Molecular Mechanisms of Neuronal Death

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Abstract Cellular homeostasis, maintenance of the balance of life and death at the cellular level, is essential for tissue integrity from development through senescence. During development of the nervous system programmmed cell death is responsible for establishing the number of neurons and shaping the nervous system. After development the majority of the postmitotic neurons should live for the life of the organism. Aberrant neuronal death occurs in neurodegenerative diseases and there is still no clear understanding of the mechanisms involved. In this chapter we discuss the molecules and pathways that regulate the life and death of cells and illustrate how these pathways are potentially involved in neurodegenerative diseases. By understanding the molecular mechanisms that regulate cell death we can then begin to identify which pathways are dysregulated in neurodegenerative diseases.

Keywords Neuron death · Caspase · IAP · Smac/DIABLO · TNF · Fas · PIDD · RAIDD · Neurodegenerative disease

Contents

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1 Introduction

Cellular homeostasis, that is, the balance of life and death at the cellular level, is a requirement for maintaining the integrity of tissues from development through maturity. During development large numbers of superfluous cells are removed by an active process termed *programmed cell death* (PCD) (Burek and Oppenheim, 1996). It was through the genetic studies of developmental death in *C. elegans* that the genes required for PCD were identified (Hengartner and Horvitz, 1994). These gene families are highly conserved from *C. elegans* to humans. Often PCD is used interchangeably with apoptosis; this is not accurate, as PCD refers specifically to developmental death. *Apoptosis* and *necrosis* were described as morphologically distinct processes (Kerr et al., 1972). In apoptosis cellular changes include cell membrane blebbing, cell shrinkage, chromatin condensation, and nuclear fragmentation (Kerr et al., 1972). Eventually the cell disintegrates, generating the so-called apoptotic bodies that will be engulfed via phagocytosis by nearby cells, thus avoiding an inflammatory response in the surrounding tissue. This lack of inflammatory response allows apoptosis to occur without damaging neighboring healthy cells. In contrast, necrosis, in which the cell suffers a major insult leading to rapid swelling, subsequent rupture of the plasma membrane and release of the intracellular contents into the surrounding cellular environment causes a strong inflammatory response. Apoptosis maintains physiological balance and its dysregulation results in pathological conditions, such as neurodegenerative diseases, cancer, and autoimmune disorders. Another mode of cell death is *autophagy*, which is characterized by the formation of large autophagic vacuoles and little inflammation (Levine and Yuan, 2005). Most autophagy does not lead to cell death but is a mechanism by which intracellular components are recycled (Yoshimori, 2007). Although the classification of the different forms of cell death seems to be clear, the boundaries are not so well defined in vivo and crosstalk can occur (Lockshin and Zakeri, 2004). With this idea in mind, we discuss the pathways of apoptotic neuronal death that occur in

acute and chronic pathological conditions such as Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke/ischemic disease, and motor neuron diseases.

2 Caspases: Key Players in Apoptosis

Caspases are the main proteins involved in the execution of apoptosis (Troy and Salvesen, 2002). They are a family of cysteine aspartate proteases with a conserved QACXG motif at the active site. To date, 13 mammalian caspases have been identified (Lamkanfi et al., 2002). Synthesized as inactive precursors or zymogens, they can be classified based on their structure, mode of activation, cleavage specificity, and function. According to their function caspases can be subdivided into three groups, shown in Fig. 1:

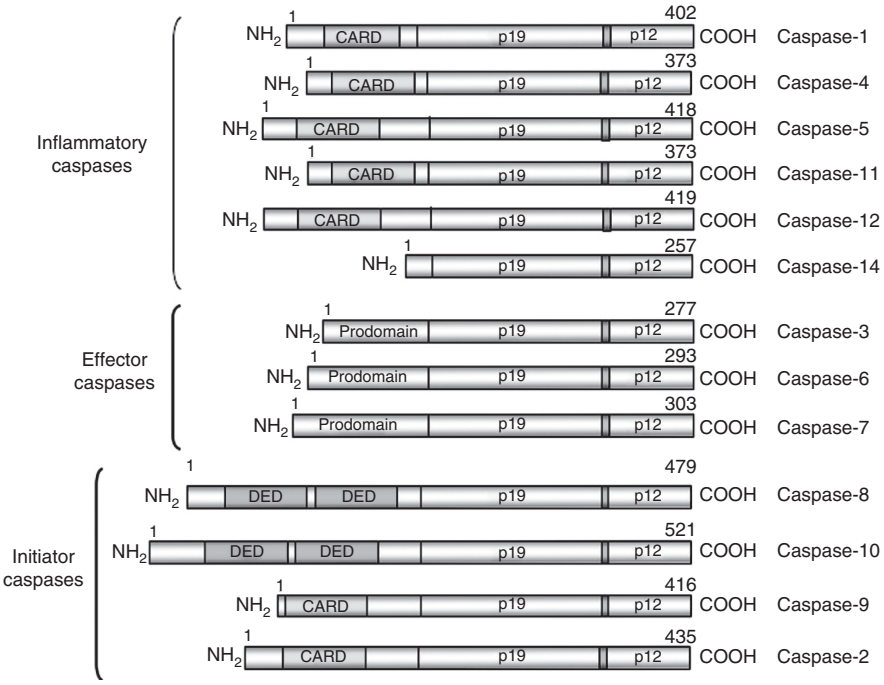

Fig. 1 Mammalian caspases

(1) Inflammatory caspases: caspase-1, -4, -5, -11, -12, and -14.
(2) Initiator caspases: involved in the apoptotic process. These caspases, also known as apical caspases, are structurally characterized by the presence of a long prodomain at the N-terminal region containing different protein–protein interaction motifs such as death effector domain (DED) found in caspase-8 and-10 or caspase recruitment domain (CARD), present in caspase-2 and -9. Via

these domains, caspases establish homotypic interactions with specific adaptor molecules.

(3) Effector caspases: this group of proteases cleave cellular substrates during apoptosis. Due to their function in the apoptotic paradigm they are also known as executioner caspases. They are characterized by the presence of short prodomains. This group contains caspase-3, -6, and -7.

2.1 Caspase Activation

Synthesized as inactive precursors or zymogens, caspases require activation to execute apoptosis (Nicholson and Thornberry, 1997). Early studies suggested that all caspases required proteolytic cleavage for their activation and that mature caspases consisted of large (p18/20) and small (p10/12) subunits arranged in heterotetramers containing two active sites (Walker et al., 1994). However, work on caspase-9 provided new insight into the mechanisms underlying caspase activation because it demonstrated that the caspase-9 zymogen could have activity without cleavage (Stennicke et al., 1999). Thus, the question, "How do caspases become activated?" is critical.

2.2 Mechanisms of Activation

2.2.1 Effector Caspases

The common mechanism of activation of effector caspases (caspases-3, -6, and -7) is through proteolytic cleavage at critical aspartic acid residues (Quan et al., 1996; Riedl et al., 2001a) shown in Fig. 2. Effector caspases are activated by other proteases, generally initiator caspases or granzyme B (an aspartate-specific serine protease), or other effector caspases. This cleavage process has two steps. First, a molecule of zymogen is cleaved at the linker region generating the p18/20 and p10/12 subunits; this structure is partially active. Then, this intermediate interacts with another heterodimer forming the active caspase. In this regard, cleavage of the effector caspase is a measure of activation. Once effector caspases become active they are able to cleave multiple substrates to induce cell death.

2.2.2 Initiator Caspases

Inactive initiator caspases exist as monomers and activation is achieved by proximity-induced dimerization (Boatright and Salvesen, 2003) shown in Fig. 2. Adaptor proteins, which interact with the prodomains of the caspases, bring the caspase molecules into proximity. When initiator caspases dimerize, they undergo conformational changes that result in an active enzyme without a requirement for

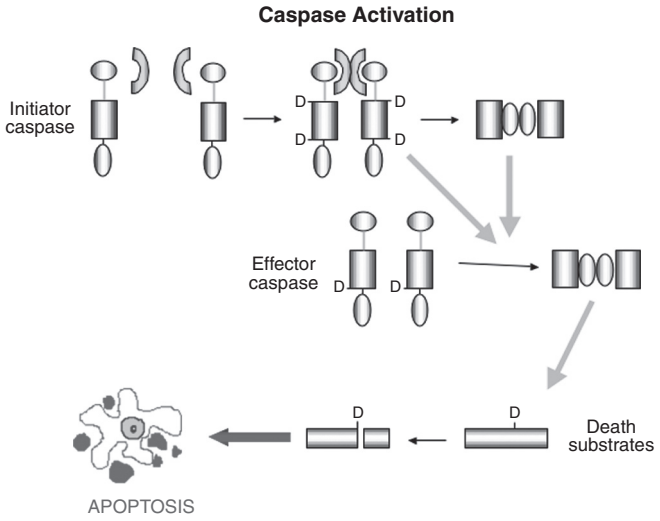

Fig. 2 Caspase activation

cleavage. Thus, cleavage cannot be used as a measure of activation when studying initiator caspases. Because caspase-9 is an initiator caspase that does not require cleavage for its activation, some studies have used the cleavage of caspase-3 as a surrogate measure for caspase-9 activation. However, caspase-8 can also cleave caspase-3. Thus, caspase-3 cleavage/activity is not a specific measurement of caspase-9 activation.

2.3 The Apoptosome

The most widely studied model is caspase-9 activation. Release of cytochrome c from the mitochondria into the cytosol promotes the assembly of the apoptosome, a complex composed of cytochrome c, Apaf-1 (Apoptosis protease-activating factor-1), and caspase-9. The presence of Apaf-1, which is the specific adaptor for caspase-9, recruits procaspase-9 to the apoptosome resulting in caspase-9 activation (Bao and Shi, 2007; Riedl and Salvesen, 2007).

2.4 The DISC

A similar process occurs for caspase-8 activation. In this case, oligomerization of the death adaptor protein Fas-Associated Death Domain (FADD) recruits procaspase-8 into the death-inducing signalling complex (DISC) allowing caspase-8 dimerization and subsequent activation (Shi, 2006).

2.5 The PIDDosome

An activating complex has also been identified for caspase-2, containing RAIDD (RIP-associated ICH-1/CED-3 homologous protein with a death domain), the specific death adaptor for caspase-2, and PIDD (p53-induced protein with a death domain) (Tinel and Tschopp, 2004; Park et al., 2007). This complex, termed the PIDDosome, has not been shown to actually mediate caspase-2 dependent death but rather, overexpression of PIDD can lead to cleavage of caspase-2 which is not necessarily an indication of activation (Tinel et al., 2007). Overexpression of PIDD does lead to death that is blocked in RAIDD-null cells (Berube et al., 2005). PIDD can also complex with RIP1 and NEMO and induce activation of NFκB, suggesting a dual function for PIDD in the regulation of survival and death (Janssens et al., 2005). Caspase-2 has been shown to be critical for both trophic factor deprivation and β-amyloid mediated neuronal death (Troy et al., 2000, 2001), shown in Fig. 3 and RAIDD is required for execution of trophic factor deprivation mediated death (Wang et al., 2006).

Once dimerized in the activating complexes there is often autocleavage of the caspase which, for caspase-2 and -8, has been shown to enhance caspase activity (Chang et al., 2003; Baliga et al., 2004). As death proceeds and effector caspases are activated there is subsequent cleavage of initiator caspases. This cleavage may lead to further enhancement of caspase activity, as in the case of caspase-9 where the initial autocleavage of caspase-9 to the p37 fragment allows XIAP to bind and inhibit activity, and the subsequent cleavage by caspase-3 to the p35 fragment relieves the XIAP inhibition thus enhancing caspase-9 activity (Denault et al., 2007).

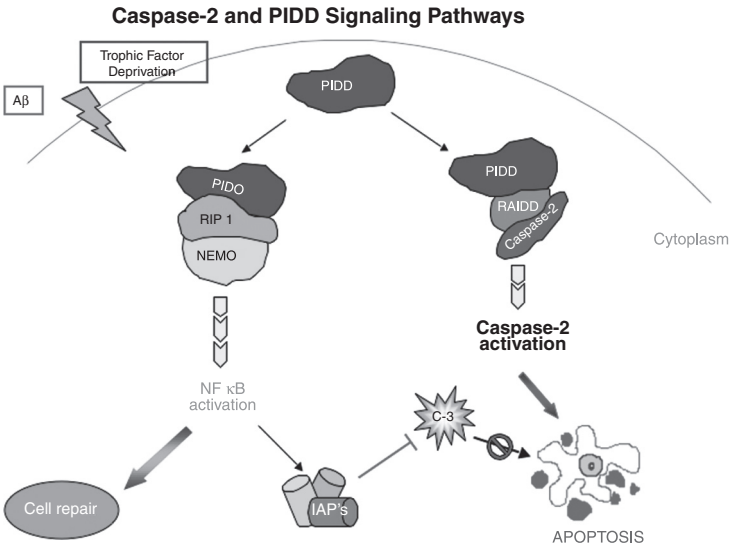

Fig. 3 Caspase-2 activation and PIDD signaling pathways

2.6 The Inflammasome

The activation of the inflammatory caspases uses a mechanism resembling that of the initiator caspases. The presence of a complex, known as the inflammasome (Martinon et al., 2002), is required for activation of this set of proteases. The recruitment of caspases into this complex results in their activation. For caspase-1, the adaptor ASC (apoptosis-associated specklike protein containing a CARD) is critical in inflammasome formation in response to a variety of stimuli, whereas involvement of the adaptors Ipaf (ICE-protease-activating factor) and NALP3 is stimulus-dependent (Mariathasan, 2007).

3 Apoptotic Routes: Intrinsic and Extrinsic Pathways

Cells undergoing apoptosis take one of two major pathways: the death receptor (extrinsic) pathway, or the mitochondrial (intrinsic) pathway. Once the cell is dead, the cellular contents form the apoptotic bodies, which are cleared by phagocytosis in a process involving neighboring cells and/or macrophages. This intricate process is tightly regulated so that there is a fine balance between prosurvival and prodeath signals for each route in the apoptotic pathway.

3.1 The Extrinsic or Receptor-Mediated Pathway

The extrinsic or receptor-mediated pathway is activated when a death ligand binds to its specific receptor on the cell membrane surface. The main death receptors are all members of the tumor necrosis factor (TNF) superfamily of receptors, which includes TNFR, Fas, p75, and TRAIL. All these receptors are characterized by the presence of domains rich in cysteine, which mediate the binding between ligand and receptor. The receptors are synthesized as transmembrane homotrimers and when they bind to their specific death ligand a DISC is formed. This complex recruits death domain (DD)-containing adaptor proteins that interact with and recruit procaspase-8, leading to caspase-8 activation. Caspase-8 activation results in the cleavage and activation of downstream effector caspases which in turn cleave a plethora of substrates, ultimately leading to cell death. Caspase-10, present only in humans, is also activated in this way.

3.1.1 TNF Pathway

TNF is a proinflammatory cytokine produced mainly by macrophages. There are two main types of receptors, TNF-R1 and TNF-R2. TNF-R2 is primarily found in the immune system and is activated by membrane-bound TNF (Wajant et al., 2003). However, TNF-R1, which is ubiquitously expressed, can be activated by both membrane-bound and soluble TNF. When TNF binds to the TNF receptor, TRADD (TNFRSF1A-associated via the death domain) is able to establish

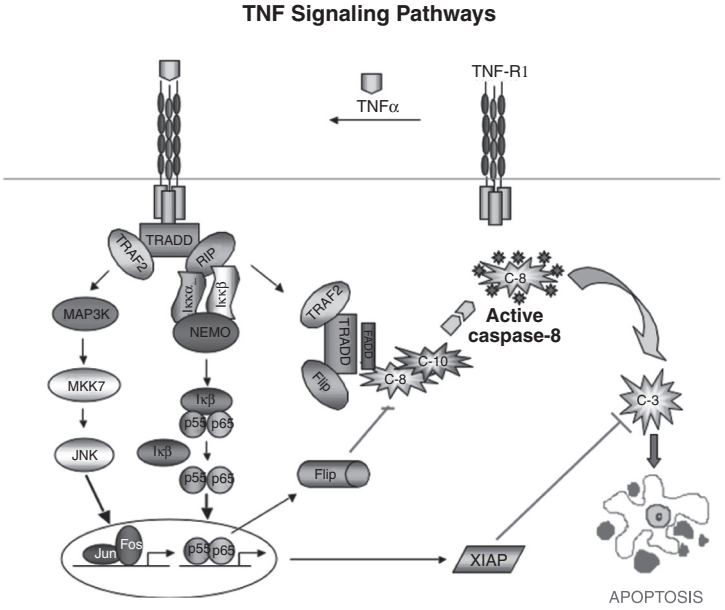

Fig. 4 TNF signaling pathways

homophilic interaction with the DD of the TNF receptor (Hsu et al., 1995), shown in Fig. 4. The binding of TRADD to the TNF receptor–ligand complex facilitates the subsequent binding of TRAF2 (TNF receptor-associated factor 2) and RIP1 (receptor-interacting kinase-1), a DD-containing serine threonine kinase.

When TRAF2 and RIP1 bind to the complex, two sequential pathways are activated: the NFκB pathway and the activated caspase-8 pathway. In the first step of the NFκB pathway, TNF activates the IκBα pathway in a process that depends on the degradation of the inhibitor IκB by the proteasome. The Iκκ complex (IκB kinase) mediates the phosphorylation of the inhibitor IκB. The Iκκ complex is formed by two related IκB kinases, IκBα and IκBβ, and NFκB essential modulator (NEMO), a regulatory protein also known as IκBγ. The roles of TRAF2 and RIP in the Iκκ complex are recruitment and stabilization, respectively (Devin et al., 2003).

In nonstimulated cells, the Iκκ complex remains inactive in the cytoplasm because of the binding of the IκB inhibitor. However, when the complex is recruited to the TNF receptor it becomes active and it is able to phosphorylate the IκB inhibitor which is in turn degraded via the proteasome (Aggarwal, 2003). The degradation of the inhibitor frees the NFκB complex to translocate to the nucleus where it activates transcription of several genes, including XIAP, c-IAP1, and c-IAP2 (Stehlik et al., 1998; Wajant, 2003).

Thus, TNF induces a strong prosurvival signal secondary to NFκB activation. This is the main difference between TNF and Fas or TRAIL, which only mediate apoptosis. TNF can have cytotoxic effects, but only when NFκB activation is

inhibited. In the second (caspase-8) part of the pathway, TRADD, which is bound to the TNF receptor, acts as a platform allowing the complex to interact with Fas-associated death domain (Yeh et al., 1998; Thorburn, 2004). Once FADD is bound to the complex, it recruits caspase-8 to form a cytoplasmic DISC protein complex that finally ends with the death of the cell (Micheau and Tschopp, 2003). The TNF receptor can also mediate an alternative pathway through the recruitment of RAIDD, which facilitates the binding of RIP1, establishing homophilic interactions via the DD found in both proteins (Duan and Dixit, 1997). This interaction mediates the recruitment of caspase-2 which in turn leads to apoptosis (Kim et al., 2000). A complex of caspase-2 with TRAF2 and RIP1 has been found that induces NFκB activation independent of caspase-2 enzymatic activity (Lamkanfi et al., 2005).

3.1.2 FAS Pathway

Fas plays a key role in the regulation of apoptosis. The Fas–Fas ligand (FasL) interaction has a special relevance because the initial characterization of the DISC formation was discovered while studying this interaction (Kischkel et al., 1995). When Fas ligand binds to its receptor, Fas, also known as CD95, a structural change takes place facilitating the trimerization of the receptor, which then mediates the recruitment of DD-containing proteins, in this case, FADD. FADD is a molecule with a double nature because it not only contains a DD but also a DED through which it establishes interactions with procaspase-8 (Chinnaiyan et al., 1995). Once procaspase-8 is recruited into the DISC complex, it is autoproteolytically processed by proximity-induced dimerization, which enhances the enzymatic activity (Fig. 5). Another study shows that the DISC complex can also contain caspase-10 but that caspase-10 cannot completely replace the caspase-8 function in apoptosis (Sprick et al., 2002). It appears that caspase-8 and -10 may have some nonredundant

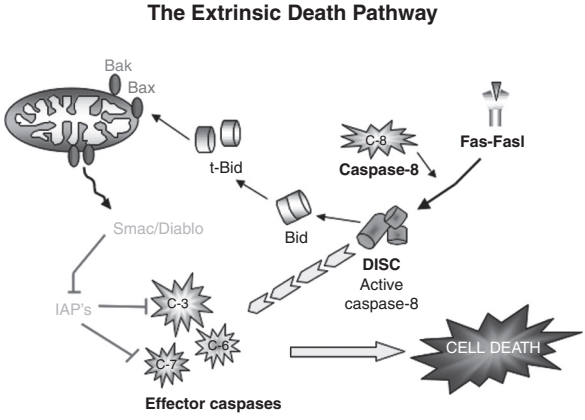

Fig. 5 The extrinsic death pathway

functions. People lacking caspase-10 can develop autoimmunolymphoproliferative syndrome II (Rieux-Laucat et al., 2003).

Modulators of caspase-8 dependent apoptosis, specifically FLIP, have also been identified in the DISC. FLIP is synthesized in two isoforms, short and long. Both have DEDs in tandem and high homology to the N-terminus of caspase-8 (Irmler et al., 1997). When FLIP is recruited into the DISC, it disrupts the complex and acts as an inhibitor of caspase-8 so that caspase-8 cannot become active. This prevents the cell from undergoing apoptosis. However, FLIP can also activate caspase-8 and caspase-10 by forming heterodimers (Boatright et al., 2004).

3.1.3 TRAIL Pathway

Although its physiological role is not completely understood, TRAIL plays a role in apoptosis in blood cells and in the immune system (Thomas and Hersey, 1998). Five TRAIL receptors have been described, which can be divided in two groups: death-inducing receptors and death-inhibitory receptors. As their own names indicate, the first group is actively involved in the apoptotic response and the second group has a defective cytoplasmic DD so they function as competitive inhibitors when they bind to TRAIL. The cascade involving TRAIL is similar to the one induced by Fas. TRAIL binds to its receptor initiating DISC formation and recruitment of caspases-8 and -10 and FLIP. DISC formation generates the active conformation of caspase-8 which in turn activates caspase-3 resulting in cell death. Although there can be an interconnection between this main pathway and the NFκB pathway, TRAIL is a weak inducer of the latter. As with the TNF receptor-mediated pathway, the activation of NFκB is mediated by RIP1 and TRAF2 (Lin et al., 2000). However, the prosurvival signal is completely masked by the strong apoptotic response.

3.2 The Intrinsic Pathway

The intrinsic pathway is the death pathway followed when apoptosis is triggered by death signals generated inside the cell (Fig. 6). In this pathway, mitochondria are the key players, controlling the cell status based on which molecules are released from the mitochondria into the cytoplasm. Because release of molecules from the mitochondria depends on the integrity of the mitochondrial membranes, mitochondrial membrane permeabilization has a key role in the origin and progression of the intrinsic pathway. The Bcl-2 family controls the regulation of mitochondrial permeability (Green and Amarante-Mendes, 1998; Green and Kroemer, 2004). This family is characterized structurally by the presence of the Bcl-2 homology (BH) domain. Family members such as Bcl-2, Bcl-Xl, or Bcl-w can have antiapoptotic effects and contain 4 BH domains (BH1, 2, 3, 4) and a transmembrane domain.

Other proteins from the Bcl-2 family are proapoptotic. The proapoptotic group is subclassified into BH3-only proteins (Bid), BH3-only with a transmembrane domain (Bad, Bim, Bik, Bmf, Hrk, Nox, or Puma), and multi-BH (BH1, 2, 3)

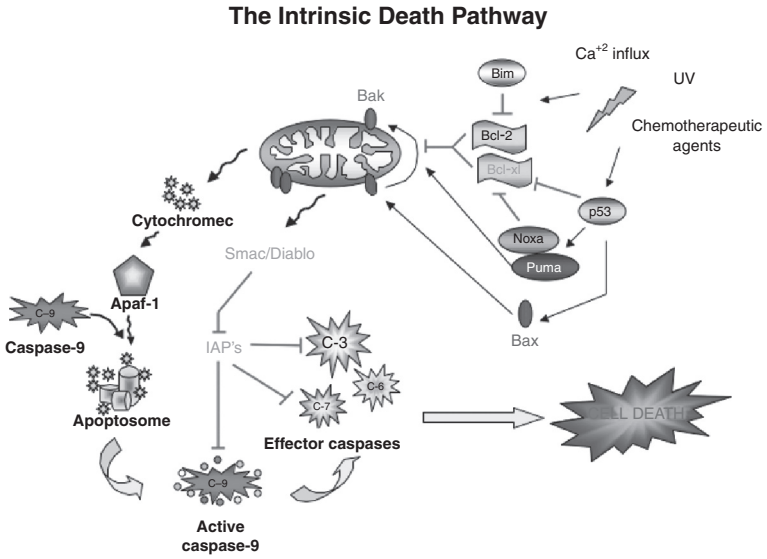

Fig. 6 The intrinsic death pathway

domains with a transmembrane domain (Bax, Bak, Bok) (Adams and Cory, 1998). The BH3-only proteins trigger apoptosis induced by the lack of trophic support or intracellular damage and thus work as damage sensors in the cell (Cheng et al., 2001). Bcl-2, the prototype family member, is found in perinuclear membranes, mitochondria, and endoplasmic reticulum (Korsmeyer et al., 1995). It has important functions in controlling both calcium and mitochondrial membrane homeostasis (Danial and Korsmeyer, 2004).

Following intracellular damage, members of the Bcl-2 family undergo oligomerization and attach to the outer mitochondrial membrane. A good example is the case of Bax and Bak. In healthy cells, Bax is present as a monomer in the cytoplasm but during the apoptotic cascade, it oligomerizes and translocates to the outer mitochondrial membrane. Bak localization seems to be mitochondrial, even in healthy cells, but undergoes conformational changes during apoptosis leading to its aggregation (Danial and Korsmeyer, 2004). Once these proteins are inserted into the outer mitochondrial membrane and become oligomerized, the mitochondrial membrane is disrupted releasing intermembrane proteins, such as cytochrome c, into the cytosol, which compromises cell viability. The involvement of cytochrome c in the apoptotic cascade was initially surprising because cytochrome c is known as an essential component of the respiratory chain. Thus, cytochrome c has a dual role. It promotes the generation of ATP and cell viability while inside the mitochondria, and, when outside the mitochondrial space in the cytosol it promotes cell death. Cytochrome c is found in the mitochondrial interspace and its release is controlled by members of the Bcl-2 family (Green and Amarante-Mendes, 1998; Chipuk et al., 2006). In this context, the antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-XL, will prevent

the release of cytochrome c whereas the proapoptotic family members, Bax, Bak, and Bid mediate its release (Kluck et al., 1997; Jurgensmeier et al., 1998; Luo et al., 2005).

The exact mechanism mediating cytochrome c release is still not fully understood. In general, it is believed that a change in the mitochondrial permeability precedes cytochrome c release. However, caspase activation and cytochrome c release can occur before detecting any mitochondrial alteration (Green and Amarante-Mendes, 1998). Because caspases can induce cytochrome c release, it also seems possible that a small initial leakage of cytochrome c could cause caspase activation, which in turn would promote the massive release of cytochrome c from the mitochondria. Either way, once cytochrome c is released into the cytoplasm it binds to Apaf-1, which is the mammalian homologue of the *C. elegans* CED-4 (Zou et al., 1997). Apaf-1 contains a CARD domain at its N-terminus that interacts with the CARD domain of procaspase-9 (Li et al., 1997). Apaf-1 interacts with dATP and cytochrome c and undergoes a conformational change forming a heptamer of APAF-1 molecules that can then complex with pro-caspase-9 (Zou et al., 1999).

This multimeric complex formed by dATP, cytochrome c, Apaf-1, and procaspase-9 is called the apoptosome. The recruitment of procaspase-9 via Apaf-1 into the apoptosome allows the activation of caspase-9 by proximity-induced dimerization. The active caspase-9 is now able to cleave downstream effector caspase-3, -6, and-7, which then cleave myriad cellular substrates involved in DNA metabolism, cytoskeletal and structural proteins, and regulators of the cell cycle, all of which compromise cell integrity and lead to cell death when disrupted (Li et al., 1997). However, cytochrome c is not the only molecule released from mitochondria during the execution of the intrinsic pathway. Smac/Diablo is also released from mitochondria into the cytoplasmic space where it binds to the BIR3 of XIAP, acts as an IAP antagonist and ultimately leads to the activation of caspase-9 and -3 (Chai et al., 2000; Verhagen et al., 2000). Omi/HtrA2 is also released from mitochondria during apoptosis and although it functions, as does Smac/DIABLO, as a competitive inhibitor of the IAPs, it seems to be a more potent inhibitor because Omi/HtrA2 not only binds to and inactivates the IAPs but can also proteolytically process them (Yang et al., 2003). Other pro-apoptotic molecules are released from the mitochondria and although their final consequences are cell disruption and death, these effects are generally considered to be caspase-independent.

4 Natural Inhibitors of Caspase Activity

4.1 The Inhibitor of Apoptosis Proteins

Caspases kill cells by cleaving a broad spectrum of cellular substrates. To ensure that the death pathway is not accidentally activated, caspase activity must be carefully regulated to prevent aberrant caspase activation. Some members of the inhibitor of apoptosis protein (IAP) family can suppress caspase activity thus avoiding unwanted

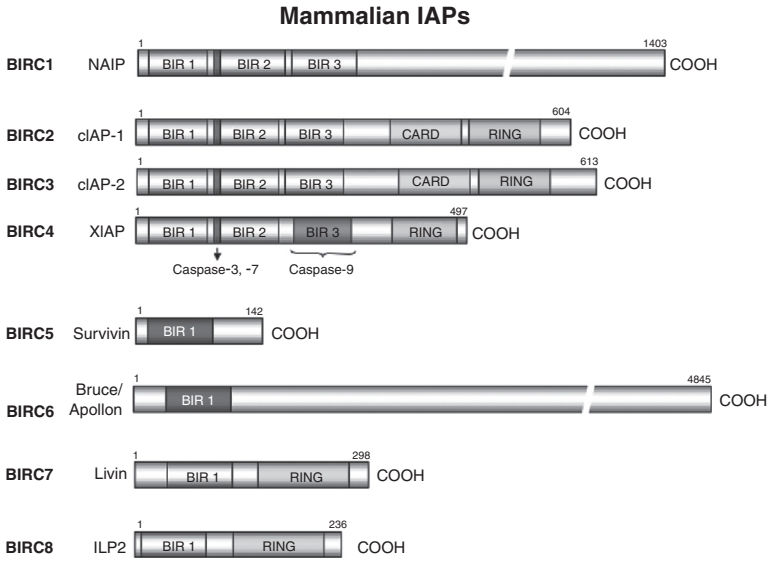

Fig. 7 Mammalian IAPs

apoptosis (Prunell and Troy, 2004). IAPs are phylogenetically highly conserved from *c. elegans* to mammals. There are eight human genes identified that belong to the IAP family (Fig. 7) (Deveraux and Reed, 1999): neuronal-apoptosis-inhibitory protein (NAIP or BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), XIAP (BIRC4), survivin (BIRC5), Apollon (BRUCE or BIRC6), melanoma-associated IAP (Livin or BIRC7), and hILP-2 (TS-IAP or BIRC8). This family of proteins is characterized by the baculovirus IAP repeat (BIR) domain. The BIR is a 65-amino-acid domain with a high cysteine and histidine content.

There are two types of BIR domains (Salvesen and Duckett, 2002). Type I binds to and inhibits caspases. Type II also binds to caspases, and in addition functions in the cell cycle. The type II BIR domains are found in two mammal IAPs, survivin (BIRC5) and BIRC6. Most of the IAPs also contain a RING domain at the carboxy-terminus region which behaves as an E3 ubiquitin ligase. The RING domain adds ubiquitin residues to target proteins so they will be degraded by the proteasome. IAP-mediated protein ubiquitination has a crucial role in the regulation of apoptosis because it can target the IAP itself and also enhance the antiapoptotic effect by targeting proapoptotic molecules for degradation. In adddition to the RING domain, c-IAP1 and c-IAP2 also contain a CARD domain located in the C-terminal region between the RING domain and BIR3. The function of CARD domains in these two IAPs is not yet known. Usually CARD motifs interact with other CARD-containing proteins, but the classical location for these protein–protein interactions is the N-terminus, not the middle of the structure as in the case of the IAPs.

The best-studied IAP is XIAP, which is the most potent IAP. It is an ubiquitously expressed 56 kDa protein with 3 BIR domains and one RING domain. XIAP has

been shown to directly bind and inhibit caspase-3, -7, and -9 (Riedl et al., 2001b). The protein–protein interactions between caspases and IAPs takes place via specific regions within the IAP structure. XIAP–BIR3 domain interacts with caspase-9 and XIAP–BIR2-linker binds caspase-3 and -7. Both BIR domains utilize a two-site binding mechanism to inhibit caspases (Scott et al., 2005). One site has been defined as the IAP-Binding Motif (IBM)–interacting groove. When caspase-3, -7, and -9 are cleaved between the large and small subunits, the new small subunit N-terminus is an IBM. This is an exosite, a functionally important site outside of the active site of the enzyme. For inhibition of caspase-3 and -7 there is also an active-site directed interaction, where a stretch of the linker domain of XIAP spans the active site of the caspase. For caspase-9, the functional inhibitory interaction is via a helix found right after the BIR3 domain. This interaction monomerizes caspase-9 and collapses the active site. Because dimerization is essential for caspase-9 activity the enzyme is inactivated (Shiozaki et al., 2003). XIAP is the most potent IAP with efficiency 100- to 1000-fold higher than the rest of the family members.

c-IAP1 and c-IAP2 are the closest paralogues of XIAP and can also bind to caspases by the IBM grooves but are relatively poor inhibitors of caspase activity. The linker region preceding the BIR2 is not a good inhibitor of caspase-3 or -7 (Eckelman and Salvesen, 2006). The BIR3 domains of cIAPs have only one of the four dimer interface–interacting residues required to inactivate caspase-9 and neither inhibits caspase-9 (Eckelman et al., 2006). IAPs can also be cleaved by caspases that may affect their activity. When XIAP is cleaved between BIR2 and BIR3, the BIR3-RING fragment becomes a more potent inhibitor of caspase-9 activity than the whole molecule (Deveraux et al., 1999). The N-t cleaved fragment of XIAP still has the ability to inhibit caspase-3 and-7, but to a much lesser extent than full-length XIAP.

IAPs have been extensively studied in the context of cancer because of the IAPs' ability to regulate members of the NFκB family and because NFκB activation seems to upregulate expression of IAPs (Stehlik et al., 1998). More recently, IAPs have been implicated in neurodegenerative diseases. In sympathetic neurons deprived of trophic factors XIAP inhibits caspase-3 activity (Troy et al., 2001) (Fig. 3). In motor neurons damaged by sciatic nerve axotomy, there is a significant decrease in the levels of endogenous XIAP and NAIP (Perrelet et al., 2004). Expression of NAIP is increased in AD, whereas that of XIAP is decreased. Treatment with glial-derived neurotrophic factor (GDNF) rescues this effect and promotes motor neuron survival (Perrelet et al., 2002). Inhibition of XIAP or NAIP blocks the neuroprotective effect of GDNF, pointing out a direct effect of IAP activity and motor neuron degeneration. Similar results have been found in the case of ischemic injury where overexpression of XIAP reduced the infarct size, the number of cells exhibiting apoptotic phenotype, and improved neurological activity (Xu et al., 1999). The fact that IAPs are endogenous inhibitors of caspase activity makes them a good therapeutic target for diseases characterized by excessive or premature cell death, such as stroke, AD, PD, and other neurodegenerative disorders. IAPs may also participate in physiological regulation of normal nervous system function. XIAP regulates activated caspase-3 in a songbird model of learning (Huesmann and Clayton, 2006).

4.2 Natural Inhibitors of the Inhibitor of Apoptosis Proteins: IAP Antagonists

After discovering that IAPs bind to and inhibit caspase activity, several studies focused on the isolation of endogenous regulators of IAP activity (Crook et al., 1993; Birnbaum et al., 1994). The first molecule identified was the second mitochondria-derived activator of caspases (Smac), also known as DIABLO, an IAP binding protein that in healthy cells is found in mitochondria (Du et al., 2000; Verhagen et al., 2000). This protein contains 239 amino acids. After stimulation, Smac/DIABLO translocates from the mitochondria to the cytosol where it binds to and blocks XIAP activity. This binding is associated with four hydrophobic residues, Ala-Val-Pro-Ile, at the Smac/DIABLO N-terminus which form the IAP-binding motif (Shi, 2002). Smac/DIABLO binds to the BIR3 domain of XIAP at the same site as caspase-9 (Liu et al., 2000; Wu et al., 2000). Therefore, the interaction of Smac/DIABLO with XIAP displaces caspase-9, thus abrogating the inhibitory effect of XIAP on caspase-9 activity.

Smac/DIABLO is not the only regulator of IAP activity. Several studies in mammalian cells have demonstrated the presence of additional molecules that suppress IAP activity in a similar fashion to Smac/DIABLO. The best-studied example is Omi/HtrA2 (Suzuki et al., 2001; Hegde et al., 2002; Martins et al., 2002; van Loo et al., 2002). This protein exhibits, as does Smac/DIABLO, mitochondrial localization with cytoplasmic release upon stimulation.

Apart from IAPs, there are several nonmammalian regulators of caspases, which are active-site specific inhibitors (Callus and Vaux, 2007). One example is a serpin from the cowpox virus, cytokine response modifier A (crmA). CrmA forms a covalent complex with the initiator caspase-1 and -8 resulting in irreversible inhibition of these caspases. It also inhibits caspase-6 but less efficiently (Dobo et al., 2006). The baculoviral protein p35 is a broad spectrum caspase inhibitor that irreversibly inactivates caspases (Bump et al., 1995; Fisher et al., 1999).

4.3 Phosphorylation

Phosphorylation is the major form of posttranslational modification. It is important to note that caspase activity differs from caspase activation. Activation refers to the conformational changes that rearrange the caspase molecule leading to the active enzyme. Caspase activity is defined as the ability of a caspase to cleave substrates. Caspase phosphorylation is able to modulate caspase activity. A clear example is the case of human caspase-9 which can be phosphorylated at a consensus sequence by Akt, a serine-threonine kinase implicated in apoptosis suppression (Cardone et al., 1998). Caspase-9 phosphorylation by Akt induces a modification in the caspase structure rendering it unable to form the tetramer required for activity. There is also evidence that phosphorylation may regulate caspase-2 activity (Nutt et al., 2005; Shin et al., 2005).

4.4 Nitrosylation

Caspases can also be modified by nitrosylation. S-nitrosylation of the active site cysteine has been shown to inactivate multiple caspases (Mannick et al., 2001). The physiological relevance of this mechanism is not yet fully understood.

5 ER-Stress

Although mitochondria are the main organelles involved in the intrinsic apoptotic pathway, the endoplasmic reticulum (ER) also plays an important role. The ER is the biggest intracellular reservoir of Ca^{2+} and Ca^{2+} functions as a second messenger interconnecting the mitochondrial pathway with the ER. When a small amount of cytochrome c is released from the mitochondria into the cytosol, there is uptake by the ER which in turn responds by releasing Ca^{2+}. This Ca^{2+} in turn disrupts the resting mitochondrial membrane potential and causes a massive release of cytochrome c that activates caspases and leads to cell death. This apoptotic activation via Ca^{2+} efflux from the ER seems to be important in disorders such as AD and stroke (Rao et al., 2004).

The main function of the ER is to ensure that only those proteins folded properly will be transported through the multivesicular secretory pathway. This property is extremely important in the case of neurodegenerative diseases because most are characterized by the presence of inclusion bodies formed from aberrantly folded protein. Amyloid plaques (β-amyloid aggregates) and neurofibrillary tangles (intracellular inclusions of hyperphosphorylated tau) are the sine qua non of Alzheimer's disease (AD), as are Lewy bodies (α-synuclein inclusions) in Parkinson's disease (PD), Pick's bodies (tau inclusions) in frontotemporal lobar degeneration, and Hirano bodies, cytoplasmatic protein aggregates of actin and actin-associated proteins, which are present in several neurodegenerative disorders such as AD and Creutzfeldt–Jacob disease. When the ER is damaged it cannot correctly regulate the accumulation of unfolded or misfolded proteins. This leads to a reduction in protein synthesis to prevent accumulation and activation of the chaperones that reside in the ER so they can contribute to the proper folding of newly synthesised proteins. There is also an increase in the degradation rate (Breckenridge et al., 2003). However, if these compensatory changes are inadequate, cell integrity will become compromised, leading to death.

Increasing evidence suggests that members of the Bcl-2 family may act not only at the mitochondrial levels but also at the ER level. There is work that suggests that Bak and Bax are involved in controlling Ca^{2+} homeostasis in the ER because double knock-out mice for Bax and Bak exhibit impaired Ca^{2+} efflux from the ER and uptake by the mitochondria; this is correlated with low levels of apoptotic cell death (Nutt et al., 2002b, a). The relevance of these data to human neurodegenerative disorders is not yet clear because so far only caspase-12 has been reported to become activated after ER stress-induced apoptosis. There is evidence showing that both Bax

and Bak are required in order to activate caspase-12 (Scorrano et al., 2003; Zong et al., 2003). In this context, both Bcl-2 family members would promote Ca^{2+} efflux from the ER, which in turn would permeabilize the outer mitochondrial membrane. Caspase-12 would then be released from the ER into cytosolic space. However, these studies were done in rodents, and there is no evidence that the caspase-12 protein is expressed in humans, although several studies suggest that human caspase-4 may have redundant functions with rodent caspase-12 (Hitomi et al., 2004).

6 Crosstalk Between the Intrinsic and Extrinsic Pathways

Although apoptosis proceeds through two major pathways in the cell that are initiated through the activation of different caspases forming different multimeric complexes, both pathways converge on the activation of downstream caspases (Danial and Korsmeyer, 2004). TRAIL employs the extrinsic pathway for triggering apoptosis, but there is also involvement of the mitochondrial pathway. In this paradigm, Smac/DIABLO is released from mitochondria, which block the inhibitory effect of XIAP on caspase-3 activity, resulting in the execution of apoptosis mediated by TRAIL. A similar situation occurs in the case of Fas-mediated apoptosis.

It is worth mentioning that in death receptor-mediated apoptosis, cells can be divided into two groups depending on the requirement for mitochondria to induce a complete apoptotic response. Type I cells do not require the mitochondrial pathway because the recruitment of procaspase-8 into the DISC complex is enough to fully activate caspase-8 which then activates effector caspases. However, Type II cells are characterized by an incomplete apoptotic response unless mitochondria are involved (Scaffidi et al., 1999). In this type of cell, efficient activation of effector caspases requires the mitochondrial amplification loop (Fig. 5). Caspase-8 cleaves cytosolic Bid, a BH3-only protein, which when cleaved to tBid is able to translocate to the mitochondria and trigger release of the proapoptotic factors cytochrome c and Smac/DIABLO (Li et al., 1998; Deng et al., 2002). The release of cytochrome c triggers apoptosome formation, subsequent caspase-9 activation, and finally the activation of effector caspases such as caspase-3.

Another positive feedback loop is established after DISC formation because this complex allows caspase-8 autoactivation which in turn cleaves downstream effector caspase-3. The cleavage of one caspase by another must be examined in relation to the timing of the ongoing cellular events in order to understand the relevance of these events. That is, as death proceeds, there is activation of initiator caspases—no cleavage necessary—leading to activation by cleavage of effector caspases. Once activated, the effector caspases may cleave initiator caspases, but this event is not necessary for activity of initiator caspases and may even decrease activity under certain conditions. Thus, as our knowledge of caspase activation increases, the prior assumptions about caspase cascades must be re-evaluated.

7 Neurodegenerative Diseases: An Example of Dysregulated Apoptosis

Because neurons are not normally replaced during the lifespan of an organism, they must possess very robust antiapoptotic mechanisms. If premature death of neurons does occur, it leads to irreversible neurodegenerative diseases. Important examples are Alzheimer's disease, Parkinson's disease, Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), all of which are characterized by the loss of neurons and the inability of the remaining ones to repopulate depleted areas of the brain. There is still debate about the mechanisms leading to neuronal death in these diseases, however, evidence is mounting that apoptosis is the major pathway (Jellinger and Stadelmann, 2001; Ayala-Grosso et al., 2002; Ugolini et al., 2003; Cribbs et al., 2004; Kermer et al., 2004). But the possibility that the apoptotic pathway coexists with necrosis cannot be excluded (Yuan et al., 2003). A major criticism of the apoptotic neuronal death hypothesis in neurodegenerative diseases is that most of the studies carried out in postmortem human tissue fail to show a significant number of neurons exhibiting the typical apoptotic phenotype. However, considering that the period of time required for neurons to die is on the order of a few hours and that brains from the end-stage of disease were losing neurons for decades, it actually seems reasonable that only a small number of neurons would be found to exhibit the morphological hallmarks of apoptotic death at any given time point.

Many reports correlate the increased expression of caspases and the presence of cleaved caspases with certain types of degenerative diseases but the causal link has not been shown. For example, the increased expression of caspase-1, -2, -3, -5, -6, -7, -8, and -9 have been reported in AD (Chan and Mattson, 1999; LeBlanc et al., 1999; Lu et al., 2000; Pompl et al., 2003), caspases-3, -8 and -9 in PD (Anglade et al., 1997), caspases-1 and -3 in ALS (Pasinelli et al., 1998), and caspases-1 and -8 in HD (Sanchez et al., 1999). Altered expression levels of receptors and death ligands suggest a role for death pathways in these disorders. It has been reported that an increase in Fas expression may be harmful to both neurons and glia, and has been associated with neurodegeneration in diseases such as AD, PD, ALS, and HD (Barone and Parsons, 2000; Ugolini et al., 2003).

Following a similar trend, an up-regulation in the expression levels of TNF receptors has been associated not only with those diseases already mentioned, but also with prion disease and ischemic brain injury. It is not simply the change in the expression levels of death ligands or receptors that is leading to increased apoptosis in these disorders. In certain cases, such as HD, spinocerebellar ataxia, and spinal muscular atrophy, the polyQ expansions introduced into the protein as a result of unstable CAG repeats in the target genes have a tendency to aggregate, forming proteinaceous inclusions in the nuclei of the affected cells ultimately leading to apoptosis (Martin et al., 1999). In these cases, the polyQ repeats are triggering ER stress because the aggregated proteins cannot be properly degraded. It has also has been reported that these aggregates can bind to procaspase-8 and that this binding leads to caspase-8 activation and subsequent cell death (Sanchez et al., 1999). Due to the increasing life expectancy in developed nations, the incidence

of neurodegenerative diseases of aging is increasing exponentially. Because AD is the primary cause of dementia among the elderly population and ALS is the most common adult onset disorder of motor neurons, we take a global overview of the molecular mechanisms leading to neuronal cell death in both diseases.

7.1 Alzheimer's Disease (AD)

Alzheimer's disease is characterized by two main histopathological hallmarks, senile plaques, which are extracellular accumulations of amyloid beta peptide (Aβ), and neurofibrillary tangles (NFT), which are intracellular inclusions of hyperphosphorylated tau protein. Accompanying these features is a profound synaptic and neuronal loss in specific vulnerable brain regions including the hippocampus and entorhinal cortex (Terry et al., 1981; Small et al., 1997). Although the pathogenesis of AD is still being debated, it is generally agreed that Aβ peptide, especially the longer 42 amino acid isoform, which is generated by proteolytic cleavage from the amyloid precursor protein (APP), is the key player in the etiopathology of AD (Hardy and Selkoe, 2002). Because the amyloid hypothesis states that the Aβ peptide is highly neurotoxic, both NFT and neuronal death are considered secondary elements caused by an imbalance between Aβ production and clearance (Hardy and Higgins, 1992). This hypothesis has been revised because it originally postulated that the most toxic species were the fibrillar peptides, but new evidence suggests that the soluble oligomeric species may play a more critical role in the pathogenesis and/or progression of the disease inasmuch as they are able to block basal synaptic transmission, alter hippocampal long-term potentiation (LTP), and mediate neuronal death (Lannfelt et al., 1995; Larson et al., 1999; Walsh et al., 2002; Walsh and Selkoe, 2007).

Multiple studies have shown that several caspases are involved in Aβ-induced neuronal cell death (Gervais et al., 1999; Troy et al., 2000; Allen et al., 2001). Experimental evidence shows that the cytoplasmic tail of APP is cleaved by caspases-3, -6, -7, and -8, and that senile plaques as well as degenerating neurons are enriched in caspase-cleaved APP (Gervais et al., 1999; Zhang et al., 2000). Moreover, both mitochondrial and ER dysfunction play an essential role in mediating cell death induced by Aβ peptides (Pereira et al., 1999). Neurons from caspase-2 null and caspase-12 null mice are resistant to Aβ-mediated neuronal cell death (Nakagawa et al., 2000; Troy et al., 2000). Caspase-2 may be involved in mitochondrial permeabilization whereas caspase-12 acts at the level of the ER (Nakagawa et al., 2000; Zhang et al., 2005).

Recent data suggest that the link between amyloid pathology and NFT degeneration may reside at the level of caspases because Aβ can promote the pathological assembly of tau filaments in vitro by triggering the activation of caspases that can cleave tau and contribute to the filament polymerization (Gamblin et al., 2003; Rissman et al., 2004; Cotman et al., 2005). Aβ accumulation also triggers caspase activation through disruption of the secretory pathway, thus generating ER stress. Caspase activation at this level also cleaves tau, which precedes tau

hyperphosphorylation, and seems to be an early event in AD tau pathology (Guo et al., 2004; Rissman et al., 2004). The accumulation of Aβ can disrupt proteasomal degradation and lead to activation of caspases (Blandini et al., 2006) which in turn are able to cleave tau, thus contributing to the formation of the NFTs (Chung et al., 2001; Gamblin et al., 2003; Rissman et al., 2004). Moreover, experimental data suggest that when caspases are activated, proteosomal degradation is inhibited in order to fully activate the apoptotic cascade, which provides an amplification loop leading unequivocally to the death of the cell (Sun et al., 2004). In addition, APP and Aβ can activate kinases (GSK-3β, SAPK/JNK, p38) that directly phosphorylate tau at certain residues contributing to tau hyperphosphorylation (Kins et al., 2003; Ferrer et al., 2005). In this context, the proteolytic cleavage of tau provides the link between Aβ and tau pathology. However, it is still unknown whether tau processing is required and causal for neurodegeneration, or is a secondary event related to caspase activation in the degenerating cells. In conclusion, multiple mechanisms coexist in the cell, which, when dysregulated, lead to neuronal degeneration.

7.2 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis is the most prevalent adult onset motor neuron disorder. The hallmark histophatological feature is the progressive loss of upper motor neurons in the motor cortex and lower motor neurons in both the spinal cord and brain stem, first described by Charcot in 1869. Accompanying the cell loss are intracellular inclusions of ubiquitinated proteins and strong reactivity to neurofilament markers in the axons (Ince et al., 1998). This is a multifactorial disorder with a diversity of etiologic mechanisms, such as genetic factors, protein aggregation, and oxidative stress, all contributing to the progression of the disease as well as cell death of the injured motor neurons via apoptotic routes.

Although the vast majority of ALS is sporadic, a small subset of familial ALS has been well studied. About 20% of the autosomal dominant familial cases have mutations in superoxide dismutase 1 (SOD1) (Rosen et al., 1993). Although other causal gene mutations have been identified in ALS, ALS 2 or alsin, ALS 4 or senataxin, and ALS 8 or VAPB, more than 100 mutations have been identified in the SOD1 gene and SOD1 mutations are the most prevalent familial form of the disease (Andersen et al., 2003). SOD1 is a 153 amino-acid-free radical scavenger whose function is to transform superoxide free radicals into hydrogen peroxide. SOD1 is a highly expressed protein representing about the 1% of total brain protein. The reason why motor neurons are susceptible to damage in the presence of SOD1 mutations remains unclear. It is thought that mutations in SOD1 do not generate a loss of function, but on the contrary, may be toxic gain of function mutations. Very recent work suggests that, although the motor neurons are more susceptible to death, the presence of mutant SOD1 in the astrocytes induces death of motor neurons that contain wild-type or mutant SOD1 (Di Giorgio et al., 2007; Nagai et al., 2007). There has been enormous interest in understanding the role of oxidative stress in

ALS because SOD1 encodes for an antioxidant enzyme. Although the relevance of oxidative stress is not fully understood, it is believed that mutations in SOD1 promote a structural change that allows a higher rate of interaction between the substrates and the active site of the enzyme, resulting in increased production of free radical species. However, there are not sufficient experimental data supporting this hypothesis because if SOD1 mutants cause peroxynitrite-dependent cell death in vitro, it would be expected that reduction in the levels of peroxynitrite by inhibition of neuronal nitric oxide synthase (nNOS) would improve the motor neuron outcomes. However, these experiments did not show a decrease in motor neuron damage (Facchinetti et al., 1999; Upton-Rice et al., 1999; Son et al., 2001).

Another possible event leading to ALS is mitochondrial dysfunction (Albers and Beal, 2000; Menzies et al., 2002). Again, several properties converge at this level because mitochondria are able to maintain Ca^{2+} homeostasis and are the source of intracellular ATP. Mitochondria generate intracellular free radicals and can also play a key role as mediators of the apoptotic pathway. Mitochondrial dysfunction has been reported in vitro as well as in vivo. Expression of mutant SOD1 (G93A) in a motor neuron cell line leads to mitochondrial abnormalities, not only at the morphological level, but also at the biochemical level, with impaired activity of complexes II and IV of the respiratory chain leading to the activation of apoptotic mechanisms and subsequent cell death (Menzies et al., 2002; Takeuchi et al., 2002; Fukada et al., 2004). In transgenic mice overexpressing mutant SOD1, mitochondrial vacuolization in motor neurons has been noted as an early event (Wong and Strong, 1998). Impaired activity in several complexes of the respiratory chain and reduced ATP synthesis have also been reported in murine models of the disease (Jung et al., 2002; Mattiazzi et al., 2002). Moreover, translocation of cytochrome c from mitochondria to the cytosolic space, triggering the apoptotic cascade, is a feature of these animals (Guegan et al., 2001; Zhu et al., 2002). Following this line of thought, it has been described that the antiapoptotic protein Bcl-2 can interact with aggregates of SOD1 in the spinal cord, thus decreasing the availability of Bcl-2 to prevent apoptosis (Pasinelli et al., 2004).

Motor neurons can have extremely long axons that travel from the spinal cord all the way to the target muscle. Preserving the morphology of these axons requires the presence of structural proteins, such as neurofilaments. Neurofilaments are the main component of the cytoskeleton in neurons and although their primary role is to maintain cell shape, they are also involved in axonal transport and influence axonal caliber. Inclusions of aberrantly assembled neurofilaments, phosphorylated or not, in the cell bodies and axons of motor neurons is one of the histopathological hallmarks of ALS (Ince et al., 1998). Transgenic mice carrying SOD1 mutations exhibit abnormalities in neurofilament organization, as well as intracellular proteinaceous inclusions, and reduced axonal transport in the ventral root (Tu et al., 1996; Zhang et al., 1997). Moreover, more than 1% of sporadic ALS cases carry deletions or expansion in the neurofilament NF-H gene (Meyer and Potter, 1995; Tomkins et al., 1998).

It is not only NF-H filaments that are involved in the disease. Transgenic mice overexpressing peripherin, an intermediate filament, develop late onset motor

neuron degeneration and altered neurofilament assembly (Beaulieu et al., 1999). This alteration in neurofilament structure, together with misfolded SOD1 proteins, may lead to cellular stress, mediated mainly by the ER. This altered situation reduces the ability of the proteasome to mediate protein degradation, thus compromising protein turnover in the cell, which in turn affects surrounding organelles, such as mitochondria, and potentially activates and/or amplifies the apoptotic cascade. Experimental evidence shows that motor neurons die mainly by apoptotic mechanisms (Martin, 1999; Guegan et al., 2001; Sathasivam et al., 2001). The study of cellular models of mutant SOD1 overexpression shows that these cells die via a programmed cell death when exposed to oxidative stress (Cookson and Shaw, 1999). Moreover, the animal models overexpressing mutant SOD1 show an up-regulation in expression and activation of caspase-1 and -3 in the spinal cord of symptomatic animals (Li et al., 2000; Vukosavic et al., 2000). Although great strides have been made in understanding the molecular mechanisms underlying the motor neuron degeneration in ALS, the complex interplay among genetic factors, altered axonal transport, oxidative stress, protein aggregation, and mitochondrial dysfunction make this multifactorial disease a very challenging disorder for therapeutic intervention.

8 Dissecting Death Pathways in Vivo

The increasing number of transgenic and knock-out murine models available in the last decade has offered the possibility of studying in vivo those proteins believed to be associated with certain neurodegenerative disorders. These models provide a more accurate view than the cellular models in which the microenvironment is abolished. However, the in vivo models must also be interpreted with caution because the knock-down of certain genes may induce genetic compensation by related family members that could mask the effect of the exogenous genes. Overexpression may be associated with lethality, or can induce artifacts due to the overexpression process and not due to the introduction of the exogenous gene per se. We also have to keep in mind the genetic background of the particular mouse because certain mutants can be lethal on one background but perfectly viable on another. If we take the results generated by these models with caution, understanding that the models try to mimic neurodegenerative disorders but are still far from perfectly reproducing the phenotype of human diseases, the models can contribute to a better understanding of the etiopathology of the disease, help untangle molecular mechanisms triggering the degenerative process, and provide tools for the identification of potential therapeutic targets. The value of culture systems in deciphering mechanisms should not be underestimated. This is well-illustrated by recent studies of the role of astrocytes in motor neuron death which showed that astrocytes expressing the mutant SOD1 protein induced death of motor neurons whether or not the neurons expressed the mutant SOD1(Di Giorgio et al., 2007; Nagai et al., 2007). It is important to remember that any of the model systems under study are approximations of the diseases and each have their own advantages and disadvantages as systems of study.

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