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Review

Apoptosis in neurodegenerative diseases: the role of mitochondria

W.G. Tatton, C.W. Olanow *

Department of Neurology, Mount Sinai School of Medicine, 1 Gustave Levy Place, Annenberg 14-94, New York, NY 10029, USA

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Abstract

Nerve cell death is the central feature of the human neurodegenerative diseases. It has long been thought that nerve cell death in these disorders occurs by way of necrosis, a process characterized by massive transmembrane ion currents, compromise of mitochondrial ATP production, and the formation of high levels of reactive oxygen species combining to induce rapid disruption of organelles, cell swelling, and plasma membrane rupture with a secondary inflammatory response. Nuclear DNA is relatively preserved. Recent evidence now indicates that the process of apoptosis rather than necrosis primarily contributes to nerve cell death in neurodegeneration. This has opened up new avenues for understanding the pathogenesis of neurodegeneration and may lead to new and more effective therapeutic approaches to these diseases.

Keywords: Apoptosis; Mitochondrion; Neurodegenerative disease; Permeability transition pore

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* Corresponding author. Fax: +1 (212) 987-7635.

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1. Morphological picture of apoptosis

In the 1960s, Kerr used newly developed lysosomal staining techniques to reveal a subpopulation of cells in the ischemic liver that were shrunken and retained intact lysosomal structure [1]. These cells contrasted with necrotic hepatic cells that were swollen and had dissolution of lysosomal organelles. He termed the process 'shrinkage necrosis'. Electron microscopy revealed that the shrunken cells had undergone marked cytoplasmic condensation with budding of condensed cytoplasm into membrane bound cell fragments, which were phagocytosed by neighboring cells. Importantly, organelle structure was maintained within the cell fragments [2]. Prednisone was subsequently shown to induce shrinkage necrosis in the adrenal cortex and adrenocorticotropic hormone was shown to prevent prednisone-induced shrinkage necrosis [3]. In 1972, Kerr et al. [4] described the ultrastructural features of shrinkage necrosis and termed the process 'apoptosis'. They noted that the nuclei showed chromatin condensation and segregated into sharply delineated masses that abutted on the inner nuclear membrane. The plasma membrane remained intact, but underwent marked blebbing. The condensed cytoplasm was fragmented into membrane-wrapped bodies that were engulfed by macrophages without an apparent inflammatory reaction. Finally, apoptotic bodies were described that contained either nuclear or cytoplasmic material, or both. The morphological picture of apoptosis described by Kerr et al. remains the standard today. Cytoplasmic and nuclear changes shown by electron microscopy provide the best evidence that cells have entered the late stages of apoptosis. The picture is slightly different in vitro where, in the absence of macrophages, apoptotic bodies persist for a longer period of time and may undergo cytolysis or secondary necrosis.

2. Nerve cell apoptosis

As early as 1951, it was known that nerve cell death played a major role in vertebrate embryogenesis [5]. It is now known that a massive death of neurons occurs during vertebrate prenatal and postnatal brain development and that this is related to failed competition for trophic factors [6] and represents a form of apoptosis [4]. The neurotrophic hypothesis proposed that nerve cells depend on trophic support from their targets for survival, and that the targets could only supply limited amounts of trophic molecules so that developing neurons were forced to compete for trophic support [7]. Elegant experiments showed that the neurons with 'the best connections' survived and maintained their connections, while weakly connected neurons died [8]. The death process in these developing neurons was termed 'programmed cell death' since it was shown to depend on activation of an intrinsic program of gene expression that led to self destruction though the synthesis of 'death proteins' [9]. The dependence on new protein synthesis was similar to that found in the apoptosis that occurs in cultured sympathetoblasts deprived of NGF [10]. It has subsequently become clear that new protein synthesis is not necessary for all forms of neuronal apoptosis [11,12]. In some forms of neuronal apoptosis, proteins that are necessary for apoptosis to occur are constitutively expressed, while they must be newly synthesized in others [13,14]. The characteristic morphologic findings of apoptosis persist in some models of nerve cell apoptosis, even when transcriptional and translational blockers are used to inhibit new protein synthesis. For example, transcriptional or translational blockers that greatly reduce new protein synthesis do not prevent apoptosis after serum withdrawal in PC12 cells that have been exposed to serum, but not to NGF [15]. Similarly, PC12 cells that have been exposed to NGF for 6 days and have initiated process growth, undergo apoptosis after serum and NGF withdrawal that is independent of new protein synthesis [16]. In contrast, apoptosis in neuronally differentiated PC12 cells caused by trophic withdrawal after 9–12 days of NGF exposure requires new protein synthesis [17]. Therefore, apoptosis can

be dependent on new protein synthesis and termed programmed cell death, or can be independent of new protein synthesis and be unprogrammed. Indeed, in some cases nerve cell apoptosis can be facilitated by the inhibition of new protein synthesis [18], likely due to loss of protection provided by newly synthesized anti-apoptotic proteins. Thus, dependence on new protein synthesis can be used as a marker for neuronal apoptosis [19,20], but its absence does not rule out apoptosis [21].

In developing neurons, apoptosis was thought to provide a counter balance for overexuberant cell replication. It therefore seemed unlikely to involve adult nerve cells, since they are unable to replicate. It is now appreciated that nerve cell apoptosis can occur in response to a variety of insults, many of which are relevant to the pathogenesis of human neurodegenerative diseases. For example, neuronal apoptosis can be caused by exposure to excitatory amino acids [22,23], the parkinsonian toxin MPTP [24] and its metabolite, MPP^+ [25–27], 6-hydroxydopamine [28], the 25–35 fragment of β-amyloid protein [29,30], mutated presenilin 2 [31], mitochondrial complex I inhibitors [32], pro-oxidants, like H_2O_2 [33], iron [34], cycad flour [35], high levels of dopamine [36] or levodopa [37], the AIDS protein gp120 [38], and overexpression of the mutant superoxide dismutase (sod-1) gene from patients with familial amyotrophic lateral sclerosis (ALS) [39].

In general, lower concentrations of toxins, particularly if delivered over a more protracted time course, induce apoptosis while higher levels induce necrosis [23]. For example, high concentrations of the β-amyloid protein fragment induce necrosis [40], while smaller concentrations cause apoptosis [29,41,42]. Intermediate concentrations often induce a mixture of necrosis and apoptosis, with the necrosis occurring in the first few hours after exposure and apoptosis appearing after a number of hours or days.

Despite the demonstration that apoptosis is an important form of nerve cell death, it did not receive recognition as a key factor in neuronal degeneration until the present decade. Part of the reason for this stems from the difficulty in demonstrating apoptotic cells because of their relatively short existence. In vivo, apoptotic nuclei and bodies are phagocytized and digested within a matter of hours. Thus, only a small number of apoptotic nuclei may be seen at one time, which creates the impression that apoptosis plays an inconsequential role. Yet, the presence of a small number of apoptotic nuclei in a tissue section at single point in time may reflect large numbers of apoptotic deaths if the process is ongoing over a period of days, weeks, or years [43]. Accordingly, in the chronic neurodegenerative diseases, massive apoptotic death may occur even though only a small number of apoptotic profiles can be seen in a single pathological specimen.

Another limitation has been the development of markers that can be used to detect apoptosis in tissue sections. In 1980, Wyllie reported that endogenous endonuclease activation in apoptosis resulted in nuclear DNA fragmentation [44]. Agarose gel electrophoresis demonstrated a 180–200 bp 'ladder' pattern of DNA degradation typical of internucleosomal DNA cleavage. DNA fragments of 300 or 500 bp in length are also formed and can be better detected with pulse field techniques [45,46]. The presence of endonuclease-mediated DNA cleavage provides conclusive evidence of apoptosis. However, large amounts of tissue are required to detect DNA cleavage with these techniques, so they are not suitable for detecting small numbers of apoptotic nuclei in a post-mortem brain. Nuclear DNA cleavage can be more readily detected using in situ end labeling (ISEL) techniques which attach a chromagen or fluorochrome to the 3′ cut ends of DNA [47,48]. Chromatin condensation can also be used as a measure of apoptosis. It ensues as a result of DNA fragmentation and can be detected using flow cytometry [49] or in situ staining with fluorescent DNA binding dyes, such as acridine orange or YOYO-1 [6,50]. The ISEL techniques have been widely used to detect apoptosis in neurodegenerative diseases, but there are concerns that in situ end labeling methods detect nuclear events other than those associated with apoptosis and that maintenance of human brain tissue in fixatives or tissue drying can induce false-positives and false-negatives [51–54]. Positive staining of individual neurons with both an ISEL technique and a dye that marks chromatin condensation has been proposed to unambiguously establish the presence of apoptosis [55,56].

3. Changes in gene expression and protein levels in apoptosis

In recent years, a number of genes and their protein products have been uncovered, which can influence or determine the progression of apoptosis and serve as markers of the process. Furthermore, a number of cytoplasmic proteins undergo nuclear translocation and mark the early stages of apoptosis. Oncoproteins, anti-oncogenes, and several protease families have received particular attention as mediators or modifiers of apoptosis. The BCL-2 homolog family of oncoproteins includes both inhibitors and promoters of apoptosis [57]. Inhibitors of apoptosis include BCL-2 itself, BCL-X (BCL-X_L, BCL-Xα, and BCL-Xβ), MCL-1, and A1, while BAX, BCL-X_S, BAD, BAK, and BIK promote apoptosis. Some of the BCL-2 family members can also interact with each other by forming homo- or heterodimers. While the subcellular location of each of the family members differs slightly, most are found in the outer mitochondrial and nuclear membranes. In normal hemopoetic cells, BCL-2 is concentrated in the outer mitochondrial membrane, while BAX is largely confined to the cytosol [58], however during apoptosis BAX becomes concentrated in mitochondrial membranes.

BCL-2, BCL-X_L, and BAX are the members of the BCL-2 family that are most prominently expressed in mammalian nervous tissue. In rodents, BCL-2 levels are high in the immature central nervous system, but decline in the adult nervous system. Small but detectable levels of BCL-2 mRNA and protein are found in neurons in a number of structures in the adult human brain, including the cerebral cortex, basal ganglia, basal nucleus of Meynert, substantia nigra, and cerebellum [59]. In contrast to neurons in the central nervous system, BCL-2 levels remain high in many adult peripheral neurons and can also be detected in microglial cells. BCL-X_L is almost entirely neuronal and remains high in many adult neurons. BAX is also expressed in some adult neurons.

Although BCL-2, BCL-X_L and BAX have been shown to be capable of altering the progression of apoptosis [60] and appear to contribute to neuronal selection during embryonic and postnatal development [57], relatively little is known about the roles of these oncoproteins in the mature nervous system. BCL-2 and BCL-X_L decrease while BAX and BCL-X_S increase in neurons entering apoptosis in vivo [61–65]. In tissue culture, we have found that primary neurons or neuron-like cells show decreases in BCL-2 and BCL-X_L in the early stages of apoptosis induced by trophic withdrawal, pro-oxidants, or mitochondrial respiratory chain inhibitors. BCL-2 and BCL-X_L levels are increased in neurons following low level damage which is insufficient to induce apoptosis. These cells are subsequently resistant to damage that normally would induce apoptosis. BCL-2 is also increased in the human nervous system in a variety of circumstances where neurons may have undergone damage, but have not yet entered into the apoptotic process. In contrast, BAX levels are increased in neurons that have sustained damage and are committed to undergo apoptosis. Accordingly, increased BCL-2 levels may reflect the induction of a survival program in neurons that have sustained damage that is not sufficient to cause apoptosis, while increased BAX levels may mean that the neuron has progressed to the early stages of apoptosis.

A number of proteases, including cysteine proteases, calpains, and proteasomes have been shown to contribute to apoptosis. Most attention has focused on the interleukin converting enzyme (ICE)-like proteases which are termed caspases, the c denoting cysteine protease and the aspase referring to the tendency of the enzymes to cleave after an aspartic acid residue [66,67]. To date, 10 different caspases have been identified and named caspases 1–10. The caspases are synthesized as inactive precursors. Several of the caspases, particularly caspase 3, have been shown to be activated in models of neuronal apoptosis [68–72]. Specific calpains have been shown to contribute to the cleavage of the cytoskeletal protein actin that occurs during apoptosis [73,74] and calpain inhibitors block actin cleavage in apoptosis [75].

A number of cell-cycle related proteins and cycle kinases increase in nerve cell apoptosis including cyclin D1, cyclin B, cyclin E, and Cdc2 kinase [76–82]. It has been proposed that the appearance of the cycle-related proteins represents an abortive attempt of the post-mitotic cells to return to the cell cycle

[83]. In this regard, the mitosis inhibitor protein, p53, increases in a number of forms of neuronal apoptosis [84,85] and has been shown to increase BAX levels in non-neuronal forms of apoptosis [86,87].

Apoptosis can be separated into p53-dependent and p53-independent forms (see [88] for a detailed review of the role of p53 in apoptosis). Recently, a large number of p53-induced genes or PIGs have been uncovered, which are proposed to mediate apoptosis by increasing reactive oxygen species (ROS) levels, thereby inducing mitochondrial damage [89]. p53-induced apoptosis can proceed after treatment with translational or transcriptional blockers [90,91], indicating that p53 can induce apoptosis through pathways that do not require new protein synthesis [92].

c-JUN, a signal-transducing transcription factor of the AP-1 family, is normally involved in cell cycle control, differentiation, and cell transformation. A variety of evidence indicates that c-JUN is involved in the induction of some forms of apoptosis [93,94], and that it is increased in a number of in vitro and in vivo models of neuronal apoptosis [30,95–99].

Another marker for apoptosis is tissue transglutaminase, which cross-links cytoplasmic proteins and is increased in apoptotic cells and apoptotic bodies [100]. Transglutaminase activity leads to the formation of high molecular mass protein polymers, which maintain the integrity of apoptotic cells and bodies and prevents leakage of their contents into the extracellular space. Fas antigen induces apoptosis that is BCL-2 independent (see [101] for a review of apoptosis mediation by Fas antigen) and its mRNA increases in ischemic nerve and glial cells [102]. Lastly, the gene for the radical scavenger protein, Cu/Zn superoxide dismutase (SOD-1), reduces neuronal apoptosis when over expressed [103,104] and increases apoptosis when under expressed [105]. Chronic inhibition of SOD1 causes spinal motoneurons to die by apoptosis [106]. Point mutations in the gene that encodes SOD-1 have been detected in patients with familial ALS [107]. In cultured neurons, these point mutations convert the anti-apoptotic action of SOD-1 to a pro-apoptotic one [39].

4. Evidence for apoptosis in neurodegenerative diseases

In recent years, apoptosis has been described in a variety of human neurodegenerative disorders, primarily based on the use of ISEL techniques to detect neuronal nuclei with apparent DNA cleavage in post-mortem brain tissue. ISEL-positive nuclei have been reported in brains or spinal cords of patients with Parkinson's disease (PD) [56,108–110], Alzheimer's disease (AD) [111–114], Huntington's disease (HD) [111,115], and ALS [115,116]. Some investigators have failed to find evidence of nuclear DNA strand breaks in some of the above conditions [52] and have challenged the reliability of ISEL techniques as a marker of apoptosis in postmortem tissue [53,54]. More definitive evidence in support of apoptosis are the electron microscopic findings of nuclear chromatin condensation in the substantia nigra pars compacta (SNc) of PD brains [117]. Changes were only detected in the SNc and were not seen in normal controls. In addition, we have studied PD postmortem brains with both an ISEL technique and the dye YOYO-1 that stains for chromatin condensation [56]. Approximately 1–2% of individual melanin-containing neurons in the SNc of PD patients stained positively with both of these techniques compared to only 0.2% in age-matched controls. Positive staining of a single neuron with both of these techniques avoids the risk of false-positive results that may occur with either one and we believe establishes the presence of apoptosis.

Another concern that has been raised is the relatively high percentage of nuclei that have DNA strand breaks in AD [118] and PD [56,109,110,117] brains using the ISEL techniques. In one study of neuronal apoptosis in the SNc of mice exposed to MPTP, about 45% of neurons died over a period of about 10 days. Nonetheless, an average of less than 1% percent of dopaminergic neurons showed ISEL and chromatin condensation positive nuclei at any one time during this period (maximum of 4–6%) [55]. The death of murine nigral neurons after MPTP exposure occurs synchronously over a short period of time [119] while the death of nigral neurons in PD likely occurs asynchronously over many years. Accordingly, the percentage of nigral ISEL-positive nu-

clei found in PD and AD brains seems high, even if some ISEL-positive nuclei are glia.

However, the large number of apoptotic cells that have been detected in the brains of patients with neurodegenerative disorders might reflect accelerated apoptosis as a result of agonal events in neurons that were pre-apoptotic and committed to undergo apoptosis at a later time point. Degenerating nerve cells may be in a pre-apoptotic state for some time before entering the end stages of apoptosis as marked by chromatin condensation and DNA cleavage. Changes in tissue perfusion and oxygen levels in the period prior to death may accelerate the entry of these cells into the final stages of apoptosis. Such a mechanism might explain the higher than expected levels of ISEL-positive nuclei and the fact that the nuclei are only found in brain regions known to be affected by the specific neurodegenerative disease.

In support of this concept are changes in apoptosis-related gene expression and protein levels in the same structures, and in some cases in the same neurons, in which nuclear DNA cleavage are found. BCL-2 was found to be decreased in neurons of AD patients with neurofibrillary tangles, but was increased or unchanged in nearby unaffected neurons [59]. Similarly, increased BCL-2 expression was noted in surviving SNc neurons in PD brains [120]. In AD postmortem brains, increased c-JUN immunoreactivity has been detected [118,121]. Staining was observed in nerve cells with ISEL-positive nuclei as well as in some nerve cells without ISEL-positive nuclei, suggesting that these nerve cells may have entered the early stages of the apoptotic process but had not yet progressed to the point of nuclear DNA cleavage. Indeed, there is evidence of increased BAX expression in dopamine neurons that have not yet undergone nuclear DNA cleavage following exposure to MPTP [61]. This may suggest that these cells were heading toward apoptotic death at the time of sacrifice. The finding of neurons in AD brains with increased levels of BAX and c-JUN, but without evidence of nuclear DNA cleavage, supports the existence of pre-apoptotic neurons in neurodegenerative diseases. Other gene and protein changes that have been found in the post-mortem brains in neurodegenerative diseases and that support the notion that apoptosis has occurred include increased transglutaminase [122] and BAX [123], the appearance of cyclin B, cyclin E, Cdc2 kinase [124], and the cyclin-dependent kinase inhibitor p16 [125], and an increase in p53 in cortical astrocytes [126]. BAX is also heavily concentrated in senile plaques and neurofibrillary tangles [121] and FAS antigen has been found in senile plaques in AD[127] and in the SNc in PD [128]. Lastly, apoptosis-like actin cleavage has been demonstrated in neurons associated with senile plaques in AD brains [129,130].

It is also possible that agonal events induce apoptosis to occur in vulnerable neurons that otherwise would not have entered apoptosis at that time. We have recently noted that mitochondrial membrane potential is reduced in fibroblasts derived from some PD patients (discussed below). If a decrease in mitochondrial membrane potential is also present in SNc neurons in PD, they may be vulnerable and prone to enter apoptosis under conditions in which normal neurons would survive. Thus, the relatively high percentages of ISEL-positive nuclei in PD and AD brains may be due to accelerated DNA cleavage in nerve cells that were already committed to undergo apoptosis at a later date or that were vulnerable to the apoptotic process.

Taken together, the evidence for nuclear changes typical of apoptosis and the finding of changes in the expression of apoptosis-related genes and their protein products makes it highly likely that apoptosis contributes to nerve cell loss in human neurodegenerative diseases.

5. The role of mitochondria in some forms of apoptosis

5.1. Mitochondrial defects and neurodegenerative diseases

Defects in mitochondrial energy metabolism have been proposed to underlie neurodegenerative diseases [131,132]. Toxins that damage the mitochondrial respiratory complexes induce deficits in animals that are very similar to those found in specific human neurodegenerative diseases. MPTP and 3-nitropropionic acid poison complexes I and II, respectively, and induce neurological deficits in monkeys that are very similar to PD and HD [133]. Agents, like coenzyme

Q_{10} and nicotinamide, that improve electron transfer in the respiratory chain, reduce parkinsonian-like deficits in MPTP monkeys [134]. Complex I activity is decreased in the SNc of PD postmortem brain tissue [135,136] while complexes II, III, and IV activity are reduced in affected regions of AD brains [137]. A decrease in cytochrome oxidase activity appears to be part of the AD complex IV deficit [138,139]. Decreases in the mRNAs for subunits I and III of cytochrome oxidase have been reported in the association cortex of AD brains [140]. Decreases in complex II and III activity have been found in the caudate nucleus in HD [141]. A complex I defect has been found in platelets and leukocytes from PD patients [142] and a decrease in cytochrome oxidase has been reported in platelets from AD patients [143]. Other studies suggest that there is also decreased complex I activity in PD fibroblasts [144]. Studies using cybrids have shown that the decreased complex I activity in PD platelets and the cytochrome oxidase defect in AD platelets can be transferred to multiple generations of recipient cells [145,146], indicating that activity decreases are intrinsic to the mitochondrial genome in PD and AD.

It has been proposed that defects in the mitochondrial respiratory chain could cause neuronal degeneration through decreased ATP formation or increased production of ROS [132,146,147]. Decreases in mitochondrial membrane fluidity in mitochondria from AD brain tissue [148] and increases in protein adducts of lipid peroxidation in PD brain tissue [149] have been interpreted as resulting from ROS-mediated membrane peroxidation. According to that view, cell death in neurodegenerative diseases could result directly from mitochondrially generated ROS.

Recent studies support another means by which mitochondrial dysfunction may induce neuronal death, namely, apoptosis. Mitochondria are now believed to play a critical role in signaling for the initiation of some forms of apoptosis. Apoptosis was initially believed to occur independently of mitochondrial factors [150,151]. The importance of mitochondria in the initiation of apoptosis is illustrated by the finding that mitochondrial factors can induce chromatin condensation and nuclear fragmentation typical of apoptosis in cell free Xenopus egg extracts [152]. Current evidence now indicates that apoptosis is associated with a sequence of events that includes a fall in mitochondrial membrane potential ($\Delta\Psi_M$), opening of a mitochondrial megapore known as the permeability transition pore (PTP), and release into the cytoplasm of small mitochondrial proteins which signal for the initiation of apoptosis known as apoptosis-initiating factors (AIF). This changing view offers new ways of interpreting the evidence for mitochondrial respiratory chain dysfunction in neurodegenerative diseases and may also offer new therapeutic targets for the treatment of neurodegenerative disease. The schema is discussed in detail in the following sections.

5.2. Mitochondrial membrane potential and apoptosis

Studies of the mitochondrial membrane potential ($\Delta\Psi_M$) have been pivotal in understanding the relationship between mitochondria and the initiation of apoptosis. An electrochemical proton gradient normally exists across the inner mitochondrial membrane resulting in a $\Delta\Psi_M$ of approximately -150 mV and a proton concentration difference (ΔpH) across the mitochondrial membrane. The $\Delta\Psi_M$ is dependent on the capacity of mitochondrial complexes I, III, and IV to use electron energy in the carrier molecules nicotinamide adenine dinucleotide (NADH), ubiquinone, and cytochrome *c* (CytC) to pump protons out of the mitochondrial matrix by transporting them across the inner mitochondrial membrane. Complex II transfers energy from $FADH_2$ to ubiquinone, but does not pump protons. The outward pumping of protons produces an electron gradient that is biochemically reflected by a pH difference (ΔpH) and electrically by a voltage across the inner mitochondrial membrane termed the mitochondrial membrane potential ($\Delta\Psi_M$) [153]. The $\Delta\Psi_M$ and the ΔpH contribute to a proton electromotive force (δp) ($\delta p = \Delta\Psi_M - 60\ \Delta$pH) where ΔpH = mitochondrial pH − cytosol pH. δp drives the conversion of ADP to ATP at complex V (ATP synthase). Since $\Delta\Psi_M$ is by far the greatest contributor to δp, in most instances it can be assumed that $\Delta\Psi_M$ varies almost linearly with the ATP/ADP ratio and provides an estimate of the ATP/ADP ratio within individual mitochondria.

Measurements of whole cell potentiometric dye fluorescence in a variety of blood, hepatic, and immune cell models have shown that $\Delta\Psi_M$ is reduced

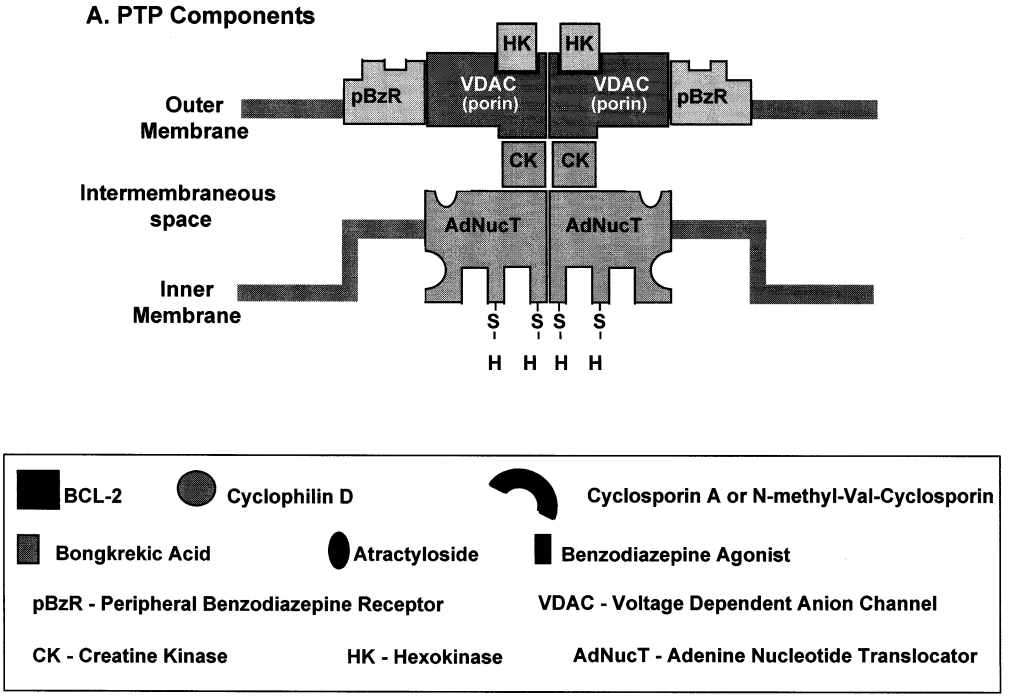
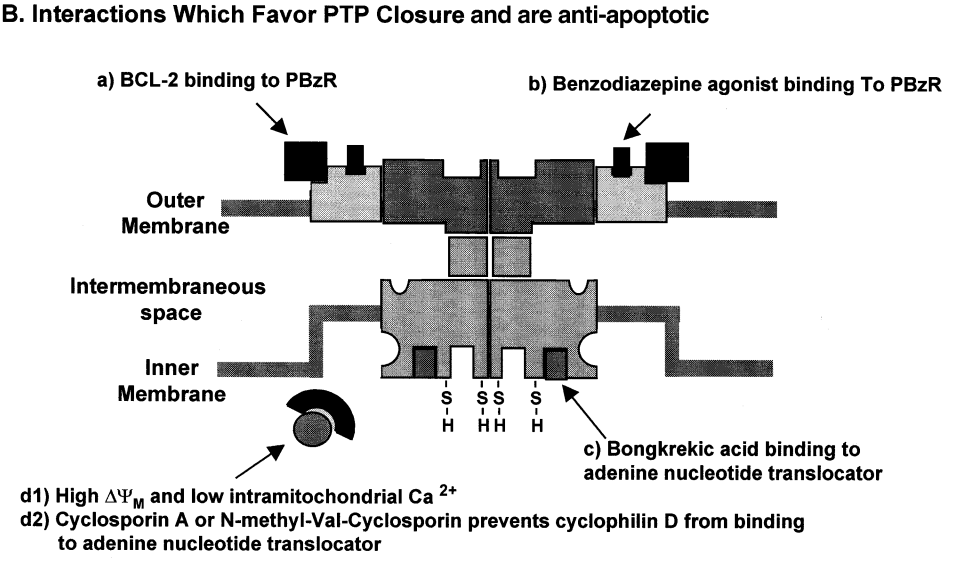


Fig. 1. Schematic representation of major components of the permeability transition pore and factors or agents which induce pore opening or pore closing. The major pore components of the PTP are shown in A in a schematic form designed to create a pore. The components are appropriately localized to the outer membrane, the intermembranous space, and the inner membrane, but their actual, relationships to each other and how those relationships create a pore spanning the inner and outer membranes are not known. The box in A defines the component names which have been abbreviated in the schematic and includes symbols for a number of agents known to influence pore opening or closing. A number of other components of the PTP are not shown in the schematic diagram but are included in the text. In B and C, the putative interactions of specific factors and agents with different components of the PTP are schematized to illustrate how these factors or agents might influence the probability of opening or closing of the pore. If a reduction in the $\Delta\Psi_M$ and opening of the PTP are factors in neuronal apoptosis in the neurodegenerative diseases, then the agents shown in B and C, and the mechanisms whereby they influence opening or closure of the PTP, may lead to the development of new agents with the capacity to block apoptosis and slow the progression of the human neurodegenerative disorders.

very early in the apoptotic process, prior to the onset of nuclear DNA fragmentation and chromatin condensation (reviewed in reference [154]). More recently, our group used laser confocal imaging to obtain direct measurements of $\Delta\Psi_M$ in living NGF-differentiated PC12 cells and showed that a decrease in $\Delta\Psi_M$ is also one of the earliest, if not the earliest, detectable event in apoptosis induced by NGF and serum withdrawal [155]. $\Delta\Psi_M$ was significantly reduced in a proportion of mitochondria 3–6 h prior to nuclear DNA fragmentation and chromatin condensation. The decrease in $\Delta\Psi_M$ was temporally correlated with an increase in intramitochondrial Ca^{2+}, but not with the increase in cytosolic ROS levels which occurred only after the decrease in $\Delta\Psi_M$ was well established. Decreases in $\Delta\Psi_M$ coupled with increases in intramitochondrial Ca^{2+} induce opening of the PTP (see below) [156]. Accordingly, changes found in early apoptosis are appropriate to open the PTP.

5.3. Permeability transition pore

A decrease in $\Delta\Psi_M$, in the presence of increased intramitochondrial Ca^{2+} [156], induces opening of a PTP which spans the inner and outer mitochondrial membranes. Details on the purification and the in vitro reconstitution of the PTP have recently been provided [157,158]. A schematic diagram illustrating the components of the PTP is provided in Fig. 1A, although the precise structure of the PTP is uncertain [159]. The adenine nucleotide translocator (AdNT) is a critical element of the PTP, but whether it forms the pore itself or is just closely associated with a



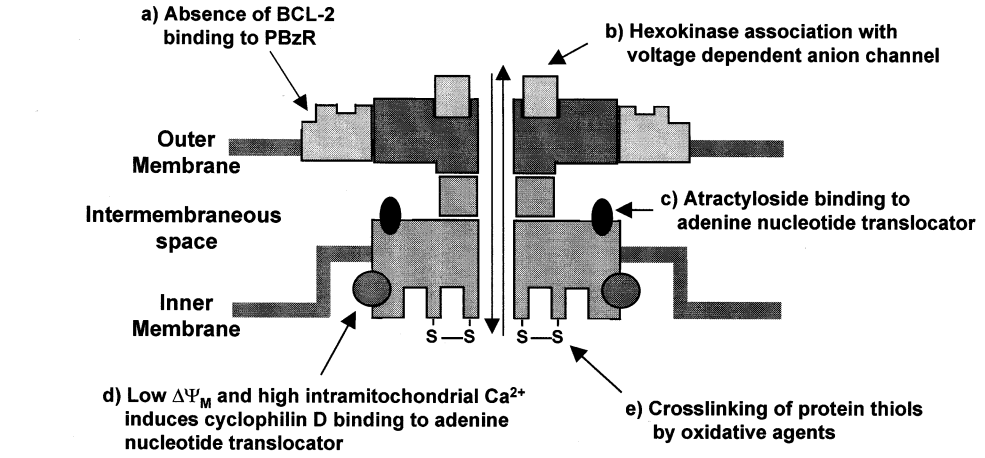


Fig. 1 (*continued*).

pore-forming protein is unknown. The PTP also includes a voltage-dependent anion channel (a porin) and a peripheral benzodiazepine receptor (PBR). As shown in the schematic drawing, these components are closely associated with hexokinase, creatine kinase, and BCL-2, as well as other elements, such as glycerol kinase, phospholipid hydroperoxidase, glutathione peroxidase, 3-b-hydroxysteroid dehydrogenase isomerase, and cardiolipin synthase, which are not shown in the diagram.

The PTP opens in response to a decrease in $\Delta\Psi_M$ in the presence of an increase in intramitochondrial Ca^{2+} [160]. Marked increases in mitochondrial Ca^{2+}, increased oxidative radical levels, or partial failure of the respiratory complexes, acting either individually or together, can induce a fall in $\Delta\Psi_M$ [161]. Opening of the PTP dissipates any remaining proton gradient across the mitochondrial membrane and further reduces the $\Delta\Psi_M$ [162]. Opening of the PTP could itself theoretically induce apoptosis, but it is more likely to lower threshold than to initiate apoptosis [160,162]. Complete opening of the PTP allows free exchange between the mitochondrial matrix and the extramitochondrial cytosol of solutes and proteins smaller

Fig. 2. Relationship of cytochrome *c* immunodensity to $\Delta\Psi_M$ and DNA fragmentation. Simultaneous images of $\Delta\Psi_M$ using the fluorochrome CMTMR (Molecular Probes, Oregon), CytC immunodensity, and YOYO-1 DNA binding in PC12 cells that had been exposed to serum and NGF for 6 days (see [155] for details). A1, A2, and A3 show identical image fields for a normal appearing, neuronally differentiated PC12 cell that was washed and then replaced in serum and NGF 6 h previously. B1, B2, and B3 show identical image fields for four PC12 cells that were washed and then placed in minimum essential media 6 h previously. Placement in minimum essential media without NGF or serum has induced apoptosis in one of the neuronally differentiated PC12 cells (see arrow), with nuclear chromatin condensation and DNA cleavage appearing between 6 and 12 h. A1 and B1 show binary addition of the images for DNA (green color) and $\Delta\Psi_M$ (red color). The upper cell in B1 (arrow) shows a nucleus with typical DNA condensation into apoptotic bodies, while the cell in A1 and the lower three cells in B1 show normal intranuclear DNA organization. Note that the apoptotic cells with nuclear DNA condensation (arrow) shows a marked reduction in $\Delta\Psi_M$, as revealed by the decrease in red intensity. A2 and B2 show binary addition of the images for cytC immunodensity (green color) and $\Delta\Psi_M$ (red color). The cell in A2 shows that most of the CytC is co-localized with $\Delta\Psi_M$ as revealed by the yellow and orange coloration resulting from red–green addition. The lower three cells in B2 show less bright yellow coloration in accordance with lower intramitochondrial CytC immunodensity. The apoptotic cell in B2 also shows increased cytoplasmic CytC immunodensity that has been shown to be extramitochondrial by co-staining for intramitochondrial biotin (not shown). A3 and B3 show binary addition of the images for cytC immunodensity (green color) and DNA (red color). A3 shows the typical discrete localization of CytC immunodensity in trophically supported cells, while B3 shows the more globular appearance of cytoplasmic CytC found in apoptotic or pre-apoptotic cells.

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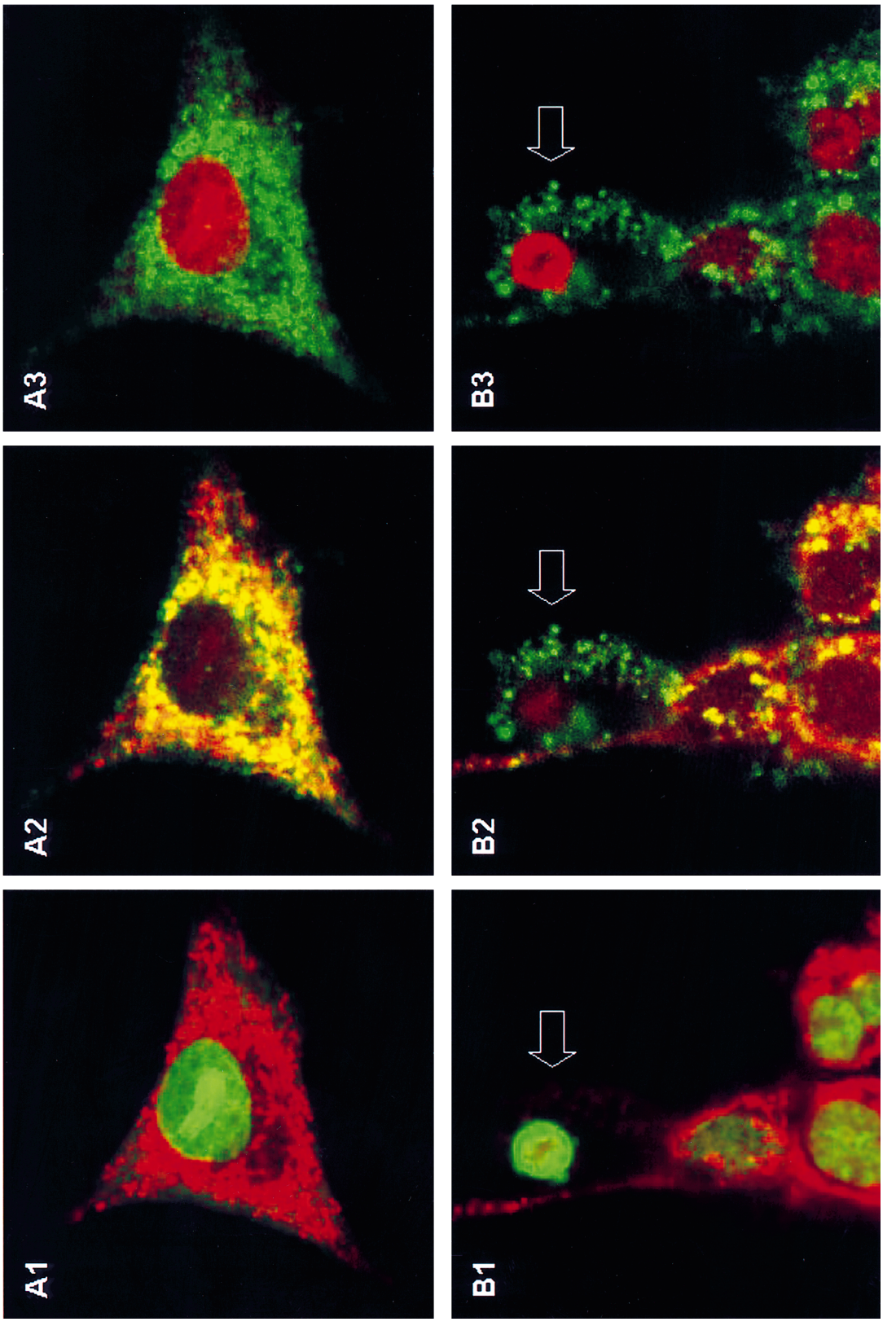
than 1500 Da [163]. This can result in mitochondrial swelling and rupture of the outer mitochondrial membrane with the release of heat labile AIFs such as cytochrome *c* from the intermembrane space of the mitochondria into the cytoplasm [164–167]. While intermembrane AIFs such as cytochrome *c* do not transit through the PTP [168], they can be released into the cytoplasm through fractures in the mitochondrial membrane. Glutathione is actively concentrated in the mitochondrial matrix and release of glutathione from mitochondria is considered to be a definitive marker of PTP opening [169].

Several factors are known to influence opening or closure of the PTP. Elements that favor pore closing are anti-apoptotic and are illustrated in Fig. 1B. Those that favor pore opening promote apoptosis and are shown in Fig. 1C. Cyclosporin A binds to the PTP and maintains it in a closed position. It also promotes pore closure by binding cyclophilins that otherwise induce PTP opening in the presence of Ca^{2+} by binding to the AdNT [160,162]. Factors like glutathione, ADP levels, and ROS levels in the mitochondrial matrix modulate the gating voltages necessary to induce PTP opening but are not sufficient in themselves to open the PTP [170]. The anti-apoptotic protein BCL-2 maintains closure of the PTP in a manner similar to cyclosporin A. Recently, it has been found that the levels of expression of BCL-2 and the PBR correlate in lymphoid cell lines and that BCL-2 co-immunoprecipitates with the PBR [171]. These findings indicate that BCL-2 likely binds to the PBR component of the mitochondrial PTP. BCL-2 has been shown to localize to the outer mitochondrial membrane [172–175] within, or near to, the mitochondrial PBR [171]. Truncated BCL-2, which cannot dock in mitochondrial membranes and remains in the cytosol, is considerably less effective in reducing apoptosis than BCL-2 located in mitochondrial membranes [176]. Richter first proposed that BCL-2 reduces apoptosis by maintaining $\Delta\Psi_M$ [161]. Zamzami and coworkers subsequently provided persuasive evidence for this concept, demonstrating that BCL-2 maintains PTP closure [177] and blocks the initiation of apoptosis by preventing the escape of heat labile molecules which signal for the onset of apoptosis. Numerous other studies have also shown that BCL-2 can prevent a decrease in $\Delta\Psi_M$ and the release of ICE-like AIFs [164,166,167]. BCL$-X_L$ similarly regulates $\Delta\Psi_M$ and matrix volume within mitochondria [178]. Thus, opening of the PTP is a critical step in many forms of apoptosis and is proposed to constitute an irreversible step in the process [179].

5.4. Apoptosis-initiating factors

The importance of mitochondrial factors in the initiation of apoptosis is demonstrated by the finding that mitochondrial homogenates are essential for the progression of the nuclear changes of apoptosis in cell free systems [152]. Several mitochondrial AIFs, which promote apoptosis, have been identified to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date. dATP, when accompanied by holocytochrome *c*, a nuclearly encoded 14 kDa protein which is normally localized to the mitochondrial intermembranous space, can promote the nuclear DNA changes of apoptosis in some cell free systems [180]. Additionally, in some forms of apoptosis, CytC can be found in the extramitochondrial cytosol in the early stages of apoptosis [181] and injection of CytC into cells induces apoptosis [182]. CytC release from mitochondria has been shown to activate a caspase 3 precursor leading to activation of an endonuclease that cleaves nuclear DNA [181,183,184]. On the other hand, the anti-apoptotic agent BCL-2 has been shown to block the release of CytC from mitochondria [181].

It has been reported that an increase in cytosolic holocytochrome *c* occurred prior to any decrease in $\Delta\Psi_M$ [185]. This finding is inconsistent with other work showing that opening of the PTP caused by a fall in $\Delta\Psi_M$ results in the escape of CytC from mitochondria [186,187]. The assertion that cytosolic CytC increases independently of $\Delta\Psi_M$ is uncertain since the measurement of $\Delta\Psi_M$ in this study employed $DiOC_6(3)$ in whole cells [185]. $DiOC_6(3)$ has a relatively low sensitivity to gradations in $\Delta\Psi_M$ [188]. It may be unsuitable for quantitative studies as whole cell measurements of $\Delta\Psi_M$ can neglect marked decreases in $\Delta\Psi_M$ in a subpopulations of mitochondria within individual cells (see [155] for details). We have examined apoptosis in partially neuronally differentiated PC12 cells induced by NGF and serum withdrawal and have simultaneously imaged CytC and $\Delta\Psi_M$ immunodensity in individual mitochondria and in the neighboring cytoplasm (Rideout, Chalmers-Redman, Pong and Tatton, unpublished observations). At high and medium levels of $\Delta\Psi_M$, mitochondrial CytC content in these locations varies randomly with $\Delta\Psi_M$. At low $\Delta\Psi_M$ levels, intramitochondrial CytC levels fall dramatically and nearby cytoplasmic CytC levels increase reciprocally (see illustrations of increases in extramitochondrial CytC immunodensity in preapoptotic and apoptotic cells in Fig. 2). Importantly, we found that losses of $\Delta\Psi_M$ in a relatively small subpopulation of a cell's mitochondria can noticeably increase CytC immunodensity in the cytoplasm. Although another study of apoptosis failed to find a decrease in modal levels of $\Delta\Psi_M$ prior to increases in levels of cytosolic CytC [189], examination of the distributions of $\Delta\Psi_M$ for the whole mitochondrial population reveals a subpopulation of mitochondria whose $\Delta\Psi_M$ loss correlates temporally with increasing cytosolic CytC levels.

Another mitochondrial AIF has been identified as an interleukin converting enzyme-like (ICE) protease, since it can be inhibited by *N*-benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone, an inhibitor specific to the ICE family [167]. BCL-2 has also been shown to prevent the release of this AIF, presumably by maintaining closure of the PTP and preserving the $\Delta\Psi_M$ [167].

Subcellular translocation of several small cytoplasmic proteins have also been seen in association with apoptosis. Hunot et al. have shown that stimulation of tumor necrosis factor-α (TNF-α) receptors on cultured dopamine neurons induces nuclear translocation of NFκ-B in association with induction of apoptosis [190]. A 70-fold increase in nuclear translocation of NFκ-B has been detected in neurons in the SNc in PD patients [190]. It remains unclear whether nuclear translocation of NFκ-B promotes cell death or represents an attempt to upregulate a survival program and protect against cell death. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an intermediary enzyme in glycolytic metabolism and a factor in mRNA translation, has also been implicated in apoptosis [191,192]. GAPDH normally exists in the cytoplasm as a tetramer bound to AU-rich regions of RNA. In some models of apoptosis, GAPDH has been shown to accumulate in the nucleus, and to be associated with transcriptional events related to apoptosis. Our preliminary data indicates that NAD^+ or ROS can displace GAPDH from cytoplasmic RNA binding sites and permit it to accumulate in the nucleus (Carlile, Borden, Chalmers-Redman, Fraser and Tatton unpublished findings). Using size affinity chromatography, we have shown that the anti-apoptotic molecules (−)-desmethyldeprenyl (DMS) and CGP 3466 (a tricyclic analog of DMS), which have neuroprotective benefits in tissue culture models of nerve cell apoptosis [193,194] bind to GAPDH and preferentially maintain it as a dimer, in which form it does not accumulate in the nucleus and does not promote apoptosis. Although the precise mechanism whereby GAPDH contributes to apoptosis is not known, re-

cent work in our laboratory suggests that nuclear accumulation of GAPDH is associated with a fall in levels of BCL-2, and that this reduction is prevented by anti-apoptotic agents such as DMS and CGP-3466. Preliminary data now indicate the presence of GAPDH nuclear translocation in the PD nigra.

It is apparent that there are many different AIFs and that different ones may be operative in different models of apoptosis. Studies in multiple myeloma cells showed that cytosolic CytC levels were increased early in apoptosis induced by ionizing radiation, but not in apoptosis induced by dexamethasone or anti-FAS antibody [195]. The apoptosis induced by ionizing radiation is likely p53-dependent, while the latter two methods of inducing apoptosis are p53-independent. Similarly, microinjection of CytC into human kidney 293 cells induces apoptosis while it does not in MCF7 breast carcinoma cells [182]. It is also clear that a variety of different AIFs that are released from mitochondria in response to a fall in $\Delta\Psi_M$, and opening of the PTP may initiate apoptosis in different cell systems. To date, there is no data pertaining to mitochondrial AIFs in human neurodegenerative diseases. The role of cytoplasmic factors, such as NFκ-B and GAPDH, also remains to be delineated.

5.5. Oxidant stress and mitochondrial apoptosis

Mitochondrial dysfunction, with a fall in ATP production, can be associated with an increase in mitochondrially derived ROS [196,197]. Increased levels of oxidative radicals, particularly in the presence of increased intramitochondrial Ca^{2+}, can induce apoptosis by causing cross linking of protein thiols in the mitochondrial inner membrane [198] with opening of the PTP [170,199]. BCL-2 has been shown to act as an anti-oxidant [176] and might provide anti-apoptotic effects through this mechanism as well as by its direct effect on the PTP. A decrease in BCL-2 levels in trophically deprived PC12 cells entering apoptosis is associated with markedly increased cytosolic levels of oxidative radicals [200]. In contrast, the reduction in apoptosis induced by bcl-2 overexpression is associated with a decrease in both ROS levels and in peroxidation of membrane lipids [176]. Similar effects have been detected with radical scavengers, such as SOD-1 and glutathione, which have been shown to prevent the direct action of oxidative radicals on PTP opening [170,199]. Thus, a defect in mitochondrial respiratory chain activity, as has been detected in PD and AD [135,137,201], might lead to apoptosis through increased production of oxidative radicals, as well as through decreased proton pumping with a fall in $\Delta\Psi_M$ and opening of the PTP. These direct and indirect actions on the PTP may reinforce each other in inducing apoptosis. A more detailed explanation of the indirect effects of BCL-2 on cellular redox equilibrium is provided in reference [202].

5.6. Mitochondria and apoptosis in neurodegenerative disease

Mitochondria were not previously thought to contribute to the initiation or progression of apoptosis. Central to that viewpoint was the finding that fibroblasts lacking mitochondrial DNA (mtDNA), ρ° cells, could die from apoptosis caused by staurosporine treatment or trophic withdrawal, and that this model of apoptosis could be blocked by bcl-2 overexpression [150]. Although an absence of mtDNA in ρ° cells compromises the synthesis of a number of proteins necessary for mitochondrial ATP production, it does not result in a loss of $\Delta\Psi_M$ or induce apoptosis consequent to a fall in $\Delta\Psi_M$. ρ° cells utilize ATP produced from glycolysis to maintain $\Delta\Psi_M$, which decreases early in apoptosis caused by exposure to TNF-α or to cycloheximide [165,203,204]. Indeed, high levels of Ca^{2+} which reduce $\Delta\Psi_M$, or treatment with atractyloside which allows the release of a soluble mitochondrial factor, have been shown to induce the nuclear stigmata of apoptosis in a cell-free system [204]. Thus, deletion of mtDNA does not appear to impair the capacity of mitochondria to maintain $\Delta\Psi_M$ or to develop apoptosis related to a fall in $\Delta\Psi_M$ with release of mitochondrial AIFs.

We believe that apoptosis is an important form of cell death in neurodegenerative disorders and that an early decrease in $\Delta\Psi_M$ is one of the central hallmarks of some forms of apoptosis, leading to opening of the PTP and release of mitochondrial AIFs. This hypothesis predicts that agents that lower $\Delta\Psi_M$ or facilitate opening of the PTP promote apoptosis while those that promote its closure are anti-apop-

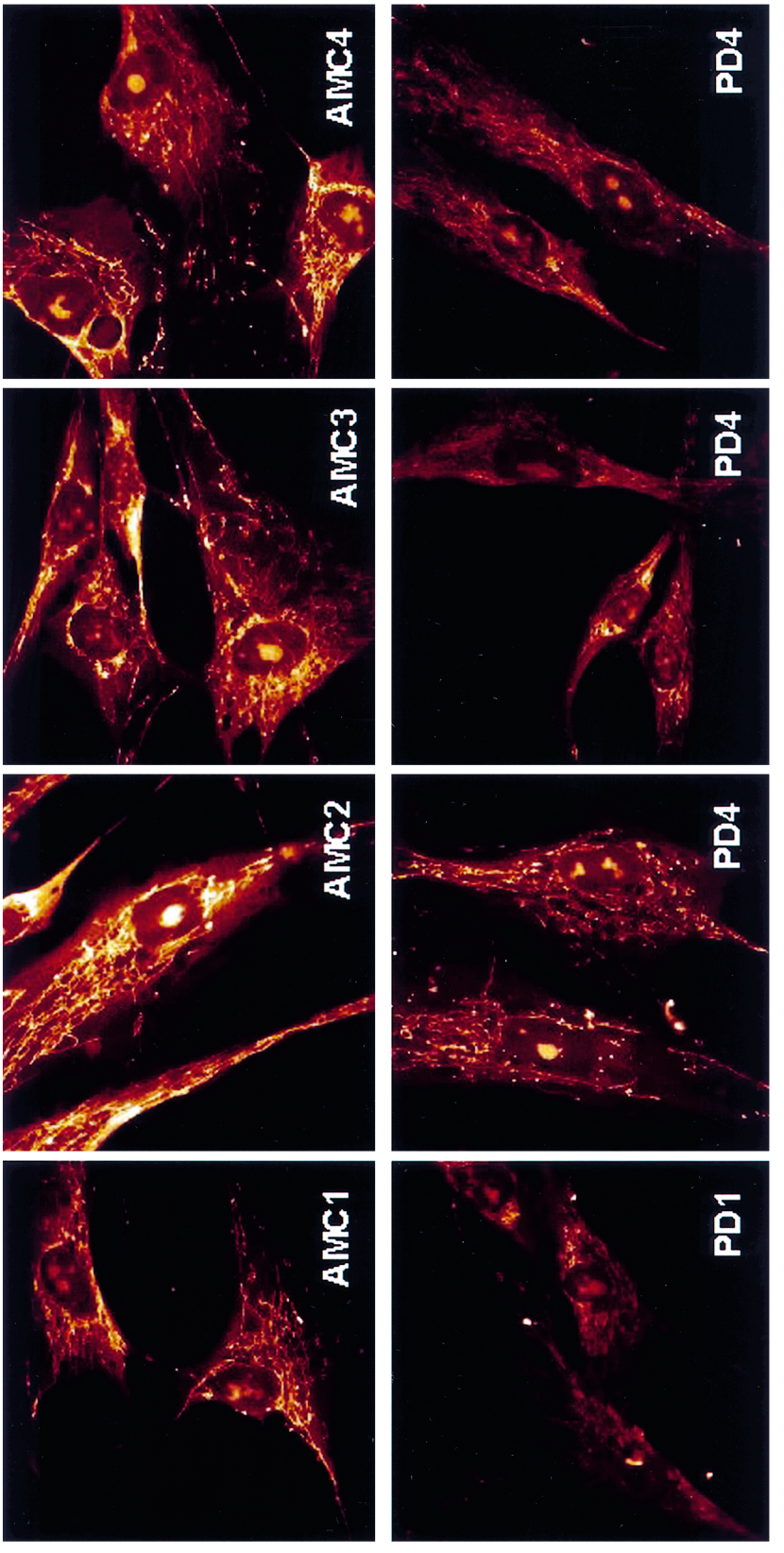
AMC4
PD4
AMC3
PD4
AMC2
PD4
AMC1
PD1

Fig. 3. Mitochondrial membrane potential in fibroblasts derived from PD patients and age-matched controls. Laser confocal scanning microscope image of mitochondrial membrane potential using chloromethyltetramethyl rosamine (CMTMR, see [155] for methodological details and interpretations) in cultured fibroblasts derived from normal, age-matched control subjects (labeled AMC1–AMC4) and PD patients (labeled PD1–PD4). Note that there is a reduction in membrane potential in PD patient fibroblasts compared to controls. To date, similar decreases have been found in about 70% of PD patients. If similar decreases in mitochondrial membrane potential are present in SNc neurons, they may make those neurons vulnerable to opening of the mitochondrial megapore and the induction of apoptosis.
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totic (see Fig. 1B,C). This theory explains why apoptosis is inhibited by agents, such as BCL-2 and cyclosporin A, which maintain PTP closure [162] and Ruthenium red, which block Ca^{2+} entry into mitochondria [205,206]. Furthermore, this hypothesis offers an explanation as to how ROS [207], mitochondrial complex I toxins like MPP^+ [20,21], and increased cytoplasmic Ca^{2+} [208] can *promote* the development of apoptosis and why increases in levels of sod-1 and glutathione can *inhibit* the likelihood of developing apoptosis [91,92,170]. Lastly, the theory would explain why a reduction in $\Delta\Psi_M$ precedes pre- and post-translational molecular events associated with apoptosis, such as cytoskeletal depolymerization, membrane blebbing and chromatin condensation with DNA strand cleavage.

At present, there is no direct evidence as to whether a decrease in $\Delta\Psi_M$ contributes to human neurodegenerative diseases. In pilot studies, we employed laser confocal microscopic techniques using the potentiometric dye, CMTMR to measure $\Delta\Psi_M$ in cutaneous fibroblasts taken from PD patients and age-matched controls (Chalmers-Redman, Olanow and Tatton, unpublished observations). Image analysis revealed a significant loss of mitochondria with high levels of $\Delta\Psi_M$ in PD fibroblasts compared to controls (Fig. 3). We hypothesize that the decrease in $\Delta\Psi_M$ in PD fibroblasts results from reduced proton pumping due to the reported complex I defect. A shift in $\Delta\Psi_M$ to lower levels could render cells vulnerable to PTP opening and the initiation of apoptosis. Recently, neuroblastoma cells transfected with a SOD-1 mutation that causes familial ALS have been shown to have decreased $\Delta\Psi_M$, similar to what we observed in the PD fibroblasts [209]. Accordingly, the decreases in mitochondrial complex activity found in PD, AD, and HD may make neurons vulnerable to apoptosis through a relative failure of proton pumping and a consequent decrease in $\Delta\Psi_M$.

6. Implications for therapy

Our current understanding suggests that apoptosis is associated with a sequence of events in which mitochondria are intimately involved. These include: (a) a fall in $\Delta\Psi_M$, coupled with a rise in intramitochondrial calcium; (b) opening of the mitochondrial PTP; and (c) release of small mitochondrial proteins, which signal the initiation of apoptosis. If a decrease in $\Delta\Psi_M$ is, in fact, a characteristic feature of mitochondria in neurodegenerative disease then studies of the complex I defect and apoptotic mechanisms in the fibroblasts and other affected disease tissues could serve to elucidate the pathogenesis of specific neurodegenerative diseases. Additionally, measurements of $\Delta\Psi_M$ in peripheral tissues could offer an early marker for one or more of the neurodegenerative diseases. A search for factors capable of inducing apoptosis in tissues from persons with neurodegenerative diseases may aid in the recognition of the agents and the mechanisms that initiate the diseases.

This schema also provides an opportunity to interfere with the cell death process and to design putative neuroprotective therapy for patients with neurodegenerative diseases. Such an approach might include agents which: (a) preserve the $\Delta\Psi_M$; (b) maintain closure of the PTP; and (c) modify the expression or the synthesis of apoptosis-related proteins or, alternatively, that bind to apoptosis-related proteins and change their shape or binding properties. Such approaches have the advantage of providing benefit regardless of the specific etiologic or pathogenic basis of neurodegeneration.

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