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In vivo cellular and molecular mechanisms of neuronal apoptosis in the mammalian CNS

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Abstract

Apoptosis has been recognized to be an essential process during neural development. It is generally assumed that about half of the neurons produced during neurogenesis die before completion of the central nervous system (CNS) maturation, and this process affects nearly all classes of neurons. In this review, we discuss the experimental data in vivo on naturally occurring neuronal death in normal, transgenic and mutant animals, with special attention to the cerebellum as a study model. The emerging picture is that of a dual wave of apoptotic cell death affecting central neurons at different stages of their life. The first wave consists of an early neuronal death of proliferating precursors and young postmitotic neuroblasts, and appears to be closely linked to cell cycle regulation. The second wave affects postmitotic neurons at later stages, and is much better understood in functional terms, mainly on the basis of the neurotrophic concept in its broader definition. The molecular machinery of late apoptotic death of postmitotic neurons more commonly follows the mitochondrial pathway of intracellular signal transduction, but the death receptor pathway may also be involved.

Undoubtedly, analysis of naturally occurring neuronal death (NOND) in vivo will offer a basis for parallel and future studies aiming to elucidate the mechanisms of pathologic neuronal loss occurring as the result of conditions such as neurodegenerative disorders, trauma or ischemia.

Contents

Abbreviations: AD, Alzheimer disease; ADP, adenosine diphosphate; AIF, apoptosis-inducing factor; Apaf, apoptosis protease activated factor; ATM, ataxia-teleangectasia gene; *ax*, ataxia mutation; BCL-2, B-cell lymphoma-2; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2′-deoxyuridine; Cdc, cell division cycle; CDK, cyclin-dependent kinase; CGC, cerebellar granule cell; Chk, checkpoint kinase; CKI, cyclin-dependent kinase inhibitor; CNS, central nervous system; CPP32, 32 kDa cysteine protease; DNA-PK, DNA-dependent protein kinase; DRG, dorsal root ganglion; E, embryonic day; E2F, early gene 2 factor; EGL, external granular layer; FADD, Fas-associated death domain; Fas, Apo-1/CD95; Fas-L, Fas ligand; FGF-2, fibroblast growth factor-2; *fh*, flathead mutation; GCL, ganglion cell layer; GCP, granule cell precursor; GRID2, δ2 glutamate receptor; HIV-1, human immunodeficiency virus-1; *hq*, harlequin mutation; IAP, inhibitor of apoptosis; ICE, interleukin-1β-converting enzyme; ICH, *ice* and *ced-3* homologue; IdU, 5-iodo-2′-deoxyuridine; IGF-I, insulin-like growth factor-I; IGL, internal granular layer; ISEL, in situ end labeling; JNK, Jun amino-terminal kinase; *lc*, lurcher mutation; MAPK, mitogen-activated protein kinase; ML, molecular layer; MPT, mitochondrial permeability transition; NGF, nerve growth factor; NO, nitric oxide; NOND, naturally occurring neuronal death; NOS, nitric oxide synthase; NT, neurotrophin; ORN, olfactory receptor neuron; P, postnatal day; $p75^{NTR}$, p75 neurotrophin receptor; PARP-1, poly(ADP-ribose) polymerase-1; PC, phaeochromocytoma; PCD, programmed cell death; PCL, Purkinje cell layer; PCNA, proliferating cell nuclear antigen; PI3K, phosphoinositide 3-kinase; PKB/Akt, protein kinase B; PNS, peripheral nervous system; PT, permeability transition; Rb, retinoblastoma protein; RGC, retinal ganglion cell; SVZ, subventricular zone; TdT, terminal deoxynucleotidyl transferase; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRAF, tumor necrosis factor receptor-associated factor; Trk, tyrosine kinase neurotrophin receptor; TUNEL, TdT-mediated dUTP nick end labeling; VZ, ventricular zone; *wb*, wobbler mutation

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1. Introduction

Programmed cell death (PCD) is a highly phylogenetically conserved mechanism by which eukaryotic cells die following a stereotyped series of molecular and cellular events commonly referred to as apoptosis (Glucksmann, 1951; Saunders, 1966). Apoptosis has been recognized to be an essential process during development where it appears to be fundamental for the control of the final numbers of neurons (and glial cells) in the central nervous system (CNS) and peripheral nervous system (PNS). Moreover, a growing body of evidence is accumulating to indicate that apoptosis is also responsible for the loss of neurons associated with physiological aging (Taglialatela et al., 1996; Kaufmann et al., 2001).

It is generally assumed that about half of the neurons produced during neurogenesis die before completion of the CNS maturation, and nearly all classes of neurons are produced in excess during development. These oversized populations of neurons are then significantly reduced during the periods of naturally occurring neuronal death (NOND), mainly upon activation of the apoptotic machinery. The neurotrophic theory has clearly established that neurons compete for limited amounts of target-derived trophic factors that have a protective role against apoptosis, and has provided a fundamental conceptual basis for a wide number of studies aiming to elucidate the mechanisms of neuron-to-target interaction eventually leading to cell survival or death. The initial concept of neurotrophic factor based on this theory has now broadened to embrace a large and molecularly heterogeneous group of surviving factors, and parallel studies have disclosed the role of electrical activity in survival during the wiring of neural networks.

Still, the general idea beyond this expanded concept is that NOND is closely related to the establishment of proper connections with targets, and under this perspective little or no attention has been dedicated to apoptosis of neural precursors and/or young neuroblasts at early developmental stages, which seems to be independent from synaptogenesis. In several areas of the brain, including the retina and the cerebellum, it appears that two subsequent periods of cell death can be observed: the first mainly occurs at the onset of neurogenesis and is not apparently related to synapse formation, while the second is linked to the wiring of young postmitotic neurons. In both periods, cell death is apoptotic (Bähr, 2000; De la Rosa and De Pablo, 2000; Lossi et al., 2002c). Why so many neurons are generated in excess and eliminated shortly thereafter remains to be understood, having also in mind that several lines of transgenic animals, in which essential cell death genes have been knocked out and/or apoptosis inhibitors have been overexpressed, display no obvious or detectable CNS defects.

In neurodegenerative disorders and traumatic neuronal injury, neuronal loss may be linked to apoptosis. However, in these conditions cell death may also be necrotic. In the latter (exogenous cell death), alterations of the cellular environment eventually result in cell swelling and disruption of the cell membrane, while the main elements of the apoptotic machinery are constitutively expressed or generated by the cell itself (cell suicide). Although the two types of cell death have initially been considered independent from each other, it is now clearly emerging that they share some cellular and molecular features, and that cells can switch from one mode of death to the other upon different conditions.

A tremendous amount of literature in the recent years has deepened our knowledge on the cellular and molecular mechanisms of apoptosis in neurons and other cells. Current knowledge about the genetic regulation of apoptosis is mainly based on studies of the nematode worm *Caenorhabditis elegans*. These studies have been extensively reviewed in numerous publications (Hengartner and Horvitz, 1994b; Hoffman and Liebermann, 1994; Stewart, 1994; Vaux et al., 1994; Yuan, 1995; Fraser et al., 1996; White, 1996; Meier and Evan, 1998; Liu and Hengartner, 1999). They will be only briefly mentioned here to put things under the right perspective. Also, the characterization of apoptosis in vertebrate

(mammalian) neurons mainly relies on in vitro studies, and readers are again invited to consult the existing reviews on this issue (Rao and White, 1997; Selimi et al., 1997; Sastry and Rao, 2000; Denecker et al., 2001; Shastry et al., 2001).

Our present work summarizes the literature on apoptosis of mammalian central neurons in vivo, with a particular attention for NOND in the postnatal cerebellum, since this is our major field of interest. In doing so we will also consider with attention the relationship of apoptosis and cell proliferation in early developmental death of neural precursors and/or young neuroblasts, and the data available on transgenic and mutant animals.

1.1. Morphological types of cell death

The issue of morphological diversity in developmental cell death has been reviewed in detail (Clarke, 1990). In an ultrastructural study on several embryonic tissues it was proposed that there are three main types of cell death during normal development, on the basis of the role of lysosomes in cell disruption (Schweichel and Merker, 1973). In the first type, cell death occurs without any detectable activation of endogenous lysosomes, but cells are eventually destroyed by phagocytosis and secondary lysosome activation by tissue macrophages. This process has also been referred to as heterophagocytosis. In the second type of cell death (autophagocytosis) cells are eliminated after activation of their own lysosomal enzymes. In the third type, there is no obvious lysosome intervention. The first two types are by far more common and have been described by various authors starting from the 1960s. The ultrastructural features of type 1 cell death in Schweichel and Merker classification correspond to the current definition of apoptosis, while at least a variant of type 3 shares several features with necrosis (Clarke, 1990).

1.1.1. Apoptosis

Apoptosis was originally defined as a distinct mode of cell death on the basis of a series of characteristic ultrastructural features (Fig. 1E and F) according to the following sequence of events: nuclear and cytoplasmic condensation, cell fragmentation and phagocytosis (Kerr et al., 1972). Initially Kerr et al. used the term “shrinkage necrosis” to describe this form of cell death. Subsequently they coined the term “apoptosis” (from the Greek = falling of the leaves), which indicates the dropping of leaves from trees or petals from flowers, to emphasize the role of this type of cell death in normal tissue turnover.

Apoptosis involves a series of stereotyped, morphologically well defined phases, that are most clearly evident at the electron microscope level.[1]

Changes in the nucleus represent the first unequivocal evidence of apoptosis (Fig. 1E). Chromatin condensation and segregation into sharply delineated masses that abut on the nuclear envelope are typically observed at the onset of apoptosis. Chromatin masses are very electrondense and often show a characteristic crescent-like appearance. High magnification electron micrographs reveal that these masses are made up of closely packed, fine granular material.

This initial condensation eventually leads to true nuclear pyknosis. In parallel with nuclear changes, cytoplasm condensation also occurs, and the cell membrane becomes convoluted with the onset of protuberances of various sizes that may give the cell a star-like appearance. As the cytoplasm density increases, some vacuoles may become evident, but the cell organelles remain unaffected, although they become abnormally closely packed, likely as a consequence of the loss of cytosol. However, ribosomes can be detached from the rough endoplasmic reticulum and from polysomes, and these latter eventually disappear. As the process continues, the cell and its nucleus assume a more irregular shape and nuclear budding occurs to produce discrete fragments, still surrounded by an intact nuclear envelope. Eventually, the cell is fragmented into membrane-bounded apoptotic bodies which still display a sharp segregation of condensed chromatin in nuclear fragments and well preserved organelles. Apoptotic bodies are rapidly cleared out in tissues by macrophages or neighboring cells, and are degraded within heterophagosomes (Fig. 1F).

We have recently described the clearance of apoptotic cells during NOND in the postnatal rabbit cerebellum in vivo (Lossi et al., 2002c) after labeling proliferating cells with a modified “window-labeling” technique (Belecky-Adams et al., 1996) to follow their fate. We have thus observed that the whole apoptotic cerebellar granule cells (CGCs) are phagocytosed by dark (medium to highly electrondense) glial elements, before being fragmented into apoptotic bodies. These glial elements likely corresponded to microglia. At early stages of apoptosis, CGCs in the external granular layer (EGL) were often seen to be contacted by glia, that bent around them and became engulfed with the entire apoptotic cell. Cells with the typical features of late apoptosis were more easily observed in the internal granular layer (IGL) and commonly fragmented into several apoptotic bodies inside the heterophagosomes. It was rather easy to spot some of these phagocytic cells engulfed with apoptotic material in close apposition to blood capillaries, and indeed we have provided ultrastructural evidence for the presence of intraluminal blood monocytes engulfed with heterophagosomes. These latter contained highly condensed nuclear DNA pre-labeled in vivo with a S-phase marker 24 h before. Since proliferation of the CGCs only occurs in the EGL, our data demonstrated that in the limited span of time between tracer administration and sacrifice some CGCs completed their division, entered the apoptotic program and were cleared by the glia. This observation gives an unequivocal demonstration of the remarkable speed by which apoptotic cells are removed from the intact nervous tissue.

[1] The morphology of physiological cell death in invertebrates somehow differs from the characteristic repetitive pattern in mammals (Harmon et al., 1998).

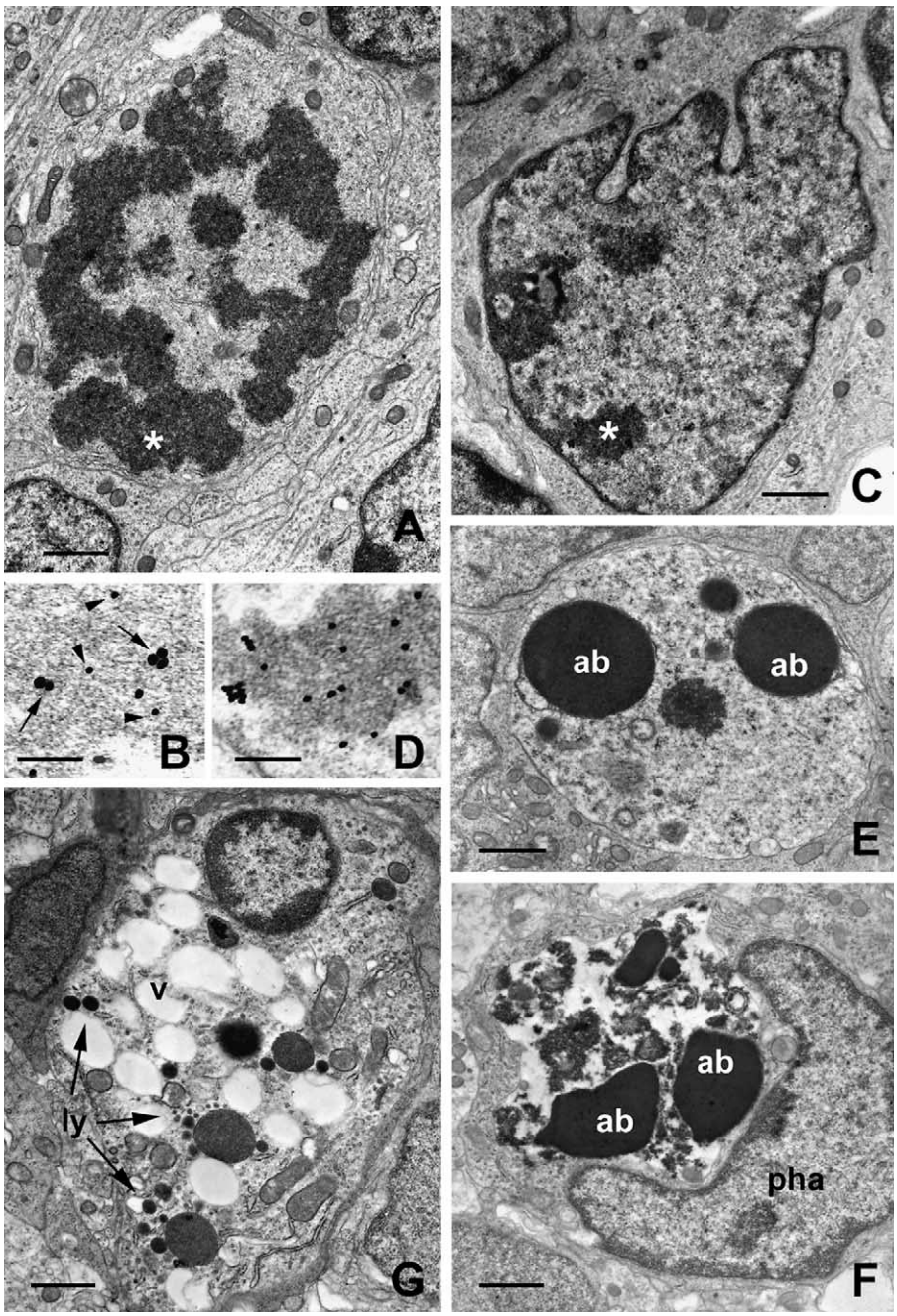

Fig. 1. Ultrastructural visualization of proliferating and apoptotic cells in the postnatal rabbit cerebellum. Proliferating cells were labeled in vivo with the S-phase markers BrdU and IdU and observed in the electron microscope following immunogold staining procedures (see also Fig. 3A). (A) Mitosis of a progenitor cell in the outer EGL. The area marked by the asterisk is shown at higher magnification in (B). (B) Chromosome labeling by BrdU (large gold particles—arrowheads) and IdU (small gold particles—arrows). (C) Precursor cell in the outer EGL. The area marked by the asterisk is shown at higher magnification in (D). (D) Intense chromatin labeling with gold particles indicative of BrdU incorporation. (E) A typical aspect of mid apoptotic cell death in the inner EGL. The nucleus is fragmented in apoptotic bodies (ab). (F) A phagocyte (pha) in the IGL is engulfed by cellular debris consisting of nuclear apoptotic bodies (ab) and highly condensed cytoplasm fragments. (G) Unconventional aspect of autophagic cell death in the EGL. The dying cell shows a normal nuclear structure and numerous vacuoli (v) in the cytoplasm, in close apposition with clusters of lysosomes (ly). Scale bars: (A, C and E–G) 0.5 μm; (B and D) 0.2 μm.

1.1.2. Necrosis

Necrosis (from the Greek = degeneration of the corpse) indicates the death of a cell or group of cells usually as a result of injury, disease or pathological state. Therefore, necrosis is traditionally associated with one or more exogenous factors leading to non physiological cell death. This latter may be divided into two main stages: the *death* of the cell that can be defined as the irreversible alteration of the cellular mechanisms that enable the cell to maintain its homeostasis, and the *subsequent degeneration* of the dead

cell (Clarke, 1990). In general terms, necrosis involves large numbers of cells which are often grouped together and is associated with an inflammatory reaction. In the irreversibly injured cells, the morphological changes characteristic of necrosis consist in clumping of the chromatin without marked changes in its distribution: usually chromatin condenses in multiple small clumps with irregular outlines, and is poorly demarcated from the surrounding nucleoplasm. Sometimes densities in the matrix of abnormally swollen mitochondria, and local membrane disruption become evident. At later stages, there is a more or less pronounced disintegration of cell organelles and membranes, although the cell somehow maintains an overall identity. The chromatin disappears at the end of the process, leaving only "ghost-like" cell residues. Usually, groups of several cells undergo necrosis in tissues, with a marked inflammatory response that eventually leads to the removal of the necrotic debris by cells of the mononuclear phagocytic system.

1.1.3. The dichotomy of apoptosis versus necrosis

Starting from pathology, but nowadays also in the broader field of cellular and developmental neurobiology, the term apoptosis rapidly caught on, in particular to emphasize the normal occurrence of this type of cell death in contrast to the association of necrosis with pathological insults (Migheli et al., 1994; Stewart, 1994; White, 1996; Marks and Berg, 1999). This dichotomy of apoptosis versus necrosis has been originally based upon the morphological differences between the two modalities of cell death, although some authors started to question such a sharp morphological distinction already at the beginning of the nineties (Clarke, 1990). In more recent times, it has become clear that, in mammalian cells, the gap between classical apoptosis and necrosis is filled by many intermediate morphological types, in which blebbing may be more or less evident and varying degree of chromatin condensation and margination may be apparent (Leist and Jaattela, 2001). In parallel, with the more and more in depth dissection of the cellular and molecular mechanisms of apoptosis (see Section 1.2) it became clear that specific cellular pathways are activated in apoptosis leading to an enforcement of the concept that, in this type of death, cells are responsible of their own demise, a reason for which apoptosis is commonly referred to as a "cell suicide". However, in more recent times this axiomatic association of apoptosis and physiological cell death turned out to be an oversimplification for several reasons. First, the intrinsically necessary elimination of specific cell populations during development[2] of multicellular organisms is often, but not always characterized by an apoptotic morphology (Schwartz et al., 1993; Leist and Jaattela, 2001). Second, apoptosis besides being relevant to an array of physiological functions (that in addition to development, comprise the differentiation and maturation of various types of cells, and several functions of the immune system), is involved with cell injury induced by a spectrum of physical and chemical agents (Boobis et al., 1990; Stewart, 1994; Ortiz et al., 2001; Yakovlev and Faden, 2001; Dainiak, 2002). Third, apoptosis is concerned with oncogenesis, tumor homeostasis, and the action of cytotoxic drugs employed in chemotherapy (Hoffman and Liebermann, 1994; Stewart, 1994; Mimeault, 2002; Singh et al., 2002). Fourth, more and more evidence is accumulating to show a role of apoptosis in several neurodegenerative disease, including Alzheimer disease (AD) and aging (Marks and Berg, 1999; Sastry and Rao, 2000), although it has been questioned whether during aging of human brain there is cell loss at all (Morrison and Hof, 1997).

In addition, a more accurate analysis of the cellular mechanisms of apoptosis suggested that at least some executioners of apoptotic and non apoptotic cell deaths may be identical (Benchoua et al., 2001; Moroni et al., 2001; Smith, 2001; Cole and Perez-Polo, 2002; Fujikawa et al., 2002; Hou and MacManus, 2002; Schwab et al., 2002).

Therefore, as the investigation of cell death in different systems proceeds, we find more and more variations to the classical concept of apoptosis versus necrosis (Lockshin and Zakeri, 2002).

1.1.4. Autophagic cell death

As already mentioned, apoptosis and necrosis are not the sole modes of cell death (Clarke, 1990). Since the discovery of caspases (see below), the most widely studied caspase-independent cell deaths were those of the autophagic type (Lockshin and Zakeri, 2002). However, long before caspases were discovered, the role of lysosomes in cell death has been extensively investigated (Clarke, 1990; Zakeri et al., 1995; Clarke et al., 1998).

The term autophagy (from the Greek = self-eating) refers to a type of cell death in which the cytoplasm is actively destroyed by lysosomal enzymes well in advance before nuclear changes become visible. The most characteristic features are the appearance of large autophagic vacuoles of lysosomal origin in the cytoplasm (Fig. 1G). In these cells, although many of the characteristic changes of apoptosis eventually become evident, they are notably delayed, and substantial cellular degradation is evident, before the typical nuclear alterations of apoptosis occur. Finally, when about three quarters of the cytoplasm has been destroyed, it begins to condense, and chromatin coalescence and margination become apparent. In parallel, agarose gel electrophoresis reveals the appearance of DNA oligomer fragmentation,

[2] This process is currently referred to as programmed cell death (PCD), and for the nervous system the term naturally occurring neuronal death (NOND) is also in use. The term PCD is often used as a synonym of apoptosis. However, for the reasons explained in the text apoptosis may be only one but several forms of cell death occurring in PCD (Alles et al., 1991; Cohen et al., 1992; Eastman, 1993). Therefore, we will use here the term PCD to indicate physiological cell death in a broad sense and not as a synonym of apoptosis. NOND will be specifically used to indicate the physiological occurrence of neuronal death in the nervous system.

and the remnants of the cell are phagocytosed as in classical apoptosis (Zakeri and Lockshin, 2002).

1.2. Cellular and molecular mechanisms of apoptosis

1.2.1. The machinery and regulators of apoptosis: lessons from the worms

Our current knowledge on the gene regulation of apoptosis in nerve cells (and other cell types) is mainly derived from studies on the nematode worm *C. elegans* (Yuan, 1995; Meier and Evan, 1998; Liu and Hengartner, 1999; Hengartner, 2001). In *C. elegans*, 131 out of 1090 somatic cells undergo developmental apoptosis. Four genes named *ced-3*, *ced-4*, *ced-9* and *egl-1* form the "death machinery" which is responsible for the execution of the cells undergoing PCD (Hengartner, 1997). Genetic studies in mutant animals have demonstrated that *ced-3* and *ced-4* are both required for cell execution. Also they have shown that *ced-9* is a survival factor that negatively regulates the activity of *ced-3* and *ced-4* (Hengartner et al., 1992). Ectopic expression of *ced-4* can induce cell death. However, the CED-4 protein is not directly responsible for the killing of cells. Rather, CED-4 acts as an adaptor molecule that regulates the killing activity of CED-3. Genetic studies in which *ced-3*, *ced-4* and *ced-9* were ectopically expressed in the six touch cells of *C. elegans* led to the demonstration that *ced-4* acts upstream of *ced-3* and may be regulated by *ced-9* (Shaham and Horvitz, 1996). In keeping, the CED-9 protein acts as a survival factor that protects cells from being killed by interacting with CED-4. A recently discovered member of the death effector family in *C. elegans* is EGL-1 (Liu and Hengartner, 1999). The loss of *egl-1* function results in survival of all cells that normally undergo apoptosis. EGL-1 might activate PCD by binding to CED-9 and inhibiting its activity. Thereby CED-4 is released from the CED-9/CED-4 containing complex (Conradt and Horvitz, 1998).

After the mammalian homologues of the death machinery genes of *C. elegans* were cloned, it became clear that these genes have similar functions in the regulation of apoptosis in all species examined so far.

1.2.1.1. CED-3 and the caspases. The mammalian homologues of CED-3 are a growing family of related proteases, which are collectively referred to as caspases (caspase = cysteine aspartate protease; Thornberry and Lazebnik, 1998). The first mammalian caspase to be identified was the interleukin-1β-converting enzyme (ICE or caspase 1; Yuan et al., 1993; Schwartz and Milligan, 1996). Later it was demonstrated that caspase 3 shows the highest sequence homology to CED-3 (Fernandez-Alnemri et al., 1994; Nicholson et al., 1995). To date the 14 members of the caspase family can be divided into three subfamilies on the basis of the peptide-sequence preferences of their substrates: (i) the ICE-protease subfamily (caspases 1, 4, 5, 13 and 14, and murine caspases 11 and 12); (ii) the CED-3 subfamily (caspases 3, 6, 7, 8, 9 and 10); and (iii) the caspase 2 subfamily (Table 1).

Table 1
Members of the caspase family

Subfamilies	Caspases	Other names
ICE-protease subfamily	Caspase 1	ICE
	Caspase 4	ICE_{rel}II, TX, ICH-2
	Caspase 5	ICE_{rel}III, TY
	Caspase 11 (murine)	
	Caspase 12 (murine)	
	Caspase 13	
	Caspase 14	
CED-3 subfamily	Caspase 3	CPP32, Yama, Apopain
	Caspase 6	Mch2
	Caspase 7	Mch3, ICE-LAP3, CMH-1
	Caspase 8	MACH, FLICE, Mch5
	Caspase 9	ICE-LAP6, Mch6
	Caspase 10	Mch4
Caspase 2 subfamily	Caspase 2	Nedd2, ICH-1

Among these, caspases 3, 6, and 7 have a short pro-domain and degrade vital cellular proteins (see also Table 2); the others have long pro-domains that mediate protein–protein interactions, and only in certain circumstances (perhaps with the exception of caspases 1 and 11) may trigger apoptosis (Thornberry and Lazebnik, 1998).

Each caspase is initially synthesized as a zymogen and requires processing at specific cleavage sites to generate the active enzyme (Stennicke and Salvesen, 1999). The caspases that are the first to be activated, in turn trigger other

Table 2
Substrates of certain mammalian caspases and related structural changes in apoptotic cells

Caspases	Substrates	Structural changes	References
Caspase 3	DFF45/ICAD; protein kinase Cδ	Chromatin condensation and fragmentation	Emoto et al. (1995), Liu et al. (1997, 1998), Cross et al. (2000)
	Fodrin, Gas2, gelsolin, PAK2, vimentin	Modifications of cell membrane and cytoskeleton	Martin et al. (1995), Kothakota et al. (1997), Rudel and Bokock (1997), Byun et al. (2001)
Caspase 6	Nuclear lamins, vimentin	Nuclear envelope breakage Alteration of cytoskeleton	Lazebnik et al. (1995), Takahashi et al. (1996), Byun et al. (2001)
Caspase 7	Gas2, vimentin	Alteration of cytoskeleton	Sgorbissa et al. (1999), Byun et al. (2001)

downstream caspases giving rise to a proteolytic cascade that culminates with the execution of apoptosis.

Evidence for the in vivo relevance of individual caspases in mammalian apoptosis within the CNS (and PNS) is mainly based upon studies on knockout mice (see Section 3).

Different subsets of caspases are activated depending upon the pro-apoptotic stimulus. For example, caspases 3, 6, and 8 are part of the Fas/TNF-mediated death pathway, while caspases 9 and 3, together with apoptosis protease activated factor 1 (Apaf1) and cytochrome *c* participate in mitochondria-associated cell death (Zimmermann et al., 2001). These two pathways do not seem to be completely independent since a link was reported through BID, a protein that mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors (Li et al., 1998; Luo et al., 1998).

The substrate specificity of CED-3 is more similar to caspase 3 than to caspase 1 or 2 (Xue et al., 1996). Therefore, the CED-3/CED-4/CED-9 complex of *C. elegans* resembles the caspase 9/caspase 3/Apaf1/cytochome *c* complex of mammals.

The differences in morphology of apoptotic cells in *C. elegans* and mammals may be related to differences in downstream events of apoptosis. In *C. elegans*, apoptotic cells show chromatin shrinkage and cytoplasm condensation. However, there are no evident apoptotic bodies as in mammals. Each of the 14 different mammalian caspases may be responsible for cleavage of several different molecules, reflecting the more complex structural morphology of apoptosis (Table 2).

1.2.1.2. CED-4 and Apaf1. The CED-4 homologue in mammals is Apaf1 (Zou et al., 1997). Apaf1 is part of a large protein complex called the apoptosome (Adams and Cory, 2002). As already mentioned, cellular demolition in apoptosis is carried out by caspases. Their activation, however, requires a number of scaffolding proteins that act in the apoptosome. In particular, biochemical studies have revealed that caspase 3 processing required not only caspase 9, but also Apaf1 and cytochrome *c* (Liu et al., 1996; Li et al., 1997). Upon binding to cytochrome *c* (also referred to as Apaf2) which is released from mitochondria at the onset of apoptosis, a series of conformational changes lead to Apaf1 multimerization and association with procaspase 9 (also referred to as Apaf3) with the generation of the about 1 MDa molecular weight apoptosome (Adams and Cory, 2002).

The biological role of Apaf1 in the nervous system is still under debate, mainly as a consequence of a series of unexpected findings derived from studies in transgenic animals (see Section 3).

1.2.1.3. CED-9 and the BCL-2 family. Since the first observation that the *ced-9* gene encodes a protein that is similar to mammalian B-cell lymphoma-2 (BCL-2) protein (Hengartner and Horvitz, 1994a), at least 15 proteins structurally related to CED-9 and BCL-2 have been identified to date, and grouped in the so-called BCL-2 protein family (Adams and Cory, 1998; Chao and Korsmeyer, 1998; Newton and Strasser, 1998; Sadoul, 1998; Sanchez and Yuan, 2001). All members of the family posses at least one of the four conserved BCL-homology (BH) domains (BH1, BH2, BH3, and BH4). Besides to BCL-2, some of these proteins (such as BCL-X_L and BCL-W) act as survival factors, whereas others (such as BAX, BAK, BAD, BID) are pro-apoptotic. CED-9 and the anti-apoptotic members of the BCL-2 family protect cells from death by at least two different mechanisms. First BCL-2 and BCL-X_L can heterodimerize and form pores, similar to those of the membrane-inserting domains of diphtheria toxin and colicins (Liang and Fesik, 1997), that act as channels for ions or even small proteins to cross the outer mitochondrial membrane. Thus, BCL-2 and BCL-X_L (located at the cytoplasmic side of the outer mitochondrial membrane) can prevent the release of cytochrome *c* from mitochondria, avoiding assembling of the apoptosome, and thereby protecting cells from being killed (Li et al., 1998; Luo et al., 1998). Second, in parallel to what happens in *C. elegans* (where CED-9 protects cells from death by interacting with CED-4, which, in turn, regulates, the activity of CED-3), BCL-X_L in mammals interacts with Apaf1. This interaction occurs via the BH4 domain and results in inhibition of the association of Apaf1 with procaspase 9, again with blockage of apoptosome formation and caspase 9 activation (Adams and Cory, 2002).

Work on distribution of the BCL-2 family members in the nervous tissue (see Sections 2 and 3) led to the conclusion that, in individual neuronal populations, there is an intricate network of interactions, with delicate balance and interchangeable functions, that modulate the pro- and anti-apoptotic function of these proteins. Noteworthy, function interchangeability of individual members of the family, such as, for example, the conversion of BCL-2 to a BAX-like death effector by caspase 2 (Cheng et al., 1997), generates further complication in our understanding of the molecular mechanisms mediated by the BCL-2 family proteins.

1.2.2. Apoptotic death pathways

Several different stimuli can initiate the apoptotic death of neurons. However, the finding that common morphological and biochemical alterations are observed independently upon the event that triggers apoptosis, suggests that most apoptotic pathways converge on a restricted number of common effector pathways (Sastry and Rao, 2000).

Basically two major pathways can be differentiated by the relative timing of caspase activation and mitochondrial release of cytochrome *c*. In the first, which is exemplified by activation of death receptors, an effector caspase is activated prior to mitochondrial alterations. In the other, cytochrome *c* is released from the mitochondrial intermembrane space prior to caspase activation.

1.2.2.1. Death receptor pathway. Death receptors are cell surface receptors that trigger apoptosis. There are several

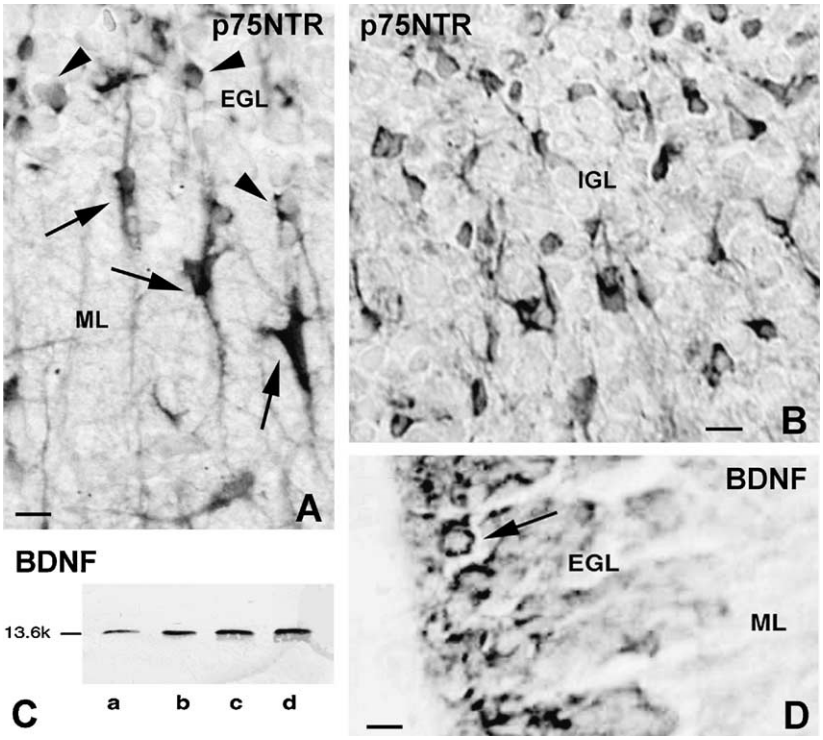

Fig. 2. Intervention of NTs in cerebellar NOND. (A and B) Localization of the low affinity $p75^{NTR}$ in the P5 rabbit cerebellar cortex suggests a role of the receptor in NOND of postmitotic CGCs. (A) Staining of premigratory CGCs in the deep EGL and ML (arrowheads). Three migrating CGCs in the ML show the typical drop-like to triangular morphology of the perikarya with initial extension of horizontal neurites (arrows). (B) staining of postmigratory CGCs in the IGL. The number of positive cells by far overcomes that of TUNEL-positive CGCs in the same location. Expression of $p75^{NTR}$ may be necessary for these neurons to receive a death signal, without implicating that presence of the receptor is per se sufficient to trigger apoptosis. (C and D) Precursor cells and premigratory CGCs contain BDNF. (C) Western blotting of proteins from CGCs isolated in Percoll gradient (lane a) or whole cerebellar extracts (lanes b–d). The 13.6 kDa band is indicative of BDNF. Protein content: (lane a) 15 μg; (lane b) 10 μg; (lane c) 20 μg; (lane d) 30 μg. (D) Immunocytochemical localization of BDNF is restricted to the outer EGL, while there is very little or absolutely no staining in the inner EGL. This observation suggests that precursor cells produce BDNF before undergoing apoptosis, thus representing an endogenous source of this trophic factor for the postmitotic CGCs. Scale bars: (A and B) 10 μm; (C) 5 μm.

types of death receptors in different tissues, but two members of the tumor necrosis factor receptor (TNFR) family were recently demonstrated to be involved in neuronal death (Raoul et al., 2000): Fas (CD95/Apo-1) and the p75 neurotrophin receptor ($p75^{NTR}$; Fig. 2A and B).

Fas. Fas has been extensively studied as a death receptor in lymphocytes. Upon exposure to cell death-triggering stimuli, lymphocytes express at the surface of their membrane the Fas ligand (Fas-L) which binds Fas through an autocrine or paracrine mode. Upon formation of the Fas/Fas-L complex, the Fas-associated death domain (FADD) adaptor protein activates the signaling procaspase 8, which eventually activates the effector caspase 3 (Blatt and Glick, 2001).

In the classical paradigm of trophic deprivation in cultured phaeochromocytoma (PC12) cells, CGCs, and spinal motorneurons, blockage of Fas/Fas-L interaction leads to reduction of cell death, indicating that Fas activation is an obligatory key step in trophic factor deprivation induced-apoptosis (Brunet et al., 1999; Le Niculescu et al., 1999; Raoul et al., 1999). Other studies have further shown that up-regulation of Fas-L expression in neurons consistently occurs as an early step in apoptosis induced by injury or stress (Raoul et al., 2000).

The control of induction of Fas-L expression is achieved by at least two pathways: the first involves Jun amino-terminal kinase (JNK; Le Niculescu et al., 1999; Martin-Villalba et al., 1999), the second protein kinase B (PKB or Akt; Francois and Grimes, 1999; Brunet et al., 1999, 2001). It seems also possible that Fas activation itself may trigger neuronal death, in the absence of Fas-L up-regulation (Raoul et al., 1999).

$p75^{NTR}$. Although $p75^{NTR}$ was the first neurotrophin receptor to be discovered, its biological role and mode of action still are not completely understood. Initially $p75^{NTR}$ was the only known receptor for nerve growth factor (NFG), but its low affinity for NGF and lack of signaling motifs in the cytoplasmic domain appeared to be major shortcomings to assign it a prominent role in the transduction of NGF biological effects. After the discovery of the high affinity tyrosine kinase neurotrophin receptors (Trks), $p75^{NTR}$ was

hypothesized to act as a Trk-co-receptor (Kaplan and Stephens, 1994; Wolf et al., 1995). More recently, attention has been focused on its putative role in neuronal apoptosis (Barker, 1998; Barrett, 2000; Raoul et al., 2000; Miller and Kaplan, 2001).

This was first suggested when it became clear that $p75^{NTR}$ was a member of the same family of transmembrane receptor as TNFR and Fas. The first reports on the apoptotic effect of $p75^{NTR}$ came from in vitro studies on neuronal cell lines (Alles et al., 1991; Barrett and Bartlett, 1994; Rabizadeh and Bredesen, 1994; Bredesen and Rabizadeh, 1997). Examples of a role for $p75^{NTR}$ as an inducer of apoptosis in vivo are still relatively few and somehow contradicting. After antisense oligonucleotide administration to rat pups it was demonstrated that $p75^{NTR}$ is necessary for post-axotomy death of sensory neurons (Cheema et al., 1996). To study the potential pro-apoptotic effects of $p75^{NTR}$, a transgenic mouse expressing the $p75^{NTR}$ intracellular domain under the control of a Tα1 α-tubulin promoter was generated (Majdan et al., 1997). Interestingly this animal model showed that the ability of $p75^{NTR}$ to cause apoptosis is restricted not only to certain neuronal types, but also to certain temporal windows during the life of these neurons. However, *$p75^{NTR}$* knockout mice show reduced neuronal death in the retina, certain spinal cord interneurons, and sympathetic neurons (Bamji et al., 1998; Frade and Barde, 1999). In the retina and spinal cord, observations in *$ngf^{-/-}$* mutants, indicate that NGF binding to $p75^{NTR}$ is the triggering event responsible for apoptosis (Frade and Barde, 1999). Sympathetic neuron cell death is instead triggered by $p75^{NTR}$ binding to brain-derived neurotrophic factor (BDNF), as suggested by analysis of *$bdnf^{-/-}$* mice (Majdan et al., 1997). Finally, it should be mentioned that additional putative $p75^{NTR}$-associated signal-transduction elements, such as the transcription factor nuclear factor kappa B, may modulate neuronal apoptosis (Taglialatela et al., 1997, 1998).

Despite of the above, still it remains difficult to assign a clear role in apoptosis to $p75^{NTR}$ as a consequence of our lack of knowledge of its intracellular signal transduction pathway (Barrett, 2000). This latter is unlikely to be the same as for Fas (or TNFR) since $p75^{NTR}$ has a different death domain (Liepinsh et al., 1997). Interestingly, a novel caspase-dependent pathway that does not involve the death domain, but BCL-2 has recently been described (Coulson et al., 1999).

1.2.2.2. Mitochondrial pathway. While in the death receptor pathway apoptosis is triggered by a relatively small number of structurally-related ligands, mitochondrial apoptosis in neurons can be triggered by a variety of structurally-unrelated agents (Sastry and Rao, 2000). This implies that mitochondrial apoptosis may be induced by more than one single mechanism.

A key event in the mitochondrial pathway is the release of cytochrome *c* into the cytosol. Experiments in cell-free systems led to hypothesize that cytochrome *c* release in mitochondrial apoptosis is either caused by a rupture of the outer mitochondrial membrane and/or by the so-called mitochondrial permeability transition (MPT), that is controlled by a voltage- and Ca^{2+}-sensitive pore, referred to as the permeability transition (PT) pore (Blatt and Glick, 2001).

Subcellular localization studies have shown that the anti-apoptotic members of the BCL-2 family (BCL-2, BCL-X_L) reside on the mitochondrial outer membrane, while the pro-apoptotic family members (BAD, BAX, BID) may be either cytosolic or present on the cytoplasmic surface of the outer mitochondrial membrane (Zimmermann et al., 2001). During apoptosis these pro-apoptotic molecules are activated and translocate to the mitochondria where they induce the release of cytochrome *c* (and other proteins) from the intermembrane space. On the other hand, the anti-apoptotic proteins BCL-2 and BCL-X_L work to prevent cytochrome *c* release from mitochondria, and thereby protect cells from death (Kluck et al., 1997; Yang et al., 1997). Subsequent events involve formation of the apoptosome and caspase activation.

Another protein that is normally located in the intermembrane space of mitochondria is the apoptosis-inducing factor (AIF). AIF is a flavoprotein which shares homology with the bacterial oxidoreductase and, that, similarly to cytochrome *c*, is a phylogenetically old, bifunctional protein (Susin et al., 1999). Upon death signaling, AIF translocates to the nucleus, binds to DNA and provokes chromatin condensation and large scale (approximately 50k bp) DNA fragmentation, apparently in a caspase-independent manner (Daugas et al., 2000). In keeping, overexpression of BCL-2 prevents the release of AIF from mitochondrion, but not its apoptogenic activity (Susin et al., 1999). Recent data show that AIF is released from mitochondria by a mechanism distinct from that of cytochrome *c*, but probably mediated by PARP-1 (Yu et al., 2002). Interestingly, in embryonic morphogenesis, genetic inactivation of AIF appears to abolish early neuronal death of proliferating precursor cells and young postmitotic neuroblasts (Joza et al., 2001). In addition, it has been shown that the phenotype of harlequin (*hq*) mutant mice, which display progressive degeneration of terminally differentiated cerebellar and retinal neurons, is due to a proviral insertion in the *aif* gene, causing about an 80% reduction in AIF expression (Klein et al., 2002). Mutant CGCs are susceptible to exogenous and endogenous peroxide-mediated apoptosis, but can be rescued by AIF expression. Overexpression of AIF in wild-type neurons further decreases peroxide-mediated cell death, suggesting that AIF serves as a free radical scavenger.

Recently, an additional protein with the dual name Smac/DIABLO, released together with cytochrome *c* during apoptosis, has been identified (Du et al., 2000; Verhagen et al., 2000). Smac/DIABLO promotes caspase activation by associating with the apoptosome and inhibiting a family of proteins that function as inhibitors of apoptosis (IAPs). In some cellular systems, cytochrome *c* is necessary but not sufficient for cell death. In these systems Smac/DIABLO

may be the second factor required for the so-called competence to die (Deshmukh and Johnson Jr., 1998).

1.3. Apoptosis and cell cycle regulation

Differentiated neurons are postmitotic cells completely devoid of replicative capability. Most mammalian CNS neurons enter the postmitotic state during embryonic life. In doing so some dividing cells exit from the cell cycle and enter a phase of mitotic quiescence commonly referred to as the G_0 phase. Indeed postmitotic neurons are believed to enter an "extended G_0 phase" which, unlike other cell types, is irreversible.[3] Investigation of the mechanisms of neuronal apoptosis has led to the unexpected discovery that, in many instances, the quiescent and dormant cell cycle machinery is "resuscitated". In this section, we present an overview of recent data suggesting that uncoordinated expression of cell cycle-related molecules is one fundamental mechanism of apoptosis in certain neurons. Readers are invited to consult a number of reviews specifically devoted to this issue for further information (White, 1996; Yoshikawa, 2000; Liu and Greene, 2001).

1.3.1. Cell cycle regulation and checkpoints

The cellular and molecular mechanisms of cell cycle regulation and checkpoint in metazoans have been extensively reviewed in the recent past (Rhind and Russell, 2000; Walworth, 2000, 2001; Bartek and Lukas, 2001; Bulavin et al., 2002; Melo and Toczyski, 2002).

Three families of proteins are primarily responsible of cell cycle regulation: the cyclins, the cyclin-dependent kinases (CDKs), and the cyclin-dependent kinase inhibitors (CKIs). Cyclins are a group of proteins whose abundance varies substantially during the cell cycle. They associate with their cognate CDKs acting as activating subunits, and eventually leading to active CDK complexes. CDKs allow progression through the different phases of the cell cycle by phosphorylating their target substrates.

For protection from a variety of different types of insults or stress resulting in DNA damage, eukaryotic cells have developed a system of checkpoints that delay progression to the next phase of the cell cycle and activates DNA repair. When DNA damage is irreparable, checkpoints eliminate potentially hazardous cells by permanent cell cycle arrest or cell death (Bartek and Lukas, 2001). During normal cell cycle progression, initiation of mitosis is triggered by a complex process of activation of the cyclin-dependent protein kinase cell division cycle 2 (Cdc2) kinase. Cdc2 kinase is the major "engine" that drives the $G_2 \rightarrow M$ transition. Prior to mitosis the Cdc2–cyclin B1 complex is held in the cytoplasm in an inactive state by Cdc2 phosphorylation at Thr14 and Tyr15. Dephosphorylation of these sites in mammals is regulated by two cell division cycle 25 (Cdc25) phosphatases, Cdc25B and Cdc25C. Cdc25B removes inhibitory phosphates in cytoplasmic Cdc2; with G_2 progression, dephosphorylated Cdc2 translocates into the nucleus where it is a target for Cdc25C, which maintains Cdc2 dephosphorylation at inhibitory sites (Bulavin et al., 2002). In mammals, the checkpoint kinase 1 (Chk1) is the most important molecule that acts upstream Cdc25C in DNA replication and damage checkpoints (Walworth, 2001). Chk1 phosphorylates Cdc25C in vitro at Ser216. Strikingly, dephosphorylation of Cdc25C coincides with mitotic entry.

G_1 phase is a period when cells make critical decisions about their fate, including the option to replicate DNA (in other words entering the S phase) and complete cell division. The decision to enter the S phase is made at the so-called restriction point in mid-to-late G_1 (Bartek et al., 1996). The genes critical for G_1/S transition and coordination of S–G_2–M progression are regulated by the parallel retinoblastoma protein (Rb) and Myc pathways (Walworth, 2000). According to the current two-wave model of the G_1 checkpoint response in mammalian cells, an initial rapid transient p53-independent response is followed by the delayed yet more sustained G_1 arrest imposed by p53 (Bartek and Lukas, 2001).

1.3.2. Retinoblastoma protein family

The retinoblastoma tumor suppressor gene encodes a nuclear protein, Rb, which plays a central role in cell cycle control. Rb and the Rb-related proteins p107 and p130 are among the best-characterized substrates of G_1 CDKs (Weinberg, 1995). Rb is a member of a family of proteins that interact with many transcription factors. The early gene 2 factor (E2F) is a transcription factor that appears to be the major physiological target of Rb.[4] The Rb family proteins bind to the transactivation domain of E2F, and strongly activate transcription of E2F-responsive genes (Stevaux and Dyson, 2002). Through activation of these genes, E2F is believed to positively regulate cell cycle progression.

Phosphorylation and dephosphorylation of Rb regulates its E2F–protein binding activity. The unphosphorylated or hypophosphorylated active form of Rb predominates in the G_1 phase and binds to E2F, to repress its transcriptional activity.

Transcriptional repression is relieved in late G_1, when Rb family proteins become highly phosphorylated by one or more CDKs (Lee et al., 1997; Zarkowska and Mittnacht, 1997; Lundberg and Weinberg, 1998). Activated E2F

[3] In the mammalian CNS, neurons differentiate from neuroepithelial stem cells, which are multipotent cells that give rise to committed progenitors of neurons and glial cells. As differentiation proceeds, neuronal progenitors transform into neuronal precursors also referred to as neuroblasts, which, in vertebrates, are incapable of cell replication. Neuronal precursors are thought to establish their future phenotype and thereby transforming into differentiated neurons, upon generation by terminal-mode symmetric divisions of committed progenitors (McKonnel, 1995; Yoshikawa, 2000).

[4] Mammalian genomes encodes at least six E2F proteins that can be subdivided into three categories. E2F1, E2F2, and E2F3 are potent transcriptional activators and interact directly with Rb; E2F4 and E2F5 are less potent and interact with all members of the Rb family; E2F6 is believed to repress transcription (Stevaux and Dyson, 2002).

triggers transcription of many genes involved in DNA replication and cell growth control.

In more recent years, the view of E2F-dependent transcription has broadened. E2F-regulated genes have a role in DNA synthesis and repair, mitosis, and, directly relevant to the purpose of the present discussion, apoptosis (Stevaux and Dyson, 2002). In particular, E2F1 has a physiological role in DNA-damage responses, perhaps through expression of DNA-repair and pro-apoptotic genes, including *Apaf1*, *casp 3* and *casp 7* (Müller et al., 2001).

In the areas of persistent neurogenesis during adulthood, Rb immunoreactivity is high in proliferating neuronal precursors, but reduced during terminal differentiation (Yoshikawa, 2000). A transient increase in the Rb protein level appears to be an important step in the initiation of terminal mitosis of neuronal progenitors, and is then followed by a drastic reduction during terminal differentiation and maintained at low levels in postmitotic neurons (Slack et al., 1998; Callaghan et al., 1999). In keeping, an increasing body of evidence is leading to the notion that postmitotic neurons generally undergo apoptosis when the cell cycle regulators that promote Rb phosphorylation are activated. Moreover, E2F family members can trigger apoptosis, and E2F1-induced apoptosis can be specifically inhibited by Rb (Liu and Greene, 2001).

The Rb-related proteins p107 and p130 appear to be able not only to substitute for many of the Rb functions in growth regulation, but also provide other fundamental functions that may extend beyond those of Rb. The abundance of p130 in differentiated neurons indicates that the growth arrest dependent upon E2F4/p130 interaction might be a key event in the maintenance of the neuronal G_0 state (Persengiev et al., 1999).

1.3.3. p53

Although there are probably multiple sensors that record and transduce DNA damage within mammalian cells, one of the most important is the p53 tumor suppressor protein. p53 plays a critical role as a transducer of damage to genomic integrity into growth arrest and/or apoptosis. DNA damage triggers stabilization and accumulation of p53, which then initiates either a G_1 cell cycle arrest or apoptosis (Fraser et al., 1996). In addition to DNA damage, several other stimuli lead to p53 activation (Blatt and Glick, 2001). In the normal state, cells contain fairly low levels of p53, since the inactive form of the protein is highly unstable. For activation, p53 must be phosphorylated. Although a large number of kinases is capable to phosphorylate p53, response to DNA damage is most likely mediated by DNA-dependent protein kinase (DNA-PK) which is a substrate of certain caspases, the product of the ataxia-teleangectasia gene (ATM), and Chk2 (Lee and McKinnon, 2000; Blatt and Glick, 2001).

When neurons mature in vitro the subcellular localization of p53 changes from the nucleus, in immature cells, to the cytoplasm, in fully differentiated neurons (Eizenberg et al., 1996). It seems that, in response to appropriate stimuli, p53 translocates to the nucleus and plays a regulatory role in directing primary neurons toward differentiation or apoptosis.

Several lines of evidence converge to indicate that neurons undergo apoptosis by p53-dependent or -independent pathways, according to the stimulus responsible for genotoxic stress. However, it is clear that p53 has a significant role in apoptosis that follows DNA damage in vivo (Wood and Youle, 1995). p53 is also a fundamental component of the $p75^{NTR}$ apoptotic signal cascade (Aloyz et al., 1998).

2. In vivo analysis of NOND in the mammalian CNS

Although there is an impressive amount of literature on the cellular and molecular mechanisms of apoptosis in neurons (and other cell types) in vitro, comparatively little work has been done to characterize NOND in intact animals. Examination of a summary table on “Nervous system cell preparations used in studies on apoptosis” in a recent review devoted to neuronal apoptosis (Sastry and Rao, 2000) is anecdotal of this situation, with works in vivo being a drop in a sea of studies on primary cultures and/or cell lines.

However, as it will be illustrated below, studies in vivo have been not only confirmatory, but provided novel and interesting information on the physiological relevance of certain apoptotic mechanisms.

2.1. Early death of proliferating precursor cells and young postmitotic neuroblasts

It is generally believed that a correct balance between cell proliferation and apoptosis during development is fundamental to determine the ultimate structure, architecture, size, and shape of tissues and organs. The existence of naturally occurring cell death in the neuroepithelium at the beginning of neurulation was first described more than 50 years ago (Glucksmann, 1951). Later, early neuronal death was described during the formation of neural crest, and neurogenesis, with a widespread distribution throughout CNS and in PNS ganglia (De la Rosa and De Pablo, 2000). In these developmental stages, differentiated neurons are very rare, if not totally absent. Therefore, although an unequivocal identification of the dead cells was not generally provided, it is reasonable to assume that they must be proliferating neuronal precursors or newly generated neuroblasts, but not neurons connected with their targets. Since proliferation, differentiation and apoptosis coexist during early neurogenesis, determination of the relationship between proliferation and apoptosis of individual cells is an important issue to be raised. Interestingly, a number of studies led to conclude that apoptotic cells enter the cell cycle (based on their capacity to synthesize DNA) shortly before death (Blaschke et al., 1996; Galli-Resta and Ensini, 1996; Thomaidou et al., 1997). For example, when the possibility that a temporal relationship existed between genesis and death of individual neurons during rat retinal development

was investigated, it was found that most neurons die in the ganglion cell layer (GCL) within a maximal interval of 5 days after their birth, irrespective of the age of genesis or of the cell type (Galli-Resta and Ensini, 1996). These findings suggested the existence of a cellular clock regulating neuronal death during early development. The same authors, after finding that neurons migrate in no less than 3 days to the GCL, where most of the cells that die remain a maximum of 2 days, concluded that the magnitude of neuronal death was likely to be far greater than previously believed.

The links between cell proliferation and apoptosis were also studied during prenatal and early postnatal development of the mouse (Blaschke et al., 1996) and rat (Thomaidou et al., 1997) cerebral cortex. In mouse embryos, during the period of cortical neurogenesis (E10–18) there was a peak of cell death (70%) at E14 with a subsequent decline up to E18 (50%). In rat neurogenesis, cell death reached a peak at E16 in the ventricular zone (VZ) and E19–P0 in the subventricular zone (SVZ), where there was a higher incidence of cell death than in other cortical regions. Gel electrophoresis of DNA extracted from the SVZ of newborn animals showed the characteristic ladder pattern of apoptosis. The number of apoptotic cells remained high in this zone for at least 2 weeks, in parallel with the continuing occurrence of mitoses. Thomaidou et al. (1997), after labeling with BrdU, showed that 71% of TdT-mediated dUTP nick end labeling (TUNEL)-labeled cells had taken up this S-phase marker before undergoing cell death. Moreover, using BrdU and tritiated thymidine in a "window-labeling" technique to identify a defined population of proliferating cells, they found that the clearance time of TUNEL-positive nuclei was 2 h and 20 min, and that most of TUNEL-positive cells were in the G_1 phase of their cell cycle.

Interestingly, these two studies lead to similar conclusions regarding the preferential localization of dying cells in the zones of cell proliferation, rather than in regions populated by postmitotic neurons.

With similar approaches, we have analyzed the relationship of cell proliferation and apoptosis in the postnatal cerebellum (Lossi et al., 2002c, 2003). The cerebellar cortex appeared to be particularly suitable to this purpose, since its developmental modalities have been thoroughly characterized. At least in altricial mammals (Lossi et al., 2002a), such as rats, mice, rabbits and humans, much of the cerebellar development occurs postnatally. In this period the forming cerebellum is covered by the EGL, a proliferative layer of cells that persists for a period of time varying according to species, but eventually becomes thinner and ultimately disappears (for review see Altman and Bayer, 1997). Extensive proliferation of the EGL during the first postnatal weeks gives rise to more than 100 million granule cells that migrate across the molecular and Purkinje cell layers to reach the IGL under the guidance of the radial glia (Rakic, 1971; Hatten, 1990). CGCs make synapses onto the Purkinje cells, and the Purkinje to CGC ratio is highly regulated, yet varies among species (Lange, 1975). The role of cell death in such a regulation is still under debate, although, starting from the 1970s, numerous studies based on counts of pyknotic nuclei in normal and experimental animals, have shown that the EGL cells of several species die during postnatal development. Work in vivo led to the demonstration, in different species of mammals including humans, of extremely high numbers of apoptotic cells in the cerebellum during the first weeks after birth (Wood et al., 1993; Krueger et al., 1995; Lossi et al., 1998). Cells undergoing PCD in the EGL have been mostly identified as immature CGCs and/or their precursors (GCPs) and correlated with the previously demonstrated pyknotic elements in the same location.

The temporal windows of cell proliferation and apoptosis overlap in P5 rabbits (Fig. 3A and B). Therefore, by sequential injections of IdU and BrdU, and different survival times up to 48 h from the administration of the first tracer, we have been able to analyze cell proliferation within the EGL in a predetermined window of time according to the interval between the administration of the two labels (Yanik et al., 1992). Moreover, by using electron microscopy and ultrastructural immunocytochemistry, we were enabled to easily identify the tracer(s) incorporated by positive cells, the type(s) of labeled cells, and the presence of cells with an apoptotic morphology (Fig. 1). After DNA fragmentation assay, we observed that within 24 h upon completion of their S phase the majority of GCPs has terminated PCD, displaying all the typical ultrastructural features of apoptosis and different patterns of incorporation of the S-phase labels according to the interval between administration of the two tracers (Fig. 3A). These data, and calculation of cell cycle parameters, demonstrated that the apoptotic machinery in GCPs is activated upon completion of the S phase and/or immediately thereafter, similarly to what happens in cortical neurogenesis. The idea that apoptosis is closely linked to cell cycle parameters was reinforced by observation of a sharp temporal sequence in the appearance of BrdU/IdU labeling in low molecular weight DNA oligomers after Southern blotting and immunodetection (Lossi et al., 2002c).

The examples given here demonstrate that early neuronal death is a widespread phenomenon in the CNS. Which is however the size the process?

A quantitative estimation of early neuronal death is not an easy job, because of the concurrent existence of cell proliferation and differentiation, because the techniques so far available to this purpose suffer from a series of drawbacks (Gilmore et al., 2000; Lossi et al., 2002b) that, at present, can only be overcome by rigorous and careful examination of results (Rakic, 2002), and because apoptotic cells are rapidly cleared from tissues.

In general terms, it is unwise to extrapolate the actual relevance of cell death upon simple estimation of the numbers of apoptotic cells labeled by in situ end labeling (ISEL) procedures. Moreover, the extremely high percentages of ISEL-positive cells in certain studies remain difficult to explain: if cell death affects half or more than a half of a proliferating population, irrespectively of the length of the cell

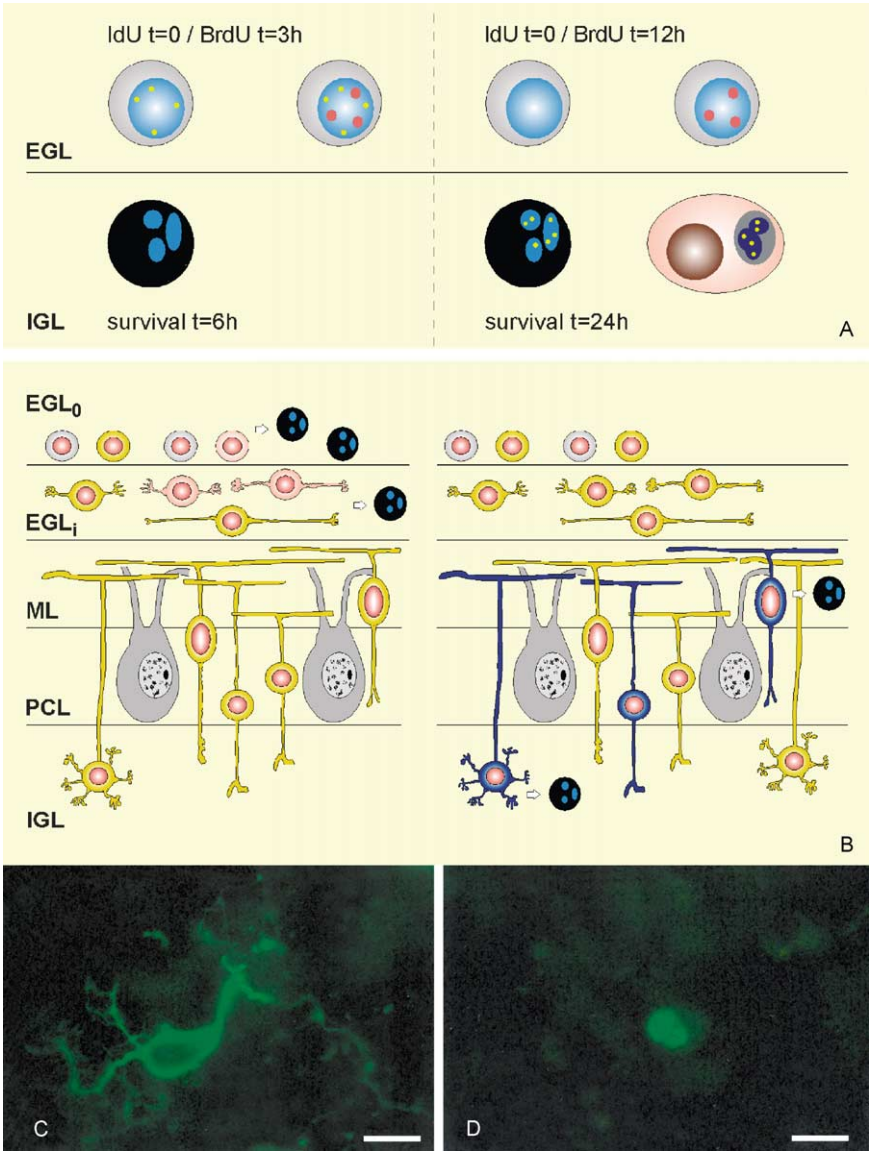

Fig. 3. NOND in the postnatal cerebellum. (A) Schematic drawing summarizing the results obtained from ultrastructural analysis of cell proliferation and apoptosis after in vivo labeling with BrdU/IdU (for details see Lossi et al., 2002c). *Left*: after a short interval between the administration of the two tracers (3 h) and a short survival time (6 h), BrdU singularly labeled and BrdU/IdU double-labeled precursors are evident in the EGL but there is no staining in the IGL, which contains unlabeled apoptotic CGCs. *Right*: after a longer interval between the administration of the two tracers (12 h) and a longer survival time (24 h), only BrdU single-labeled precursors are evident in the EGL, while the IGL displays IdU-positive apoptotic CGCs and phagocytes engulfed with heterophagosomes containing IdU-labeled DNA remnants. Small yellow dots indicate small-sized colloidal gold particles after immunocytochemical labeling (IdU), while large red dots indicate large-sized colloidal gold particles after immunocytochemical labeling (BrdU). These results demonstrate that between 6 and 12 h from S-phase marker incorporation precursor cells undergo early apoptosis in the EGL, while apoptosis of postmitotic CGCs occurs at least 24 h after DNA replication. (B) Death pathways related to proliferation, differentiation, and migration of CGCs (redrawn from Lossi et al., 1998). For the sake of clarity early neuronal death of precursor cells/young postmitotic neuroblasts (*left*) and death of postmitotic CGCs (*right*) are represented separately, but in actual terms there is a temporal overlap. In both cases apoptotic cells are TUNEL-positive. Precursor cells (gray cytoplasm) in the outer EGL (EGL_o) do not/or only express a limited subset of differentiation-specific antigens (see Lossi et al., 1998). *Left*: as differentiation proceeds, a subpopulation of precursor cells express anti-apoptotic modulators (such as BCL-2). They become young postmitotic neuroblasts (yellow cytoplasm), migrate across the molecular (ML) and Purkinje cell (PCL) layers, and give rise to mature CGCs that will eventually survive in the IGL. However, other precursor cells express pro-apoptotic modulators (pink cytoplasm), and undergo apoptosis throughout the outer and inner EGL (EGL_o and EGL_i) according to their differentiation stage. Apoptotic cells in this location express high levels of Chk1 and Rb protein, but not activated caspases/PARP-1 (Lossi et al., 2003). Therefore, their death mechanism appears to be linked to cell cycle regulation, but caspase independent. *Right*: in the IGL some postmitotic CGCs (blue cytoplasm) undergo delayed apoptosis as a result of failure to make proper synaptic contacts in the ML (Lossi et al., 2002c). In these neurons, there is expression of cleaved caspases and p85 (Lossi et al., 2003). Therefore, death of postmitotic CGCs is dependent upon caspase activation. (C) and (D) Ex vivo visualization of caspase 3 cleavage in P8 rats confirms the selective activation of caspase 3 in the IGL. Cerebellar slices were transfected by a particle-mediated biolistic procedure with a construct that allows for the detection of the onset caspase 3 activity, by visualizing the translocation of enhanced yellow fluorescent protein (EYFP) from the cytosol to the nucleus (Lossi and Merighi, 2003). (C) A fully morphologically differentiated control CGC in the IGL displays cytoplasmic fluorescence, indicative that caspase 3 is not activated in this cell. (D) Translocation of EYFP into the nucleus 6 h after staurosporin (3 μM) challenge indicates activation of caspase 3. Bars = 5 μm.

cycle, the total cell population will remain stable (as one of the two daughter cells will be eliminated) or even decrease.

Since the principle at the basis of the ISEL procedures relies on addition of reporter molecules to DNA strand breaks, one is led to hypothesize that a misleading evaluation of cell death is linked to detection of DNA modifications that are *not* related to apoptosis. Interestingly, substantial death of migrating and differentiating postmitotic neurons occurs by E15.5 during CNS development in mice deficient of XRCC4 and ligase-IV, two proteins required for repair of double-stranded DNA breaks (Gilmore et al., 2000). The most obvious explanation of these findings is that the occurrence of double-stranded DNA breaks in early postmitotic and differentiating neurons physiologically affects large numbers of nerve cells during development. Although the significance of this observation remains obscure, it is important to remark that cell death does not necessarily occur in neurons with double-stranded DNA breaks. However, double-stranded DNA breaks may be detected by the ISEL techniques and misinterpreted to indicate cell death.

In other studies, estimation of cell death has been carried out indirectly, by calculating the rate of neurogenesis with respect to the length of the cell cycle, and still figures were around 50–70% (Caviness Jr. et al., 1995).

All the above observations converge to suggest that the proportion of early neuronal death might be close to that of late neuronal death of postmitotic neurons (see Section 2.2), although much work still has to be done to further clarify this issue. In particular, it will be also important to establish the reason(s) why so many neurons are generated to die immediately thereafter. For example, since precursor cells in the EGL produce BDNF (Fig. 2C and D), and the peak in cerebellar BDNF mRNA coincides with that of apoptosis (Rocamora et al., 1993; Lindolm et al., 1997), on a purely speculative basis one could hypothesize that the high rate of proliferation and apoptosis in the EGL maintains a proper supply of endogenous BDNF for the developing cerebellar cortex.

2.1.1. *Molecular control of early neuronal death*

Although it seems clear that early neuronal death is apoptotic and affects significant numbers of cells, the question of what apoptotic pathways are activated in vivo during this process remains open.

Due to the functional importance of caspases and members of the BCL-2 family, distributional studies in normal animals have focused onto the localization of these proteins and/or their mRNAs, mainly in primates (Sohma et al., 1996; Lichnovsky et al., 1998; Lossi et al., 1998; Bernier et al., 2000; Vinet et al., 2002) and rodents (Castren et al., 1994; Frankowski et al., 1995; Ishii et al., 1996; Mizuguchi et al., 1996; Srinivasan et al., 1998; De Bilbao et al., 1999; Mooney and Miller, 2000). As discussed in Section 3, additional information from several strains of transgenics has further increased our knowledge on this matter. However, since early neuronal death has received little consideration until recently, little work has been done to thoroughly characterize the molecular pathways involved.

Caspases 3 and 9 are expressed in the telencephalic VZ (Sommer and Rao, 2002). Experimental activation of caspase 3 leads to progenitor cell death which is blocked by a pan-caspase inhibitor (D'Sa-Eipper and Roth, 2000). This indicates that neural progenitor can activate a caspase-dependent apoptotic pathway.

In the cerebellum, although caspases represent major actors in apoptosis, some experiments in vitro showed that caspase inhibitors were unable to protect CGCs from KCl withdrawal (Miller et al., 1997; Padmanabhan et al., 1999). Since apoptosis could be blocked by transcriptional inhibitors, the question of whether PCD might be related to activation of certain components of the cell cycle machinery was raised (Ferrari et al., 1995).

We have shown that premigratory CGCs with typical apoptotic morphologies are stained in vivo with antibodies against phospho-Chk1 and two different forms of phospho-Rb (Lossi et al., 2003). In keeping with the notion that the more superficial part of the EGL is populated by proliferating progenitors, while premigratory CGC reside in the inner EGL, we obtained evidence for selective cytoplasmic localization of phospho(Tyr15) Cdc2 in numerous cells within the inner EGL. As expected, the distribution of the proliferating cell nuclear antigen (PCNA), which shows a nuclear localization in cells from G_1/S to G_2/M (Takahashi and Caviness Jr., 1993), was nearly specular to that of phospho-Cdc2. Two major conclusions can at present be drawn from these results. First, the extremely selective localization of phospho-Chk1 in our model of early NOND suggests that activation of this effector kinase is associated with the execution of apoptosis in GCPs and premigratory CGCs.

Second, one is led to conclude that hyperphosphorylation of Rb at specific sites is required for PCD. Notably, Rb phosphorylation is regulated by G_1 phase cyclins and CDKs (Bartek et al., 1996), and functional inactivation of Rb requires sequential modifications by at least two distinct cyclin–CDK complexes (Lundberg and Weinberg, 1998). Even more interestingly, phosphorylation at different sites is required for inhibiting specific Rb activities (Zarkowska and Mittnacht, 1997), and an array of different functions for Rb has clearly been demonstrated in nerve cells (Lee et al., 1994; Slack et al., 1998; Callaghan et al., 1999). In support, numerous experiments in vitro (Park et al., 1997; Padmanabhan et al., 1999) and in transgenic and mutant animals (see Section 3) indicate a role in cerebellar apoptosis for G_1/S checkpoint controllers and Rb.

Nonetheless, we do not know if the observations in cerebellum may be generalized to the other systems where early neuronal death is a prominent feature during development.

2.2. *Death of postmitotic neurons*

Death of postmitotic neurons is a widely recognized phenomenon that plays a crucial role in sculpting and

maintaining the architecture of the mature CNS. A relatively large apoptotic loss of different types of neurons (and glial cells) occurs during vertebrate development, and the general explanation for this phenomenon is that the survival of nerve cells depends on specific neurotrophic factors which are synthesized by their targets (Oppenheim, 1985; Johnson and Oppenheim, 1994; Raff et al., 1994; Miller and Kaplan, 2001). Additional signals from neighboring neurons and glial cells are also required for the developing neurons to survive (Raff et al., 1994), but ultimately the survival of neurons depends on a complex interaction of several factors, that upon imbalance lead to cell death. Therefore, although many neuronal types are produced in excess, only a portion of them get sufficient proper support for survival, and the other die facilitating appropriate neuron–target innervation.

After the recognition of the neurotrophin (NT) family of neurotrophic factors (NGF, BDNF, NT-3, NT-4/5), several other molecules influencing the early development of the nervous system were identified. Therefore, it was not surprising that, for example, NGF withdrawal from culture medium led to apoptosis in several types of neurons and neural cell lines (Sastry and Rao, 2000). It is interesting to note that, in general terms, only selected CNS neuronal populations in selected temporal windows seem to be affected by experimental mutations of the genes encoding for NT and/or their receptors, indicating that there may be some compensatory effects between related molecules in vivo.

It is worth mentioning that the adult brain also requires a constant trophic input for appropriate function. Although the main source of trophic factors for mature neurons is considered to arise locally from glial cells and synaptic partners, recent evidence suggests that hormonal-like influences from distant sources may also be important. These include not only relatively well-characterized steroid hormones that cross the brain barriers, but also blood-borne protein growth factors able to cross the barriers and exert unexpected, albeit specific, trophic actions (Torres-Aleman, 2000). Among these the insulin-like growth factor-I (IGF-I), fibroblast growth factor-2 (FGF-2) or the NTs, exert a tonic trophic input on brain cells and likely are important players in the regulation of cell death of postmitotic neurons (Torres-Aleman et al., 1998).

Different culturing conditions and numerous molecules, often structurally unrelated among each others, have been shown to be able to induce apoptosis of postmitotic neurons (and, at times, glial cells) in vitro (Sastry and Rao, 2000). Among these one can mention low $[K^+]$, alterations in Ca^{2+} homeostasis, oxidative stress, nitric oxide (NO), ultraviolet and X-ray irradiation, modulators of protein phosphorylation (such as staurosporine, okadaic acid), DNA-damaging agents and nucleosides (such as camptothecin, mitoxantrone, etoposide, arabinonucleosides, 2-chloroadenosine), neurotransmitters and related agonists (glutamate, NMDA, kainic and quisqualic acids), neurotoxicant drugs, peptides and proteins (β-amyloid, HIV-1 gp120), lipids (ceramide, retinoic acid, phosphatidylinositol-3-phosphate). Some of these molecules are physiologically present in the nervous tissue and therefore may play a role in postmitotic neuronal death in normal animals.

Moreover, the importance of normal electrical activity has been recognized to play a crucial role in cell death protection (Linden, 1994; Cameron et al., 1998; Weiss et al., 1998).

Lesions in the proximity to the cell body of postnatal or adult CNS neurons have widely been used as an experimental paradigm. Reviewing these data is beyond our present purpose, also considering that, until quite recently, most of them have been generated with the purpose of characterize the mode of action of neurotrophic factors, rather than focusing on cell death morphologies and pathways in vivo. As an example we will briefly mention below some data on optic nerve injury which is one of the favorite experimental models to study neurotrophic factor effects on retinal neurons. After crushing or axotomy of the optic nerve the almost entire population of retinal ganglion cells (RGCs) die by apoptosis within 14 days (Bähr, 2000). Under this experimental conditions it was shown that NGF and BDNF enhance the survival of RGCs (Carmignoto et al., 1989; Maffei et al., 1990; Mey and Thanos, 1993). Subsequent studies led to assume that BDNF was the molecule effective in protecting RGCs from death, although its effects appeared to be transient and limited to a subset of RGCs. Interestingly, BDNF, as a side effect, was capable to induce nitric oxide synthase (NOS) in the retina with subsequent NO production and increased cell loss (Klöcker et al., 1998). This fact was held to be at the basis of the relatively limited effect of BDNF in promoting survival. Relevant to the present discussion, some unexpected findings in vivo could be explained considering the wide range of molecules that are able to induce neuronal apoptosis in vitro, and the likely possibility that more than one single pathway is activated in the more complex in vivo situation.

2.2.1. *Molecular control of postmitotic neuronal death*

In general terms, cell death of postmitotic neurons appears to follow the mitochondrial pathway of apoptosis. The signal transduction pathways acting upstream formation of the apoptosome are however still not completely characterized. In the case of NT, which exert both anti- and pro-apoptotic effects, how signals for neuronal survival/death actually operate is still a matter of debate. It is in fact possible that cells are instructed to die by switching on the apoptotic machinery, or cells intrinsically committed to death are rescued by switching off their suicide program (Bredesen and Rabizadeh, 1997). Studies in vitro have shown that upon binding to the high affinity Trk receptors, NT activated different signal transduction pathways such as the phosphoinositide 3-kinase (PI3K) and the Raf/Raf/mitogen-activated protein kinase (MAPK) pathways (Segal and Greenberg, 1996). The PI3K pathways eventually leads to activation of PKB/Akt, which interacts with caspase 9 (at least in certain species), and BAD (Bähr, 2000). Activation of procaspase 9 (and thus formation of the apoptosome) appeared to be a point of convergence in the molecular pathways of apoptosis

induced not only by growth factor withdrawal, but also by most stimuli that may be effective in injuring postmitotic neurons. For example, adult rat RGCs displayed increased cleavage of caspase 3 after optic nerve section as assessed by Western blotting and TUNEL labeling (Kermer et al., 1999a,b, 2000a). Caspase 9 became activated following axotomy, and reduction of caspase 9 activity by repeated intraocular injections of specific inhibitors significantly prevented RGC death (Kermer et al., 2000a). Both BDNF and IGF-I were protective against RGC apoptosis, by inhibition of caspases 3 and 9 (Kermer et al., 2000b), but they apparently activated different intracellular pathways. In fact, IGF-I, but not BDNF protected RGCs via PI3K-dependent PKB/Akt phosphorylation (Klöcker et al., 2000).

As an additional example, when the temporal and spatial aspects of caspase 9 and 3 activation were studied in olfactory receptor neurons (ORNs) undergoing apoptosis after target removal in vivo, enhanced expression of procaspases 9 and 3 was observed (Cowan et al., 2001). Activation was initially evident at the level of the lesion (the axonal terminals), then in axons, and only later in the ORN soma. These observations indicated a retrograde propagation of pro-apoptotic signals from synapse, through the axon, to the ORN cell body. In keeping, mature ORNs of *casp 3* knockouts did not undergo caspase-dependent TUNEL-positive apoptosis after olfactory bulb removal. These results demonstrated that ORNs require caspase 3 activation to undergo normal developmental and mature target-deprived apoptosis. Giving that activation of the mitochondrial apoptotic pathway is a well established phenomenon in all above experimental conditions, one is led to ask whether or not the same pathway is activated under more physiological circumstances.

We have recently demonstrated that postmitotic neurons in the normal rabbit cerebellum IGL undergo apoptosis in vivo as a consequence of failing to make proper synaptic contacts with the Purkinje cell dendrites (Lossi et al., 2002c). Therefore, we have further investigated the molecular mechanisms underlying apoptosis in this model of NOND.

Numerous studies have shown that caspase 3 activation is a critical step in executing CGC apoptosis in vitro (see, for example, Tanabe et al., 1998; Allsopp et al., 2000). Unsurprisingly, we obtained evidence for specific cleavage of caspase 3 and other caspases in our experimental conditions. Caspase/PARP-1 cleavage selectively occurred within the IGL, which is populated by postmitotic, postmigratory CGCs that reached this location after traveling across the more superficial layers of the forming cerebellar cortex. We have confirmed this result after biolistic transfection of acute cerebellar slices (Lossi and Merighi, 2003) with a vector that allows for the direct visualization of caspase 3 activation in vivo (Fig. 3C and D).

The pattern of immunostaining for cleaved caspase 3 was almost identical to that obtained after in situ labeling of cells with fragmented DNA. As previously reported by others (Srinivasan et al., 1998), our observations indicated that caspase 3 is cleaved in the cytosol and then rapidly translocated into the nucleus of cells undergoing PCD. A remarkable observation was the lack of immunostaining for the inactive zymogen. Biochemical analysis of rat cerebellar extracts at different developmental stages has recently shown that the level of expression of both uncleaved and cleaved caspase 3 in the postnatal period (P0–30) was rather low compared to other areas of the brain (Mooney and Miller, 2000). In addition, an investigation of the expression of caspase 3, BAX and BCL-X_L mRNAs by in situ hybridization showed that whereas BAX and BCL-X_L mRNAs were expressed widely in neonate and adult mice, caspase 3 mRNA levels were strongly reduced after birth (De Bilbao et al., 1999). These authors found that in early postnatal days, caspase 3 mRNA was expressed at particularly high levels in the areas of active neurogenesis, where a positive correlation with the distribution of apoptotic nuclei also was shown. In the adult, caspase 3 mRNA was restricted to the piriform and entorhinal cortices, the neocortex, and to areas where neurogenesis is still present (SVZ, olfactory bulb and dentate gyrus). These results demonstrate that the expression of caspase 3 mRNA is highly regulated in parallel with occurrence of postnatal apoptosis.

As to the localization of active caspases upstream to caspase 3, the demonstration of cleaved caspases 9 and 7 in postmitotic CGCs indicated these two caspases and caspase 3 act in cascade to execute PCD in vivo (Lossi et al., 2003). Detection of positive staining for cleaved caspase 9 was consistent with some experiments in vitro using potassium-deprived CGCs (Gerhardt et al., 2001), but not after withdrawal of trophic support (Allsopp et al., 2000). Although in cultured CGCs it appears that other caspases, namely caspase 6, contribute to early caspase 3 activation besides to caspase 9 (Allsopp et al., 2000), we have been unable to detect cleaved caspase 6 immunoreactivity in vivo. Therefore, our results speak in favor of the idea that, in postmitotic CGCs, the apoptotic machinery is primarily triggered by caspase 9 cleavage.

It is well known that activated caspase 3 is responsible for proteolytic cleavage of many key proteins in apoptosis, among which PARP-1 (Smith, 2001). We have studied the distribution of PARP-1 in postnatal rabbits, and obtained evidence that cleavage at P5 was restricted to the cerebellum, although the zymogen had a widespread distribution in several other areas of the brain. Previous biochemical data from our laboratory indicated that there is an up-regulation of PARP-1 in the postnatal rabbit cerebellum, in parallel with the peak of CGC death in P5 animals (Lossi et al., 2002c). While expression of PARP-1 occurred in postmitotic neurons populating the inner premigratory EGL and other layers of the forming cerebellar cortex, immunocytochemical labeling for p85 (the active PARP-1 fragment) was restricted to the nucleus of postmitotic postmigratory CGCs undergoing delayed apoptosis within the IGL (Fig. 3A and B).

An additional substrate for caspase 3 in cultured CGCs appeared to be Rb (Boutillier et al., 2000), although this finding is somehow controversial (Padmanabhan et al., 1999). Rb cleavage by caspases is cell specific, being an important event only in certain types of apoptosis (Tan and Wang, 1998). Overexpression of a cleavage-resistant Rb mutant prevented nuclear fragmentation, suggesting that cleavage of Rb was an obligatory step for cultured CGCs to enter apoptosis (Boutillier et al., 2000). However, it remained unclear whether or not phosphorylation of Rb was required for caspase recognition and further degradation. It is known that caspases preferentially degrade the underphosphorylated form of Rb in cycling cells (Dou and An, 1998). Nonetheless, in postmitotic neurons, phosphorylation of Rb upon apoptosis entry has been described on unidentified residues or on Ser795 (Copani et al., 1999; Padmanabhan et al., 1999). The pattern of in vivo staining for phospho-Rb (Ser807–811) and phospho-Rb (Ser795), would favor the possibility that the first, but not the latter is a substrate for caspase 3 in the rabbit IGL (Lossi et al., 2003). Considering that early neuronal death of proliferating GCPs and young postmitotic neuroblasts displays a partial temporal overlap to that of fully differentiated postmitotic CGCs (Fig. 3B), the presence of phospho-Rb (Ser795) immunoreactive CGCs at boundaries with the Purkinje cell layer may not be related to apoptosis, but rather to retention of high levels of proliferation proteins upon exit from the cell cycle, such is for example the case of PCNA (Lossi et al., 2002b). In keeping with this possibility, there is a critical temporal requirement for Rb during neuronal determination (Slack et al., 1998), and the protein is subsequently down-regulated.

3. Transgenic models and mutant animals

With the advent of transgenic technology we have gained a more in depth knowledge about the function of many genes related to apoptosis. Tables 3 and 4 summarize the main data on transgenic and mutant animals displaying neural phenotypes related to cell death. These animal models have proved to be very useful to confirm in vivo the biological functions of several molecules involved in PCD. We will briefly consider below the three groups of transgenic animals more relevant to the present discussion: (i) those carrying caspase (and related molecules) transgenes; (ii) the BCL-2 family transgenics; and (iii) animals with cell cycle-related protein mutations.

Analysis of caspase knockouts has been fundamental to prove the crucial role of some members of this protease family in neuronal apoptosis (Nicholson and Thornberry, 1997), while others, such as caspases 1 and 2, were shown to be only indirectly involved (Kuida et al., 1995; Li et al., 1995; Bergeron, 1998; Troy et al., 2000, 2001), depending upon the type of apoptotic stimulus and the regulated relative expression of components of the different pathways which may be theoretically activated to execute apoptosis.

CNS alterations in mice deficient in caspase 3 have been demonstrated to reflect the failure of apoptosis during normal neurogenesis, and the reduction of cell death has been associated with suppression of the mitochondrial apoptotic pathway (Kuida et al., 1996). The CNS phenotype of caspase 9 deficient mice consisted of an excess of cells in most regions including the periventricular cerebral wall, i.e. the area in which progenitor cells are generated (Hakem et al., 1998; Kuida et al., 1998). In keeping with the idea that significant cell death affects proliferating neuronal precursors and young neuroblasts (De la Rosa and De Pablo, 2000), the caspase 9 knockout phenotype did not argue for a lack of cell death in the VZ during neurogenesis, but rather for an augmented number of precursor cells prior to neurogenesis. This concept was supported by the observation that there were more BrdU-incorporating cells in the VZ of *casp* $9^{-/-}$ (and *apaf1*$^{-/-}$) mice compared to wild-types (Hakem et al., 1998; Yoshida et al., 1998). Considering that 100% cortical precursor cells are mitotically active in wild-type mice (Takahashi et al., 1993; Miyama et al., 1997), CNS alterations in these transgenics indicated that, during development, not only decreased cell death, but also increased cell proliferation prior to the onset of neurogenesis may be responsible for oversized neuronal populations. Due to centrality of Apaf1 in the apoptotic machinery, targeted mutations of this adapter protein had profound effects on development (Cecconi, 1999). Interestingly, *apaf1* knockout did not perfectly overlap *casp 3* and *casp 9* knockouts, which, as discussed above, predominantly exhibit a neuronal phenotype. In *apaf1*-deficient embryos, the number and severity of developmental alterations were higher, more severe, and distributed all over the organism. Besides to reduction in apoptosis, the excess of CNS neurons could be related to an increased mitotic activity, in keeping with the recently hypothesized role for Apaf1 in cell proliferation (Cecconi, 1999; Cecconi and Gruss, 2001).

Studies in transgenic animals showed unequivocally that endogenous BCL-2 is crucial for the maintenance of specific populations of neurons during the early postnatal period (Michaelidis et al., 1996), impedes neuronal death induced by various stimuli, and regulates neuronal survival in a dose-dependent manner (Tanabe et al., 1997). Thus, overexpression of BCL-2 led to significantly increased neuronal numbers in several areas of the CNS, as a consequence of inhibition of apoptosis in NOND (Martinou et al., 1994; Zanjani et al., 1996, 1997) and experimental injury (Bonfanti et al., 1996; Yakura et al., 2002). Likewise, transgenic animals carrying mutations for one or more genes encoding for the BCL-2 family proteins demonstrated the synergistic/antagonistic effects of these molecules on spontaneous and experimental neuronal apoptosis (Lindsten et al., 2000; Fan et al., 2001).

The overall results of transgenesis experiments aiming to modify cell cycle-related protein levels converged to demonstrate an important role of these molecules in CNS apoptosis. *rb*$^{-/-}$ mice died by E16 and showed alterations of the nervous system (Clarke et al., 1992; Jacks et al., 1992; Lee

Table 3
Transgenic animals displaying neuronal death phenotypes

Transgenic animals	Phenotype	References
Dominant negative mutant of caspase 1	Reduced sensitivity to ischemia; DRG neurons resistant to trophic factor deprivation	Friedlander and Yuan (1998)
casp 2$^{-/-}$	Sympathetic neurons more susceptible to trophic factor deprivation; cell death of facial motorneurons augmented	Bergeron (1998)
casp 3$^{-/-}$	Pronounced effects on the development of the CNS and premature lethality; diffuse hyperplasia, ectopic cell masses, abnormal structural organization and augmented numbers of neurons in the cortex, striatum, hippocampus, cerebellum and retina	Kuida et al. (1996), Pompeiano et al. (2000)
casp 9$^{-/-}$	Excess of cells in most CNS regions	Hakem et al. (1998), Kuida et al. (1998)
casp 12$^{-/-}$	Reduced sensitivity to certain apoptotic stimuli	Nakagawa et al. (2000)
apaf1$^{-/-}$	Open brain (and/or spina bifida) and forebrain overgrowth; retinal overgrowth; delayed cortical layering and enlargement of the mantle layer in the brain; thickening of the hindbrain walls	Cecconi et al. (1998), Cecconi (1999), Yoshida et al. (1998), Cecconi and Gruss (2001)
bcl-2 deficient	Reduction of neuronal survival	Michaelidis et al. (1996), Tanabe et al. (1997)
bcl-2 overexpressing	Reduced neuronal loss during the NOND period, leading to hypertrophy of several neuronal types including cerebellar Purkinje neurons and RGCs; neurons were more resistant to permanent ischemia	Martinou et al. (1994), Zanjani et al. (1996, 1997), Yakura et al. (2002)
bax$^{-/-}$	Numerical reduction of specific neuronal populations (?)	Bernard et al. (1998), Brady and Gil-Gomez (1998), Bar-Peled et al. (1999), Fan et al. (2001)
bax$^{-/-}$/*bak*$^{-/-}$	Accumulation of excess cells within CNS	Lindsten et al. (2000)
BCL-X_L deficient	Extensive apoptotic cell death of postmitotic immature neurons in brain, spinal cord, and DRGs	Motoyama et al. (1995)
BCL-X_L and BAX deficient	Greatly reduced levels of apoptosis both in vivo and in vitro	Shindler et al. (1997)
bcl-x$_L$$^{-/-}$/*casp-9*$^{-/-}$	Expanded VZ; neuronal phenotype identical to that of mice lacking only caspase 9	Zaidi et al. (2001)
BCL-X_L overexpressing	Reduced susceptibility to apoptosis	Parsadanian et al. (1998)
rb$^{-/-}$	Aberrant mitoses and marked neuronal death in several areas of the developing brain, spinal cord, and sensory ganglia; increased cell death was detected mainly in the intermediate zone but not in the VZ	Clarke et al. (1992), Jacks et al. (1992), Lee et al. (1992)
p107 and Rb deficient	Early embryonic death and accelerated apoptosis	Yoshikawa (2000)
p130$^{-/-}$	Varying degrees of nervous system disorganization; massive apoptosis and increased numbers of proliferating cells in the brain; marked neuronal loss in the spinal cord and DRGs	LeCouter et al. (1998)
Double-targeted mutation of Jnk1 and Jnk2	Increased PCD in the forebrain leading to precocious degeneration of cerebral precursors	Haydar et al. (1999)
traf6$^{-/}$	Failure of neural tube closure and exencephaly; reduction of apoptosis in the ventral diencephalon and mesencephalon	Lomaga et al. (2000)
Cystatin B deficient	Loss of CGCs	Pennacchio et al. (1998)

Table 4
Mutant animals displaying neuronal death phenotypes

Mutation	Phenotype	References
Ataxia (*ax*)	Increased numbers of apoptotic cells in cerebellum	Ohgoh et al. (2000)
Flathead (*fh*)	40% of normal brain size at birth; dramatic increase in apoptotic cell death in the E16–P1 interval mainly affecting neocortex, hippocampus, cerebellum and retina; no anomalies in PNS	Roberts et al. (2000)
hoxc-8	Enhanced apoptosis of C7-T1 motoneurons	Tiret et al. (1998)
Harlequin (*hq*)	Degeneration of cerebellar and retinal neurons; reduction in AIF expression	Klein et al. (2002)
Lurcher (*lc*)	Primary cell death of cerebellar Purkinje neurons; secondary cell death of CGCs and olivary neurons	Wüllner et al. (1995, 1998), Zanjani et al. (1998), Doughty et al. (2000), Selimi et al. (2000b)
Weaver	Increased apoptosis of cerebellar EGL neurons	Gillardon et al. (1995), Migheli et al. (1995, 1999), Owens et al. (1995), Wüllner et al. (1998), Selimi et al. (2000b)
Weeble	Significant neuronal loss in the cerebellum and in the hippocampal CA1 field	Nystuen et al. (2001)
Wobbler (*wb*)	Significant motor neuron loss in the cervical, but not lumbar spinal cord segments; gliosis	Festoff et al. (2000)

et al., 1992): cell death in the developing CNS of $rb^{-/-}$ embryos seemed to involve postmitotic neuroblasts shortly after they entered an ectopic S phase, and was associated with high levels of p53 and p21 (Macleod et al., 1996), suggesting that apoptosis in $rb^{-/-}$ mice is p53 dependent.

$p107^{-/-}$ mice displayed no obvious anomalies. However, mice deficient in both p107 and Rb died at about E11.5 with a CNS phenotype displaying accelerated apoptosis, consistent with the idea that the two proteins have cooperative and overlapping functions (Yoshikawa, 2000). The phenotype of $p130^{-/-}$ displayed massive apoptosis and increased numbers of proliferating cells, but with a strong dependence upon the genetic background, probably because of the existence of p130 modifier genes (LeCouter et al., 1998).

Although the examples given so far speak in favor of the recombinant DNA approach, transgenic animals have at times raised new questions, rather than offering definitive answers. Moreover, knockout animals often provided problems of their own. Redundancy is a first problem in knockouts, so that overexpression of compensatory molecules often yields a normal phenotype, although the developmental dynamics may be only slightly altered (Pamfer and Donnay, 1999). A second problem is that the knockout may produce a number of unintended and not recognized consequences. For instance, the knockout of individual caspases, in general, does not seriously affect embryonic development (Pamfer and Donnay, 1999), but the knockout of caspase 9 is lethal, having as a consequence a massive forebrain overgrowth, with exencephaly (Zheng et al., 1999; Oppenheim et al., 2001). The first interpretation of this finding was that excess cell number derived from blockage of apoptosis (Chun and Schatz, 1999). However, an excess of neurons was present as early as E10 in *casp* $9^{-/-}$ mice, well before the developmental stages in which one can observe heavy ISEL-positive staining of apoptotic cells (Blaschke et al., 1996; Kuida et al., 1998), and it was subsequently observed that cell death in the brain and spinal cord were quantitatively normal, but dying cells did not appear to be apoptotic (Oppenheim et al., 2001). The cause of brain overgrowth remained unexplained so far, and this example tell us about the importance of an in vivo confirmation in normal animals (possibly from a variety of different species) of the results obtained in transgenics.

Similar problems have been found in the interpretation of the results deriving from analysis of spontaneous mutants (Table 4). In addition, these animals often display multifaceted phenotypes, and the mutated gene(s) has(have) not always been identified.

In general terms, studies on spontaneous mutants have been helpful to better characterize the importance of cell-autonomous and target-related neuronal death. Among these mutations, lurcher (*lc*), an autosomal dominant mutation in mice that resembles human adult dominant ataxia, is the best characterized. Initially it was shown that, in *lc*, Purkinje cells undergo apoptosis during postnatal cerebellar development (Wüllner et al., 1995). Then, it was demonstrated that the gain-of-function mutation in the δ2 glutamate receptor (GRID2) results in the cell-autonomous death of cerebellar Purkinje cells in heterozygous *lc* mice. This, in turn, triggered the massive loss of afferent CGCs and olivary neurons during the first few postnatal weeks (Selimi et al., 2000a). Evidence suggested that the death of Purkinje cells was a direct consequence of GRID2 activation, while death of CGCs and olivary neurons was a consequence of target deprivation. Cell death in these three neuronal types occurred by apoptosis, and activated caspase 3 was detected in all these neurons before their death (Doughty et al., 2000; Selimi et al., 2000a).

In parallel, the neurochemistry of Purkinje cells in *lc* mice was significantly modified compared to that of normal animals. These neurons exhibited increased BAX, BCL-X and procaspase 3 expression, while no loss of BCL-2 was evident (Wüllner et al., 1998; Selimi et al., 2000a). Moreover, mice carrying null mutations of both the *bax* and *p53* genes displayed delayed Purkinje cell death in response to the GRID2 (*lc*) mutation and no secondary death of CGCs (Doughty et al., 2000). In contrast, the p53 deletion had no effect on either cell death pathway. In keeping, *bax* inactivation in *lc* mutants rescued CGCs but not Purkinje neurons upon inhibition of caspase 3 cleavage, while *p53* inactivation did not affect neuronal loss or caspase 3 activation in mutants (Selimi et al., 2000a). Finally, adult *lc* mutants expressing a human *bcl-2* transgene still displayed Purkinje cell degeneration, but most olivary neurons were rescued from target-related cell death. Although the number of olivary neurons was equivalent to that in wild-type animals, the inferior olive nucleus was significantly shorter in its rostrocaudal extent (Zanjani et al., 1998).

4. Concluding remarks

Although we are still far form a overall coherent view on NOND from evidence in normal and transgenic animals, the emerging picture is that of a dual wave of apoptotic cell death which affects central neurons at different stages of their life. Early neuronal death of proliferating precursor cells and young postmitotic neuroblasts is assuming a more detailed contour, and an in depth analysis of the spatial, temporal and molecular links of proliferation and apoptosis will be surely much informative to better clarify the physiological role of this phenomenon. In particular, a careful examination of transgenic models and/or spontaneous mutants showing alterations in the number of neural cells during neurogenesis, and ex vivo approaches to modify gene expression will be helpful to give an answer to a number of yet unsolved questions, the ultimate of which is why so many neurons are generated in excess and die immediately thereafter.

The death of postmitotic neurons is much better understood in functional terms, mainly on the basis of the neurotrophic concept in its broader definition. Nonetheless a

number of issues remain unsolved, such as for example the effective role of $p75^{NTR}$ in neuronal apoptosis.

The availability of models of NOND in vivo such as the cerebral and cerebellar cortices, the olfactory system and the retina is and will be of importance to the definition of the biological function(s) of NOND. The cerebellar cortex, which has a relatively simple architecture and has been widely characterized in terms of neuronal proliferation, migration and circuitry, appears to be particularly suitable to this purpose since the extent of cell death is significantly higher compared to other brain and spinal cord areas.

Undoubtedly, analysis of NOND in vivo will offer a basis for parallel and future studies involving secondary neuronal loss in neurodegenerative disorders, trauma or ischemia, which are, at present, the major causes of human neurological deficiencies.

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