post-mitotic neurons, but that Bax and Bak combine to regulate programmed cell death in at least a subpopulation of neural precursor cells.

Apaf-1, Caspase-9, and Caspase-3

In *C. elegans*, the downstream mediators of CED-9 action are CED-4 and CED-3. In the mammalian nervous system, the immediate downstream mediators of Bcl-2 family effects are Apaf-1, caspase-9, and caspase-3 (Joza *et al*., 2002). The targeted disruptions of *apaf-1*, *caspase-9*, and *caspase-3* resulted in similar pathological changes consistent with the functional relationship between Apaf-1-dependent apoptosome formation, caspase-9, and caspase-3 activation (Kuida *et al*., 1996, 1998; Cecconi *et al*., 1998; Hakem *et al*., 1998). Apaf-1-, caspase-9-, and caspase-3-deficient mice exhibited extensive perinatal lethality and gross structural neuropathology which included cranial facial abnormalities, exencephaly, neural precursor cell hyperplasia, and ectopic neural masses (Fig. 6). Examination of gene-disrupted embryos revealed a striking decrease in the number of cells undergoing neuronal programmed cell death. Neither *apaf-*$1^{-/-}$- nor *caspase-*$9^{-/-}$-deficient cells exhibited caspase-3 activation *in vivo* or *in vitro* indicating that these three molecules participate in a linear death pathway (Kuan *et al*., 2000). This conclusion is further supported by data demonstrating that the increased neuronal cell death observed in Bcl-X_L-deficient embryos could be completely prevented by concomitant deficiency in any one of these three genes (Kuan *et al*., 2000; Roth *et al*., 2000; Zaidi *et al*., 2001). Thus, in the developing mouse brain, Bcl-X_L regulates immature neuron programmed cell death

through its ability to inhibit Bax-dependent apoptosome formation and caspase-3 activation. This pathway, however, cannot account for all programmed cell death in the developing nervous system for several reasons. First, the striking expansion of neural precursor cells observed in *apaf-1*$^{-/-}$, *caspase-9*$^{-/-}$, and *caspase-3*$^{-/-}$ mice is not observed in *bax*$^{-/-}$ mice or *bax*$^{-/-}$/*bak*$^{-/-}$ mice. Thus, the upstream molecular mediators of caspase-3 activation in neural precursor cells are different from those in post-mitotic neurons. Second, the neurodevelopmental effects of Apaf-1, caspase-9, and caspase-3 deficiency are incompletely penetrant and are influenced by strain-specific genetic factors. For example, caspase-3-deficient 129X1/SvJ mice uniformly die during the perinatal period and have severe neurodevelopmental pathology, while caspase-3-deficient C57BL/6J mice survive into adulthood and have minimal neuropathological complications (Leonard *et al*., 2002). Intercrosses of these two strains suggest the presence of strain-dependent genetic modifiers that influence the significance of caspase-3 activation in nervous system development cell death. Identification of these genes should provide new insights into the molecular regulation of nervous system programmed cell death.

DEVELOPMENTAL PHASES OF PROGRAMMED CELL DEATH

Programmed cell death in the mammalian nervous system involves an orderly progression of developmental phases (Fig. 7). As cells transition from one phase to the next, the stimuli triggering cell death change and the critical intracellular molecular regulators of death undergo revision.

Neural Precursor Cell

One of the most exciting and controversial areas of modern developmental neurobiology research is determination of the extent and significance of programmed cell death in neural precursor cells (Voyvodic, 1996; Sommer and Rao, 2002). Several techniques are available to identify degenerating and/or apoptotic cells in the developing nervous system and these have been applied by various investigators to determine the frequency of programmed cell death in mammalian neural precursor cells. Unfortunately, the resultant estimates have varied widely. Investigators using a technique called *in situ* end labeling plus (ISEL+) to detect DNA double strand breaks in apoptotic cells have estimated that between 50% and 70% of the proliferating cells in the embryonic mouse brain are destined to die (Blaschke *et al*., 1996, 1998). In contrast, other investigators using the TUNEL method to detect apoptotic cells reported a death rate of 0.3–1.7% in the proliferative zone of the E14 rat forebrain (Thomaidou *et al*., 1997). Low rates of neural precursor cell death were also identified by detection of activated caspase-3 immunoreactivity, *in vivo* infusion of annexin V, and ultrastructural examination of the developing mammalian brain (Ferrer *et al*., 1992; Srinivasan *et al*., 1998; van den Eijnde *et al*., 1999; Rakic and Zecevic, 2000). These disparate results have led to interesting discussions on cell death detection methodologies and the possible role of DNA strand breaks in nervous system development (Chun and Schatz, 1999; Chun, 2000; de la Rosa and de Pablo, 2000; Gilmore *et al*., 2000).

Regardless of the extent of cell death in the neural precursor cell population, the dramatic expansion of this population in mice lacking specific apoptotic effector molecules (e.g., *apaf-1*$^{-/-}$ or *caspase-9*$^{-/-}$ mice) demonstrates that a reduction in neural precursor cell programmed cell death significantly affects

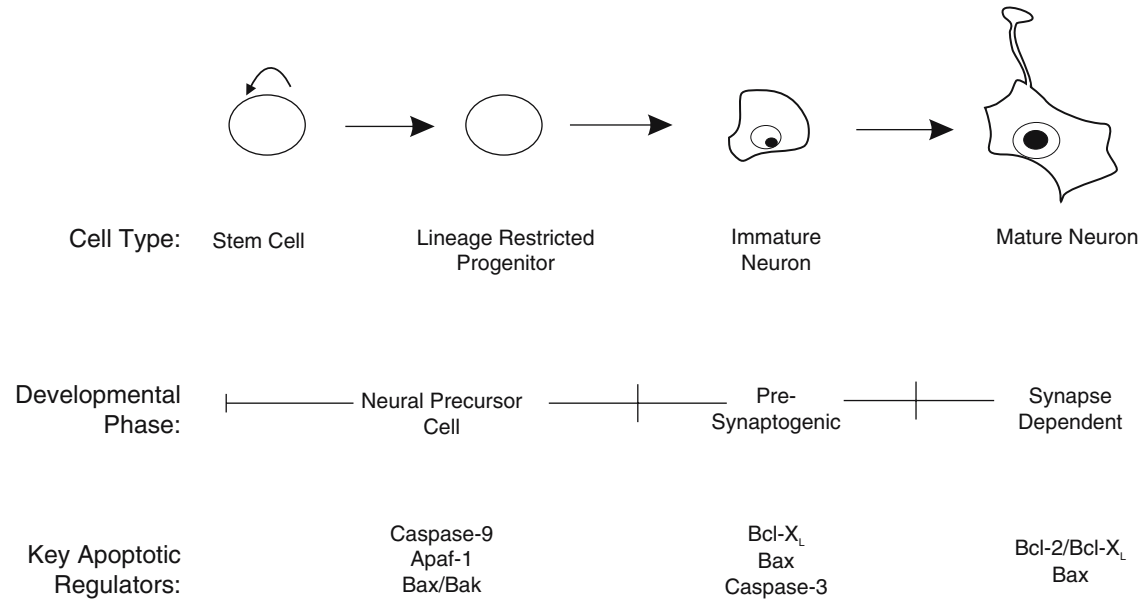

FIGURE 7. Diagrammatic representation of the cellular phases of neuronal programmed cell death.

nervous system development. In formulating hypotheses about neural precursor cell death and its role in mammalian nervous system development, it is important to recognize that neural precursor cells are heterogeneous, consisting of self-replicating multipotent stem cells and several types of lineage-restricted progenitors (Sommer and Rao, 2002). In addition, the neural precursor cell population size is affected not only by cell death but by cell proliferation. A recent study demonstrated that transgenic overexpression of β-catenin in neural precursor cells produced a marked expansion of this cell population (Chenn and Walsh, 2002). This effect was mediated by β-catenins ability to promote cell cycle reentry in dividing neural precursor cells and occurred despite a two-fold increase in apoptosis in the β-catenin transgenic brains. In contrast with the neural precursor cell expansion observed in *apaf-1*$^{-/-}$, *caspase-9*$^{-/-}$, and *caspase-3*$^{-/-}$ embryos, which results in thickening of the developing forebrain and expansion of the neural precursor cell population into the lateral ventricles, β-catenin transgenic mice exhibited no increase in cortical thickness or ventricular ingrowth, but a striking "horizontal expansion" of the cerebral hemispheres resulting in numerous cortical convolutions. These results with genetically modified mice indicate at least two mechanisms by which final cortical size can be regulated by the neural precursor cell population. First, increased numbers of neural stem cells ("founder" cells), as seen in β-catenin transgenic mice, can produce dramatic horizontal expansion of the cerebral cortical surface; and second, decreased death of neural stem cells and/or "daughter cells" (lineage-restricted progenitors), as seen in *apaf-1*$^{-/-}$, *caspase-9*$^{-/-}$, and *caspase-3*$^{-/-}$ mice, can produce a similarly dramatic increase in vertical expansion and cortical thickness. The balance between these two processes ultimately determines the size and shape of the developing brain.

A great deal remains to be learned about the stimuli triggering programmed cell death in neural precursor cells. Likely, a combination of cell intrinsic signals, for example, DNA damage or abnormal cell cycle progression, and extrinsic signals; for example, limited trophic factor support, combine to regulate neural precursor cell programmed cell death (Voyvodic, 1996; de la Rosa and de Pablo, 2000). Recent studies have revealed a significant degree of DNA aneuploidy in embryonic cortical neuroblasts which might trigger an apoptotic response in neural precursor cells similar to that observed when these cells are exposed to DNA damaging agents *in vivo* or *in vitro* (D'Sa-Eipper *et al*., 2001; Rehen *et al*., 2001). Defective cytokinesis of neuronal precursor cells may also be a stimulus for programmed cell death since mutations in the gene-encoding Citron-Kinase, which is normally expressed at high levels in the ventricular zone and is involved in neural precursor cell division, resulted in extensive apoptosis in the developing nervous system (Di Cunto *et al*., 2000). It is likely that additional neural precursor cell death signals will be identified as developmental neurobiologists focus their attention on this intriguing cell population.

Pre-Synaptogenic

One of the most revealing outcomes of recent investigations of neuronal programmed cell death is the remarkable sensitivity of newly post-mitotic neurons to apoptosis. In addition to Bcl-X_L deficiency, targeted gene disruptions in *rb*, *DNA ligase IV*, and *XRCC4* all produced extensive immature neuron death (Jacks *et al*., 1992; Lee *et al*., 1992; Frank *et al*., 1998; Gao *et al*., 1998; Slack *et al*., 1998). The protein product of the retinoblastoma tumor suppressor gene is an important regulator of the cell cycle and XRCC4 and DNA ligase IV are involved in DNA double strand break repair. Rb-deficient mice show numerous ectopic mitotic figures and apoptotic neurons in the developing central and peripheral nervous systems. Similarly, XRCC4- and DNA ligase IV-deficient embryos show abundant death of immature neurons suggesting that both cell cycle dysregulation and inadequate repair of DNA double strand breaks are apoptotic stimuli for immature neurons. The response of immature neurons to DNA damage and cell cycle dysregulation is regulated by p53 since p53 deficiency completely blocked the increased neuronal apoptosis observed in XRCC4- and DNA ligase IV-deficient mice and inhibited the death of Rb-deficient neurons in the central nervous system (Morgenbesser *et al*., 1994; Macleod *et al*., 1996; Frank *et al*., 2000; Gao *et al*., 2000). In contrast, the increased death of immature neurons in Bcl-X_L-deficient embryos was unaffected by concomitant p53 deficiency (Klocke *et al*., 2002). These findings suggest that multiple death pathways exist in immature neurons and that different apoptotic stimuli may selectively engage different components of the death machinery. The relative contribution of different programmed cell death stimuli, including DNA damage, cell cycle dysregulation, and inadequate trophic factor support, to immature neuron death and normal nervous system development remains to be determined.

Synapse Dependent

Neurons that have survived earlier phases of programmed cell death extend neurites, form synapses, and enter a phase of development that is characterized by extensive death. Neuron death at this stage is typically triggered by inadequate neurotrophic support but other death stimuli, such as a lack of electrical activity and/or death receptor signaling, may also kill synapse-bearing neurons (Cowan *et al*., 1984; Burek and Oppenheim, 1996; Raoul *et al*., 2000). The importance of target-derived neurotrophic factors during this phase is well documented and has been the focus of intense investigation (Bibel and Barde, 2000); the significance of the latter two stimuli during normal development requires additional investigation.

During the maturation of neurons, a change occurs in the molecular regulation of programmed cell death. Unlike neural precursor cells and immature neurons which typically require Apaf-1-dependent caspase-9 and caspase-3 activation for programmed cell death, neuronal death due to neurotrophin deprivation, as well as programmed cell death of motor neurons *in vivo*, becomes Apaf-1- and caspase-independent (Honarpour *et al*., 2001; Oppenheim *et al*., 2001). These alterations emphasize the fact that programmed cell death pathways are remarkably cell-specific and differentiation-dependent. Mature neurons remain dependent on Bcl-2 family members for regulating viability and it is clear that Bax- and Bak-mediated mitochondrial dysfunction

is a potent death stimulus independent of downstream caspase activation (Wei *et al*., 2001).

GLIAL CELL DEATH

Like neurons, glial cells also undergo programmed cell death in the developing mammalian nervous system (Raff *et al*., 1993). Many parallels exist between neuronal cell death and that of oligodendrocytes and astrocytes. Glial cell death is similarly extensive; for example, over half of the oligodendrocytes present in the rat optic nerve undergo programmed cell death as do numerous astrocytes in the developing cerebellum (Barres *et al*., 1992; Krueger *et al*., 1995). Glial cell death may also be regulated by trophic molecules, is typically apoptotic in appearance, may involve competition for limited quantities of trophic molecules, and is regulated by Bcl-2 and caspase family members (Barres *et al*., 1993; Burne *et al*., 1996; Jacobson *et al*., 1997). Thus, the maturation-dependent phases of programmed cell death and the molecular regulation of death described earlier in this chapter can be equally applied to cells of glial lineage as to those of neuronal lineage.

SUMMARY

Programmed cell death research has had an arguably unparalleled impact on the field of developmental neurobiology. For over one hundred years, investigations of neuronal cell death have challenged our assumptions about nervous system development and its cellular and molecular regulation. Current studies on programmed cell death, including investigations of neural stem cell death, hold tremendous promise for elucidating the complex processes involved in normal mammalian nervous system development.

REFERENCES

Allsopp, T.E., Wyatt, S., Paterson, H.F., and Davies, A.M., 1993, The proto-oncogene *bcl-2* can selectively rescue neurotrophic factor-dependent neurons from apoptosis, *Cell* 73:295–307.

Barres, B.A., Hart, I.K., Coles, H.S.R., Burne, J.F., Voyvodic, J.T., Richardson, W.D. *et al*., 1992, Cell death and control of cell survival in the oligodendrocyte lineage, *Cell* 70:31–46.

Barres, B.A., Jacobson, M.D., Schmid, R., Sendtner, M., and Raff, M.C., 1993, Does oligodendrocyte survival depend on axons? *Curr. Biol.* 3:489–497.

Beard, J., 1896, The history of a transient nervous apparatus in certain Ichthyopsida. An account of the development and degeneration of ganglion-cells and nerve fibres, *Zool. Jahrbüchér Abt. Morphol.* 9:1–106.

Bibel, M. and Barde, Y.-A., 2000, Neurotrophins: Key regulators of cell fate and cell shape in the vertebrate nervous system, *Genes Dev.* 14:2919–2937.

Blaschke, A.J., Staley, K., and Chun, J., 1996, Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex, *Development* 122:1165–1174.

Blaschke, A.J., Weiner, J.A., and Chun, J., 1998, Programmed cell death is a universal feature of embryonic and postnatal neuroproliferative regions throughout the central nervous system, *J. Comp. Neurol.* 396:39–50.

Boise, L.H., González-García, M., Postema, C.E., Ding, L., Linsten, T., Turka, L.A. *et al*., 1993, *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death, *Cell* 74:597–608.

Bouillet, P., Cory, S., Zhang, L.-C., Strasser, A., and Adams, J.M., 2001, Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim, *Dev. Cell* 1:645–653.

Burek, M.J. and Oppenheim, R.W., 1996, Programmed cell death in the developing nervous system, *Brain Pathol.* 6:427–446.

Burne, J.F., Staple, J.K., and Raff, M.C., 1996, Glial cells are increased proportionally in transgenic optic nerves with increased numbers of axons, *J. Neurosci.* 16:2064–2073.

Bursch, W., 2001, The autophagosomal-lysosomal compartment in programmed cell death, *Cell Death Differ.* 8:569–581.

Cecconi, F., Alvarez-Bolado, G., Meyer, B.I., Roth, K.A., and Gruss, P., 1998, Apaf1 (CED-4 Homolog) regulates programmed cell death in mammalian development, *Cell* 94:727–737.

Chai, J., Shiozaki, E., Srinivasula, S.M., Wu, Q., Dataa, P., Alnemri, E.S. *et al*., 2001, Structural basis of Caspase-7 inhibition by XIAP, *Cell* 104:769–780.

Chen, D.F., Schneider, G.E., Martinou, J.-C., and Tonegawa, S., 1997, Bcl-2 promotes regeneration of severed axons in mammalian CNS, *Nature* 385:434–439.

Cheng, E.H. Y., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T. *et al*., 2001, BCL-2, BCL-X_L sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis, *Mol. Cell* 8:705–711.

Chenn, A. and Walsh, C.A., 2002, Regulation of cerebral cortical size by control of cell cycle exit in neural precursors, *Science* 297:365–369.

Chi, S., Kitanake, C., Noguchi, K., Mochizuki, T., Nagashima, Y., Shirouzu, M. *et al*., 1999, Oncogenic ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells, *Oncogene* 18:2281–2290.

Chun, J., 2000, Cell death, DNA breaks and possible rearrangements: An alternative view, *Trends Neurosci.* 23:407–408.

Chun, J. and Schatz, D.G., 1999, Rearranging views on neurogenesis: Neuronal death in the absence of DNA end-joining proteins, *Neuron* 22:7–10.

Clarke, P.G.H., 1990, Developmental cell death: Morphological diversity and multiple mechanisms, *Anat. Embryol.* 181:195–213.

Cowan, W.M., Fawcett, J.W., O'Leary, D.D.M., and Stanfield, B.B., 1984, Regressive events in neurogenesis, *Science* 225:1258–1265.

D'Mello, S.R., Kuan, C.-Y., Flavell, R.A., and Rakic, P., 2000, Caspase-3 is required for apoptosis-associated DNA fragmentation but not for cell death in neurons deprived of potassium, *J. Neurosci. Res.* 59:24–31.

D'Sa-Eipper, C., Leonard, J.R., Putcha, G., Zheng, T.S., Flavell, R.A., Rakic, P. *et al*., 2001, DNA damage-induced neural precursor cell apoptosis requires p53 and caspase-9 but neither Bax nor caspase-3, *Development* 128:137–146.

de la Rosa, E.J. and de Pablo, F., 2000, Cell death in early neural development: Beyond the neurotrophic theory, *Trends Neurosci.* 23: 454–458.

Deckwerth, T.L., Elliott, J.L., Knudson, C.M., Johnson, Jr., E.M., Snider, W.D., and Korsmeyer, S.J., 1996, Bax is required for neuronal death after trophic factor deprivation and during development, *Neuron* 17: 401–411.

Deiss, L.P., Galinka, H., Berissi, H., Cohen, O., and Kimchi, A., 1996, Cathepsin D protease mediates programmed cell death induced by interferon-γ, Fas/APO-1 and TNF-α, *EMBO J.* 15:3861–3870.

Deveraux, Q.L. and Reed, J.C., 1999, IAP family proteins—suppressors of apoptosis, *Genes Dev.* 13:239–252.

Di Cunto, F., Imarisio, S., Hirsch, E., Broccoli, V., Bulfone, A., Migheli, A. *et al*., 2000, Defective neurogenesis in citron kinase knockout mice by altered cytokinesis and massive apoptosis, *Neuron* 28:115–127.

Dunn, Jr., W.A., 1994, Autophagy and related mechanisms of lysosome-mediated protein degradation, *Trends Cell Biol.* 4:139–143.

Ernst, M., 1926, Über Untergang von Zellen während der normalen Entwicklung bei Wirbeltieren, *Z. Anat. Entwicklungsgesch* 79: 228–262.

Ferrer, I., Soriano, E., Del Rio, A., Alcántara, S., and Auladell, C., 1992, Cell death and removal in the cerebral cortex during development, *Progress in Neurobiol.* 39:1–43.

Frank, K.M., Sekiguchi, J.M., Seidl, K.J., Swat, W., Rathbun, G.A., Cheng H.-L. *et al*., 1998, Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV, *Nature* 396:173–177.

Frank, K.M., Sharpless, N.E., Gao, Y., Sekiguchi, J.M., Ferguson, D.O., Zhu, C. *et al*., 2000, DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via the p53 pathway, *Mol. Cell* 5:993–1002.

Gao, Y., Ferguson, D.O., Xie, W., Manis, J.P., Sekiguchi, J., Frank, K.M. *et al*., 2000, Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development, *Nature* 404:897–900.

Gao, Y., Sun, Y., Frank, K.M., Dikkes, P., Fujiwara, Y., Seidl, K.J. *et al*., 1998, A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis, *Cell* 95:891–902.

Garcia, I., Marinou, I., Tsujimoto, Y., and Martinou, J.-C., 1992, Prevention of programmed cell death of sympathetic neurons by the *bcl-2* proto-oncogene, *Science* 258:302–304.

Gilmore, E.C., Nowakowski, R.S., Caviness, Jr., V.S., and Herrup, K., 2000, Cell birth, cell death, cell diversity and DNA breaks: How do they all fit together? *Trends Neurosci.* 23:100–105.

Glücksmann, A., 1951, Cell deaths in normal vertebrate ontogeny, *Bio. Rev.* 26:59–86.

González-García, M., Garcia, I., Ding, L., O'Shea, S., Boise, L.H., Thompson, C.B. *et al*., 1995, *bcl-x* is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death, *Proc. Natl. Acad. Sci. USA* 92:4304–4308.

González-García, M., Thompson, C.B., Ding, L., Duan, L., Boise, L.H., and Nuñez, G., 1994, *bcl-*x_L is the major *bcl-x* mRNA form expressed during murine development and its product localizes to mitochondria, *Development* 120:3033–3042.

Götz, R., Karch, C., Digby, M.R., Troppmair, J., Rapp, U.R., and Sendtner, M., 2000, The neuronal apoptosis inhibitory protein suppresses neuronal differentiation and apoptosis in PC12 cells, *Hum. Mol. Genet.* 9:2479–2489.

Häcker, G., 2000, The morphology of apoptosis, *Cell Tissue Res.* 301: 5–17.

Hakem, R., Hakem, A., Duncan, G.S., Henderson, J.T., Woo, M., Soengas, M.S. *et al*., 1998, Differential requirement for caspase-9 in apoptotic pathways *in vivo*, *Cell* 94:339–352.

Hamburger, V., 1992, History of the discovery of neuronal death in embryos, *J. Neurobiol.* 23:1116–1123.

Harlin, H., Reffey, S.B., Duckett, C.S., Lindsten, T., and Thompson, C.B., 2001, Characterization of XIAP-deficient mice, *Mol. Cell. Biol.* 21:3604–3608.

Holcik, M., Thompson, C.B., Yaraghi, Z., Lefebvre, C.A., MacKenzie, A.E., and Korneluk, R.G., 2000, The hippocampal neurons of neuronal apoptosis inhibitory protein 1 (NAIP1)-deleted mice display increased vulnerability to kainic acid-induced injury, *Proc. Natl. Acad. Sci. USA* 97:2286–2290.

Honarpour, N., Tabuchi, K., Stark, J.M., Hammer, R.E., Südhof, T.C., Parada, L.F. *et al*., 2001, Embryonic neuronal death due to neurotrophin and neurotransmitter deprivation occurs independent of Apaf-1, *Neuroscience* 106:263–274.

Horvitz, H.R., 1999, Genetic control of programmed cell death in the nematode *Caenorhabditis elegans*, *Cancer Res.* 59:1701s–1706s.

Huang, Y., Park, Y.C., Rich, R.L., Segal, D., Myszka, D.G., and Wu, H., 2001, Structural basis of caspase inhibition by XIAP: Differential roles of the Linker versus the BIR domain, *Cell* 104:781–790.

Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A., and Weinberg, R.A., 1992, Effects of an *Rb* mutation in the mouse, *Nature* 359:295–300.

Jacobson, M., 1991, *Developmental Neurobiology*, 3rd edn, Plenum Press, New York.

Jacobson, M.D., Weil, M., and Raff, M.C., 1997, Programmed cell death in animal development, *Cell* 88:347–354.

Joza, N., Kroemer, G., and Penninger, J.M., 2002, Genetic analysis of the mammalian cell death machinery, *Trends Genet.* 18:142–149.

Kerr, J.F.R., Wyllie, A.H., and Currie, A.R., 1972, Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics, *Br. J. Cancer* 26:239–257.

Klocke, B.J., Latham, C.B., C. D'Sa, and Roth, K.A., 2002, p53 deficiency fails to prevent increased programmed cell death in the Bcl-XL-deficient nervous system, *Cell Death Differ* 9:1063–1068.

Knudson, C.M., Tung, K.S.K., Troutellotte, W.G., Brown, G.A.J., and Korsmeyer, S.J., 1995, Bax-deficient mice with lymphoid hyperplasia and male germ cell death, *Science* 270:96–99.

Korsmeyer, S.J. 1999, *BCL-2* gene family and the regulation of programmed cell death, *Cancer Res.* 59:1693s–1700s.

Krajewski, S., Krajewska, M., Shabaik, A., Miyashita, T., Wang, H.-G., and Reed, J.C., 1994, Immunohistochemical determination of *in vivo* distribution of Bax, a dominant inhibitor of Bcl-2, *Am. J. Path.* 145:1323–1328.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W., and Reed, J.C., 1993, Investigations of the subcellular distribution of the Bcl-2 oncoprotein residence in the nuclear envelope, endoplasmic reticulum, and other mitochondrial membranes, *Cancer Res.* 53:4701–4714.

Krueger, B.K., Burne, J.F., and Raff, M.C., 1995, Evidence for large-scale astrocyte death in the developing cerebellum, *J. Neurosci.* 15: 3366–3374.

Kuan, C.-Y., Roth, K.A., Flavell, R.A., and Rakic, P., 2000, Mechanism of programmed cell death in the developing brain, *Trends Neurosci.* 23: 287–293.

Kügler, S., Straten, G., Kreppel, F., Isenmann, S., Liston, P., and Bähr, M., 2000, The X-linked inhibitor of apoptosis (XIAP) prevents cell death in axotomized CNS neurons *in vivo*, *Cell Death Differ.* 7:815–824.

Kuida, K., Haydar, T.F., Kuan, C.-Y., Gu, Y., Taya, C., Karasuyama, H. *et al*., 1998, Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase-9, *Cell* 94:325–337.

Kuida, K., Zheng, T.S., Na, S., Kuan, C.-Y., Yang, D., Karasuyama, H. *et al*., 1996, Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice, *Nature* 384:368–372.

Lee, C.-Y. and Baehrecke, E.H., 2001, Steroid regulation of autophagic programmed cell death during development, *Development* 128: 1443–1455.

Lee, E.Y.H.P., Chang, C.-Y., Hu, N., Wang, Y.-C.J., Lai, C.-C., Herrup, K. *et al*., 1992, Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis, *Nature* 359:288–294.

Leonard, J.R., D'Sa, C., Cahn, R., Korsmeyer, S., and Roth, K.A., 2001, Bid regulation of neuronal apoptosis, *Dev. Brain Res.* 128:187–190.

Leonard, J.R., Klocke, B.J., D'Sa, C., Flavell, R.A., and Roth, K.A., 2002, Strain-dependent neurodevelopmental abnormalities in caspase-3-deficient mice, *J. Neuropathol. Exp. Neurol.* 61:673–677.

Liang, X.H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H. *et al*., 1999, Induction of autophagy and inhibition of tumorigenesis by *beclin 1*, *Nature* 402:672–676.

Lindsten, T., Ross, A.J., King, A., Zong, W.-X., Rathmell, J.C., Shiels, H.A. *et al*., 2000, The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues, *Mol. Cell* 6:1389–1399.

Liston, P., Fong, W.G., Kelly, N.L., Toji, S., Miyazaki, T., Conte, D. *et al*., 2001, Identification of XAF1 as an antagonist of XIAP anti-caspase activity, *Cell Biol. Nat.* 3:128–133.

Lomaga, M.A., Henderson, J.T., Elia, A.J., Robertson, J., Noyce, R.S., Yeh, W.-C. *et al*., 2000, Tumor necrosis factor receptor-associated factor 6 (TRAF6) deficiency results in exencephaly and is required for apoptosis within the developing DNS, *J. Neurosci.* 20:7384–7393.

Macleod, K.F., Hu, Y., and Jacks, T., 1996, Loss of *Rb* activates both *p53*-dependent and independent cell death pathways in the developing mouse nervous system, *EMBO J.* 15:6178–6188.

Martinou, J.-C., Dubois-Dauphin, M., Staple, J.K., Rodriguez, I., Frankowski, H., Missotten, M. *et al*., 1994, Overexpression of Bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia, *Neuron* 13:1017–1030.

Mercer, E.A., Korhonen, L., Skoglösa, Y., Olsson, P.-A., Kukkonen, J.P., and Linkholm, D., 2000, NAIP interacts with hippocalcin and pro-tects neurons against calcium-induced cell death through caspase-3-dependent and -independent pathways, *EMBO J.* 19: 3597–3607.

Merry, D.E. and Korsmeyer, S.J., 1997, Bcl-2 gene family in the nervous system, *Annu. Rev. Neurosci.* 20:245–267.

Merry, D.E., Veis, D.J., Hickey, W.F., and Korsmeyer, S.J., 1994, *bcl-2* protein expression is widespread in the developing nervous system and retained in the adult PNS, *Development* 120:301–311.

Michaelidis, T.M., Sendtner, M., Cooper, J.D., Airaksinen, M.S., Holtmann, B., Meyer, M. *et al*., 1996, Inactivation of *bcl-2* results in progressive degeneration of motoneurons, sympathetic and sensory neurons during early postnatal development, *Neuron* 17:75–89.

Morgenbesser, S.D., Williams, B.O., Jacks, T., and DePinho, R.A., 1994, *p53*-dependent apoptosis produced by *Rb*-deficiency in the develop-ing mouse lens, *Nature* 371:72–74.

Motoyama, N., Wang, F., Roth, K.A., Sawa, H., Nakayama, K.-I., Nakayama, K. *et al*., 1995, Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice, *Science* 267:1506–1510.

Nakayama, K., Nakayama, K.-I., Negishi, I., Kuida, K., Sawa, H., and Loh, D.Y., 1994, Targeted disruption of Bcl-2 αβ in mice: Occurrence of gray hair, polycystic kidney disease, and lymphocytopenia, *Proc. Natl. Acad. Sci. USA* 91:3700–3704.

Nicholson, D.W., 1999, Caspase structure, proteolytic substrates, and function during apoptotic cell death, *Cell Death Differ.* 6:1028–1042.

Nicotera, P., 2000, Caspase requirement for neuronal apoptosis and neuro-degeneration, *IUBMB Life* 49:421–425.

Oltvai, Z.N., Milliman, C.T., and Korsmeyer, S.J., 1993, Bcl-2 hetero-dimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death, *Cell* 74:609–619.

Oppenheim, R.W., 1991, Cell death during development of the nervous system, *Annu. Rev. Neurosci.* 14:453–501.

Oppenheim, R.W., Flavell, R.A., Vinsant, S., Prevette, D., Kuan, C.-Y., and Rakic, P., 2001, Programmed cell death of developing mammalian neurons after genetic deletion of caspases, *J. Neurosci.* 21: 4752–4760.

Perrelet, D., Ferri, A., MacKenzie, A.E., Smith, G.M., Korneluk, R.G., Liston, P. *et al*., 2000, IAP family proteins delay motoneuron cell death *in vivo*, *Eur. J. Neurosci.* 12:2059–2067.

Putcha, G.V., Moulder, K.L., Golden, J.P., Bouillet, P., Adams, J.A., Strasser, A. *et al*., 2001, Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis, *Neuron* 29: 615–628.

Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y., and Jacobson, M.D., 1993, Programmed cell death and the control of cell survival: Lessons from the nervous system, *Science* 262:695–700.

Rakic, S. and Zecevic, N., 2000, Programmed cell death in the developing human telencephalon, *Eur. J. Neurosci.* 12:2721–2734.

Raoul, C., Pettmann, B., and Henderson, C.E., 2000, Active killing of neurons during development and following stress: A role for $p75^{NTR}$ and Fas? *Curr. Opin. Neurobiol.* 10:111–117.

Rehen, S.K., McConnell, M.J., Kaushal, D., Kingsbury, M.A., Yang, A.H., and Chun, J., 2001, Chromosomal variation in neurons of the develop-ing and adult mammalian nervous system, *Proc. Natl. Acad. Sci. USA* 98:13361–13366.

Riedl, S.J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C., Fesik, S.W. *et al*., 2001, Structural basis for the inhibition of caspase-3 by XIAP, *Cell* 104:791–800.

Robertson, G.S., Crocker, S.J., Nicholson, D.W., and Schulz, J.B., 2000, Neuroprotection by the inhibition of apoptosis, *Brain Pathol.* 10: 283–292.

Roth, K.A., 2002, *In situ* detection of apoptotic neurons. In *Neuromethods, Vol. 37: Apoptosis Techniques and Protocols* (A.C. LeBlanc, ed.), Humana Press, Inc., Totowa, NJ, pp. 205–224.

Roth, K.A., Kuan, C.-Y., Haydar, T.F., D'Sa-Eipper, C., Shindler, K.S., Zheng, T.S. *et al*., 2000, Epistatic and independent apoptotic func-tions of Caspase-3 and Bcl-X_L in the developing nervous system, *Proc. Natl. Acad. Sci. USA* 97:466–471.

Roth, K.A., Motoyama, N., and Loh, D.Y., 1996, Apoptosis of *bcl-x*-deficient telencephalic cells *in vitro*, *J. Neurosci.* 16:1753–1758.

Roy, N., Mahadevan, M.S., McLean, M., Shutler, G., Yaraghi, Z., Farahani, R. *et al*., 1995, The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy, *Cell* 80: 167–178.

Saeki, K., You, A., Okuma, E., Yazaki, Y., Susin, S.A., Kroemer, G. *et al*., 2000, Bcl-2 down-regulation causes autophagy in a caspase-independent manner in human leukemic HL60 cells, *Cell Death Differ.* 7:1263–1269.

Sedlak, T.W., Oltvai, Z.N., Yang, E., Wang, K., Boise, L.H., Thompson, C.B. *et al*., 1995, Multiple Bcl-2 family members demonstrate selective dimerizations with Bax, *Proc. Natl. Acad. Sci. USA* 92:7834–7838.

Seglen, P.O. and Bohley, P., 1992, Autophagy and other vacuolar protein degradation mechanisms, *Experientia* 48:158–172.

Shibata, M., Kanamori, S., Isahara, K., Ohsawa, Y., Konishi, A., Kametaka, S. *et al*., 1998, Participation of cathepsins B and D in apoptosis of PC12 cells following serum deprivation, *Biochem. Biophys. Res. Commun.* 251:199–203.

Shindler, K.S., Latham, C.B., and Roth, K.A., 1997, *bax* deficiency prevents the increased cell death of immature neurons in *bcl-x*-deficient mice, *J. Neurosci.* 17:3112–3119.

Shindler, K.S., Yunker, A.M.R., Cahn, R., Zha, J., Korsmeyer, S.J., and Roth, K.A., 1998, Trophic support promotes survival of *bcl-x*-deficient telencephalic cells *in vitro*, *Cell Death Differ.* 5: 901–910.

Slack, R.S., El-Bizri, H., Wong, J., Belliveau, D.J., and Miller, F.D., 1998, A critical temporal requirement for the retinoblastoma protein family during neuronal determination, *J. Cell Biol.* 140:1497–1509.

Snider, W.D., 1994, Functions of the neurotrophins during nervous system development: What the knockouts are teaching us, *Cell* 77: 627–638.

Sommer, L. and Rao, M., 2002, Neural stem cells and regulation of cell number, *Progress in Neurobiol.* 66:1–18.

Srinivasan, A., Roth, K.A., Sayers, R.O., Shindler, K.S., Wong, A.M., Fritz, L.C., and Tomaselli, K.J., 1998, *In situ* immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system, *Cell Death Differ.* 5:1004–1016.

Srinivasula, S.M., Hegde, R., Saleh, A., Datta, P., Shiozaki, E., Chai, J. *et al*., 2001, A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis, *Nature* 410: 112–116.

Stoka, V.V., Turk, B., Schendel, S.L., Kim, T.W., Cirman, T., Snipas, S.J. *et al*., 2001, Lysosomal protease pathways to apoptosis: Cleavage of bid, not pro-caspases is the most likely route, *J. Biol. Chem.* 276:3149–3157.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M. *et al*., 1999, Molecular characterization of mitochondrial apoptosis-inducing factor, *Nature* 397:441–446.

Thomaidou, D., Mione, M.C., Cavanagh, J.F.R., and Parnavelas, J.G., 1997, Apoptosis and its relation to the cell cycle in the developing cerebral cortex, *J. Neurosci.* 17:1075–1085.

Tolkovsky, A.M., Xue, L., Fletcher, G.C., and Borutaite, V., 2002, Mitochondrial disappearance from cells: A clue to the role of autophagy in programmed cell death and disease? *Biochemie* 84: 233–240.

Uchiyama, Y., 2001, Autophagic cell death and its execution by lysosomal cathepsins, *Arch. Histol. Cytol.* 64:233–246.

van den Eijnde, S.M., Lips, J., Boshart, L., Marani, E., Reutelingsperger, C.P.M., and De Zeeuw, C.I., 1999, Spatiotemporal distribution of dying neurons during early mouse development, *Eur. J. Neurosci.* 11:712–724.

Veis, D.J., Sorenson, C.M., Shutter, J.R., and Korsmeyer, S.J., 1993, Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair, *Cell* 75:229–240.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E. *et al*., 2000, Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins, *Cell* 102:43–53.

Voyvodic, J.T., 1996, Cell death in cortical development: How much? Why? So what? *Neuron* 16:693–696.

Wei, M.C., Zong, W.-X., Cheng, E.H.Y., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A. *et al*., 2001, Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death, *Science* 292:727–730.

White, F.A., Keller-Peck, C.R., Knudson, C.M., Korsmeyer, S.J., and Snider, W.D., 1998, Widespread elimination of naturally occurring neuronal death in *Bax*-deficient mice, *J. Neurosci.* 18:1428–1439.

Wu, G.S., Saftig, P., Peters, C., and El-Deiry, W.S., 1998, Potential role for cathepsin D in p53-dependent tumor suppression and chemosensitivity, *Oncogene* 16:2177–2183.

Xue, L., Fletcher, G.C., and Tolkovsky, A.M., 1999, Autophagy is activated by apoptotic signalling in sympathetic neurons: An alternative mechanism of death execution, *Mol. Cell. Neurosci.* 14: 180–198.

Yu, S.-W., Wang, H., Poitras, M.F., Coombs, C., Bowers, W.J., Federoff, H.J. *et al*., 2002, Mediation of poly(ADP-Ribose) polymerase-1-dependent cell death by apoptosis-inducing factor, *Science* 297: 259–263.

Zaidi, A.U., D'Sa-Eipper, C., Brenner, J., Kuida, K., Zheng, T.S., Flavell, R.A. *et al*., 2001, Bcl-XL-Caspase-9 interactions in the developing nervous system: Evidence for multiple death pathways, *J. Neurosci.* 21: 169–175.

Zaidi, A.U., McDonough, J.S., Klocke, B.J., Latham, C.B., Korsmeyer, S.J., Flavell, R.A. *et al*., 2001, Chloroquine-induced neuronal cell death is p53 and Bcl-2 family-dependent but caspase-independent, *J. Neuropathol. Exp. Neurol.* 60:937–945.

Zheng, T.S. and Flavell, R.A., 2000, Divinations and surprises: Genetic analysis of caspase function in mice, *Exp. Cell Res.* 256:67–73.

Zheng, T.S., Hunot, S., Kuida, K., Momoi, T., Srinivasan, A., Nicholson, D.W. *et al*., 2000, Deficiency in caspase-9 or caspase-3 induces compensatory caspase activation, *Nat. Med.* 6:1241–1247.

Zheng, T.S., Schlosser, S.F., Dao, T., Hingorani, R., Crispe, I.N., Boyer, J.L. *et al*., 1998, Caspase-3 controls both cytoplasmic and nuclear events associated with Fas-mediated apoptosis *in vivo*, *Proc. Natl. Acad. Sci. USA* 95:13618–13623.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A., and Wang, X., 1997, Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3, *Cell* 90:405–413.

Zou, H., Li, Y., Liu, X., and Wang, X., 1999, An APAF-1 cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9, *J. Biol. Chem.* 274:11549–11556.