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Review

Apoptosis of the cerebellar neurons

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Summary. Naturally occurring neuronal death (NOND) is an essential phenomenon during the course of normal development of the nervous system. Studies *in vivo* and on organotypic cultures have helped to elucidate the basic histological and ultrastructural features, as well as the main cellular mechanisms of NOND in several areas of the brain. This review examines the existing evidence about the two waves of apoptotic cell death that affect the different types of cerebellar neurons in normal development and certain pathological conditions. The first wave regards neuronal progenitors and pre-migratory neuroblasts, the second post-migratory neuroblasts and mature neurons. The underlying cellular and molecular mechanisms are discussed critically also in the light of their relevance to neurodegenerative diseases.

Key words: Apoptosis, Programmed cell death, Cerebellum, Neurons, Organotypic cultures

Introduction

Two parallel, intrinsically linked processes occur during development of the nervous system: the generation of new neurons and glia, and the death of cells that are no longer required and/or are produced in excess. The elimination of specific cell populations is a general process that takes place in virtually all tissues and organs during development of multicellular organisms. Because this process of cell death occurs under physiological conditions and follows a complex framework of regulated events, it is commonly referred to as programmed cell death (PCD) (Holtzman et al., 1992; Migheli et al., 1994; Stewart, 1994; White, 1996; Marks and Berg, 1999).

PCD takes several different forms, but, most commonly, neurons die following a stereotyped and phylogenetically conserved series of molecular and cellular modifications, usually referred to as apoptosis (Glucksmann, 1951; Saunders, 1966). Nonetheless PCD in neurons is not always characterised by an apoptotic morphology (Alles et al., 1991; Cohen et al., 1992; Eastman, 1993; Leist and Jaattela, 2001).

Apoptosis was originally defined as a form of cell death independent from pathological insults. More recently, however, it has also been implicated in the loss of neurons associated with physiological ageing, many neurodegenerative disorders and traumatic injuries.

The characterisation of apoptosis in mammalian neurons mainly relies on *in vitro* studies, although analysis of intact animals under normal and/or experimental conditions and transgenic models would be of paramount importance to our understanding of relevance of apoptosis *in vivo*. This review is focused on data available in current literature on apoptosis of mammalian cerebellar neurons *in vivo*. It aims to put together a comprehensive discussion of data obtained from studies other than those derived from isolated cells from primary cultures and or neuronal cell lines. The idea beyond this is that a correct understanding of the role of apoptosis in cerebellar biology may be better achieved when cell to cell interactions are preserved as it occurs *in vivo* and/or in experimental models that are closer to the *in vivo* conditions.

Some basic concepts and definitions

The nervous system differentiates from the 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. Neuroblasts are thought to establish their future phenotype, and thereby transform into differentiated neurons, upon generation by terminal-mode symmetric divisions of committed progenitors (McConnel, 1995; Yoshikawa, 2000). Thus, differentiated neurons are postmitotic cells completely

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devoid of replicative capability. Most mammalian CNS neurons reach such a 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 which, differently from other cell types, is irreversible.

For neurons, the term naturally occurring neuronal death (NOND) is also in use as a synonym of PCD. NOND will be used in this paper to indicate physiological neuronal death under normal conditions.

The term apoptosis will be used specifically to indicate a type of cell death which, as will become clear below, is characterised by a very well defined series of morphological and biochemical hallmarks but not necessarily independent from pathological insults.

Specific cell pathways are activated in apoptosis, and, in this type of death, cells are responsible for their own demise, a reason why apoptosis is commonly considered to be a form of ''cell suicide''.

The discovery of apoptosis

Given the widespread occurrence of apoptosis in multicellular organisms identification of molecules that control the process and analysis of their biological effects is obviously becoming more and more important.

Current knowledge about the genetic regulation of apoptosis is mainly based on studies of the nematode worm *Caenorhabditis elegans* (Hengartner and Horvitz, 1994; Hoffman and Liebermann, 1994; Stewart, 1994; Vaux et al., 1994; Yuan, 1995; Fraser et al., 1996; Meier and Evan, 1998; Liu and Hengartner, 1999).

After the discovery that an essential protein for developmental death in *Caenorahbitis elegans* (*Caenorhabditis elegans* death protein-3= CED-3) shared considerable homology with the human and murine interleukin-1ß converting enzyme (ICE - Yuan et al., 1993), and subsequent recognition of the ICE/CED-3 or caspase family of cysteine proteases (for review see Alnemri et al., 1996, Fraser et al., 1996), these proteins have been extensively investigated among those producing proapoptotic signals (Allsopp et al., 2000; Gerhardt et al., 2001). Nowadays, it is fully established that mammalian caspases are synthesised as inactive precursors which are then activated by proteolysis (Stennicke and Salvesen, 1999; Katchanov et al., 2001). In particular, recent studies have shown that caspase 3 cleavage triggers apoptosis in cerebellar granule cells (CGCs) as the endpoint of a cascade that involves other caspases *in vitro* and *in vivo* (Allsopp et al., 2000; Lossi et al., 2004a).

Histologically, apoptosis was originally defined as a distinct mode of cell death on the basis of a series of characteristic ultrastructural features according to a well defined sequence of events (nuclear and cytoplasmic condensation, cell fragmentation and phagocytosis) which affect cells during the course of their elimination (Kerr et al.,1972). Since these ultrastructural changes were similar, but not identical to those occurring in necrosis, initially Kerr et al. used the term "shrinkage necrosis" to describe this type of cell death.

Subsequently, they adopted the term "apoptosis" (from the Greek=falling of the leaves), which indicates the dropping of leaves from trees or petals from flowers, to emphasise the role of this type of cell death in normal tissue turnover.

Apoptosis involves a series of stereotyped, morphologically well defined phases, which are most clearly evident at the electron microscope level and eventually result in cell shrinkage (Fig. 1).

Changes in the nucleus represent the first unequivocal evidence of apoptosis. Chromatin condensation and segregation into sharply delineated masses that abut on the nuclear envelope are a typically initial observation. These masses are made up of closely packed, fine, granular material and thus are very electrondense. They often show a characteristic crescent-like appearance. This initial chromatin condensation eventually leads to true nuclear pyknosis. 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 cell organelles remain constantly unaffected, although abnormally closely packed, probably as a consequence of the loss of cytosol. Ribosomes detach from the rough endoplasmic reticulum and from polysomes. As the process proceeds, 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. In tissues, apoptotic bodies are rapidly cleared out by macrophages or neighboring cells, and are degraded within heterophagosomes.

Specifically regarding CGCs, we have recently demonstrated that individual granules are phagocytosed by glial cells before being fragmented into apoptotic bodies (Lossi et al., 2002b). Apoptotic CGCs in the external granular layer (EGL) are internalized as a whole within the microglia. This latter bends around the CGC and becomes engulfed with the apoptotic cell. In the internal granular layer (IGL) CGCs are commonly fragmented into several apoptotic bodies before being retrieved inside the heterophagosomes. It was easy to spot some phagocytic cells engulfed with apoptotic material in close apposition to blood capillaries. In experiments aiming to establish the temporal relationship of proliferation and apoptosis, we have observed intraluminal blood monocytes engulfed with heterophagosomes that contained highly condensed nuclear DNA pre-labelled *in vivo* as little as 24 hours before. Since proliferation of CGCs only occurs in the EGL, these observations demonstrate that in the limited span of time between tracer administration and sacrifice,

some CGCs completed their division, entered the apoptotic program and are cleared by the glia.

Apoptosis versus necrosis

It is often assumed that apoptosis (being a gene regulated process) is a completely different form of cell death compared to necrosis, which is consequent to external insults that compromise cell integrity. This concept was 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).

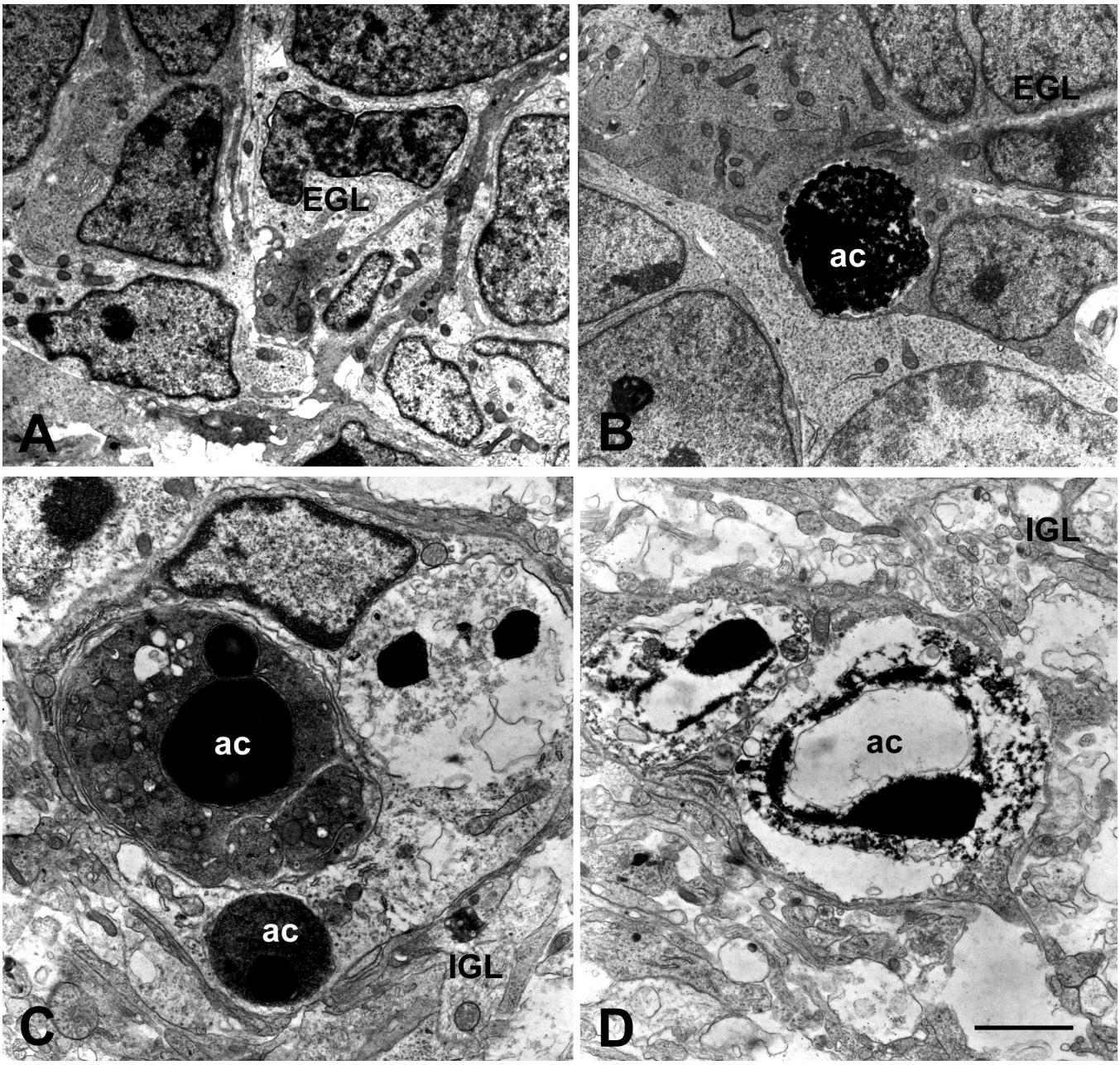

Fig. 1. Ultrastructure of apoptotic neurons in the rabbit cerebellum at post-natal day 5. **A.** Healthy CGCs in the EGL. **B.** A late apoptotic cell the border between the EGL and the Purkinje cell layer. Note the very high degree of nuclear and cytoplasm condensation. **C.** A phagocytic cell in the IGL is engulfed with apoptotic cell residues. **D.** Two mid-to-late apoptotic cells in the IGL display advanced nuclear condensation and initial cytoplasm shrinkage with intact organelles. ac: apoptotic cell; EGL: external granular layer; IGL: internal granular layer. Bar: 2 µm.

In more recent times, it has become clear that, in mammalian cells, the gap between apoptosis and necrosis is filled by many intermediate morphological cell types, in which blebbing may be more or less evident, and varying degrees of chromatin condensation and margination may be apparent (Leist and Jaattela, 2001).

Additionally, the association of apoptosis and physiological cell death also turned out to be an oversimplification for several reasons. First, the intrinsically necessary elimination of specific cell populations during development of multicellular organisms is often, but not always, characterised 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 additional 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, tumour homeostasis, and the action of cytotoxic drugs employed in chemotherapy (Hoffman and Liebermann, 1994; Stewart, 1994; Mimeault, 2002; Singh et al., 2002). Fourth, 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; (Moroni et al., 2001; Cole and Perez-Polo, 2002; Fujikawa et al., 2002; Hou and MacManus, 2002; Schwab et al., 2002).

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 effectors (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, cytochrome *c* is released from the mitochondrial intermembrane space prior to caspase activation. In the second, which is exemplified by activation of death receptors, an effector caspase is activated prior to mitochondrial alterations. Death receptors are cell surface receptors that trigger apoptosis. There are several types of death receptors in different tissues, but two members of the tumour 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 ($p75^{NTR}$). 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 hypothesise 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), which is controlled by a voltage- and Ca^{2+}-sensitive pore, referred to as the permeability transition (PT) pore (Blatt and Glick, 2001). Enhanced K^+ efflux has also been shown to be an essential mediator, not only for early apoptotic cell shrinkage, but also of downstream caspase activation and DNA fragmentation (Remillard and Yuan, 2004).

Caspases (caspase = cysteine aspartate protease - Thornberry and Lazebnik, 1998; Blatt and Glick, 2001) play a crucial role in dismantling cell structures and organelles during the course of apoptosis. Among the 14 members of the caspase family identified to date, only caspases 3, 6, and 7 degrade vital cellular proteins and thus are directly involved in apoptosis. The others mediate protein–protein interactions, and may only occasionally trigger apoptosis (Thornberry and Lazebnik, 1998). Each caspase is initially synthesised as a zymogen and requires processing at specific cleavage sites to generate the active enzyme (Stennicke and Salvesen, 1999). The first caspases to be activated, in turn, trigger other downstream caspases giving rise to a proteolytic cascade that culminates with the execution of apoptosis.

Caspase 3 activation depends on activity of a large protein complex (apoptosome) which plays a fundamental role in apoptosis (Adams and Cory, 2002). The apoptosome consists of apoptosis protease activated factor 1 (APAF 1 - Zou et al., 1997), cytochrome c (also referred to as APAF 2), and procaspase 9 (also referred to as APAF 3). Biochemical studies have revealed that caspase 3 processing requires not only the up-stream caspase 9, but also APAF 1 and cytochrome c (Liu et al., 1996; Li et al., 1997). Once cytochrome c is released from mitochondria at the onset of apoptosis, a series of conformational changes lead to APAF 1 multimerisation and association with procaspase 9 with the generation of the about 1 MDa molecular weight apoptosome (Adams and Cory, 2002).

Another important group of apoptosis-related proteins is formed by the so-called mammalian B-cell lymphoma- 2 (BCL-2) protein family (Adams and Cory, 1998; Chao and Korsmeyer, 1998; Newton and Strasser, 1998; Sanchez and Yuan, 2001). Besides BCL-2, some other protein members of the family (such as BCL-XL and BCL-W) act as survival factors, whereas others (such as BAX, BAK, BAD, BID) are pro-apoptotic. The anti-apoptotic members of the BCL-2 family protect cells from death by at least two different mechanisms. First, BCL-2 and BCL-XL 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, BCL-XL interacts with APAF 1, and inhibits association of APAF 1 with pro-caspase 9, again with blockage of apoptosome formation and inhibition of caspase 9 activation (Adams and Cory, 2002). Subcellular localization studies have shown that the anti-apoptotic members of the BCL-2 family (BCL-2, BCL-XL) 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.

Another protein that is normally located in the intermembrane space of mitochondria is the apoptosis-inducing factor (AIF). AIF is a flavoprotein that, upon apoptotic signalling, 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). Overexpression of BCL-2 prevents the release of AIF from mitochondria, but not its apoptogenic activity (Susin et al., 1999). Recent data show that AIF is released by a mechanism distinct from that of cytochrome *c*, probably mediated by poly-adenyl ribose polymerase 1 (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). 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 *hq* 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.

An additional protein with the dual name Smac/DIABLO, released together with cytochrome c during apoptosis, (Du et al., 2000; Verhagen et al., 2000), 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. Therefore, in these systems, Smac/DIABLO may be the second factor required for the so-called competence to die (Deshmukh and Johnson jr., 1998). One of these IAPs, called survivin has recently been demonstrated to be essential for normal CNS development (Jiang et al., 2005).

Identification of apoptotic cells *in vivo*

Apoptosis is a very quick phenomenon, and apoptotic cells *in vivo* are very efficiently removed by macrophages. As a consequence, while it is relatively simple to study apoptosis in cell cultures, especially if cell death is experimentally triggered by simple addition of apoptogenic drugs to culture medium, identification of apoptotic cells in tissue sections is a rather demanding task, as a consequence of the non simultaneous occurrence of death in different cellular types and/or even within a given cell population. Moreover, when apoptosis is affecting precursors that are still capable of replication, analysis of PCD is made even more complicated (Fig. 2). Further difficulties are added by the possibility that some cell death processes in PCD are not apoptotic, and that some molecules and/or mechanisms of apoptotic and non-apoptotic cell death may be shared. The discussion of the technical approaches for study of neural apoptosis *in vivo* is beyond the scope of the present paper, but we have reviewed this issue in a book devoted to methodological aspects of CNS analysis (Lossi et al., 2002a), and several other relevant reviews may be found in the literature (Harmon et al., 1998; Marks and Berg, 1999; Sastry and Rao, 2000; Blatt and Glick, 2001).

As an alternative to the *in vivo* approach, the use of organotypic cultures (Fig. 3) is particularly appealing, since cultures display a normal architecture and neurochemical differentiation, maintain the connections between different neuronal populations in a quasi physiological fashion, and can be experimentally manipulated by gene transfer procedures (see Savill et al., 2005).

Apoptosis of cerebellar neurons *in vivo*

It is generally assumed that about half of the neurons produced during neurogenesis die before completion of 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 PCD. In several areas of the brain, including the cerebellum, there are two subsequent periods of apoptotic cell death: the first 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 (De la Rosa and De Pablo, 2000; Lossi et al., 2002b).

We will review below the data available for specific types of cerebellar neurons.

Apoptosis of stellate and basket cells

Stellate and basket cell progenitors proliferate in the white matter during development and finally reach the molecular layer. They are generated post-natally from the ventricular zone (VZ) at the roof of the fourth ventricle between P2/3 and P16/17 with a peak at P6/7 in rodents (Altman and Bayer, 1997). In the first postnatal week these neurons are present in white matter, whereas in the following days they proliferate and move from the

white matter to the molecular layer. Migration is completed at the third week when these neurons are totally absent in the white matter.

During their proliferation/migration, the stellate and basket cells undergo apoptosis as demonstrated in the GAD67/GFP mice (Yamanaka et al., 2004). In these animals, all GABAergic neurons express the fluorescent marker GFP (green fluorescent protein) under the control of the GAD67 promoter that is specifically turned on in GABAergic neurons. In transgenics, fluorescent cells with an apoptotic morphology are present in all layers of cerebellar cortex between P5 and P21. Five to ten percent of the total number of GFP-positive apoptotic cells are located in the molecular layer, the Purkinje cell layer and the white matter. These observations indicate, although on indirect bases, that the stellate and basket cells suffer apoptosis, both during their migration from the white matter across the Purkinje cell layer, and after they reach their final destination in the molecular layer.

Apoptosis of Purkinje cells

Purkinje cells are the key neurons of the cerebellar cortex and its only output. These neurons have been the target of many neurological mutations in the mouse and mutants turned out to be very useful animal models to study Purkinje cell death (Dusart et al., 2006).

It is still debated whether or not Purkinje cells undergo apoptosis during development, because they normally do not exhibit the morphological features typical of PCD (Norman et al., 1995). However, these cells are not easily recognisable until P4, when they reach their final location between the molecular layer and IGL, and start forming a monocellular layer in cerebellar cortex (Fig. 3A,B). Quantification studies show that from P4 to adulthood there is no variation in the Purkinje cell total number, but that developmental cell death also affects these neurons. In mouse embryos, pyknotic figures are present in the area of the cortical plate where Purkinje cells originate, and immuno-

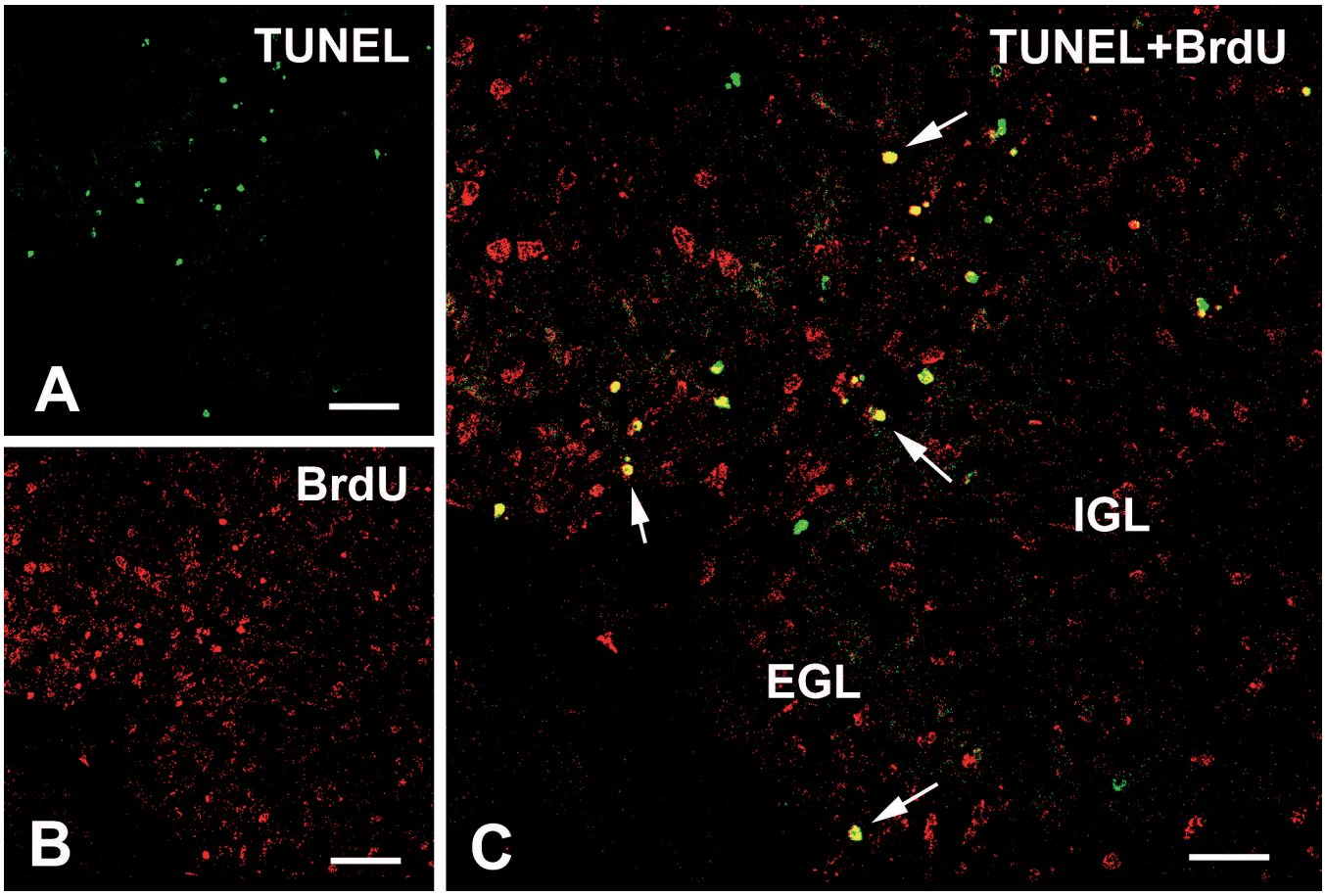

Fig. 2. Relationship of apoptosis and proliferation in the murine cerebellum at post-natal day 10. Apoptotic cells were labelled according to the TUNEL procedure (green), and proliferating cells were identified after multiple injections of the nucleotide analogue BrdU and subsequent immunostaining (red). Apoptotic cells are mainly detected in the outer portion of EGL, together with numerous BrdU positive cells. Note the presence of several double-labelled nuclei (yellow), some of which are marked by arrows. Confocal stacks of 10 optical z sections (green channel: Alexa Fluor 488; red channel: Alexa Fluor 594). BrdU: 5-bromodeoxyuridine; EGL: external granular layer; IGL: internal granular layer. Bars: A, B, 10 μm; C, 20 μm.

cytochemical studies showed active caspase-3 positivity, together with TUNEL positivity in these neurons (Marin-Teva et al., 2004). In parallel, transgenic mice that overexpress the antiapoptotic molecule BCL-2 have 40% more Purkinje cells than wild type mice (Zanjani et al., 1996), and, finally, Purkinje cells undergo apoptosis at P1-P5 when cultured *in vitro*. Taken together, all these studies confirm the occurrence of two periods of apoptotic programmed Purkinje cell death: a first embryonic period around E15 (Ashwell, 1990) and a second postnatal period around P3.

In the recent years, many genes were demonstrated

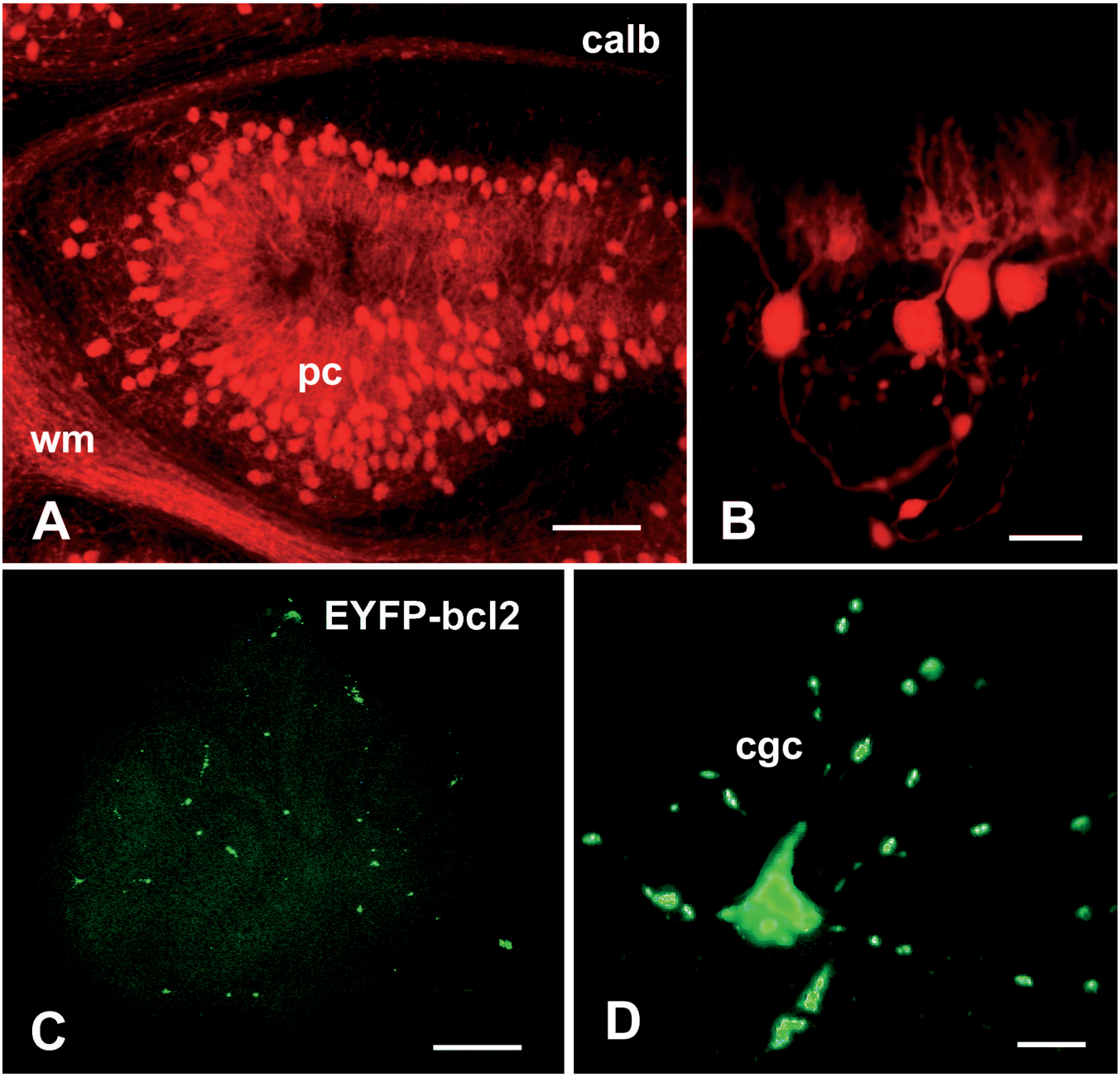

Fig. 3. Organotypic cultures of the murine post-natal cerebellum. **A-B.** A culture maintained *in vitro* for 8 days (8DIV) displays normal neurochemical differentiation as exemplified by immunostaining with an antibody directed against 28 kDa calbidin, a calcium-binding protein that specifically labels the Purkinje cells. **C.** Cerebellar slice at 8DIV after biolistic transfection with a vector that encodes for a fusion protein consisting of the antiapoptotic modulator bcl-2 tagged with EYFP. **D.** At higher magnification the fluorescent tag is clearly detected in a cell with the typical morphology of a granule neuron. bcl-2: B-cell lymphoma 2 protein; calb: calbindin; cgc: cerebellar granule cell; EYFP: enhanced yellow fluorescent protein; EGL: external granular layer; IGL: internal granular layer. Bars: A, 250 µm; B, 100 µm; C, 500 µm; D, 15 µm.

to be involved in control of Purkinje cell death, and, at the same time, the microglia was also implicated in the regulation of this process (Marin-Teva et al., 2004). Purkinje cell death is also present in many naturally occurring mouse mutations, where it generally takes the form of some type of cell degeneration (Guillardon et al., 1995). Toppler and Woozy are mutants in which Purkinje cells undergo PCD following apoptosis, sometimes with the concurrent activation of an autophagic mechanism. In these mutants, the death pathway seems to be different from that followed during conventional PCD (Dusart et al., 2006). On the other hand, many Purkinje cells die during normal ageing with degenerative changes similar to apoptosis.

Apoptosis of granule cells

Among cerebellar neurons, apoptosis of CGCs has been the most widely studied both *in vivo* and *in vitro*. *In vivo* analysis of granule cell apoptosis is made easier by the fact that this neuronal population is made up of billions of cells. Therefore, although NOND is not synchronised throughout the entire cerebellum, and apoptotic cells are rapidly removed from tissue, it is possible to localize this type of apoptotic cerebellar neuron in tissue sections with better chances of success.

The existence of two consequent phases of cell death that affect the pre-and post migratory CGCs respectively has been clearly demonstrated (see also Lossi and Merighi, 2003 for review). The first phase that affects the progenitor cells in the EGL has been neglected until recently. Nonetheless, the existence of NOND in the neuroepithelium at the beginning of neurulation was first described more than 50 years ago (Glucksmann, 1951). Later, early neuronal death was observed during the formation of the neural crest and neurogenesis, with a widespread distribution throughout the 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, it was reasonably assumed that dying cells were proliferating neuronal precursors, or newly generated neuroblasts at a stage in which they were totally disconnected from the target. A number of studies led to the conclusion that these early apoptotic neurons entered the cell cycle (based on their capacity to synthesize DNA, i.e. after BrdU labelling) shortly before death (Blaschke et al., 1996; Galli-Resta and Ensini, 1996; Thomaidou et al., 1997). For example, after labelling neurons born during rat development in limited time intervals, it was found that most retinal ganglion cells (RGCs) died within a maximal interval from 5 days after they have been generated (Galli-Resta and Ensini, 1996).

We have analyzed the relationship between proliferation and apoptosis of the granule cells in the postnatal cerebellum of rabbits and mice (Lossi et al., 2002b, 2004a). In these, as well as in other altricial mammals, such as rats, and humans, much of the cerebellar development occurs post-natally in parallel with massive cell death (Lossi et al., 2002b). The occurrence of cell death in the developing cerebellum has been originally inferred starting from the numerous studies based on counts of pyknotic nuclei in normal and experimental animals, which have shown that EGL cells of several species die during post-natal development. Subsequent work *in vivo* led to the demonstration, in different species of mammals, including humans, that death is of the apoptotic type (Wood et al., 1993; Krueger et al., 1995; Lossi et al., 1998). Cells undergoing apoptosis in the EGL have been mostly identified as immature CGCs and/or their precursors (GCPs).

Which apoptotic pathways are activated *in vivo* during the process of early NOND is still another open question. Due to the functional importance of caspases and members of the BCL-2 family, distributional studies have been carried out and focused onto the localisation of these proteins and/or their mRNAs, mainly in primates (Sohma et al., 1996; Lichnovsky et al., 1998; Lossi et al., 1998; Bernier and Parent, 1998; 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). These studies led to the demonstration that caspases 3 and 9 are expressed in the telencephalic ventricular zone (Sommer and Rao, 2002) and that experimental activation of caspase 3 is responsible for progenitor cell death, which is blocked by a pan-caspase inhibitor (D'Sa-Eipper and Roth, 2000). This indicated that neural progenitors can activate a caspase-dependent apoptotic pathway. However, in the cerebellum some experiments *in vitro* showed that caspase inhibitors were unable to protect CGCs from death (Miller et al., 1997; Padmanabhan et al., 1999). Since apoptosis could be blocked by transcriptional inhibitors, the question of whether it could be related to activation of certain components of the cell cycle machinery was raised (Ferrari et al., 1995).

We have analyzed the expression of a number of proteins involved in cell cycle control in the postnatal rabbit cerebellum, and have demonstrated that pre-migratory CGCs with typical apoptotic morphologies are stained *in vivo* with antibodies against phospho-Chk1 and two different forms of phospho-Rb (Lossi et al., 2004a). Moreover, in organotypic cultures transfected with a vector that enables us to follow the process of caspase 3 activation in living cells, we have proved that caspase 3 is not activated during apoptosis of GCPs/pre-migratory CGCs (Lossi et al., 2004b). Therefore, early neuronal death of GCPs/pre-migratory CGCs appears to be caspase 3-independent.

Although these studies have shed additional light on the first wave of apoptotic cell death affecting the CGCs, its functional significance remains elusive. The CGC-to-Purkinje cell ratio varies among species, yet it is highly regulated (Lange, 1975). There is thus the possibility that NOND of GCPs/pre-migratory CGCs, is related to the establishment of a correct ratio between the two neuronal types. In keeping, there are several data indicating that the survival of CGCs and Purkinje

neurons is based upon a reciprocal trophic support. Another possible explanation is that the massive cell death of the EGL neurons is related to the process of fissuration of the cerebellar cortex that eventually leads to formation of the folia. Although there is no direct evidence in support of this hypothesis, it is of interest to note that the process of cell death does not occur synchronously throughout the cerebellum, and that in certain lobes it appears that the number of apoptotic neurons is higher than in the others (Lossi et al., 2004a).

During the second apoptotic wave in CGCs, NOND affects postmitotic neurons. This is a widely recognised phenomenon that has been demonstrated to play a crucial role in sculpting and maintaining the architecture of the mature CNS since the establishment of the neurotrophic theory (Oppenheim, 1985; Johnson and Oppenheim, 1994; Miller and Kaplan, 2001). We recently demonstrated that postmitotic granule cells in the rabbit cerebellum undergo apoptosis as a consequence of failing to make proper synaptic contacts with the Purkinje cell dendrites (Lossi et al., 2002b). We further investigated the molecular mechanisms underlying the process of cell death of these neurons and obtained evidence for specific cleavage of several caspases and PARP-1, the most biologically relevant substrate of caspase 3 (Smith, 2001). Caspase/PARP-1 cleavage selectively occurs within the IGL, which is known to be populated by postmitotic, post-migratory CGCs (Lossi et al., 2004a). Therefore, in this case, PCD is again apoptotic, but, differently from early NOND in CGPs, is caspase-dependent.

Apoptosis of neurons of deep cerebellar nuclei

Data from literature demonstrate that deep cerebellar nuclei neurons die by apoptosis after neonatal rats were treated with ethanol (Dikranian et al., 2005). In other experiments, unilateral penduculotomy causes deafferentation of the hemicerebellum and axotomy in the efferent pathway from the ipsilateral deep cerebellar nuclei. The effects of the axotomy on cerebellar nuclear neurons begin within 3 hours after lesion, and neurodegeneration occurs within 48 hous with clear apoptotic signs (Sherrard and Bower, 1997). There are not data showing apoptosis during normal development of deep cerebellar nuclear neurons.

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, although the use of transgenic models and knockout animals is not free from a series of drawbacks a correct interpretation of results that often make very difficult.

Numerous experiments in which the number of copies of caspase genes has been altered by recombinant DNA technology led to the demonstration that caspases 1, 2 are not essential for apoptosis at least in CNS (Kuida et al., 1995; Friedlander and Yuan, 1998; Troy et al., 2000, 2001). On the other hand, mice deficient in caspases 3, 9 and APAF 1 showed striking phenotypes, with pronounced effects on the development of the CNS and premature lethality (Kuida et al., 1996; Bergeron and Yuan, 1998; Hakem et al., 1998; Cecconi et al., 1998, Cecconi, 1999; Yoshida et al., 1998; Pompeiano et al., 2000; Cecconi and Gruss, 2001). Diffuse hyperplasia, ectopic cell masses, abnormal structural organisation and augmented numbers of neurons in the cortex, striatum, hippocampus, cerebellum and retina were observed (Kuida et al., 1996; Pompeiano et al., 2000). These alterations 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).

Also, several strains of transgenic animals have been generated with altered expression of members of the BCL- 2 family.

Altogether, the studies carried out on these animal models have confirmed the anti- or pro-apoptotic roles of numerous protein members of the group, and indicated that they often impede/ promote neuronal cell death induced by various stimuli in a dose-dependent manner, and that the endogenous levels of these proteins are able to regulate neuronal survival (Martinou et al., 1994; Bonfanti et al., 1996; Michaelidis et al., 1996; Zanjani et al., 1996; Shindler et al., 1997; Tanabe et al., 1997; Bernard et al., 1998; Brady and Gil-Gomez, 1998; Parsadanian et al., 1998; Bar-Peled et al., 1999; Lindsten et al., 2000; Fan et al., 2001; Leonard et al., 2001; Zaidi et al., 2001; Yakura et al., 2002).

Moreover, the generation of transgenics with altered expression of some genes involved in cell cycle regulation, particularly those encoding for the proteins of the Rb family, confirmed the involvement of these proteins in neural apoptosis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Field et al., 1996; Macleod et al., 1996; Yamasaki et al., 1996; LeCouter et al., 1998; Yoshikawa, 2000).

A striking number of spontaneous mutations in which the number of CNS neurons is altered in relation to apoptosis, have been studied. These include the ataxia (Ohgoh et al., 2000), flathead (Roberts et al., 2000), harlequin (Klein et al., 2002), hoxc-8 (Tiret et al., 1998), lurcher (Doughty et al., 2000; Selimi et al., 2000), weaver (Selimi et al., 2000), weeble (Nystuen et al., 2001), and wobbler mutations (Festoff et al., 2000).

Apoptosis and Alzheimer

In the last decade apoptosis was considered the main cause of neuronal death in some human neuro-degenerative disorders, such as Alzheimer disease (AD). Albeit the cerebellum is not usually affected in AD, several studies *in vitro* have indicated the possibility that CGCs may be a target for the toxic effects of amyloid-ß (Aß) – a peptide that accumulates in the brains of people with AD and forms amyloid plaques (Canu et al., 1998; Engidawork et al., 2001a,b; Tsuchiya et al., 2004).

The association between AD and apoptosis was based on the following observations: (1) A, directly induces apoptosis of cultured neurons; (2) fragmentation of nuclear DNA after TUNEL labelling, is detected in AD brains; (3) activated caspases 3, 8 and 9 are present in AD hippocampal neurons; (4) pharmacological or molecular inhibition of certain members of the caspase family offers partial or complete protection against A,-induced apoptotic cell death *in vitro*; (5) the amyloid precursor protein can be cleaved by caspases; (6) caspase 3-cleaved fragments of tau, a microtubule-associated protein that is the primary protein component of the filaments found in the brains of people with AD have also been detected in post-mortem samples.

More recently, however, some researchers demonstrated the lack of cells with typical apoptotic morphology in human AD post-mortem brain tissue (Jellinger, 2006; Zhu et al., 2006). These authors confute the simple use of the TUNEL assay for DNA fragmentation to assess apoptosis in post-mortem material, where tissues are often not optimally fixed and DNA fragmentation frequently occurs as post-mortem autolysis (see also Lossi et al., 2002a, for technical discussion).

In a brain area such as the hippocampus, which is primarily affected by neuronal death in AD, these researchers detected only rare neurons with morphologic signs of apoptosis. In the same neurons, evidence of caspase activation and accumulation of cleaved amyloid precursor protein (APP), a 90 kD transmembrane protein whose cleavage leads to production of A,, was indicative for apoptotic neuronal degeneration in one study (Jellinger, 2006). However, Zhu and co-workers (2006) deny a role for apoptosis in AD, based on quantitative estimation of the numbers of degenerating neurons. They claim that whereas the end stage manifestation of apoptosis requires only up to 24 hours to be completed, AD is a chronic disease with a clinical duration of almost 10 years. If all neurons in AD died with an apoptotic mechanism, they calculated that, in 10 years, about 4000 cells at any given time should be undergoing apoptosis in the hippocampus, which would be rapidly of neurons leading to an acute disease (Zhu et al., 2006).

In the last years numerous mice models have been developed to reproduce the biochemical and histopathological aspects of AD, in particular to understand more precisely the roles of APP and tau. However, there is no evidence of caspase activation or apoptotic cell death in currently available animal models of AD, and there is therefore no evidence *in vivo* for a potentially beneficial effect of blocking apoptosis in AD.

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