Mechanisms of programmed cell death in the developing brain

Chia-Yi Kuan, Kevin A. Roth, Richard A. Flavell and Pasko Rakic

Programmed cell death (apoptosis) is an important mechanism that determines the size and shape of the vertebrate nervous system. Recent gene-targeting studies have indicated that homologs of the cell-death pathway in the nematode *Caenorhabditis elegans* have analogous functions in apoptosis in the developing mammalian brain. However, epistatic genetic analysis has revealed that the apoptosis of progenitor cells during early embryonic development and apoptosis of postmitotic neurons at later stage of brain development have distinct roles and mechanisms. These results provide new insight on the significance and mechanism of neural cell death in mammalian brain development.

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CELL DEATH has long been recognized to occur in most neuronal populations during normal development of the vertebrate nervous system (reviewed in Ref. 1). Traditionally, the investigation of neural death in development focused on the role of target-derived survival factors such as NGF and related neurotrophins (see Ref. 2 for a review). However, in the past few years, the genetic analysis of programmed cell death in the nematode *Caenorhabditis elegans* has inspired new approaches to study this phenomenon (see Box 1 for the developmental functions of programmed cell death). It is through such gene-targeting studies that recent insights into the molecular regulation of mammalian programmed cell death have been obtained.

The integration of specification and execution phases of cell death in *C. elegans*

During the development of an adult *C. elegans* hermaphrodite, 131 out of the total 1090 cells undergo programmed cell death in a lineage-specific and mostly cell-autonomous manner. Three groups of genes involved in this process have been identified by genetic screening[3]. The first group of genes includes *ces-1* and *ces-2* (ces, cell-death specification) and affects the death of specific types of cells. The second group of genes affects most, if not all, of the 131 cells undergoing cell death, and is therefore involved in the execution phase of cell death. These global regulators, which include *egl-1* (egl, egg-laying defective), *ced-9* (ced, cell-death abnormal), *ced-4* and *ced-3*, form an obligate cell-death pathway (Fig. 1a). The last group of genes, which includes *ced-1*, *ced-6*, *ced-7*, *ced-2*, *ced-5*, *ced-10* and *nuc-1* (nuc, nuclease abnormal), is involved in the degradation of DNA and phagocytosis of the cell corpses. Among the three groups of cell-death genes, those involved in the execution phase of apoptosis have been the most extensively studied. Cumulative biochemical studies suggest that EGL-1 triggers programmed cell death by binding to CED-9 and thus releasing the cell-death activator CED-4 from a CED-9–CED-4 protein complex, which leads to activation of CED-3 (Ref. 4).

Remarkably, structural homologs of all the genes involved in the execution phase of cell death in *C. elegans* have been identified in mammals. The mammalian homologs of *ced-3* comprise a family of cysteine-containing, aspartate-specific proteases called caspases[5]. The *ced-4* homolog is identified as one of the apoptosis protease-activating factors (APAFs)[6]. The mammalian homologs of *ced-9* belong to a growing family of Bcl2 proteins, which share the Bcl2-homology (BH) domain and are either pro- or anti-apoptotic[7]. The cloning of *egl-1* indicates that it is similar to the BH3-domain-containing, pro-apoptotic subfamily of Bcl2 proteins[4].

What determines the fate of cell-autonomous apoptosis? Molecular cloning in *C. elegans* reveals that *ces-2* encodes a basic-leucine-zipper (bZIP) transcription-factor motif, suggesting that programmed cell death could be regulated by differential gene expression[8]. Indeed, recent study suggests that the sex-determination protein, TRA-1A, represses *egl-1* transcription in the hermaphrodite-specific neurons (HSNs) and prevents apoptosis of these neurons, which would normally die in a male worm[9]. One intriguing implication of this study is that, although the anti-apoptotic (for example, CED-9) and pro-apoptotic (for example, CED-3 and CED-4) molecules are always present in living cells, the most-upstream molecule of the pathway (for example, EGL-1) could integrate various regulation signals to determine cell fate. Presumably, there are similar mechanisms in mammalian cells to integrate the regulatory signals at the initiation point of the apoptosis cascade. Such an integration point might reside at the transcription level of the pro-apoptotic Bcl2-family genes. Alternatively, the integration mechanisms might include posttranslational modifications or translocation of the Bcl2-family proteins between cellular organelles.

Caspase 9 and caspase 3 in the developing mammalian brain

The mammalian homologs of *ced-3* comprise a family of cysteine-containing, aspartate-specific proteases called caspases. These are present in living cells as proenzymes that contain three domains: an N-terminal domain, a large subunit and a small subunit. Activation of caspases involves proteolytic processing between domains followed by association of the large and small subunits to form an active heterodimer or tetramer[5]. Once activated, caspases cleave other caspases

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Box 1. Multiple functions of programmed cell death in brain development

The biological functions of cell death during normal development have captured the imagination of embryologists since its discovery in the 19th century[a]. In 1926, Ernst summarized three main types of developmental cell death: the first occurring during regression of vestigial organs; the second during morphogenesis of organ anlage; and the third during remodeling of tissues. These were later named phylogenetic, morphogenetic and histiogenetic degeneration by Glucksmann in a scheme designed to explain the biological functions of developmental cell death[b]. Phylogenetic degeneration, exemplified by regression of pronephros and mesonephros in higher vertebrates, rarely occurs in the developing nervous system. By contrast, cell death in many places of the embryonic brain, such as at the edge of the neural plate and within the optic stalk was considered to be related to the morphogenetic process. The prime example of histiogenetic degeneration was described in the spinal ganglia of chick embryos. By quantitative and experimental methods, Hamburger and Levi-Montalcini showed that the spinal ganglia corresponding to the limbs are larger than the adjacent ganglia, and many ganglial neuroblasts degenerate if the limb bud is removed early[c]. These results suggested that neurons compete for a limited supply of peripherally derived surviving 'trophic' factors and their death is the consequence of an initially surplus neuronal population.

The subsequent discovery of NGF by Levi-Montalcini and colleagues consolidated the trophic theory, which became one of the most-influential concepts of neural development of this century[d]. Consequently, the predominant view is that cell death occurs mainly to match the size of each neuronal population to the magnitude of its target fields and to eliminate neurons with erroneous or inadequate projections[e,f]. By contrast, the possibility that cell death can be a mechanism of morphogenesis of the nervous system has been relatively unexplored. In recent years, mutant mice that overexpress an anti-apoptotic gene *Bcl2* or lack the pro-apoptotic gene *Bax* have been generated, both of which exhibit increases in selected neuronal subpopulations without gross malformations of the nervous system[g,h]. These consequences of reduced developmental cell death, consistent with the idea of social control of cell survival or death, seemingly exclude a significant role of cell death in the morphogenesis of the nervous system.

It was therefore surprising that mutant mice deficient in the pro-apoptotic genes *Casp3*, *Casp9* and *Apaf1* all showed severe malformations of the nervous system because of a reduction of developmental cell death[i–m]. Moreover, mice with deficiency of the protein kinases Jnk1 and Jnk2 exhibited pronounced neurulation defects, which were preceded by reduction of cell death prior to the closure of the hindbrain neural tube (Fig. I)[n,o]. Together, these findings strongly implicate a role of cell death during morphogenesis of the developing brain. The apparently discrepant consequences of reduced developmental cell death could be reconciled by the demonstration of caspase-3-mediated, and Bax- and Bcl-X_L-independent apoptosis of neural founder cells, followed by Bax- and Bcl-X_L-regulated and caspase-3-dependent death of postmitotic neurons[p].

In summary, cell death shall no longer be considered merely a mechanism to match the neuron population to its target fields. Rather, cell death has an addditional important role in adjusting the initial progenitor pool needed for proper morphogenesis of the nervous system, the mechanism of which remains to be investigated.

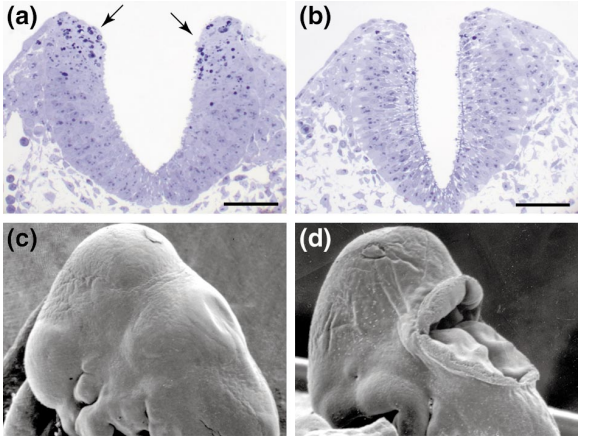

Fig. I. ***Brain region-specific programmed cell death is required for the closure of the hindbrain neural tube.*** *Pyknotic cell deaths are characteristically located at the lateral edges of the hindbrain neural tube prior to closure in wild-type embryos* **(a)** *and* **(c)** *but are greatly reduced in mouse mutants deficient in both Jnk1 and Jnk2 protein kinases* **(b)**. *As a consequence of this reduction in region-specific apoptosis, Jnk1 and Jnk2 dual-deficient embryos exhibited neurulation defects at the hindbrain* **(d)**. *Scale bar, 100 μm in* (a) *and* (b). Modified, with permission, from Ref. n.

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and various cellular substrates, including the DNA fragmentation factor 45/inhibitor of caspase-activated deoxynuclease (DFF45/ICAD), which leads to the ultrastructural changes that typify apoptosis[10]. To date, more than 14 caspase proteases have been isolated, among which only null mutants of *Casp3* and *Casp9* showed severe defects of programmed cell death in the nervous system[11–13].

The majority of homozygous *Casp3* and *Casp9* null mutants are embryonic lethal or die shortly after birth. A general reduction of pyknotic cell death is found in the embryonic brain tissue of the mutants[11–13]. As a consequence of the reduction of developmental cell death in the nervous system, multiple indentations of the cerebrum and periventricular masses constituted by supernumerary neurons were generated (Fig. 2a,b). However, despite the severe defects of programmed cell death in the brain, the developmental apoptosis of thymocytes in the *Casp3* and *Casp9* null mutants is largely preserved[11–13]. Similarly, other lines of caspase-deficient mice (*Casp1*, *Casp2*, *Casp8* and *Casp11*) all show preferential apoptosis defects rather than a global suppression of cell death, indicating that individual members of the caspase family have a dominant and non-redundant role in apoptosis in a tissue-selective or stimulus-dependent manner.

The similar phenotypes of null mutations of *Casp3* and *Casp9* suggest that these two caspases might function along the same cell-death pathway in brain development. Consistent with this idea, capsase 9 was previously identified as an upstream activator of caspase 3 in a biochemical study using human HeLa cells[14]. In this study, it was shown that caspase 9 bound to Apaf1, the human homolog of *ced-4*, and cytochrome c through a caspase-recruitment domain (CARD) motif in its N-terminal sequence, forming an active apoptosome. By contrast, caspase 3 lacks the CARD motif and does not bind to Apaf1 directly. These results suggest a linear activation cascade between caspase 9 and caspase 3 in response to cytochrome c released from the mitochondria during apoptosis. Indeed, biochemical assays demonstrate that the cytochrome-c-mediated cleavage of pro-caspase 3 is defective in the cytosolic fractions from *Casp9* null mutants, but is restored by adding *in vitro* transcribed and translated caspase 9 (Fig. 2c,d). Furthermore, the requirement of caspase 9 for normal caspase 3 processing *in vivo* is confirmed by immunofluorescence studies that show the absence of activated caspase 3 in the nervous tissue of *Casp9*-deficient embryos[12]. Together, these results have established a linear apoptosis cascade in caspase 9 to caspase 3 during the normal development of the mammalian brain.

It remains unclear what key factors lie downstream of caspase 3 and lead to apoptosis in brain development. Although caspase 3 is essential for the cleavage of DFF45/ICAD in DNA fragmentation, mice that lack DFF45/ICAD are surprisingly viable without defects of brain development[15]. Thus, either DFF45/ICAD is not the key downstream target of caspase 3 or there are redundant pathways for DNA fragmentation in developmental apoptosis. Moreover, a small number of *Casp3* null mice survive to adulthood without obvious defects, even though their offspring show developmental defects (C-Y. Kuan, unpublished observations). These observations indicate other compensatory mechanisms for developmental apoptosis in the absence of caspase 3 that remain to be identified.

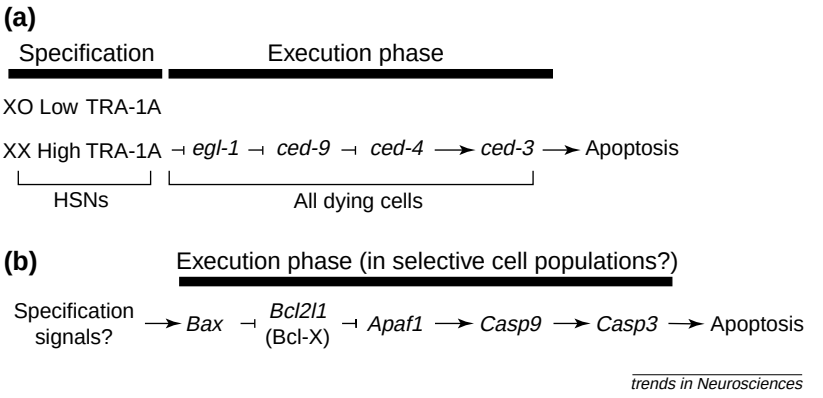

Fig. 1. ***Comparison of the cell-death pathway in*** **Caenorhabditis elegans** ***and in mammals.*** *In* C. elegans **(a)**, egl-1, ced-9, ced-4 *and* ced-3 *form a linear cascade involved in most, if not all, cells that undergo programmed cell death during development (execution phase). The apoptosis versus survival fate of hermaphrodite-specific neurons (HSNs) in a male (XO) or hermaphrodite (XX) worm depends on the levels of the sex-determination protein TRA-1A (specification phase). In mammals* **(b)**, *gene-targeting studies identified* Bax, Bcl2l1 *(*Bcl-X*)*, Apaf1, Casp9 *and* Casp3 *as the key components of the programmed cell death pathway in brain development. However, whether these mammalian homologs constitute an obligate execution cascade for apoptosis in selective or all cell populations is under investigation. Moreover, the specifying signals that trigger brain-region-specific apoptosis in the early embryogenesis remain to be identified.*

Bax and Bcl-X_L are key pro- and anti-apoptotic Bcl2-family proteins in mammalian brain development

The Bcl2 family consists of approximately 15 members and share sequence homology with CED-9 at one or more Bcl2 homology domains (BH1–BH4). The family can be divided into anti-apoptotic [Bcl2, Bcl-X_L (the long isoform encoded by *Bcl2l1*), Bcl-W (also known as Bcl2l2), Mcl1 and A1/Bfl1 (also known as Bcl2a1)] and pro-apoptotic (Bax, Bak, Bok, Bid, Bad, Bcl-X_S, Bim (also known as Bcl2l11), Bik, Blk, Hrk] subgroups[7]. The anti-apoptotic family members all possess BH1 and BH2 domains, and some (Bcl2, Bcl-X_S and Bcl-W (Bcl2l2)] contain additional BH3 and BH4 domains.

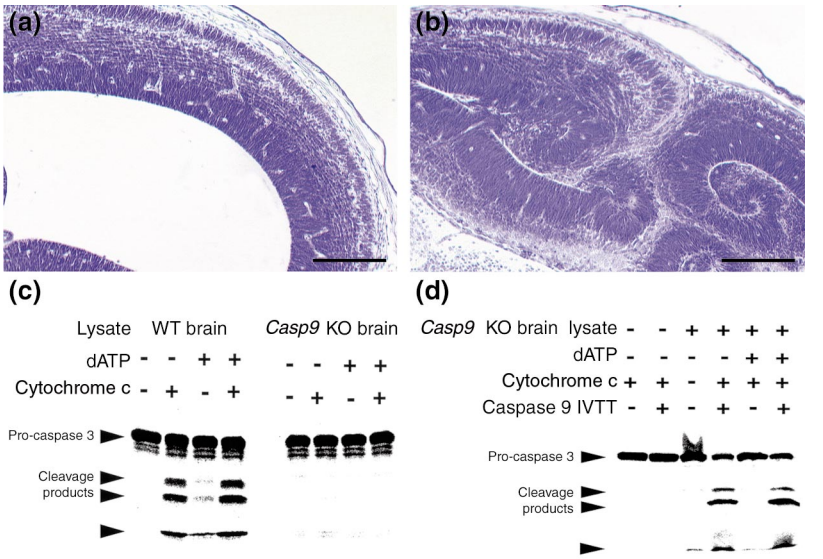

Fig. 2. ***Caspase 9 and caspase 3 form a linear apoptosis cascade in the developing brain.*** *Compared with a wild-type embryo* **(a)**, Casp9 *null mice exhibit increased cortical thickness and multiple indentations in the embryonic brain* **(b)**, *which is similar to the phenotype of* Casp3 *deficiency. Biochemical studies showed that the cytochrome-c-dependent cleavage of pro-caspase 3 is detected using the cytosol extracts from the embryonic* Casp9 *null mouse brain* **(c)**. *The cytochrome-c-dependent cleavage of pro-caspase 3 using lysate from* Casp9 *null mice, however, is restored by the addition of* in vitro *transcribed-translated (IVTT) caspase 9, indicating the specificity of the genetic defects* **(d)**. *Scale bar, 200 μm.* Modified, with permission, from Ref. 12.

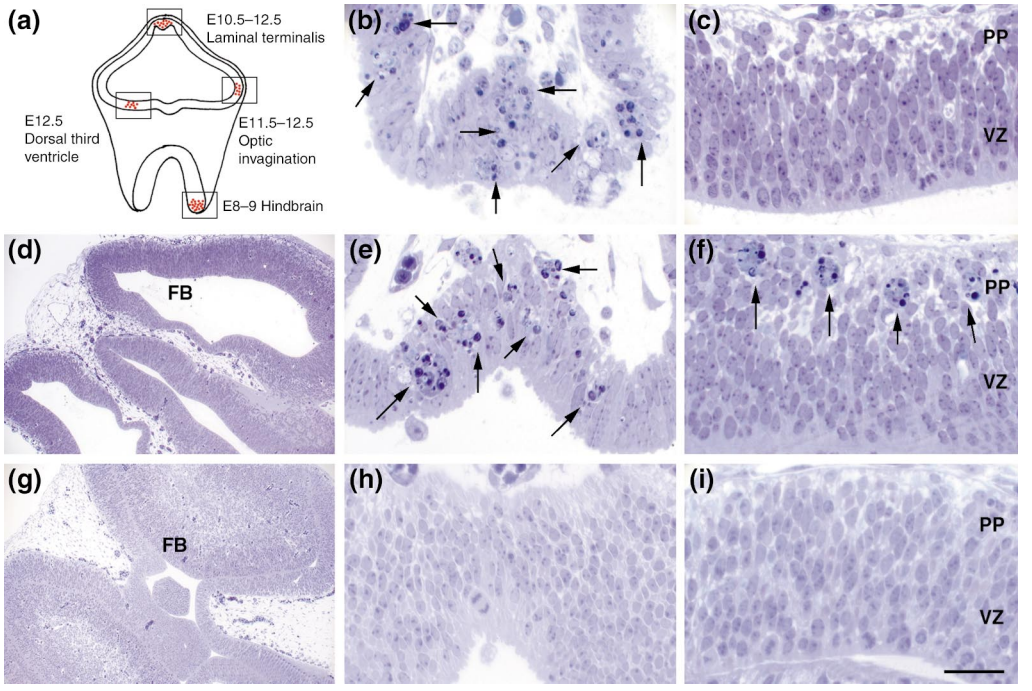

Fig. 3. ***Comparison of the developmental brain apoptosis in wild-type,* Bax*-deficient,* Bcl2l1*-deficient,* Casp3*-deficient and* Bcl2l1/Casp3 *double-mutant embryos.*** *In normal embryogenesis, intense apoptosis occurs in a spatially and temporally precise manner* **(a)**. *For example, clusters of pyknotic cells (indicated by arrows) are found in wild-type embryos in the laminal terminalis* **(b)** *but not in either the postmitotic preplate (PP) or the proliferative ventricular zone (VZ) of the developing cortical wall* **(c)**. *Mice deficient in Bax exhibit an apparently normal forebrain (FB) formation* **(d)** *and retain numerous pyknotic cells in the laminal terminalis* **(e)** *in embryonic (E) day 12. The* Bcl2l1 *deficiency causes ectopic death of postmitotic neurons in the preplate but not in the proliferative ventricular zone in the developing cortex of E12.5 mice* **(f)**. *In contrast to the phenotype of Bax deficiency, mice that are deficient in caspase 3 show severe hyperplasia of the embryonic forebrain* **(g)** *and absence of pyknotic cells in the laminal terminalis* **(h)**. *Moreover, the ectopic pyknotic cell death in the developing cortex caused by* Bcl2l1 (Bcl-X) *deficiency is rescued by the* Bcl2l1 (Bcl-X)/Casp3 *double deficiency* **(i)**. *Scale bar, 1 mm in* (d) *and* (g), *and 125 μm in* (b), (c), (e), (f), (h) *and* (i). Modified, with permission, from Ref. 36.

The pro-apoptotic family members all possess a BH3 domain, which in most cases is essential for their pro-apoptotic effect, but can be subdivided based on the presence (Bax, Bak and Bok) or absence (Bid, Bad, Bcl-X_L Bik, Blk and Hrk) of BH1 and BH2 domains. The 'BH3 only' subset is homologous to the recently described pro-apoptotic EGL-1 in *C. elegans*. Interactions between pro- and anti-apoptotic Bcl2-family members appear to establish the baseline sensitivity to apoptosis. If anti-apoptotic members predominate, sensitivity to apoptotic stimuli is low; the opposite is true when pro-apoptotic molecules are in excess. Although Bcl2 family members affect a variety of intracellular processes, increasing evidence suggests that regulation of the release of cytochrome c from mitochondria has an important role in its effects on apoptosis. Bax, Bak and Bid are known cytochrome-c-releasing factors, whereas Bcl2 and Bcl-X_L can block cytochrome-c redistribution[16–18].

Of the anti-apoptotic *Bcl2* gene family, only *Bcl2l1* (Bcl-X) disruption has been reported to produce a dramatic neurodevelopmental phenotype[19]. *Bcl2l1* (Bcl-X) can undergo alternative splicing to produce two major protein isoforms, Bcl-X_L and Bcl-X_S (Ref. 20). Bcl-X_L inhibits apoptosis, whereas Bcl-X_S is pro-apoptotic because of its ability to antagonize the actions of Bcl2 and Bcl-X_L. In the mouse, Bcl-X_L is the predominant transcript and is found at high levels in both embryonic and adult brain[21]. In the developing brain, little Bcl-X_L immunoreactivity is detected in neural precursor cells located in the ventricular zone but it is present at high levels in immature neurons in the intermediate and marginal zones[19]. Targeted disruption of *Bcl2l1* (Bcl-X) causes a dramatic increase in apoptosis of immature neurons throughout the embryonic nervous system but fails to affect apoptosis of neural precursor cells in the ventricular zone. In addition, Bcl-X_L-deficient embryos die at approximately E13.5, secondary to increased hematopoietic apoptosis. Targeted disruption of *Bcl2* affects only programmed cell death in specific neuronal subpopulations during embryogenesis and after the period of naturally occurring cell death[22,23]. Thus, Bcl2 might complement Bcl-X_L in promoting the survival of this subpopulation. Consistent with this idea, neurons that lack both Bcl-X_L and Bcl2 are more susceptible to apoptosis *in vitro* than those that lack Bcl-X_L or Bcl2 alone[24]. Similarly, Bcl-X_L/Bcl2 double deficient embryos show increased immature neuron apoptosis compared with embryos that lack only Bcl-X_L. Whether Bcl2l2 (Bcl-W) or Bcl2a1 (Bfl1/A1) or both (the targeted disruptions of which have no effect on neuronal programmed cell death) have a similar role in neural development is unclear.

Just as Bcl-X_L appears to be the key anti-apoptotic Bcl2-family member that regulates neuronal programmed cell death, Bax has emerged as the crucial pro-apoptotic family member during nervous system development. Targeted disruption of *Bax* dramatically decreases programmed cell death in the developing nervous system, which results in increased numbers of neurons in selected neuronal populations[25,26]. Bax-deficient neurons show decreased susceptibility to trophic-factor withdrawal both *in vivo* and *in vitro*. Bax is capable of forming heterodimers with Bcl-X_L *in vitro*, and thus Bax and Bcl-X_L might interact to regulate neuron survival[27,28]. Indeed, the generation of Bax/Bcl-X_L double deficient embryos demonstrated that Bax deficiency could prevent the increased apoptosis of Bcl-X_L-deficient neurons both *in vivo* and *in vitro*[29]. Thus, the relative levels and interaction between Bax and Bcl-X_L appear to determine neuronal susceptibility to apoptosis during development.

Although several other pro-apoptotic Bcl2-family members including Bid, Bad, Bak, Hrk and Bok are produced during nervous-system development, their role in regulating neuronal cell death is as yet unclear. We have examined both Bid-deficient and Bad-deficient embryonic nervous systems and have detected no alterations in caspase-3 activation and programmed cell death *in vivo* or *in vitro*. The other pro-apoptotic members can affect neuronal apoptosis indirectly through their interaction with Bax or be found to affect specific subpopulations of neurons.

Apaf1, mammalian homolog of *ced-4*, links the functions of Bcl2-family proteins and caspases

The important role of CED-4 in mediating programmed cell death in *C. elegans* has been known for many years; however, only recently have mammalian (Apaf1) and *Drosophila* (Dark) homologs been described[6,30]. Apaf1 promotes caspase-3 activation though its effects on pro-caspase 9 via formation of a multiprotein complex termed an apoptosome[14,31]. In addition, several Apaf1 isoforms and additional mammalian CED-4-like molecules have been identified and further investigations are required to determine their possible roles in apoptosis[32,33].

Several investigators have suggested that Bcl2-family members might affect apoptosis through a direct interaction with Apaf1, as has been demonstrated for CED-9 and CED-4 in *C. elegans*. However, others have not found a direct physical interaction between Apaf1 and Bcl2-family members in mammalian cells. Regardless of the mechanisms, the significance of Apaf1 in neuronal programmed cell death has been amply demonstrated in mice with mutated *Apaf1*. Insertional mutagenesis or targeted disruption of *Apaf1* produced perinatal lethality that was associated with marked developmental abnormalities[34,35]. Apaf1-deficient mice exhibit craniofacial abnormalities, alterations in the eye and retina, and a variety of brain abnormalities, including exencephaly, hyperplasia and ectopic neural masses. These abnormalities appear to result from decreased programmed cell death, shown by a marked reduction in terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelled (TUNEL)-positive cells and histopathologically apoptotic cells. Apaf1-deficient cells fail to activate caspase 3 *in vivo* or *in vitro*, which presumably accounts for the similar developmental abnormalities seen in Apaf1-, caspase-9- and caspase-3-deficient embryos.

Caspase 3 and Bcl-X_L have both independent and epistatic apoptotic functions

The above-mentioned gene-targeting studies thus identify Bax, Bcl-X_L, Apaf 1, caspase 9 and caspase 3 as key regulators of programmed cell death in neural development. Moreover, on the basis of the sequence homology and analogous functions, Bax, Bcl-X_L, Apaf1, caspase 9 and caspase 3 might form an evolutionary-conserved cell-death pathway in the mammalian nervous system (Fig. 1b). Consistent with this hypothesis, it has been shown that *Bax* deficiency prevented increased immature neuron death caused by the *Bcl2l1* (Bcl-X) mutation[28]. In addition, null mutations of either *Apaf1* or *Casp9* disrupted the activation of caspase 3 *in vivo*[12,34]. Most recently, genetic studies were conducted to test whether the anti-apoptotic function of Bcl-X_L is mediated specifically through inhibition of the pro-apoptotic effects of caspase 3, as predicted by an epistatic relationship of these two molecules, which are similar to their counterparts in *C. elegans*[36].

In normal development, clusters of pyknotic cells are confined to specific locations of the nervous system at precise times of development (Fig. 3a). This brain-region-specific apoptosis presumably affects neuronal progenitor cells given its occurrence during the period of active neurogenesis. Except for these brain-region-specific cell deaths, only a few pyknotic cells are sparsely distributed in the rest of the developing nervous system (Fig. 3b,c). By contrast, *Bcl2l1* (Bcl-X) deficiency causes widespread pyknotic clusters in the postmitotic population, in addition to the region-specific cell death in the developing brain (Fig. 3f). When *Bcl2l1* (Bcl-X) mutant mice are crossed wirh the *Casp3* deficiency genetic background, the double mutation virtually abrogates the ectopic cell death caused by the *Bcl2l1* (Bcl-X) deficiency alone (Fig. 3i). Similarly, the concomitant *Casp3* deficiency prevents the increased apoptosis of *Bcl2l1* (Bcl-X)-deficient cortical neurons in response to serum deprivation *in vitro*[36]. These results suggest that *Bcl2l1* (Bcl-X) deficiency causes apoptosis of postmitotic neurons primarily through uninhibited activation of caspase 3. However, the epistatic relationship is clearly not universal, as *Casp3* deficiency does not prevent increased hematopoietic cell apoptosis and embryonic lethality seen in *Bcl2l1* (Bcl-X)-deficient mice, suggesting a caspase-3-independent apoptotic pathway that is normally suppressed by Bcl-X_L during development of the hematopoietic system.

If Bax and caspase 3 are both pro-apoptotic in an obligate, epistatic cell-death pathway, the phenotype of *Bax* deficiency should be identical to that of *Casp3* deficiency. On the contrary, there are significant differences between the phenotypes of *Bax*- and *Casp3*-deficient embryos. There are no signs of hyperplasia or malformations of the nervous system, and the apoptosis of neuronal progenitor cells, which is exemplified by brain-region-specific apoptosis, is preserved in the *Bax*-deficient embryos (Fig. 3d,e). By contrast, the *Casp3* deficiency greatly reduces the brain-region-specific apoptosis and shows marked hyperplasia of the embryonic nervous tissue (Fig. 3g,h). These results indicate that, though downstream of Bax and Bcl-X_L in the apoptosis of postmitotic neurons, caspase 3 has a unique function in regulating the size of the progenitor pool during early development, even before neurogenesis in a given region begins.

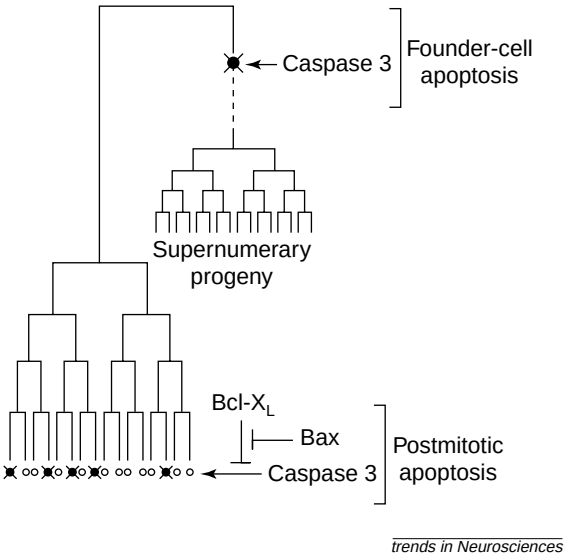

Fig. 4. ***Distinct molecular mechanism of apoptosis of the neuronal founder cells and postmitotic neurons in the developing mammalian nervous system.*** *Gene-targeting studies and epistatic genetic analysis indicate that Bax, Bcl-X_L and caspase 3 form an obligate cell-death pathway in the postmitotic neurons. By contrast, neither Bax nor Bcl-X_L is involved in the apoptosis of neuronal founder cells, which is greatly reduced by the caspase-3 deficiency, resulting in the generation of supernumerary progeny and severe brain malformations as a consequence. Crossed circles indicate naturally occurring programmed cell death in brain development.* Modified, with permission, from Ref. 36.

Box 2. The magnitude of programmed cell death in neural development

Despite general agreement on the occurrence of programmed cell death in neural development, the estimates of the magnitude of cell death vary widely. Cell death was initially detected by classical histological stains but in recent years the understanding of the molecular mechanism of apoptosis inspired several new, sensitive methods for detecting programmed cell death. One important breakthrough was the discovery of endogenous endonuclease activity during apoptosis leading to DNA fragmentation and formation of a characteristic 'ladder' pattern in agarose gels[a]. On the basis of this observation, a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method was developed for *in situ* detection of apoptosis at the single-cell level[b]. Using the TUNEL method, Thomaidou *et al.* reported a death rate of 0.3–1.7% of cells in the proliferative zone and even fewer dying cells in the developing cortical plate in embryonic-day (E) 14 rat embryos[c]. As caspase 3 is the predominant apoptotic caspase executioner in the nervous system, cell death can be detected using an antibody that recognizes the cleaved fragment but not the inactive proenzyme form of caspase 3 (Ref. d). A relatively small number of apoptotic cells were detected in the developing mouse brain by this method, which, in addition, revealed intense capsase-3 activation associated with a high density of TUNEL-positive and toluidine-blue stained pyknotic cells at sites of known morphogenetic cell death[e]. Moreover, apoptotic cells could be detected by the Ca^{2+}-dependent, phospholipid-binding protein annexin V, which labels the phosphatidylserine residues exposed on the cell surface in the early phase of apoptosis[f]. *In vivo* infusion of biotin-conjugated annexin V into E9–E14 mouse embryos also revealed extensive labeling at sites of morphogenetic cell death but only a few dispersed cells in the developing telencephalon[g].

In contrast to these findings, up to 70% of cells in the E14 mouse embryonic cerebral cortex were reported to undergo apoptosis using a variation of the TUNEL method called *in situ* end-labeling plus (ISEL+)[h]. Such a high incidence of developmental programmed cell death, if proven accurate, would challenge many assumptions and conclusions of previous cortical neurogenesis studies. As TUNEL and traditional histological methods have never revealed such extensive cell death, the higher incidence of cell death detected by the ISEL+ method cannot be explained by a long clearance time of apoptotic corpses. Therefore, either the ISEL+ method, which employs TdT to label DNA breaks with digoxigenin-conjugated dUTP, is far more sensitive in detecting the early phase of DNA breaks in apoptosis or it gives 'false-positive' results, indicating transient DNA breaks in cells not committed to apoptosis. In this regard, the recent discovery of massive neuronal apoptosis in mice that lack XRCC4, a ligase needed for end-joining of the double-stranded DNA breaks that typically occurs in V(D)J recombination in the immune system, is particularly intriguing[i]. On the basis of this finding, it was suggested that the extensive neuronal 'apoptosis' revealed by the ISEL+ method reflects a selection process that sorts those cells making advantageous recombination products (survive) from those that do not (undergo apoptosis)[j]. Alternatively, the highly sensitive ISEL+ method might label both apoptotic cells and cells with transient DNA breaks, which are subsequently end-joined by the XRCC4 ligase in normal development, resulting in an over-estimation of programmed cell death. This possibility can be determined only when advances in techniques permit a pulse labeling of ISEL+ cells and a prospective analysis of their fates.

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Apoptosis of neuronal progenitor cells affects mammalian brain formation

On the basis of the phenotype of individual mutants and epistatic genetic analysis, a scheme is proposed to explain the interactions of Bax, Bcl-X_L and caspase 3 during mammalian brain development (Fig. 4). Bcl-X_L inhibits the pro-apoptotic effect of caspase 3 in the postmitotic neuronal population, and therefore *Bcl2l1* (Bcl-X) deficiency causes increased apoptosis of postmitotic neurons, which is prevented by the additional absence of *Casp3*. Bax modulates the anti-apoptotic effects of Bcl-X_L; the null mutation of *Bax* therefore reduces the normally occurring developmental death of postmitotic neurons without affecting the global formation of the nervous system. The unique feature of caspase 3 in this scheme is its dual function in both postmitotic and neuronal progenitor apoptosis. Although *Casp3* deficiency results in decreased apoptosis of postmitotic neurons in the developing cortex, given the normal brain organization in *Bax*-deficient mice, this effect is insufficient to cause gross malformations (see Box 2 for the magnitude of programmed cell death in neural development). Rather, caspase-3 deficiency rescues a number of progenitor cells from programmed cell death, which results in an exponential expansion of the progeny, ultimately leading to marked dysplasia and malformations of the nervous system.

The identification of caspases as regulators of founder-cell numbers in the neural tube has important implications for the development and evolution of the mammalian forebrain. Telencephalic expansion during mammalian evolution resulted from massive enlargement and complex morphogenesis of the forebrain portion of the neural tube, which is visible even before the onset of postmitotic neuron generation. Although neurogenesis is the main engine for this telencephalic expansion, the increased forebrain size in caspase-3- and caspase-9-deficient embryos suggests that programmed cell death might participate in determining the production of specific progenitor populations while sparing others[37,38]. Therefore, the precise coordination of proliferation and differential apoptosis mediated by caspases during early neurogenesis is crucial for the proper regulation of cortical size and shape in the mammalian brain.

Concluding remarks

The genetic studies of programmed cell death in the nematode *C. elegans* have provided new approaches to study the mechanism of apoptosis in mammalian

neural development. A cumulative body of evidence, as reviewed in this article, indicates that mammalian homologs of the cell-death genes in *C. elegans* have analogous functions in apoptosis and form a similar epistatic pathway in brain development. Moreover, these studies also implicate casapse 3, presumably caspase 9 and Apaf1 as well, in the apoptosis of neuronal progenitor cells, a function that is distinct from the classical role of programmed cell death in matching postmitotic neuronal population with postsynaptic targets. These findings, provide new insights to the biological functions of programmed cell death in brain development and raise some intriguing questions. As the Bcl2-family proteins Bax and Bcl-X_L are not involved in the caspase-3-mediated early progenitor cell death, how is the early brain-region-specific apoptosis regulated so precisely, especially given the ubiquitous presence of caspases throughout the nervous system? In theory, there could be novel signal-transduction mechanisms to trigger apoptosis at specific locations or general cytoprotective mechanism to prevent excessive activation of caspases in the rest of the nervous system. The identification of the cytoprotective mechanism and brain-region-specific apoptotic signaling inductions raises new challenges in developmental biology. Moreover, it remains to be seen whether unidentified new members of the Bcl2 family that are homologous to *ced-9* and *egl-1* in *C. elegans* are integrated in these apoptotic and cytoprotective signal-transduction mechanisms.

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