Neuronal cell death in neurodegenerative diseases: recurring themes around protein handling

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Abstract

Neuronal cell death plays a role in many chronic neurodegenerative diseases with the loss of particular subsets of neurons. The loss of the neurons occurs during a period of many years, which can make the mode(s) of cell death and the initiating factors difficult to determine. *In vitro* and *in vivo* models have proved invaluable in this regard, yielding insight into cell death pathways. This review describes the main mechanisms of neuronal cell death, particularly apoptosis, necrosis, excitotoxicity and autophagic cell death, and their role in neurodegenerative diseases such as ischaemia, Alzheimer's, Parkinson's and Huntington's diseases. Crosstalk between these death mechanisms is also discussed. The link between cell death and protein mishandling, including misfolded proteins, impairment of protein degradation, protein aggregation is described and finally, some pro-survival strategies are discussed.

Introduction: neurodegeneration in disease

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and ischaemia, are characterized by the loss or dysfunction of particular groups of neurons. Neuronal dysfunction may not necessarily be the result of neuronal cell death. It can be caused by synaptic loss, impairment of long-term potentiation (LTP) or disruption of neuronal signalling as a result of disease pathology. These events often precede neuronal cell loss in chronic neurodegenerative diseases [1]. Aging is a factor in these diseases, suggesting that accumulation of neuronal stresses over time causes cell death. However, during normal

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aging there is relatively little neuronal loss, which is in contrast to a high degree of neuronal death in neurodegenerative diseases [2]. This review will focus on mechanisms of cell death in neurons and will attempt to bring together some common themes around protein handling that may help to shed light on the causes of neuronal cell death in neurodegenerative diseases. The underlying genetic basis of some neurodegenerative diseases such as familial AD, familial PD and HD has helped greatly in furthering our understanding of what causes neuronal cell death in these diseases and the type of cell death that is induced.

Mechanisms of cell death in neuronal cells

Although it is recognized that neurons die in neurodegenerative diseases, the mode of cell death is often unclear. There are a number of recognized ways in which neuronal cells can die, including apoptosis, necrosis, autophagic cell death (ACD) and excitotoxicity. Other forms of cell death such as oncosis and paraptosis have not been studied much in neurons, and a role for these modes of cell death in neurodegenerative diseases is not known, largely because of the lack of specific markers [1].

Necrosis

Necrosis is an acute form of cell death. It is characterized by loss of ATP, dissipation of ion gradients, cell swelling and ultimately cell lysis (Fig. 1A). Necrosis thus causes inflammation in the surrounding region due to release of cell contents into the environment. Necrotic cell death is usually caused by severe cellular stress, *e.g.* by high levels of toxins. Necrosis is not a programmed form of cell death in that there is no identifiable biochemical pathway, and once initiated, this form of cell death does not offer much potential for therapeutic intervention since it tends to be acute in nature.

Apoptosis

Apoptosis was originally defined as 'shrinkage necrosis', exhibiting condensed and fragmented nuclei in shrunken cells. In contrast to necrosis, apoptosis is characterized by maintenance of cellular ATP and the initiation of a biochemical pathway leading to cell shrinkage, budding off of apoptotic bodies and phagocytosis of these cell particles. There is no associated cell lysis or inflammation. Biochemically, apoptosis is associated with the activation of caspase proteases that exist in a functional hierarchy of initiator and downstream effector caspases (Fig. 1B). Effector caspases cleave a wide variety of protein substrates to cause the degradation and demise of the cell [3]. There are at least two well-characterized

ways in which caspase activation is initiated in mammalian cells. These are the intrinsic (or mitochondrial) pathway and the extrinsic (or death receptor) pathway.

In the intrinsic pathway, caspase activation is triggered by the release of cytochrome c from the mitochondria into the cytosol, where it plays an essential role in the formation of a multimeric caspase activation complex called the apoptosome [4, 5]. Specifically, cytosolic cytochrome c interacts with Apaf-1 which, in the presence of dATP, leads to clustering and autoactivation of initiator caspase-9 [4, 6]. Active caspase-9 causes cleavage and activation of downstream effector caspases, such as caspase-3. This occurs in response to several apoptotic stimuli including neurotoxins such as colchicine, glutamate, 6-hydroxydopamine [7–9]. It should be noted that other pro-apoptotic factors such as Smac/Diablo, apoptosis-inducing factor (AIF) and Omi/Htra2 are also released from the mitochondria during apoptosis [10]. One of these, AIF can induce chromatin condensation without caspase activation (Fig. 1C) and in fact, apoptotic nuclear morphology in neuronal cells is not always associated with caspase activity [11, 12]. In the extrinsic pathway, ligand binding of death receptors (*e.g.* Fas, TNF-receptor, TRAIL receptors) leads to trimerization of these receptors and the formation of an intracellular multiprotein complex. The initiator caspases-8 or -10 are part of this complex, and proximity-induced autoactivation leads to their cleavage and full activation (Fig. 1B) [13].

Apoptosis can be regulated at various points in the pathway. Mitochondrial integrity, and thus the intrinsic pathway, is regulated by the Bcl-2 family, which includes both pro- (*e.g.* Bax, Bak) and anti-apoptotic (*e.g.* Bcl-2, Bcl-x$_L$) members, which either promote or inhibit release of pro-apoptotic factors into the cytosol (Fig. 1B) [14–16]. Downstream of the release of cytochrome c, caspase activation and activity are regulated by the inhibitor of apoptosis protein (IAP) family of proteins [17].

Excitotoxicity

Particular to neuronal cells is death by excitotoxicity, which is toxicity caused by excessive stimulation of the NMDA subtype of glutamate receptors. This form of neuronal cell death occurs as a result of ATP depletion that occurs during ischaemia. The loss of ATP causes general depolarization of neurons, thus stimulating neurotransmitter release, and impairing ATP-dependent neurotransmitter re-uptake systems. Since glutamate is the most abundant excitatory neurotransmitter in the brain, this leads to overexcitation of NMDA receptors (Fig. 1C). These ligand-gated ion channels allow influx of Ca^{2+}. Cytosolic Ca^{2+} is then sequestered by the mitochondria and by the endoplasmic reticulum (ER). If excessive, this can cause ER stress and/or mitochondrial permeability transition [18]. It is open to discussion, however, whether excitotoxicity can really be considered a separate form of cell death, as it is linked to activation of both apoptosis and necrosis (Fig. 1C). For example, *in vitro* evidence shows that glutamate can cause both early necrosis and delayed apoptosis in neurons [19].
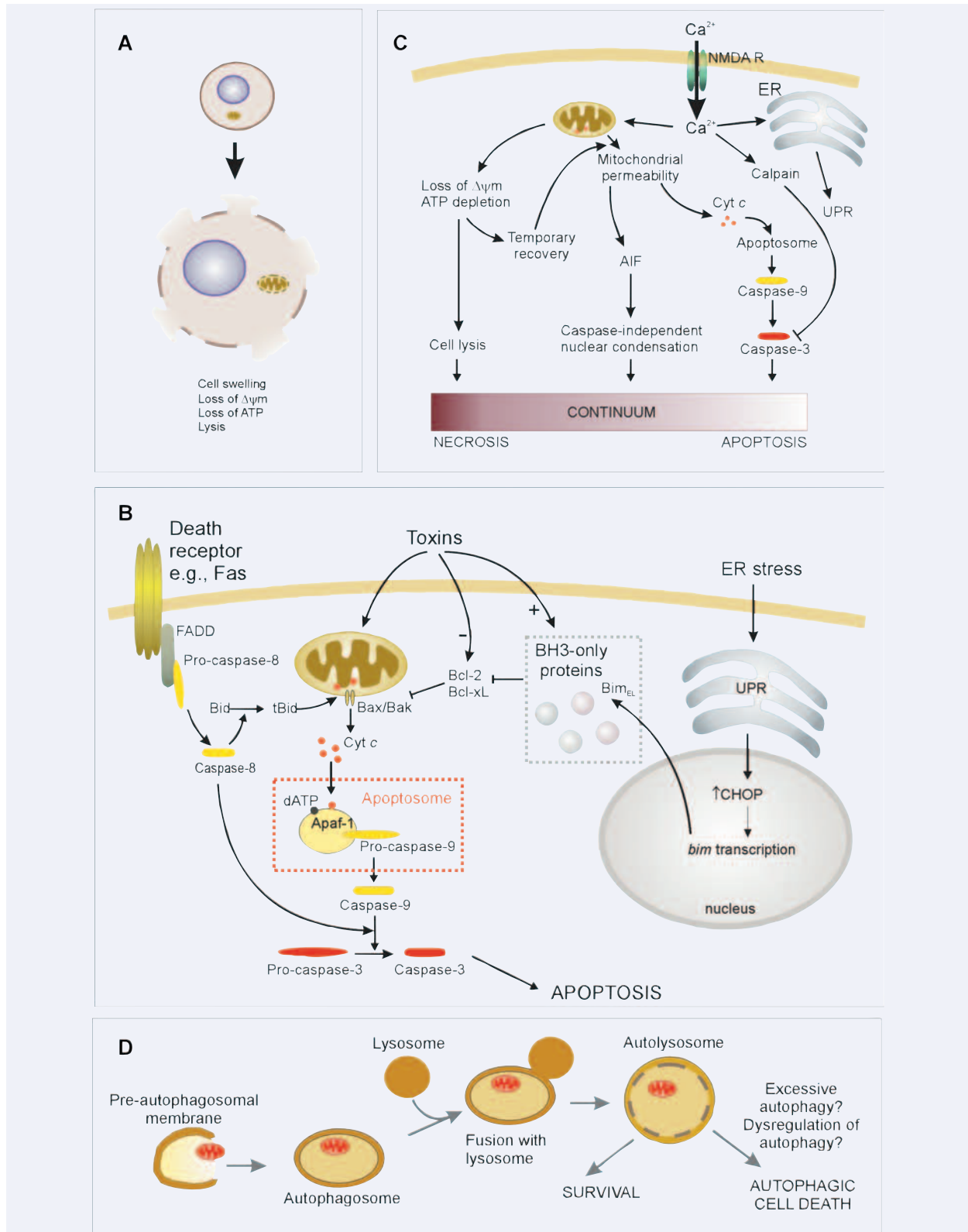
Fig. 1 Mechanisms of cell death. See text for details. (**A**) Necrosis is characterized by loss of ATP, which leads to cell lysis and release of cellular contents into the extracellular environment. (**B**) Apoptosis induction involves different pathways leading to caspase activation. In the extrinsic pathway, ligand binding to death receptors (*e.g.* Fas) induces their trimerization and recruitment of caspase-8 through death domain-containing adaptor proteins (*e.g.* FADD). This leads to auto-activation of caspase-8, which can directly activate effector caspases. Alternatively, in some cells Bid cleavage by caspase-8 is required for death. Truncated Bid translocates to the mitochondria and activates the intrinsic pathway. The intrinsic pathway is activated by toxins or conditions that cause stress to the mitochondria or endoplasmic reticulum (ER). Such stresses often alter the levels of, or cause post-translational modification of, Bcl-2 family proteins, initiating the release of cytochrome *c* from the mitochondria. For example, ER stress up-regulates the unfolded protein response (UPR), which increases expression of Bim_{EL}, a BH3-only protein that antagonizes Bcl-2 and Bcl-xL, permitting Bax-mediated cytochrome *c* release. Once in the cytosol, cytochrome *c* stimulates formation of the apoptosome complex, leading to caspase-9 activation. Initiator caspases (*e.g.* caspase-8 and -9) are shown in yellow, downstream effector caspases (*e.g.* caspase-3) are shown in red. (**C**) Excitotoxicity is caused by the influx of Ca^{2+} through the NMDA receptor. This can result in apoptosis, necrosis, or cell death that exhibits apoptotic nuclear morphology without caspase activation, suggesting a continuum of death patterns. (**D**) Autophagic cell death may be due to excessive autophagic digestion, as a result of dysregulation of the pathway by unknown mechanisms.

Autophagic cell death

The word autophagy comes from the Greek meaning 'self-eating'. It is primarily a mechanism by which organelles, protein aggregates and some proteins are degraded by the cell (see section below on autophagy). It may also be a pathway to cell death termed ACD, which is thought to due to excessive autophagy (Fig. 1D) [20]. ACD has a distinct morphology, including organelle swelling, vacuolation of the cytoplasm and membrane disruptions leading to local inflammation [21]. Although autophagy is generally associated with conditions of starvation, where it plays a pro-survival role in providing amino acids and other building blocks of cellular macromolecules, markers of autophagy have been observed during cell death. ACD has mainly been associated with developmental cell death, where massive autophagy causes irreversible cellular atrophy, leading to cell death of large numbers of cells [22]. Recently, induction of cell death that is dependent on autophagy (Atg) genes, such as Atg7 or Beclin1 has been reported [23, 24].

Crosstalk between different cell death mechanisms

Many cell death stimuli can induce more than one mode of cell death depending on the conditions, such as the severity of the stress, duration of the stress, the underlying 'health' of the cell, particularly with regard to redox levels and mitochondrial integrity. Some researchers have proposed that there is a continuum of cell death mechanisms in ischemic and excitotoxic death, with apoptosis and necrosis at opposite ends of a scale that incorporates cell death mechanisms featuring some but not all of the characteristics of apoptosis and necrosis [25–27]. Notably, certain neurotoxins, such as glutamate that induces excitotoxicity, and 6-hydroxydopamine that is a Parkinson's mimetic, can induce both apoptosis and necrosis *in vitro* [8, 19]. The mode of death induced by glutamate has been shown to depend on mitochondrial transmembrane potential ($\Delta\Psi$m) [19, 28]. The $\Delta\Psi$m controls three interrelated mitochondrial functions of relevance to neuronal survival: namely,

ATP synthesis, Ca^{2+} accumulation and superoxide generation [29]. During exposure of cerebellar granule cells to glutamate, both $\Delta\Psi$m and ATP levels decline rapidly (Fig. 1C) [19]. In those neurons with irreversibly dissipated $\Delta\Psi$m, necrosis rapidly ensues, while the surviving population recover both $\Delta\Psi$m and energy levels and subsequently undergo delayed apoptosis [19]. Since many of the cytoplasmic and nuclear changes observed during apoptosis require energy in the form of ATP, the ability of mitochondria to generate sufficient ATP may be one important factor that directs neurons towards necrosis or apoptosis [19, 30], such that if ATP levels are at least partially maintained, the cell is capable of undergoing apoptosis and if ATP levels fall profoundly, necrotic killing ensues [31]. This is supported by the observation that while treatment of cerebellar granule cells with glutamate induces delayed apoptosis, treatment with a combination of glutamate and the irreversible mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) results in necrosis [19].

There is also mounting evidence of crosstalk between apoptosis, autophagy and ACD. Inhibition of caspases induces cell death that is dependent on the autophagy genes *Atg7* and *Beclin 1* (also known as *Atg6*) [23]. Conversely, inhibition of autophagy can trigger apoptosis [32]. At a basic level autophagic digestion of injured mitochondria may also help to ensure that only healthy mitochondria endure within cells. The intrinsic pathway of apoptosis requires the presence of Bax/Bak, but the mode of cell death switches to ACD in the absence of Bax/Bak [33]. Similarly, in apoptosis-deficient cells that lack Bax and Bak, prolonged stress to the ER results in cell death that resembles necrosis and is associated with autophagy [34]. Inhibition of autophagy with 3-methyladenine or Atg5 knockdown in these cells promoted survival, which suggests that in apoptosis-competent cells autophagy has a pro-survival function, while in apoptosis-deficient cells autophagy is part of the cell death process, *i.e.* ACD [34].

During recent years, details of the molecular mechanisms that mediate crosstalk between different cell death mechanisms are beginning to be elucidated. One group of molecules that play a role at the intersection of apoptosis, autophagy and ACD is the Bcl-2 family. The best understood function of Bcl-2 proteins is in the regulation of apoptosis through control of the release of

pro-apoptotic factors from mitochondria. Recently, Bcl-2 has been shown to also inhibit autophagy [32, 35] and ACD [36]. It has been suggested that the anti-autophagy function of Bcl-2 may help maintain autophagy at levels that are compatible with cell survival, rather than cell death [35]. However, the effect of Bcl-2 on autophagy is controversial as it has also been reported to sensitize cells to autophagy and permit autophagy to develop through inhibition of apoptosis [33, 37]. Bcl-2 inhibition of autophagy is mediated through its interaction with Beclin 1 [35]. Beclin 1 was the first identified mammalian autophagy gene product and plays an essential role in autophagosome formation [39]. However, it was originally identified in a screen for Bcl-2-interacting proteins [39]. Recently, Beclin 1 has been shown to be a novel BH3-only protein and to interact with a number of anti-apoptotic Bcl-2 family members including Bcl-2, Bcl-xL, Bcl-w and Mcl-1 [40–43]. Thus, one possible scenario is that a Bcl-2:Beclin 1 rheostat acts as a switch between autophagy and apoptosis.

The autophagy gene product, Atg5, is another protein having roles in both autophagy and apoptosis. Atg5 is involved in the early stages of autophagosome formation [44]. Atg5 has been reported to interact with FADD (Fas-associated protein with death domain), mediating interferon-γ-induced cell death [45]. Atg5-mediated cell death, but not vacuole formation, was blocked in FADD-deficient cells [45]. In contrast, inhibition of autophagy with 3-methyladenine or expression of an Atg5 mutant blocks both cell death and vacuole formation [45]. A more recent study shows that calpain-mediated cleavage of Atg5 produces a truncated form of Atg5 that can translocate from the cytosol to mitochondria, associate with Bcl-xL and trigger cytochrome *c* release [46]. Thus, truncated Atg5 may promote apoptosis by acting in a manner reminiscent of some BH3-only proteins that inactivate anti-apoptotic Bcl-2 proteins and permit Bax/Bak-mediated cytochrome *c* release [47]. Since Ca^{2+} influx is a major factor in excitotoxicity it will be particularly interesting to observe the role, if any, of truncated Atg5 in excitotoxic neuronal cell death. One might speculate that calpain-mediated cleavage of Atg5 during excitotoxicity diverts cytoprotective autophagy and promotes cell death. However, calpains have also been shown to inactivate caspases and in this way, convert apoptotic death to necrotic death [48]. Therefore, it is difficult to predict whether apoptosis or necrosis would be the outcome of calpain-mediated cleavage of Atg5 and caspases within the same neuron. It is likely that mitochondrial outer membrane permeabilization (even perhaps involving truncated Atg5) permits the release of pro-apoptotic factors such as AIF, which induces nuclear condensation. This scenario would support the suggested continuum model of cell death patterns (Fig. 1C) and the experimental observation of neurons that exhibit characteristics of both apoptosis (condensed nuclei) and necrosis (cell lysis, lack of caspase activity) [11, 26, 27].

Increasingly, we are learning that there are compensatory cell death pathways that are induced if one route to cell death is blocked. Research in this area is gathering momentum, particularly with regard to crosstalk between apoptosis and autophagy (Fig. 2). In addition to the Bcl-2 family, Beclin 1 and Atg5 there continue to be new proteins identified that participate in both
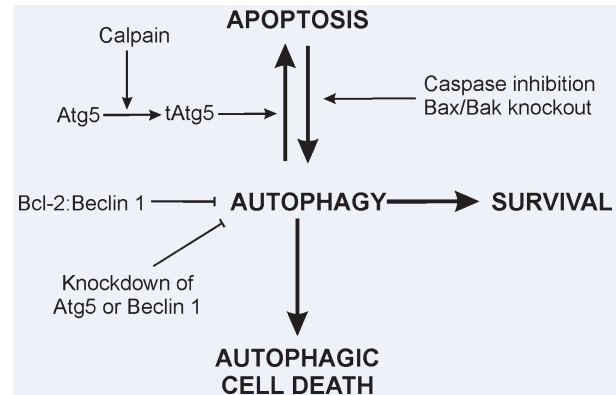

Fig. 2 Crosstalk between apoptosis, autophagy and autophagic cell death. The schematic depicts some points of crosstalk between these pathways (see text for details).

apoptosis and ACD, *e.g.* the novel transmembrane protein TMEM166 [49]. The current models suggest that apoptosis and autophagy may be mutually exclusive pathways, which would further suggest that apoptosis and ACD are also mutually exclusive. This idea is supported by the accumulating evidence such as the effect of Bcl-2:Beclin 1 interaction and calpain-mediated cleavage of Atg5 . However, at least one study has reported the accumulation of autophagic vacuoles preceding apoptotic cell death [50]. Is this indicating that there is also a continuum of death patterns between apoptosis and ACD? It will be interesting during the coming years to learn the role, if any, of crosstalk between different cell death pathways in neurodegenerative diseases.

Mechanisms of neuronal cell death in neurodegenerative disease: the evidence

There is growing evidence from post-mortem patient tissue describing the modes of cell death observed in neurodegenerative diseases. Such evidence has been difficult to accumulate, partly due to the protracted nature of most neurodegenerative diseases, such that only a tiny proportion of the whole cell population is in the process of dying at any one time. Since cell death occurs over many years, the snap-shot representation obtained from such samples can only reveal cells that have recently died. Usually these tissues are representative of the end-stage of the disease, far-removed in time from when we would ideally like to be treating such patients to attempt to prevent neuronal cell death. Often the dying/dead cells display mixed signals and defy classification into distinct patterns of cell death. Another difficulty with patient samples is that markers used for different modes of cell death can be

overlapping, non-specific, and therefore, prone to misinterpretation. For example, terminal transferase dUTP nick-end labelling (TUNEL staining) has been used as a marker of apoptosis in human brain tissue. However, TUNEL can also label necrotic cells [51]. Therefore, it is imperative that the correct criteria are used to monitor different modes of cell death. Usually more than one characteristic feature of the type of cell death should be considered. TUNEL staining needs to be combined with others, *e.g.* ultrastructural analysis of nuclear morphology, or demonstration of activated or cleaved caspases, such as caspase-3 or -8, using specific antibodies for the identification of apoptosis [52]. Although criteria for the identification of autophagy have recently been published, and markers for autophagy have been identified in different neurodegenerative diseases [53], it is not yet possible to be certain that these are indicative of the occurrence of ACD.

Much evidence regarding mechanisms of cell death in neurodegenerative diseases comes from models, either *in vitro* cell culture systems or *in vivo* models of the disease. These models use acute toxin exposure and/or genetic mutations found in disease and have contributed much to promote the view that apoptosis and other forms of cell death play important roles in neuronal cell death in neurodegenerative diseases. However, models are limited since they tend to examine the effect of just one toxin or one gene on cell death, and they generally involve acute exposure to insults, which may not be relevant to the protracted degeneration observed in patients. Nevertheless, they have greatly increased our knowledge of the biochemical mechanisms of neuronal cell death in neurodegenerative diseases. In the following section, some of the major neurodegenerative diseases and the link to neuronal cell death mechanisms are described.

Alzheimer's disease

AD is the most common neurodegenerative disease. It is clinically characterized by progressive memory loss and cognitive dysfunction, resulting in severe dementia. The pathology of AD includes the loss of medium and large pyramidal neurons from hippocampal regions [54–56] and the presence of extracellular placques containing amyloid filaments and intracellular neurofibrillary tangles composed of hyperphosphorylated tau [57]. Familial AD is caused by mutations in amyloid precursor protein or presenilin-1 or -2. Presenilins play a crucial role in γ-secretase activity, which is responsible for proteolytic processing of amyloid precursor protein, producing the β-amyloid peptide [58]. AD mutations lead to altered processing that is accompanied by increased production of a slightly larger peptide, β-amyloid-42, which is prone to oligomerization, forming fibrils and placques [59]. It is uncertain what leads to neuronal cell death in AD.

Studies of post-mortem brain tissue indicate that neuronal apoptosis occurs in AD [60]. Active caspases have been detected in post-mortem tissue from AD brains [61–63]. However, this has been disputed by others who report no apoptotic morphology in AD cases [64]. *In vitro* models show that β-amyloid can induce apoptosis [65, 66]. Presenilin mutations sensitize neurons to

induction of apoptosis and to excitotoxicity [67, 68]. In fact, it has been suggested that accumulation of β-amyloid leads to increased sensitivity to excitotoxicity in AD [69] and excessive activation of NMDA receptors has been specifically linked to β-amyloid-induced degeneration in a rat model [70]. It has also been suggested that excitotoxicity might have a more important role in later stages of AD [71]. Although autophagy has been linked to models of AD, there is no evidence that this is part of a cell death pathway [72].

Parkinson's disease

PD is the second most common neurodegenerative disease, mainly affecting people over 55 years. Pathologically it is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta and the presence of eosinophilic intracytoplasmic inclusions (Lewy bodies) within the surviving neurons. This leads to dopamine depletion in the striatum, which is the direct cause of the clinical features of bradykinesia, resting tremor and rigidity. The clinical symptoms of PD become evident when there is a loss of about 50–60% of the dopaminergic neurons within the substantia nigra. Thus, PD is a good example of a neuro-degenerative disease where neuronal cell loss is directly respon-sible for the clinical features observed. The cause of PD is most probably multifactorial, with genetic predisposition, environmental toxins and aging being important factors in disease initiation and progression for both hereditary and sporadic PD. To date, mutations in at least 13 PARK genes have been linked to the pathogenesis of familial PD [73]. These include mutations in genes that encode the proteins α-synuclein, parkin, PTEN-induced kinase 1 (PINK1), DJ-1, leucine-rich repeat kinase2 (LRRK2) and ubiquitin carboxy-terminal hydrolase L1 (UCHL1) [73]. Of these, parkin and UCHL1 are linked to the ubiquitin-proteasome system (UPS) that degrades damaged or misfolded proteins [74]. In addition, several of these genes, including parkin, PINK1, DJ-1 and Omi/Htra2 are linked to the mitochondria, and may have roles in mitochondrial function and resistance to oxidative stress [75]. The molecular mechanisms that initiate dopaminergic neuron loss in PD are not known. Evidence from various sources suggest a role for toxin-induced death, oxidative stress, defects in mitochondrial complex I, protein aggregation and impairment of the UPS [76–78].

The presence of apoptotic chromatin clumps along with TUNEL staining in neuromelanin-containing cells in the substantia nigra PD post-mortem tissue suggests that apoptotic cell death contributes to neuronal loss in PD [79–82]. However, others have been unable to confirm these findings [83, 84]. Activated caspase-3 has also been observed in these cells [81]. There is also evidence for a role for the extrinsic apoptotic pathway in post-mortem studies of PD brain. These are related to the increased levels of TNFα, soluble Fas and the Fas adaptor protein FADD in the midbrain of PD patients [85–87]. Importantly, increased active caspase-8 has been detected in neuromelanin-containing substantia nigra neurons in PD brains [88, 89]. *In vitro* models of PD using the Parkinson mimetics MPP$^+$ (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-hydroxydopamine have reported both

apoptosis and necrosis [8, 90–92]. The pesticide rotenone induces caspase-3-mediated apoptosis in ventral mesencephalic dopaminergic neurons [93]. Inducible expression of mutant α-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis [94]. Targeted overexpression of α-synuclein in the nigrostriatal system has provided a new animal model of PD [95]. In this model there is selective degeneration of nigral dopamine neurons with activation of caspase-9 [96]. Some early studies proposed a role for excitotoxicity in PD although subsequent research has not supported this hypothesis [97, 98]. However, there may be a role for ACD in PD. Ultrastructural examination revealed autophagosome-like structures in melanized neurons of the substantia nigra in PD patients [99–101]. It has been reported that the neuromelanin granules in catecholaminergic neurons of the substantia nigra are delimited by double membranes, and may in fact be autophagosomes sequestering dopamine and metabolites to protect the cell from their lethal accumulation in the cytosol [101]. In support of these findings, dopamine and MPP$^+$ have been shown to induce ACD in human neuroblastoma cells [102, 103].

Huntington's disease

HD is one of a number of trinucleotide repeat disorders. It is caused by a dominantly inherited expansion of a translated CAG repeat in the N-terminus of the huntingtin protein. The length of the CAG expansion inversely correlates with age of onset of disease symptoms [104]. The main clinical symptom is the occurrence of generalized involuntary movements, which is a consequence of the selective loss of the medium spiny neurons in the neostriatum [104, 105]. The disease pathology is characterized by the presence of nuclear inclusions containing mutant huntingtin [106]. Neuronal cell death in HD may be linked to the accumulation of toxic mutated huntingtin fragments since expansion of the huntingtin CAG repeat above 35 results in neuronal cell death [107–109]. Although a number of interacting proteins have been identified, the physiological role of huntingtin is not known.

Caspases have been shown to be activated in HD brains [110, 111]. Interestingly, huntingtin itself is a substrate for caspases, generating truncated fragments containing the polyglutamine tract that may be toxic [112–114]. Excitotoxicity has been suggested to play a role in the pathogenesis of HD. The medium spiny neurons that are lost in HD are innervated by glutamate-releasing neurons from the cortex, and they selectively degenerate upon the administration of glutamate receptor agonists into the rat striatum [115]. Post-mortem analysis of the early stage HD patient brains indicates increased levels of the NMDA receptor agonist, quinolinic acid, and its precursor 3-hydroxy-kynurenine, suggesting that it may act as an endogenous excitotoxin in HD [116]. Moreover, expression of mutant huntingtin is related to alterations in glutamate neurotransmission, including decreased glutamate uptake, altered trafficking and functionality of glutamate receptors [117]. Although cultured medium spiny from transgenic mice expressing different mutations in huntingtin are more sensitive to excitotoxic

damage [118], *in vivo* experiments have produced conflicting results [119, 120]. However, a direct link between mutant huntingtin expression and excitotoxicity in HD patients has not been shown. Autophagy is stimulated by huntingtin, but it is not known if this is related to ACD [121]. Paradoxically, certain mutant forms of huntingtin can protect neurons from cell death [122].

Ischaemia

Ischaemia, or stroke, is an acute condition caused by an interruption in blood supply, leading to localized deprivation of oxygen, glucose and trophic support in the brain. Since neurons are reliant on a constant supply of oxygen and glucose to generate ATP, the loss of ATP during ischaemia causes rapid dissipation of ion gradients, leading to uptake of water and cell swelling, lysis and cell death.

Ischaemia is characterized by acute neuronal cell death and delayed death that occurs many days after the initial insult [123, 124]. The initial phase of cell death is necrotic and the delayed death is apoptotic. Neuronal cell death at the ischemic core tends to be necrotic, while in the penumbra region it is apoptotic, suggesting that the severity of the insult determines the mode of cell death [123, 124]. Both phases of cell death can be blocked by antagonism of NMDA receptors [125]. This cell death is excitotoxic and thus shares the same characteristics in terms of a continuum of necrosis and apoptosis being induced [25, 26]. Upregulation of Beclin 1 is observed in the ischemic penumbra, along with and subcellular redistribution of the autophagy marker LC3 to vacuolic structures in ischemic neurons indicating that autophagy is induced during ischaemia [126–128]. However, ACD has not been conclusively shown.

Recurring themes around protein mishandling in neurodegenerative diseases

Key to the prevention and treatment of neurodegenerative diseases is an understanding of the mechanisms that trigger neuronal cell death. Notably, different neurodegenerative diseases, with different causes, genetic mutations, different patterns of neuronal death, often display a number of common features that include ER stress, oxidative stress, mitochondrial dysfunction, impairment of the proteasome and protein aggregation. These changes are often interrelated, causing disruption of normal neuronal function and eventually leading to cell death. The picture is further complicated by the fact that neurons mount pro-survival responses to protect themselves from these detrimental, disease-related changes. Therefore, neurons in the final stages of their demise often present a complex picture reflecting a struggle between pro-death factors and pro-survival responses, and teasing out the sequence of events can be a

challenge. Here we will discuss neurodegenerative diseases with relation to problems in protein handling.

Protein homeostasis in cells is a balance between protein synthesis and protein degradation. Disruption of this equilibrium leads to problems of accumulation and aggregation of excess or misfolded proteins. Many neurodegenerative diseases including AD, PD and HD are characterized by the accumulation and aggregation of misfolded proteins. Misfolding and/or aggregation of proteins can be the result of problems during synthesis due to impairment of the synthesis/folding machinery, or mutations in the sequence of the protein causing incorrect folding or a tendency to aggregate. Proteins can also become unfolded at some point during their life cycle, or undergo abnormal cleavage generating proteins that cannot fold properly or have a tendency to aggregate. Similarly, mutations in proteins such as proteases can cause the inappropriate accumulation of proteins that cannot be processed further. In addition, proteasome impairment can render a cell unable to degrade misfolded proteins efficiently, thus compounding the problem.

Endoplasmic reticulum stress, the unfolded protein response and endoplasmic reticulum stress-induced cell death

The ER is the site of synthesis and folding of secreted, membrane-bound and some organelle-targeted proteins. It also acts as a major store for intracellular Ca^{2+}. The protein-folding capacity of the ER is highly sensitive to alterations in cellular ATP levels, Ca^{2+} levels, and the redox state of the cell, all of which result in the accumulation and aggregation of unfolded proteins, a condition referred to as ER stress [129]. In response cells mount the unfolded protein response (UPR) [129]. In mammalian cells, this is a three-pronged protective strategy resulting in halt of general protein synthesis to reduce the backlog of proteins, induction of Grp78 and Grp94 chaperone proteins to aid folding of proteins already in the system and ER-associated degradation to remove proteins that cannot be refolded by the ER. This concerted cellular response is mediated through three ER transmembrane receptors: pancreatic ER kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring enzyme 1 (Ire1) [130]. In resting cells, all three ER stress receptors are maintained in an inactive state through their association with the ER chaperone, Grp78. On accumulation of unfolded proteins, Grp78 dissociates from the three receptors, which leads to their activation and triggers the UPR.

Although UPR activation is primarily a protective response by the cell, if the stress is unresolved, prolonged activation of the UPR may trigger cell death [129]. In fact, in recent years, a third apoptosis pathway has been proposed, whereby ER stress leads to direct activation of initiator caspase-12 that is located at the ER [131]. However, since full-length caspase-12 is not expressed in the majority of human beings, and since many reports demonstrate that mitochondrial release of cytochrome c, apoptosome formation and activation of caspase-9 are required for ER stress-induced apoptosis, it is likely that this death occurs *via* the intrinsic pathway [132–134]. For example, we and others have recently shown that ER stress-induced apoptosis requires up-regulation of the Bcl-2 family member Bim that further implicates a requirement for the mitochondrial pathway [133, 135]. The details of how UPR signalling is switched from pro-survival to pro-death is unclear and is a subject of much research, reviewed in [129]. Induction of the transcription factor, CHOP, has been shown to be required for ER stress-induced apoptosis [136]. Recently, the attenuation of IRE1 activity by persistent ER stress has been identified as a potential switch [137]. If this is involved in neuronal cell death in neurodegenerative diseases that exhibit ER stress, it suggests the exciting possibility of altering disease pathogenesis by manipulating the UPR.

ER stress is linked to the pathogenesis of several different chronic neurodegenerative disorders, where UPR markers are seen to be up-regulated. For example, markers of UPR activation (phosphorylated PERK and Grp78) are increased in AD brains [138]. These changes were seen in normal-appearing neurons, suggesting a role for the UPR early in AD neurodegeneration. Furthermore, presenilin-1 and -2 reside in the ER, and AD mutations appear to impair the ER stress response and enhance vulnerability to stress-induced apoptosis [67, 68]. It has also been suggested that presenilin-1 mutations may deregulate ER Ca^{2+} stores [139]. Immunoreactivity for phosphorlyated PERK and phosphorylated eIF2α has also been detected in neuromelanin-containing dopaminergic neurons in the substantia nigra of PD cases [140]. The phospho-PERK immunoreactivity was colocalized with increased α-synuclein immunoreactivity in dopaminergic neurons [140]. *In vitro* models using two PD mimetic compounds, 6-hydroxydopamine and MPP$^+$, trigger ER stress in dopaminergic neurons [141, 142]. Furthermore, neuronal cultures from PERK knockout mice, display an increased sensitivity to 6-hydroxydopamine [142], while a null mutation in CHOP results in a reduction in 6-hydroxydopamine-induced apoptosis *in vivo* [143]. However, protection was not observed in the chronic MPTP model, despite robust expression of CHOP [143]. In trinucleotide repeat disorders such as HD, the intracellular accumulation of polyglutamine triggers ER stress by inhibiting protein degradation and has been linked to the induction of neuronal cell death by N-terminal mutant huntingtin [144–146]. ER stress is also a factor in neuronal cell death during ischaemia, with upregulation of CHOP [147, 148].

Protein degradation: the ubiquitin-proteasome system and autophagy

Misfolded or unwanted proteins are degraded within the cell *via* two pathways. The ubiquitin-proteasome system (UPS) is the main degradatory pathway for the majority of intracellular proteins. This system is linked to ER stress responses since ER-associated degradation feeds proteins into the UPS for degradation. Secondly, autophagy is used by the cell to eliminate unwanted or damaged organelles *via* lysosomal degradation and can also be used for degradation of proteins.

Fig. 3 Protein metabolism in neurons. Misfolded proteins can occur as a result of mutations, oxidative damage, erroneous proteolysis or other post-translational modifications. These can be refolded by chaperones such as Hsps, or targeted for proteasomal degradation by the addition of ubiquitin (Ub). Impairment of UPS-mediated degradation can lead to the formation of protein aggregates, which may be degraded *via* autophagy. The accumulation of misfolded proteins or protein aggregates leads to neuronal cell death by an unknown mechanism.
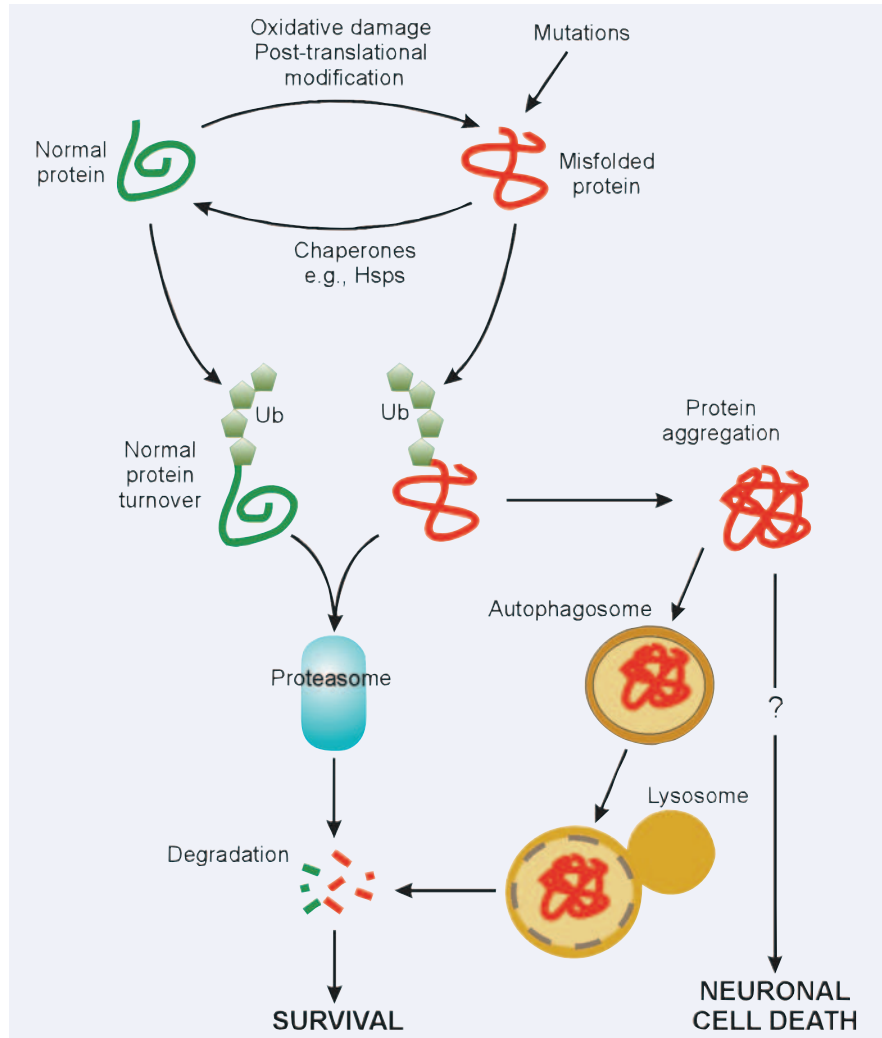
Ubiquitin-proteasome system

The UPS involves conjugation of ubiquitin to target substrates, followed by destruction of the tagged protein by the 26S proteasome system [149]. Conjugation of ubiquitin to proteins involves a series of enzyme reactions. Ubiquitin-activating enzyme E1 generates a high-energy intermediate, E1-ubiquitin. Next, E2 enzymes transfer ubiquitin from E1 to E3 ligase that is bound to specific substrates, to which it ligates activated ubiquitin [149]. Cells usually contain just one E1, a few E2 and many E3s, the latter of which are specific for just a small number of substrates. Tagged proteins are then degraded into peptides by the proteasome, a barrel-shaped, multiprotein, proteolytic complex.

Alterations in the UPS have been connected to several neuro-degenerative diseases [150, 151]. Proteasome inhibition leads to the intracellular accumulation of ubiquitinated proteins and causes cell death [152, 153]. The accumulation of ubiquitin conjugates is often found in protein aggregates, which include the neurofibrillary tangles of AD, Lewy bodies in PD and nuclear inclusions in

HD [154, 155]. It is likely that accumulation of ubiquitin conjugates reflects failed attempts by the UPS to remove the damaged/abnormal proteins that have been tagged for destruction (Fig. 3). This could be caused by malfunction or overload of the UPS, or from structural changes in specific protein substrates, halting their degradation.

Proteasome activity is impaired in AD [156]. In the brains of some AD patients a mutated ubiquitin with an extra 20 amino acids at its C-terminus has been observed [157]. This mutated ubiquitin forms polyubiquitin chains that cannot be disassembled and potently inhibit proteasomal degradation of proteins to which they are attached [158]. UPS impairment may also play a role in PD. The PD-associated gene parkin is an E3 ligase, involved in the addition of ubiquitin chains to a select number of protein substrates [159, 160]. It is frequently mutated in early onset PD with loss of ligase function and a reduction in degradation of target proteins [159, 161, 162]. One target of parkin is Pael-R (Parkin-associated endothelin receptor-like receptor) and loss of parkin

function results in accumulation of Pael-R, ER stress and neuronal cell death [163, 164]. Conversely, overexpression of parkin suppresses ER stress-induced cell death [164]. Parkin is abundantly expressed within mesencephalic dopaminergic neurons that may explain part of their selective vulnerability [159]. Another mutated-in-PD gene, UCH-L1, was originally characterized as a deubiquitinating enzyme [165]. However, it has also been found to act as a ubiquitin ligase [166] and a mono-ubiquitin stabilizer [167]. Discovery of these mutations in genes linked to UPS function has led to the hypothesis that some PD mutations cause aberrant accumulation of substrates that may be toxic [168]. In addition, reports that α-synuclein is degraded through the proteasome led to the idea that abnormalities in UPS-mediated degradation of α-synuclein underlie PD [169–171]. Systemic administration of proteasome inhibitors produces pathological features that are similar to those found in PD [172, 173]. However, some groups have been unable to reproduce these results [174]. The intracellular accumulation of polyglutamine in HD inhibits protein degradation and has been linked to the induction of neuronal cell death by N-terminal mutant huntingtin [144–146].

Autophagy

Autophagy mediates the intracellular degradation of organelles and protein complexes that are too big to pass through the narrow pore of the proteasome. It is normally associated with nutrient and growth factor deprivation where it promotes cell survival through provision of molecular building blocks [20]. However, it can also be induced by the presence of protein aggregates such as are found in neurodegenerative diseases (Fig. 3). Three types of autophagy have been described in mammalian cells: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [175]. They all share the lysosome as a common end-point, but differ in the substrates targeted, their regulation and the conditions under which they are preferentially activated. In macroautophagy, intracellular components are sequestered by a limiting membrane to form an autophagic vacuole, the autophagosome, that then fuses with lysosomes to degrade the contents [176]. In microautophagy, substrates are directly internalized through invaginations of the lysosomal membrane. CMA is specialized for the degradation of selective substrate proteins that contain a KFERQ (or similar) motif that is recognized by heat shock cognate protein, HSC70, which translocates the proteins into the lysosomes [177].

Autophagy has been linked to different neurodegenerative diseases. However, it is unclear if this phenomenon is the result of increased autophagic activity, or decreased fusion of autophagosomes with lysosomes [178]. It has been reported that β-amyloid generation may occur in autophagic vacuoles [179]. Autophagy is also linked to PD [99, 101]. α-Synuclein is degraded by both CMA and the proteasome [180, 181]. Pathogenic α-synuclein mutations block CMA-mediated degradation of α-synuclein and of other substrates which may underlie the toxic gain-of-function of these mutants [181]. Increased autophagosomes are seen in HD [121], which may be linked to the processing of huntingtin

fragments by autophagy [182]. Autophagic neuronal death may play a role in neonatal brain ischaemia [127, 128].

The presence of autophagosomes in neurons in neurodegenerative disorders has raised questions about whether they are contributing to neuronal cell death or protecting against it. Emerging evidence supports the view that induction of autophagy is a neuroprotective response and that inadequate or defective autophagy, rather than excessive autophagy, promotes neuronal cell death in most of these disorders. A cytoprotective role for autophagy by catabolizing intracellular substrates for energy, and by removing damaged mitochondria and other factors that trigger apoptosis, is clear [183]. Autophagy also prevents toxic effects of misfolded or abnormal proteins by sequestering them inside autophagosomes delivering them to the lysosome for degradation [184]. This suggests that increased autophagy observed in neurodegenerative diseases may be linked to an increase in the levels of misfolded and aggregated proteins, and/or to increased numbers of damaged mitochondria, both of which are associated with aging.

Although the UPS and autophagy were thought to work in parallel, recent investigations suggest a functional link between the two. For example, proteasome inhibition activates autophagy and the suppression of autophagy causes polyubiquitinated protein aggregates [185]. Toxicity due to proteasome inhibition can be rescued by autophagy induction [152, 186]. The obvious interpretation is that autophagy upregulation simply provides an alternative route for protein degradation when the UPS is impaired. However, it is also possible that by removing mitochondria from the cell autophagy induction reduces cellular susceptibility to subsequent apoptotic stimuli mediated by the mitochondrial pathway [32, 187].

Protein aggregation

Proteins that are misfolded and escape degradation may be prone to aggregation. This occurs because misfolded proteins often expose hydrophobic surfaces that would normally be protected within the interior of the protein. This leads to abnormal protein–protein interactions and formation of intracellular inclusions [188]. The formation of intracellular inclusions may result from the build-up of high concentrations of proteins in the cell, and/or aberrant protein folding.

Many neurodegenerative diseases are characterized by the presence of aggregated protein deposits, such as the extracellular amyloid plaques and intracellular neurofibrillary tangles found in AD, Lewy bodies found in most forms of PD nuclear inclusions found in HD. Protein aggregates often contain high levels of ubiquitin, as well as of misfolded proteins. [154, 155]. The two most striking pathological features of AD are aggregated extracellular deposits of β-amyloid plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau proteins. Both of these are caused by the misfolding and conversion of highly soluble proteins into insoluble, filamentous polymers. Mutations in the amyloid precursor protein in certain forms of familial AD lead to its aberrant cleavage by the γ-secretase complex, resulting in the

production of β-amyloid42 that is prone to aggregation and leads to formation of extracellular amyloid plaques. β-amyloid42 has been shown to induce apoptosis in neurons in culture, and to cause oxidative stress. In PD α-synuclein is recognized as a major component of Lewy bodies [189]. It has a tendency to form protein aggregates that are increased in mutant forms of the protein [190, 191].

It is likely that the accumulation of protein aggregates is linked in some way to neurodegeneration. However, a direct link between aggregate formation and cell death has not been shown (Fig. 3). Protein aggregates may disrupt normal functioning of neurons, and this stress may lead to the initiation of cell death. For example, protein aggregation can cause UPS impairment [145]. It has also been suggested that the accumulation of mutant protein into cellular aggregates may be neuroprotective, probably depending on the nature and the function of the protein in question. In this scenario intracellular protein deposits would become deleterious only after reaching a certain level within the cells.

Towards pro-survival therapies for neurodegenerative diseases

There is a large body of evidence linking impairment of protein handling with neuronal loss in neurodegenerative diseases. Model systems clearly demonstrate that neuronal cell death can be induced acutely by excessive ER stress, inhibition of the UPS, inhibition of autophagy or excessive autophagy. The protracted nature of many neurodegenerative diseases indicates that neurons can protect themselves from a certain amount of damage before there is severe disruption to function and viability. This suggests that the life or death of an individual neuron is dependent upon the overall burden of accumulated misfolded or aggregated proteins, balanced against the overall capacity of the cell to deal with this effectively and safely.

Therapeutic approaches aimed at modulating the activity of protein degradation systems, the UPS and autophagy, are therefore of particular relevance to neurodegenerative disease. However, since the UPS has roles in synaptic activity and neuronal plasticity, proteasome-modulating drugs could have deleterious effects in the nervous system [150, 192]. Macroautophagy-activating drugs such as rapamycin have been shown to increase clearance of aggregate-prone polyglutamine mutant proteins and mutant α-synuclein [193, 194]. In fact, in a transgenic HD mouse model, autophagy-enhancing drugs decrease aggregate formation and improved HD-associated behavioural tasks [193]. Thus, this class of drugs may prove particularly useful in promoting degradation of protein aggregates, and thus allowing improved neuronal function.

Neurons themselves mount protective responses to various stresses. Increases in chaperone proteins such as heat shock proteins (Hsps) are observed in the presence of misfolded proteins to help with refolding. In several neurodegenerative diseases there is

evidence for increased expression of Hsps [195]. For example, focal cerebral ischaemia increases the levels of several classes of Hsps and their corresponding mRNAs [196]. Notably, the inducibility of Hsps decreases with aging, which may contribute to the inability of aged neurons to fully protect themselves from stresses such as protein misfolding, aggregation and oxidative stress [197]. Hsps promote survival *via* several different mechanisms [198]. They aid in the refolding and/or degradation of misfolded proteins [199]. Hsp27 and Hsp70 can regulate apoptosis through their ability to inhibit release of pro-apoptotic factors from the mitochondria and to interact with key components of the apoptosome [200–204]. The small Hsps, Hsp27 and αB-crystallin can also modulate intracellular redox potential and help maintain cytoskeletal integrity [198]. Hsps can promote survival in models of various neurodegenerative diseases [195]. Hsps reduce neuronal cell death in models of PD. Heat shock (which leads to elevated expression of inducible Hsp27 and Hsp70) is protective in *in vitro* models of PD [8, 90] while Hsp27 itself is protective against 6-hydroxydopamine-induced apoptosis *in vitro* [8]. Hsp27 but not Hsp70 is protective against α-synuclein-induced cell death in neuronal cells [205]. Viral vector-delivered Hsp70 is protective against MPTP toxicity in an *in vivo* model of PD [206]. Hsp27 also protects against polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin [207], while Hsp70 can alter the endosome-lysosomal localization of huntingtin [208]. Hsp70 overexpression is neuroprotective in a model of cerebral ischaemia [209].

Currently, available therapies for neurodegenerative diseases treat only the symptoms, but not the cause of the disease. As we understand more clearly the cause of these diseases, and improve diagnostic ability at pre-symptomatic and early stages, we may be able to develop therapies that slow or halt the underlying neurodegeneration. From a therapeutic point of view, the use of apoptosis inhibitors such as caspase inhibitors has failed to prevent neurodegeneration. Likely, the damage is too far advanced for the inhibition of these late-stage participants to be effective and the cell will find another way to die, *e.g.* by necrosis. For example, recent clinical failures with apoptosis inhibitors in PD underscore the need for a shift in thinking with regard to drug discovery for neurodegenerative diseases [210]. Targeting the causes of cell death is the next major challenge in generating effective therapies, and in this regard, strategies incorporating chaperones and/or protein degradation-enhancing drugs may prove a worthwhile endeavour.

Acknowledgements

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