Minireview

Proteolytic mechanisms in necrotic cell death and neurodegeneration

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Abstract Programmed neuronal cell death is required during development to achieve the accurate wiring of the nervous system. However, genetic or accidental factors can lead to the premature, non-programmed death of neurons during adult life. Inappropriate death of cells in the nervous system is the cause of multiple neurodegenerative disorders. Pathological neuronal death can occur by apoptosis, by necrosis or by a combination of both. Necrotic cell death underlies the pathology of devastating neurological diseases such as neurodegenerative disorders, stroke or trauma. However, little is known about the molecular mechanisms that bring about necrotic cell death. Proteases play crucial roles in neuron degeneration by exerting both regulatory and catabolic functions. Elevated intracellular calcium is the most ubiquitous feature of neuronal death with the concomitant activation of cysteine calcium-dependent proteases, calpains. Calpains and lysosomal, catabolic aspartyl proteases, play key roles in the necrotic death of neurons. In this review, we survey the recent literature on the role of cysteine and aspartyl proteases in necrosis and neurodegeneration, aiming to delineate common proteolytic mechanisms mediating cellular destruction.

Keywords: *Caenorhabditis elegans*; Calpain; Caspase; Cathepsin; Necrosis; Neurodegenerative disorders; Stroke

1. Introduction

It is becoming increasingly clear that multiple mechanisms of cell death, as well as a crosstalk between different death pathways, contribute to neurodegeneration. Specific mechanisms dictate cell death in particular neurodegenerative diseases depending on the nature and severity of the insult to the cell, as well as on the cellular context [1,2]. Moreover, different death mechanisms may operate in different parts of the same stressed neuron [3,4]. Acute and chronic neurodegenerative conditions have been associated with both, apoptotic and necrotic cell death [5–8]. While necrosis and apoptosis can be distinguished in some situations, in others the distinction is not so clear [3] and certain dying cells might show distinctive features of both apoptosis and necrosis [9,10]. The idea of a continuum of responses ranging from apoptosis to necrosis is emerging, where the relative contributions of each depends on several factors, including the energy content of the cell and the severity of the insult [3]. Neurons at the core of an ischemic lesion undergo necrotic death and are resistant to caspase inhibitors, whereas neurons at the periphery show apoptotic features and can be partially rescued by caspase inhibitors [11].

Apoptosis is a genetically regulated process of cell-suicide that is modulated by a variety of cellular signalling pathways that integrate common death effectors with a range of different stimuli [12]. The morphologic features of apoptosis include nuclear and cytoplasmic condensation, internucleosomal DNA cleavage and packaging of the cell into apoptotic bodies that are engulfed by phagocytes, preventing release of intracellular components [13]. Two main pathways lead to apoptotic neuronal cell death. (1) Mitochondrial damage, due to reactive oxygen species (ROS), increased Ca^{2+} influx, hypoxia, UV, etc. leads to cytochrome *c* release into the cytosol, with the subsequent formation of the apoptosome and the activation of effector caspases (caspase-3, -7). This is frequently referred to as the 'intrinsic pathway'. (2) The 'extrinsic pathway', where binding of ligands to death receptors of the tumour necrosis factor family induces the activation of effector caspases through the c-Jun-N-terminal kinase. Activation of caspase-8 [14] results in cleavage of BID and cytochrome *c* release [15]. Caspase-8 activation can also directly stimulate effector caspases [16]. Although mediated by diverse mechanisms, apoptosis always requires the proteolytic activation of effector caspases (caspase-3 and caspase-7), caspase-3 being a key molecule [17]. Canonical caspase-dependent neuronal apoptosis shapes the nervous system during development. However, things get more complicated for neuronal death during adulthood [2]. Neurons often show caspase-independent apoptotic features and in some cases, co-activation of several parallel death pathways is required before cell death can take place [18,19]. Other than caspases, calpains and cathepsins have also been implicated in apoptotic neuronal death (see below).

Contrary to apoptosis, necrosis is not a developmentally programmed type of cell death. Instead, necrotic cell death occurs by deregulation of normal cellular activities when cells are exposed to extreme stress conditions. Necrosis is morphologically characterised by extensive vacuolation of the cytoplasm, mitochondrial swelling, dilatation of the endoplasmic reticulum and plasma membrane rupture. The cell is lysed without formation of vesicles. As a consequence, cellular contents are liberated into the intracellular space, which might precipitate damage to neighbouring cells and evoke inflammatory

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responses [20]. Necrosis typically occurs following ischaemia, hypoxia, stroke or trauma [21] but it has also been reported in Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [4,22–25]. Although the molecular mechanisms that bring about necrotic cell death are not fully elucidated, experimental evidence indicates that necrosis of adult neurons is mediated by increase of intracellular Ca^{2+}, is independent of caspases, and instead, cytosolic calpains and spilled lysosomal cathepsins are the major players in necrotic neuronal death (see below).

Energy depletion is a potent trigger of necrosis. During ischemic and hypoglycaemic episodes, lack of oxygen or essential nutrients leads to a shortage of energy to sustain the membrane potential. As a consequence, plasma membrane depolarization occurs, which results in massive release of the excitatory neurotransmitter glutamate at synaptic clefts. Overstimulation of the glutamate *N*-methyl-D-aspartate (NMDA) receptor and the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor channel leads to Ca^{2+} and Na^{+} overload in postsynaptic neurons [26] (Fig. 1). Additionally, the secondary activation of voltage-gated calcium channels [27] also contributes to Ca^{2+} overload and ultimately, the necrotic death of postsynaptic neurons. This phenomenon is known as excitotoxicity in mammals and primarily involves the elevation of intraneuronal calcium concentration. Excitotoxicity is not only related to acute neurodegenerative conditions such as ischaemia/anoxia, epilepsy, brain trauma or spinal cord injury (SCI), but also to chronic neurodegenerative conditions such us AD [28–30]. Apart from excitotoxicity, other neurotoxic processes involved in neuronal death have recently been unravelled in mammalian systems. Using cultured murine cortical neurons subjected to oxygen/glucose deprivation as a model of hypoxia, Aarts et al. [31] discovered a new lethal pathway involving a Ca^{2+}-permeable non-selective cation conductance. The authors observed that this cation conductance results in Ca^{2+} overload and requires the TRPM7 ion channel. TRPM7 is activated by reactive oxygen/nitrogen species and allows Ca^{2+} entry into the neuron. Ca^{2+} overload further stimulates free radical production and additional Ca^{2+} entry through this channel despite blockade of NMDA receptors. Suppression of TRPM7 conductance prevented neuronal death both in the presence and in the absence of NMDA blockade (Fig. 1). Acidification, which is a consequence of oxygen depletion, also plays an important role in necrotic neuronal death [32]. A recent report shows that acidosis activates Ca^{2+}-permeable acid sensing ion channels (ASICs) resulting in glutamate receptor-independent neuronal injury due to Ca^{2+} toxicity [33] (Fig. 1). Injection of ASIC1a blockers or knockout of the ASIC1a gene protects the brain from ischemic injury more potently that glutamate antagonism [33].

In *Caenorhabditis elegans*, altered ion homeostasis results in the necrotic death of specific neurons. Necrotic cell death can be triggered by toxic mutations in several genes. The best characterize case involves gain-of-function mutations in genes encoding the ion channel proteins termed degenerins [34,35].

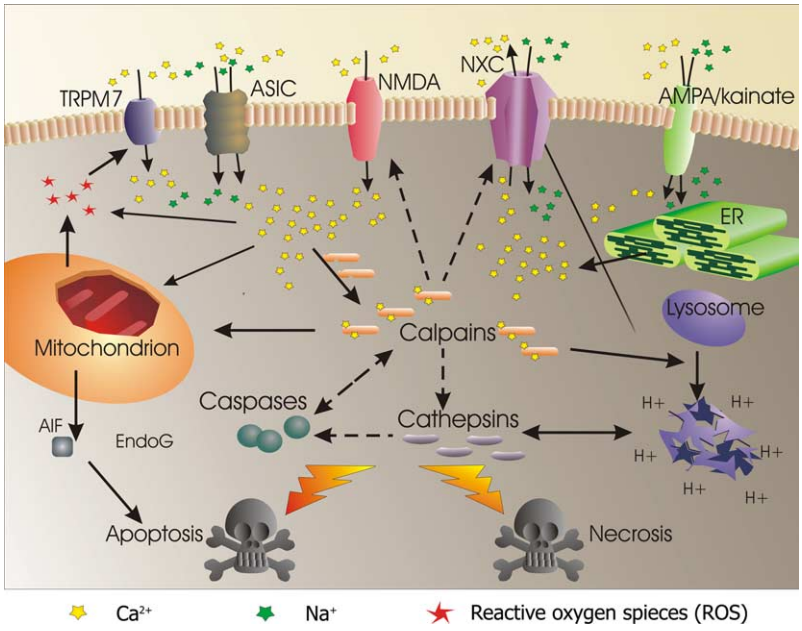

Fig. 1. The central role of calcium in neurodegeneration. Some of the membrane receptor channels involved in necrotic cell death and neurodegeneration are depicted. Gating of AMPA, NMDA, ASIC and TRPM7 channels in postsynaptic neurons provokes an influx of calcium ions inside the cell. The sharp increase of intracellular calcium concentration is the principal death-signalling event in both necrosis and apoptosis, resulting in the subsequent activation of calpains. Activated calpains cleave NMDA [86] and NCX channels [87]. NMDA remains active, whereas NCX, which is involved in calcium extrusion, becomes inactivated. Both these events contribute to further elevation of intracellular calcium. Depending on the level of calpain activation, neurons might undergo apoptotic or necrotic death. The left side of the figure illustrates events underlying neuronal death showing predominantly apoptotic features. The direct link between calpains and cathepsins (right side) is supported by findings in nematode and primate neurons undergoing necrotic cell death [88,128]. However, lysosomal damage and activation of cathepsins might occur independently of calpains. Dashed arrows point to identified protease targets (see text for details). Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; NMDA, *N*-methyl-D-aspartate; ASIC, acid sensing ion channel; TRPM7, transient receptor potential cation channel and NXC, Na^{+}/Ca^{2+} exchanger.

Fig. 2. Neurodegenerative disease in *Caenorhabditis elegans*. A nematode neuron undergoing necrosis as a result of degenerin ion channel hyperactivation. The degenerating cell swells to several times its normal diameter, whereas the nucleus is distended and has a distorted morphology. Black arrow points to the degenerating neuron.

Cells that express mutated degenerins undergo a type of cell death with morphologic and mechanistic features of mammalian excitotoxic cell death [36] (Fig. 2). Degenerins share similarity with mammalian epithelial sodium channels (ENaCs) [37]. In addition to degenerins, mutations that hyperactivate the α-subunit of the nicotinic acetylcholine receptor (nAChR) [38], or constitutively activate G-protein α-subunit [39,40] also result in alterations of cellular ionic homeostasis and induce necrotic cell death in *C. elegans*. Recent studies demonstrate that Ca^{2+} is also a central effector of *C. elegans* neurodegeneration, since proteins of the endoplasmic reticulum that regulate intra-cellular Ca^{2+} levels are required for necrotic cell death. These include the calcium-binding chaperones calreticulin and calnexin, and the calcium channels InsP3R and RyR [41].

Elevated intracellular Ca^{2+} is the most ubiquitous feature of neuronal death, which can result in both apoptotic- and necrotic-like type of cell death. The contribution of each type of cell death appears to correlate with the severity of the insult [42]. Calcium overload triggers lethal downstream events, including oxidative stress, mitochondrial dysfunction and calcium-dependent protease activation. Proteases can be considered as the executioners of cell death, and when a lethal protease-cascade is set in motion, cell demise becomes inevitable. In the following section we review the current state of our understanding about the proteolytic mechanisms involved in necrotic cell death and neurodegeneration.

2. Proteases in neurodegeneration and necrosis

A wide variety of proteases are engaged in cell death processes through both non-specific and limited proteolysis. These include cytosolic cysteine and aspartyl proteases, lysosomal proteases, microglial proteases [43] and the ubiquitin proteasome system [44]. In this section, we mainly focus on cytosolic cysteine-proteases (caspases and calpains) and lysosomal aspartyl-proteases (cathepsins) involved in necrotic cell death and neurodegeneration.

2.1. Caspases

Caspases are the main executioners of apoptotic cell death. Caspases constitute a family of cysteine proteases which specifically cleave after an aspartic residue in target proteins. Apoptotic caspases are classified as initiator caspases (caspase-2, -8, -9, and -10) and effector or executioner caspases, which include caspase-3, -6, and -7. Caspases are produced as inactive zymogens and must undergo activation during apoptosis. The activation of effector caspases is performed by an activated initiator caspase, through cleavage at specific internal Asp residues. The activation mechanism of initiator caspases is autocatalytic. Whether this requires cleavage or just dimerization is still under debate [45]. Activation of an initiator caspase triggers the apoptotic caspase-cascade and it is, therefore, tightly regulated and often requires the assembly of a multicomponent complex such as the apoptosome, where caspase-9 binds to mitochondrially released cytochrome *c* and APAF1 to form a 1.4 MDa complex. Activation of caspases requires ATP. Once activated, the executioner caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular targets (see Table 1) leading ultimately to cell death. Among all caspases caspase-3 is of particular interest because it appears to be a common downstream effector. Caspase-3 substrates include cytoskeletal proteins such as spectrin, DNA-repairing enzymes, cell-cycle proteins and enzymes involved in signal transduction.

Caspase-mediated proteolysis also appears to have a role in necrosis. Caspases can activate calpain proteases by mediating degradation of calpastatin, an endogenous inhibitor of calpain [46] and caspases might become activated by calpain pro-

Table 1
Protease classes implicated in neurodegeneration

Protease family	Activation mechanism	Substrates	Reference
Caspase	Proteolytic processing;	Cytoskeletal proteins	[120]
	Autoproteolysis or proteolysis by other	Signal transduction proteins	[120]
	proteases (caspases,cathepsins)	PM Ca^{2+}pump	[50]
	Phosphorylation?	Calpastatin	[46]
		Polyglutamine-containing proteins	[51]
		APP, Presenilin	[51]
Calpain	Ca^{2+}	Cytoskeletal proteins	[83–85]
	Autoproteolysis (Ca^{2+}-dependent)	Signal transduction proteins	[61,120]
	Membrane translocation	Receptor ion channels	[86,87]
		Caspases	[69,70]
		Apoptotic regulatory factors	[68]
		Polyglutamine-containing proteins	[67]
		APP	[120]
Cathepsin	Acidic pH	Non specific cleavage	
	Proteolytic processing by other lysosomal enzymes	Proinflammatory caspases	[98]
		APP	[106]

Abbreviations: PM, plasma membrane; APP, amyloid-β precursor protein.

teases [47]. Excess intracellular calcium results in mitochondrial damage and cytochrome *c* release, which might ultimately lead to caspase activation [48]. Caspase-3 has been demonstrated to be involved in neuronal death after transient cerebral ischaemia [49] and the plasma-membrane Ca^{2+} pump in neurons is a substrate for caspase-mediated cleavage and inactivation [50]. In addition, caspases might contribute to the neuronal death associated with the formation of intracellular polypeptide aggregates by cleaving the amyloid-β precursor protein (APP), presenilins, huntingtin (htt), tau and α-synuclein [51], which in turn become more toxic after cleavage.

2.2. Calpains

Calpains are a family of calcium-dependent cysteine proteases that perform limited proteolytic cleavage of a variety of cellular substrates [52]. There are two ubiquitous isoforms of calpains, μ-calpain (calpain I) and m-calpain (calpain II) that are activated by micro- and milli-molar concentrations of Ca^{2+} in vitro, respectively. Tissue-specific isoforms sum up a total of 14 members [53]. The ubiquitous isoforms are heterodimers composed of a distinct 80 kDa catalytic subunit and an identical 30 kDa regulatory subunit. Both, the large and the small subunit contain multiple calcium-binding sites [54,55]. Deletion of the shared 30 kDa subunit in mice results in embryonic lethality [56] whereas deletion of the 80 kDa subunit of μ-calpain does not precipitate any severe phenotype [57], indicating that m-calpain plays an important role during early development. Additional functions have been ascribed to calpains in cell motility and growth cone motility and guidance in neurons [58]. Mutations in Calpain III, a skeletal muscle-specific calpain, result in a recessive form of limb-girdle muscular dystrophy [59].

The role of calpains in neuronal cell death has been examined in a number of neuropathological conditions [60–62]. Inhibition of calpains prevents neuronal and behavioural defects in a mouse model of PD and calpain activation was evident in post-mortem midbrain tissues from cases of PD [63]. In the case of AD, calpain activation can be detected before abnormalities in the microtubule-associated protein tau occur. Activated calpain associates with neurofibrillary tangles, which are abnormal aggregates of hyperphosphorylated tau and a major pathological feature of AD [64]. Calpains also appear to function as upstream activators of the ERK1,2 MAPK signalling pathway and mediate in part the hyperphosphorylation of tau and neurofilaments [65]. Calpains are also activated in HD [66] and htt is degraded to small fragments by calpain after ischemic injury in rat brains [67].

Calpain activation has also been reported in a number of in vivo and cell culture models of apoptosis. Members of the Bcl-2 protein family of cell death regulators can be processed by calpains [68]. Calpain-mediated cleavage of caspases that can result in both, caspase inhibition [69] and activation [70] has also been reported. Conversely, caspases might regulate calpain activity by mediating degradation of calpastatin, the endogenous inhibitor of calpain. Calpains have also been implicated in the activation of p53 [71]. The authors examined the requirement of calpain activation in the death of cultured cortical neurons evoked by DNA damage. Inhibition of calpain by either association with its intrinsic inhibitor calpastatin or by pharmacological inhibitors results in reduced p53 activation and cytochrome *c* release, preventing death of embryonic cortical neurons.

There is also evidence of caspase-independent contribution of calpains to apoptotic events that accompany excitotoxicity [72,73]. Calpain inhibition with leupeptin has been shown to improve survival of primary rat motoneurons exposed to AMPA-mediated excitotoxicity, as well as motoneuron survival and muscle function in 3-day-old rats following neonatal nerve injury [74]. Recent in vitro experiments show that, in isolated liver and brain mitochondria, release of the apoptosis inducing factor (AIF) requires active calpain whereas release of cytochrome *c* does not. Calpeptin-mediated inhibition of calpain precludes AIF release [75]. AIF has been shown to play a role in excitotoxicity initiated by NMDA receptors independent of caspases [76] and has been found to translocate to the nucleus prior to cytochrome *c* release from mitochondria [76–78]. It remains to be determined whether AIF cleavage by calpain plays a role in Ca^{2+}-mediated excitotoxicity. Studies in mutant mice lacking or overexpressing the endogenous calpain inhibitor calpastatin suggest that calpain mediates excitotoxic signals through mobilization of pro-apoptotic factors [79]. The authors report that modulation of calpastatin had no effect under normal conditions suggesting that calpastatin inhibits calpains only under pathological conditions. After kainite-evoked excitotoxicity at concentrations that mimic transient ischaemia associated with apoptotic features, the authors observed activation of Bid, nuclear translocation of mitochondrial AIF and EndoG, DNA fragmentation and nuclear condensation without evidence of caspase-3 activation (Fig. 1). Those apoptotic features were enhanced in calpastatin deficient mice and suppressed in calpastatin overexpressing mice. Moreover, comparison of transgenic mice overexpressing calpastatin with mice overexpressing a viral inhibitor of caspases after kainite-mediated excitotoxixity revealed a prominent role for calpains in excitotoxic cell death. Hippocampal neurons are rescued from neuritic cytoskeleton disruption in mice overexpressing calpastatin, whereas caspase suppression had no effect on the neuritic pathologies [80]. The authors demonstrate that kainite-evoked sub-acute neuronal death involves calpain-mediated cytoskeletal disorganization independently of DNA fragmentation and that caspases do not play major roles in the disorganization of cytoskeletal networks. Additionally, the contribution of the caspase system appears negligible in the adult brain as manifested by the apparent absence of caspase-3.

The importance of calpain activation in acute cell injury and necrotic cell death triggered by calcium influx has been largely established. One of the mechanisms by which calpain activation contributes to cell demise is the cleavage of several essential cytoskeletal proteins of neuronal axons, such us neurofilaments, cain/cabin 1 and fodrin. Oxygen/glucose deprivation during anoxia and ischaemia of isolated rat optic nerves causes degradation of neurofilaments. Degradation of neurofilaments could be attenuated by Ca^{2+} removal, blockade of voltage-gated Na^{+} channels or inhibition of calpains [81]. Cain/cabin 1 contains a calcineurin-binding domain at the C-terminal region and calpain cleaves cain/cabin 1 in this region. It has been suggested that calpain-mediated cleavage of cain/cabin 1 results in calcineurin activation, which mediates calcium-triggered cell death [82]. Calpain-mediated proteolysis of fodrin has also been observed during necrotic cell death induced by hypoxia in rat cardiomyocytes [83]. Titin, the largest myofila-

ment protein in vertebrate striated muscle, is also target of calpain-mediated degradation in cardiomyocytes undergoing necrotic cell death [84]. Myosin Va belongs to the class V myosin proteins that move cargo along actin filaments and is involved in vesicle transport. In cerebellar granule neurons, myosin Va is cleaved by calpain I after exicitotoxicity. Calpain inhibitors prevent myosin Va proteolysis and improve morphological appearance of neurons, probably by preserving the integrity of the cytoskeleton [85]. Another mechanism by which calpain contributes to cell death is the cleavage of membrane channels. Calpain has been shown to cleave the subunit NR2B of the NMDA receptor under excitotoxic conditions in vitro and in vivo [86] (Fig. 1). Cleaved NR2B remains on the extracellular surface and it is predicted to be active. However, the contribution of the cleaved form to excitotoxicity has not been determined. A recent report provides insight into the molecular mechanisms by which calpains contribute to excitotoxic cell death. During excitotoxicity, Ca^{2+} influx through glutamate receptors is followed by a second, delayed, uncontrolled Ca^{2+} increase that leads to neuronal demise. Bano et al. [87] show that the plasma membrane Na^{+}/Ca^{2+} exchanger (NCX) is cleaved by calpains during brain ischaemia in neurons undergoing excitotoxicity. NCX operates in cellular Ca^{2+} extrusion, and its proteolytic inactivation seems to be responsible for the delayed excitotoxic Ca^{2+} upregulation and the death of the neurons. Inhibition of calpains by overexpression of calpastatin, or an NCX isoform not cleaved by calpains, prevents secondary Ca^{2+} overload and rescues neurons from excitotoxic death (Fig. 1).

Genetic studies in *C. elegans* demonstrate the requirement for calpain activation in the execution of neurodegenerative cell death inflicted by various genetic lesions [88]. Suppression of necrosis by systematic RNAi-mediated knock down of calpains encoded in the nematode genome coupled with analysis of double mutants showed that two specific calpain proteases (CLP-1 and TRA-3) are mostly required for proper execution of necrotic cell death (see Fig. 3).

2.3. Cathepsins

The lysosomal system plays a key role in cellular destruction during cell death. Two classes of lysosomal proteolytic enzymes appear to be the most active in neurodegeneration – aspartyl (cathepsin D) and cysteine (cathepsin B, H and L) proteases. Cathepsin proteases are implicated in both intracellular proteolysis and extracellular matrix remodelling and play important roles in numerous cellular processes by exerting degradation and regulatory functions. Lysosomal cathepsins play a role in both apoptosis and necrosis [89]. In addition to lysosomal cathepsins, cathepsins released from microglial cells also play an important role in neuronal death by degrading extracellular matrix proteins [90]. Deregulation or absence of cathepsins has important consequences on the maintenance and function of the nervous system. Mice lacking cathepsins B and L die during the second to fourth week of life and display neuronal loss and brain atrophy [91]. Cathepsin D deficiency induces a lysosomal storage disease associated with ceroid lipofuscin in mouse CNS neurons, and degeneration of neurons in the mouse retina [92]. Similarly, mutated cathepsin D causes lysosomal storage disease and profound neurodegeneration in mammals [93]. Leakage of cathepsins due to damaged lysosomal membranes during ageing also contributes to neuron degeneration. Cathepsins have also been directly implicated in long term memory (LTM) formation. Mutations in the *cer* gene, encoding an inhibitor of cathepsins in *Drosophila melanogaster*, result in LTM defects [94]. *cer* expression increases after LTM conditioning, suggesting that cathepsins are activated early in LTM formation.

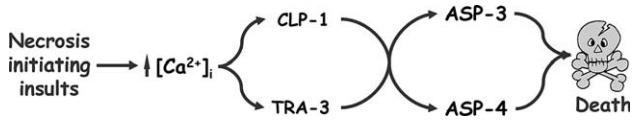

Fig. 3. A proteolytic cascade mediating necrosis in *Caenorhabditis elegans*. Execution of necrotic cell death in the nematode requires the activity of both calpain and cathepsin proteases. Two specific calpain proteases, TRA-3 and CLP-1, function redundantly upstream of aspartyl proteases ASP-3 and ASP-4 to mediated necrotic death [88]. Such an arrangement is consistent with a function of calpains in the activation of cathepsins, in accordance with the calpain–cathepsin hypothesis [128].

Several studies report differential expression of lysosomal cysteine proteases in models of neurological disorders. Lysosomal cathepsins B and L have been implicated in delayed neuronal death after global and focal cerebral ischaemia. Increased amount and activity of cathepsin B has been reported in hippocampal neurons after global ischaemia, and specific inhibitors of cathepsins B and L effectively reduce ischaemic cerebral damage [95]. Involvement of cathepsin B in motor neuron degeneration in cases of ALS has also been reported [96].The immunoreactivity of cathepsins H, L and D was not significantly different between control and ALS cases, whereas cathepsin B was downregulated. Cystatin C is an endogenous inhibitor of lysosomal cysteine proteases. Larger brain infarcts were found in cystatin C knockout mice, following focal ischaemia. In contrast, brain damage after global ischaemia was diminished in cystatin C knockout mice [97]. Cathepsin B release is an early event following occlusion of cerebral arteries, which eventually triggers the activation of pro-inflammatory caspases (caspase-1 and -11) in focal cerebral ischaemia [98]. In a cell culture model of sub-lethal NMDA-induced injury, specific blockade of cathepsin B/L type protease activity significantly attenuated spine damage [99]. Microglial-secreted cathepsin B has also been identified as a mediator of neuronal death induced by the amyloid-β peptide in vitro [100].

Cathepsin D is involved in neuronal death induced by ageing, transient forebrain ischaemia and excessive stimulation of glutamate receptors during excitotoxicity [101]. Increased cathepsin D expression has been observed in both neuronal and glial cells of rat brain after treatment with kainate, particularly in regions that show features of neurodegeneration [102]. Age-related changes in the subcellular localization of cathepsin D in rat cerebral cortex indicate that leakage of cathepsin D into the cytoplasm in old rats is closely associated with neurodegeneration [103], while reduction of lysosomal membrane stability and increased cytosolic cathepsin D is observed in aged brain and in AD. Similarly, distribution of cathepsin D-immunoreactive cell bodies in aged dogs resembles that in AD, reflecting a conserved pattern of brain ageing in mammals [104]. The implication of cathepsin D in AD has also been established. Lysosome numbers and the concentration of cathepsin D increase in neurons that are vulnerable to AD before the onset of pathology [101,105]. Cathepsin D

has also been proposed to function as γ-secretase, converting the APP into β-amyloid [106]. Cathepsin D and B activity is increased in familial early-onset AD (FAD) caused by mutations in presenilin. Presenilin modulates proteolysis and turnover of several signalling molecules, and appears to have a direct effect on the lysosomal system [107]. Presenilin 1 (PS1) interacts with telencephalin (TLN) and the APP via their transmembrane domain. Cathepsin D deficiency results in the localization of TLN to autophagic vacuoles, similarly to what is observed in PS1 (−/−) neurons [108]. A mouse model of Niemann-Pick type C disease shows a mild age-related increase in cathepsin-D content within nerve cells in many brain regions, with cathepsin-D elevation being greatest in microglial cells [109]. Cathepsin D is also found in early endosomes of cultured cortical neurons with Niemann-Pick type C defects [110]. Cathepsin D is differentially expressed in macrophages and microglia in a mouse model of SCI [111]. Cathepsin D is also involved in HD. When truncated htt is expressed in vitro, immunoreactive cytoplasmic bodies containing htt are formed (htt bodies). The fibrillar core of these htt bodies contains cathepsin D [112].

The requirement of aspartyl proteases in necrotic cell death and neurodegeneration has also been demonstrated in *C. elegans*. Reduction of aspartyl protease activity by specific mutations, chemicals or starvation protected against neurodegeneration inflicted by various insults, including hyperactivated degenerins, the nAChR subunit, DEG-3 and the G-protein subunit $G\alpha_s$ [88]. While at least six aspartyl protease genes (*asp-1* to *asp-6*) are expressed in *C. elegans* [113], two specific cathepsins, ASP-3 and ASP-4, appear to synergistically mediate necrotic cell death. Moreover, overexpression of ASP-3 and ASP-4 is sufficient to induce necrosis [88] (Fig. 3).

3. Protease activation in neurodegeneration

What signals trigger protease mal-activation during pathological cell death? Elevation of intracellular calcium concentration during the initial stages of cell death elicits activation of calpain proteases. Since raise of intracellular calcium is the most ubiquitous feature in neurodegeneration, calpain activation represents a critical step in both, apoptotic and necrotic neuronal death (Fig. 1). Nonetheless, the physiological role of calpains is not to contribute to the unwanted death of neurons. Rather, upon activation, the precise and limited cleavage of key structural and signalling proteins by calpains serves to modulate protein function. As discussed above, mouse m-calpain plays an important role during early development [57], and calpains have been ascribed functions in cell motility and guidance [58,114]. Mutations in *Drosophila* calpain D results in a rudimentary optic lobe and in altered flying and walking behaviours due to the absence of certain neurons [115]. Similarly, one of the *C. elegans* calpains required for neurodegeneration (TRA-3) is involved in sex determination [116]. However, influx of Ca^{2+} to the cytoplasm results in aberrant calpain activation and concomitant cellular degeneration. An increase in intracellular Ca^{2+} may occur in response to diverse necrosis-initiating stimuli such as excess glutamate, acidosis [33] or reactive oxygen/nitrogen species (ROS) [31]. It is conceivable that the degree of Ca^{2+} elevation and ensuing calpain activation will determine whether cells dye by apoptosis or necrosis. Mild Ca^{2+} elevation favours apoptosis [117], whereas acute calpain activation precipitates necrosis probably via catastrophic cleavage of regulatory and structural proteins [88].

Cathepsins play important roles during the development and in the function of the nervous system through regulated intracellular and extracellular proteolysis. However, the inappropriate activation of cathepsins has devastating consequences on cell viability. How are cathepsins mal-activated or mis-localized during inappropriate cell death? Studies in primates indicate that damage to the lysosomal membrane is inflicted enzymatically by activated calpains (Fig. 1). Calpains localize to lysosomal membranes after the onset of ischemic episodes with subsequent spillage of cathepsins to the cytoplasm [118]. This observations lead to the formulation of the 'calpain–cathepsin' hypothesis, whereby Ca^{2+}-mediated activation of calpains results in rupture of lysosomes and leakage of killer cathepsins that eventually dismantle the cell. In *C. elegans*, two specific calpains, TRA-3 and CLP-1, have been shown to function upstream of cathepsins ASP-3 and ASP-4 during degeneration inflicted by various necrosis initiators [88] (Fig. 3). Permeabilization of lysosomal membranes has also been reported in cases of apoptosis [20]. In a recent screen for drugs that activate p53-independent apoptosis in colon cancer, several compounds have been found to induce lysosomal membrane permeabilization, mis-localization of cathepsins B and D, and apoptotic cell death. However, at higher concentrations many of these compounds induce loss of cell membrane integrity, diminished caspase activity and reduced cell viability, suggesting a shift from apoptosis to necrosis [119]. Although, a further increase in cytosolic cathepsins at higher drug concentrations has not been reported, the results suggest that mild leakage of cathepsins from lysosomes results in apoptosis, while unrestrained leakage leads to catastrophic cellular destruction and necrosis.

Nevertheless, the molecular pathways leading to the activation of the different proteases involved in neurodegeneration are certainly more complex due to crosstalk between proteolytic mechanisms; cathepsins activate caspases [98], caspases activate calpains [46], and vice versa [70]. Moreover, activating pathways are likely to differ depending on the neuronal population and the nature or severity of the insult.

4. Concluding remarks and future prospects

Enzymatic proteolysis is involved in diverse neuropathological conditions including acute and chronic neurodegenerative diseases. Several lines of investigation converge to implicate caspases, calpains, cathepsins and other proteases in both, apoptotic and necrotic neuronal death. However, the molecular mechanisms that govern the erroneous activation of proteases in neurodegenerative processes as well as the downstream cascades that are set in motion remain largely elusive. Several difficulties have hampered progress towards elucidation of the proteolytic pathways involved in neurodegeneration. Among them is the fact that caspases and calpains cleave many common substrates including cytoskeletal and regulatory proteins [46,120] (see Table 1), and also the extensive crosstalk between different proteolytic systems [117]. The capacity of proteases to modulate signal transduction pathways [61] further complicates the delineation of their precise roles in vivo due to pleotropic effects. Another important issue is that synthetic protease inhibitors initially thought to be specific for a given

protease class were subsequently found to have a broader inhibition range. For example, calpain-specific synthetic inhibitors also act on other cysteine proteases, such as cathepsin B [121] and several caspase inhibitors including Z-VAD-fmk and Z-DEVD-fmk have been shown to directly block calpain activity and necrotic cell death after traumatic brain injury [122,123]. Therefore one should be cautious when drawing conclusions based on experiments with synthetic inhibitors.

Disruption of calcium homeostasis plays a key role in neuronal injury. Elucidating the function of calpains, which are tightly regulated by intracellular calcium, is of great importance. The recent generation of genetically engineered mice overexpressing the endogenous calpain inhibitor calpastatin [80], as well as the progress in imaging calpain protease activity in living mice [124] will certainly contribute to enhance our understanding of the specific roles of calpain proteases in different neurodegenerative processes in vivo.

As discussed above, disturbance of cathepsin enzymatic activities accompanies brain ageing and neurodegeneration. Apart from leakage of lysosomal cathepsins due to rupture of lysosomal membranes or to aberrant calpain activation, microglial cathepsins also contribute to neurodegeneration [90]. In addition, it is worth mentioning that lysosomal cathepsins have been found to undergo alternative trafficking routes. An alternatively spliced form of cathepsin B localizes to mitochondria and provokes cell death [125], whereas another cysteine protease, cathepsins L, derived from translation ititiation at a downstream AUG site and devoid of a signal peptide, is targeted to the nucleus [126]. This suggests additional pathophysiological functions of cathepsins at non-lysosomal sites.

Another important step in the cascade leading to neuronal cell death is degradation of extracellular matrix proteins by microglia-derived proteases other that cathepsins. It has been shown that excitotoxic cell death of murine hippocampal neurons can be prevented by inhibitors of tissue plasminogen activator (tPA) or by inhibitors of plasmin in vivo. Plasmin is a potent activator of the matrix metalloproteinases (MMPs). MMP's are upregulated after excitotoxic challenge and inhibition of MMP's prevents kainite-induced excitotoxicity in the rat brain [127].

As research in the field of neurodegeneration moves forwards, it becomes apparent that an intricate network of signalling pathways and the variety of cell death mechanisms are involved in neuronal degeneration. Cytosolic, lysosomal and microglial proteases emerge as key players in the process. Our growing understanding of the proteolytic mechanisms mediating necrosis and neurodegeneration holds promise of facilitating the development of novel neuroprotective agents in an effort to battle neurodegenerative disorders.

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References

[1] Yuan, J., Lipinski, M. and Degterev, A. (2003) Diversity in the mechanisms of neuronal cell death. Neuron 40, 401–413.
[2] Benn, S.C. and Woolf, C.J. (2004) Adult neuron survival strategies – slamming on the brakes. Nat. Rev. Neurosci. 5, 686–700.
[3] Nicotera, P., Leist, M. and Manzo, L. (1999) Neuronal cell death: a demise with different shapes. Trends Pharmacol. Sci. 20, 46–51.
[4] Mattson, M.P. (2000) Apoptosis in neurodegenerative disorders. Nat. Rev. Mol. Cell Biol. 1, 120–129.
[5] Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P. and Lipton, S.A. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc. Natl. Acad. Sci. USA 92, 7162–7166.
[6] Davis, J.N. and Antonawich, F.J. (1997) Role of apoptotic proteins in ischemic hippocampal damage. Ann. N.Y. Acad. Sci. 835, 309–320.
[7] MacManus, J.P. and Buchan, A.M. (2000) Apoptosis after experimental stroke: fact or fashion?. J. Neurotrauma 17, 899–914.
[8] Martin, L.J., Al-Abdulla, N.A., Brambrink, A.M., Kirsch, J.R., Sieber, F.E. and Portera-Cailliau, C. (1998) Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A perspective on the contributions of apoptosis and necrosis. Brain Res. Bull. 46, 281–309.
[9] Sperandio, S., de Belle, I. and Bredesen, D.E. (2000) An alternative, nonapoptotic form of programmed cell death. Proc. Natl. Acad. Sci. USA 97, 14376–14381.
[10] Wyllie, A.H. and Golstein, P. (2001) More than one way to go. Proc. Natl. Acad. Sci. USA 98, 11–13.
[11] Le, D.A., et al. (2002) Caspase activation and neuroprotection in caspase-3- deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. Proc. Natl. Acad. Sci. USA 99, 15188–15193.
[12] Danial, N.N. and Korsmeyer, S.J. (2004) Cell death: critical control points. Cell 116, 205–219.
[13] Hengartner, M.O. (2001) Apoptosis: corralling the corpses. Cell 104, 325–328.
[14] Micheau, O. and Tschopp, J. (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114, 181–190.
[15] Esposti, M.D. (2002) The roles of Bid. Apoptosis 7, 433–440.
[16] Cryns, V. and Yuan, J. (1998) Proteases to die for. Genes Dev. 12, 1551–1570.
[17] Hengartner, M.O. (2000) The biochemistry of apoptosis. Nature 407, 770–776.
[18] Deshmukh, M. and Johnson Jr, E.M. (1998) Evidence of a novel event during neuronal death: development of competence-to-die in response to cytoplasmic cytochrome *c*. Neuron 21, 695–705.
[19] Raoul, C., Estevez, A.G., Nishimune, H., Cleveland, D.W., deLapeyriere, O., Henderson, C.E., Haase, G. and Pettmann, B. (2002) Motoneuron death triggered by a specific pathway downstream of Fas potentiation by ALS-linked SOD1 mutations. Neuron 35, 1067–1083.
[20] Leist, M. and Jaattela, M. (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nat. Rev. Mol. Cell. Biol. 2, 589–598.
[21] Lee, J.M., Zipfel, G.J. and Choi, D.W. (1999) The changing landscape of ischaemic brain injury mechanisms. Nature 399, A7–A14.
[22] Guo, Q., Fu, W., Sopher, B.L., Miller, M.W., Ware, C.B., Martin, G.M. and Mattson, M.P. (1999) Increased vulnerability of hippocampal neurons to excitotoxic necrosis in presenilin-1 mutant knock-in mice. Nat. Med. 5, 101–106.
[23] Martin, J.B. (1999) Molecular basis of the neurodegenerative disorders. N. Engl. J. Med. 340, 1970–1980.
[24] Martin, L.J. (2001) Neuronal cell death in nervous system development, disease, and injury (Review). Int. J. Mol. Med. 7, 455–478.
[25] Taylor, J.P., Hardy, J. and Fischbeck, K.H. (2002) Toxic proteins in neurodegenerative disease. Science 296, 1991–1995.
[26] Choi, D.W. (1992) Excitotoxic cell death. J. Neurobiol. 23, 1261–1276.
[27] Fern, R. and Moller, T. (2000) Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop. J. Neurosci. 20, 34–42.

[28] Mattson, M.P. (2003) Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. Neuromol. Med. 3, 65–94.
[29] Rao, S.D. and Weiss, J.H. (2004) Excitotoxic and oxidative cross-talk between motor neurons and glia in ALS pathogenesis. Trends Neurosci. 27, 17–23.
[30] Young, C., Tenkova, T., Dikranian, K. and Olney, J.W. (2004) Excitotoxic versus apoptotic mechanisms of neuronal cell death in perinatal hypoxia/ischemia. Curr. Mol. Med. 4, 77–85.
[31] Aarts, M., Iihara, K., Wei, W.L., Xiong, Z.G., Arundine, M., Cerwinski, W., MacDonald, J.F. and Tymianski, M. (2003) A key role for TRPM7 channels in anoxic neuronal death. Cell 115, 863–877.
[32] Ding, D., Moskowitz, S.I., Li, R., Lee, S.B., Esteban, M., Tomaselli, K., Chan, J. and Bergold, P.J. (2000) Acidosis induces necrosis and apoptosis of cultured hippocampal neurons. Exp. Neurol. 162, 1–12.
[33] Xiong, Z.G., et al. (2004) Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. Cell 118, 687–698.
[34] Chalfie, M. and Wolinsky, E. (1990) The identification and suppression of inherited neurodegeneration in *Caenorhabditis elegans*. Nature 345, 410–416.
[35] Driscoll, M. and Chalfie, M. (1991) The mec-4 gene is a member of a family of *Caenorhabditis elegans* genes that can mutate to induce neuronal degeneration. Nature 349, 588–593.
[36] Hall, D.H., Gu, G., Garcia-Anoveros, J., Gong, L., Chalfie, M. and Driscoll, M. (1997) Neuropathology of degenerative cell death in *Caenorhabditis elegans*. J. Neurosci. 17, 1033–1045.
[37] Canessa, C.M., Horisberger, J.D. and Rossier, B.C. (1993) Epithelial sodium channel related to proteins involved in neurodegeneration. Nature 361, 467–470.
[38] Treinin, M. and Chalfie, M. (1995) A mutated acetylcholine receptor subunit causes neuronal degeneration in *C. elegans*. Neuron 14, 871–877.
[39] Berger, A.J., Hart, A.C. and Kaplan, J.M. (1998) G αs-induced neurodegeneration in *Caenorhabditis elegans*. J. Neurosci. 18, 2871–2880.
[40] Korswagen, H.C., Park, J.H., Ohshima, Y. and Plasterk, R.H. (1997) An activating mutation in a *Caenorhabditis elegans* Gs protein induces neural degeneration. Genes Dev. 11, 1493–1503.
[41] Xu, K., Tavernarakis, N. and Driscoll, M. (2001) Necrotic cell death in *C. elegans* requires the function of calreticulin and regulators of Ca(2+) release from the endoplasmic reticulum. Neuron 31, 957–971.
[42] Sattler, R. and Tymianski, M. (2000) Molecular mechanisms of calcium-dependent excitotoxicity. J. Mol. Med. 78, 3–13.
[43] Nakanishi, H. (2003) Microglial functions and proteases. Mol. Neurobiol. 27, 163–176.
[44] Ardley, H.C., Hung, C.C. and Robinson, P.A. (2005) The aggravating role of the ubiquitin–proteasome system in neurodegeneration. FEBS Lett. 579, 571–576.
[45] Boatright, K.M. and Salvesen, G.S. (2003) Mechanisms of caspase activation. Curr. Opin. Cell Biol. 15, 725–731.
[46] Wang, K.K. (2000) Calpain and caspase: can you tell the difference?. Trends Neurosci. 23, 20–26.
[47] Yamashima, T. (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. Prog. Neurobiol. 62, 273–295.
[48] Zhu, L.P., Yu, X.D., Ling, S., Brown, R.A. and Kuo, T.H. (2000) Mitochondrial Ca(2+) homeostasis in the regulation of apoptotic and necrotic cell deaths. Cell Calcium 28, 107–117.
[49] Chen, J., Nagayama, T., Jin, K., Stetler, R.A., Zhu, R.L., Graham, S.H. and Simon, R.P. (1998) Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J. Neurosci. 18, 4914–4928.
[50] Schwab, B.L., et al. (2002) Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis. Cell Death Differ. 9, 818–831.
[51] Wellington, C.L. and Hayden, M.R. (2000) Caspases and neurodegeneration: on the cutting edge of new therapeutic approaches. Clin. Genet. 57, 1–10.
[52] Goll, D.E., Thompson, V.F., Li, H., Wei, W. and Cong, J. (2003) The calpain system. Physiol. Rev. 83, 731–801.
[53] Suzuki, K. and Sorimachi, H. (1998) A novel aspect of calpain activation. FEBS Lett. 433, 1–4.
[54] Hosfield, C.M., Elce, J.S., Davies, P.L. and Jia, Z. (1999) Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. EMBO J. 18, 6880–6889.
[55] Strobl, S., et al. (2000) The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. Proc. Natl. Acad. Sci. USA 97, 588–592.
[56] Arthur, J.S., Elce, J.S., Hegadorn, C., Williams, K. and Greer, P.A. (2000) Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. Mol. Cell. Biol. 20, 4474–4481.
[57] Azam, M., Andrabi, S.S., Sahr, K.E., Kamath, L., Kuliopulos, A. and Chishti, A.H. (2001) Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. Mol. Cell. Biol 21, 2213–2220.
[58] Robles, E., Huttenlocher, A. and Gomez, T.M. (2003) Filopodial calcium transients regulate growth cone motility and guidance through local activation of calpain. Neuron 38, 597–609.
[59] Richard, I., et al. (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell 81, 27–40.
[60] Kim, Y.J., et al. (2001) Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. Proc. Natl. Acad. Sci. USA 98, 12784–12789.
[61] Lee, M.S., Kwon, Y.T., Li, M., Peng, J., Friedlander, R.M. and Tsai, L.H. (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 405, 360–364.
[62] Ray, S.K., Hogan, E.L. and Banik, N.L. (2003) Calpain in the pathophysiology of spinal cord injury: neuroprotection with calpain inhibitors. Brain Res. Brain Res. Rev. 42, 169–185.
[63] Crocker, S.J., et al. (2003) Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease. J. Neurosci. 23, 4081–4091.
[64] Grynspan, F., Griffin, W.R., Cataldo, A., Katayama, S. and Nixon, R.A. (1997) Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease. Brain Res. 763, 145–158.
[65] Veeranna, et al. (2004) Calpain mediates calcium-induced activation of the erk1,2 MAPK pathway and cytoskeletal phosphorylation in neurons: relevance to Alzheimer's disease. Am. J. Pathol. 165, 795–805.
[66] Gafni, J. and Ellerby, L.M. (2002) Calpain activation in Huntington's disease. J. Neurosci. 22, 4842–4849.
[67] Kim, M., Roh, J.K., Yoon, B.W., Kang, L., Kim, Y.J., Aronin, N. and DiFiglia, M. (2003) Huntingtin is degraded to small fragments by calpain after ischemic injury. Exp. Neurol. 183, 109–115.
[68] Gil-Parrado, S., et al. (2002) Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members. J. Biol. Chem. 277, 27217–27226.
[69] Bizat, N., et al. (2003) In vivo calpain/caspase cross-talk during 3-nitropropionic acid-induced striatal degeneration: implication of a calpain-mediated cleavage of active caspase-3. J. Biol. Chem. 278, 43245–43253.
[70] Blomgren, K., Zhu, C., Wang, X., Karlsson, J.O., Leverin, A.L., Bahr, B.A., Mallard, C. and Hagberg, H. (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia: a mechanism of pathological apoptosis? J. Biol. Chem. 276, 10191–10198.
[71] Sedarous, M., Keramaris, E., O'Hare, M., Melloni, E., Slack, R.S., Elce, J.S., Greer, P.A. and Park, D.S. (2003) Calpains mediate p53 activation and neuronal death evoked by DNA damage. J. Biol. Chem. 278, 26031–26038.
[72] Chen, M., He, H., Zhan, S., Krajewski, S., Reed, J.C. and Gottlieb, R.A. (2001) Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion. J. Biol. Chem. 276, 30724–30728.
[73] Chen, M., Won, D.J., Krajewski, S. and Gottlieb, R.A. (2002) Calpain and mitochondria in ischemia/reperfusion injury. J. Biol. Chem. 277, 29181–29186.

[74] Kieran, D. and Greensmith, L. (2004) Inhibition of calpains, by treatment with leupeptin, improves motoneuron survival and muscle function in models of motoneuron degeneration. Neuroscience 125, 427–439.
[75] Polster, B.M., Etxebarria, A., Basanez, G., Hardwick, J.M. and Nicholls, D.G. (2004) Calpain I induces cleavage and release of apoptosis inducing factor from isolated mitochondria. J. Biol. Chem. 280, 6447–6454.
[76] Yu, S.W., et al. (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297, 259–263.
[77] Cande, C., Vahsen, N., Garrido, C. and Kroemer, G. (2004) Apoptosis-inducing factor (AIF): caspase-independent after all. Cell Death Differ. 11, 591–595.
[78] Zhu, C., Qiu, L., Wang, X., Hallin, U., Cande, C., Kroemer, G., Hagberg, H. and Blomgren, K. (2003) Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. J. Neurochem. 86, 306–317.
[79] Takano, J., Tomioka, M., Tsubuki, S., Higuchi, M., Iwata, N., Itohara, S., Maki, M. and Saido, T.C. (2005) Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: Evidence from calpastatin-mutant mice. J. Biol. Chem., PMID: 15691848.
[80] Higuchi, M., et al. (2005) Distinct mechanistic roles of calpain and caspase activation in neurodegeneration as revealed in mice overexpressing their specific inhibitors. J. Biol. Chem., PMID: 15699033.
[81] Stys, P.K. and Jiang, Q. (2002) Calpain-dependent neurofilament breakdown in anoxic and ischemic rat central axons. Neurosci. Lett. 328, 150–154.
[82] Kim, M.J., et al. (2002) Calpain-dependent cleavage of cain/cabin1 activates calcineurin to mediate calcium-triggered cell death. Proc. Natl. Acad. Sci. USA 99, 9870–9875.
[83] Aki, T., Yoshida, K. and Fujimiya, T. (2002) Phosphoinositide 3-kinase accelerates calpain-dependent proteolysis of fodrin during hypoxic cell death. J. Biochem. (Tokyo) 132, 921–926.
[84] Lim, C.C., et al. (2004) Anthracyclines induce calpain-dependent titin proteolysis and necrosis in cardiomyocytes. J. Biol. Chem 279, 8290–8299.
[85] Alavez, S., Moran, J., Franco-Cea, A., Ortega-Gomez, A., Casaletti, L. and Cameron, L.C. (2004) Myosin Va is proteolysed in rat cerebellar granule neurons after excitotoxic injury. Neurosci. Lett. 367, 404–409.
[86] Simpkins, K.L., Guttmann, R.P., Dong, Y., Chen, Z., Sokol, S., Neumar, R.W. and Lynch, D.R. (2003) Selective activation induced cleavage of the NR2B subunit by calpain. J. Neurosci. 23, 11322–11331.
[87] Bano, D., et al. (2005) Cleavage of the plasma membrane Na(+)/Ca(2+) exchanger in excitotoxicity. Cell 120, 275–285.
[88] Syntichaki, P., Xu, K., Driscoll, M. and Tavernarakis, N. (2002) Specific aspartyl and calpain proteases are required for neurodegeneration in *C. elegans*. Nature 419, 939–944.
[89] Ferri, K.F. and Kroemer, G. (2001) Organelle-specific initiation of cell death pathways. Nat. Cell Biol. 3, E255–E263.
[90] Nakanishi, H. (2003) Neuronal and microglial cathepsins in aging and age-related diseases. Ageing Res. Rev. 2, 367–381.
[91] Felbor, U., Kessler, B., Mothes, W., Goebel, H.H., Ploegh, H.L., Bronson, R.T. and Olsen, B.R. (2002) Neuronal loss and brain atrophy in mice lacking cathepsins B and L. Proc. Natl. Acad. Sci. USA 99, 7883–7888.
[92] Koike, M., et al. (2003) Involvement of two different cell death pathways in retinal atrophy of cathepsin D-deficient mice. Mol. Cell. Neurosci. 22, 146–161.
[93] Tyynela, J., Sohar, I., Sleat, D.E., Gin, R.M., Donnelly, R.J., Baumann, M., Haltia, M. and Lobel, P. (2000) A mutation in the ovine cathepsin D gene causes a congenital lysosomal storage disease with profound neurodegeneration. EMBO J. 19, 2786–2792.
[94] Comas, D., Petit, F. and Preat, T. (2004) *Drosophila* long-term memory formation involves regulation of cathepsin activity. Nature 430, 460–463.
[95] Seyfried, D.M., et al. (2001) A selective cysteine protease inhibitor is non-toxic and cerebroprotective in rats undergoing transient middle cerebral artery ischemia. Brain Res. 901, 94–101.
[96] Kikuchi, H., Yamada, T., Furuya, H., Doh-ura, K., Ohyagi, Y., Iwaki, T. and Kira, J. (2003) Involvement of cathepsin B in the motor neuron degeneration of amyotrophic lateral sclerosis. Acta Neuropathol. (Berl) 105, 462–468.
[97] Olsson, T., Nygren, J., Hakansson, K., Lundblad, C., Grubb, A., Smith, M.L. and Wieloch, T. (2004) Gene deletion of cystatin C aggravates brain damage following focal ischemia but mitigates the neuronal injury after global ischemia in the mouse. Neuroscience 128, 65–71.
[98] Benchoua, A., Braudeau, J., Reis, A., Couriaud, C. and Onteniente, B. (2004) Activation of proinflammatory caspases by cathepsin B in focal cerebral ischemia. J. Cereb. Blood Flow Metab. 24, 1272–1279.
[99] Graber, S., Maiti, S. and Halpain, S. (2004) Cathepsin B-like proteolysis and MARCKS degradation in sub-lethal NMDA-induced collapse of dendritic spines. Neuropharmacology 47, 706–713.
[100] Gan, L., et al. (2004) Identification of cathepsin B as a mediator of neuronal death induced by Abeta-activated microglial cells using a functional genomics approach. J. Biol. Chem. 279, 5565–5572.
[101] Adamec, E., Mohan, P.S., Cataldo, A.M., Vonsattel, J.P. and Nixon, R.A. (2000) Up-regulation of the lysosomal system in experimental models of neuronal injury: implications for Alzheimer's disease. Neuroscience 100, 663–675.
[102] Hetman, M., Filipkowski, R.K., Domagala, W. and Kaczmarek, L. (1995) Elevated cathepsin D expression in kainate-evoked rat brain neurodegeneration. Exp. Neurol. 136, 53–63.
[103] Jung, H., Lee, E.Y. and Lee, S.I. (1999) Age-related changes in ultrastructural features of cathepsin B- and D-containing neurons in rat cerebral cortex. Brain Res. 844, 43–54.
[104] Bi, X., Head, E., Cotman, C.W. and Lynch, G. (2003) Spatial patterns of mammalian brain aging: distribution of cathepsin D-immunoreactive cell bodies and dystrophic dendrites in aging dogs resembles that in Alzheimer's disease. J. Comp. Neurol. 464, 371–381.
[105] Nixon, R.A. (2000) A protease activation cascade in the pathogenesis of Alzheimer's disease. Ann. N.Y. Acad. Sci 924, 117–131.
[106] Crawford, F.C., Freeman, M.J., Schinka, J., Abdullah, L.I., Richards, D., Sevush, S., Duara, R. and Mullan, M.J. (2000) The genetic association between Cathepsin D and Alzheimer's disease. Neurosci. Lett. 289, 61–65.
[107] Cataldo, A.M., Peterhoff, C.M., Schmidt, S.D., Terio, N.B., Duff, K., Beard, M., Mathews, P.M. and Nixon, R.A. (2004) Presenilin mutations in familial Alzheimer disease and transgenic mouse models accelerate neuronal lysosomal pathology. J. Neuropathol. Exp. Neurol. 63, 821–830.
[108] Esselens, C., et al. (2004) Presenilin 1 mediates the turnover of telencephalin in hippocampal neurons via an autophagic degradative pathway. J. Cell Biol. 166, 1041–1054.
[109] German, D.C., Liang, C.L., Song, T., Yazdani, U., Xie, C. and Dietschy, J.M. (2002) Neurodegeneration in the Niemann-Pick C mouse: glial involvement. Neuroscience 109, 437–450.
[110] Jin, L.W., Shie, F.S., Maezawa, I., Vincent, I. and Bird, T. (2004) Intracellular accumulation of amyloidogenic fragments of amyloid-β precursor protein in neurons with Niemann-Pick type C defects is associated with endosomal abnormalities. Am. J. Pathol. 164, 975–985.
[111] Hashimoto, M., et al. (2005) Gene expression profiling of cathepsin D, metallothioneins-1 and -2, osteopontin, and tenascin-C in a mouse spinal cord injury model by cDNA microarray analysis. Acta Neuropathol. (Berl.) 109, 165–180.
[112] Qin, Z.H., et al. (2004) Huntingtin bodies sequester vesicle-associated proteins by a polyproline-dependent interaction. J. Neurosci. 24, 269–281.
[113] Tcherepanova, I., Bhattacharyya, L., Rubin, C.S. and Freedman, J.H. (2000) Aspartic proteases from the nematode *Caenorhabditis elegans*. Structural organization and developmental and cell-specific expression of asp-1. J. Biol. Chem. 275, 26359–26369.
[114] Glading, A., Lauffenburger, D.A. and Wells, A. (2002) Cutting to the chase: calpain proteases in cell motility. Trends Cell Biol. 12, 46–54.

[115] Delaney, S.J., Hayward, D.C., Barleben, F., Fischbach, K.F. and Miklos, G.L. (1991) Molecular cloning and analysis of small optic lobes, a structural brain gene of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 88, 7214–7218.
[116] Barnes, T.M. and Hodgkin, J. (1996) The tra-3 sex determination gene of *Caenorhabditis elegans* encodes a member of the calpain regulatory protease family. EMBO J. 15, 4477–4484.
[117] Neumar, R.W., Xu, Y.A., Gada, H., Guttmann, R.P. and Siman, R. (2003) Cross-talk between calpain and caspase proteolytic systems during neuronal apoptosis. J. Biol. Chem 278, 14162–14167.
[118] Yamashima, T., Tonchev, A.B., Tsukada, T., Saido, T.C., Imajoh-Ohmi, S., Momoi, T. and Kominami, E. (2003) Sustained calpain activation associated with lysosomal rupture executes necrosis of the postischemic CA1 neurons in primates. Hippocampus 13, 791–800.
[119] Erdal, H., Berndtsson, M., Castro, J., Brunk, U., Shoshan, M.C. and Linder, S. (2005) Induction of lysosomal membrane permeabilization by compounds that activate p53-independent apoptosis. Proc. Natl. Acad. Sci. USA 102, 192–197.
[120] Chan, S.L. and Mattson, M.P. (1999) Caspase and calpain substrates: roles in synaptic plasticity and cell death. J. Neurosci. Res. 58, 167–190.
[121] Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. and Goldberg, A.L. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78, 761–771.
[122] Knoblach, S.M., Alroy, D.A., Nikolaeva, M., Cernak, I., Stoica, B.A. and Faden, A.I. (2004) Caspase inhibitor z-DEVD-fmk attenuates calpain and necrotic cell death in vitro and after traumatic brain injury. J. Cereb. Blood Flow Metab. 24, 1119–1132.
[123] Waterhouse, N.J., et al. (1998) Calpain activation is upstream of caspases in radiation-induced apoptosis. Cell Death Differ. 5, 1051–1061.
[124] Stockholm, D., Bartoli, M., Sillon, G., Bourg, N., Davoust, J. and Richard, I. (2005) Imaging calpain protease activity by multiphoton FRET in living mice. J. Mol. Biol. 346, 215–222.
[125] Muntener, K., Zwicky, R., Csucs, G., Rohrer, J. and Baici, A. (2004) Exon skipping of cathepsin B: mitochondrial targeting of a lysosomal peptidase provokes cell death. J. Biol. Chem. 279, 41012–41017.
[126] Goulet, B., Baruch, A., Moon, N.S., Poirier, M., Sansregret, L.L., Erickson, A., Bogyo, M. and Nepveu, A. (2004) A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor. Mol. Cell 14, 207–219.
[127] Campbell, S.J., Finlay, M., Clements, J.M., Wells, G., Miller, K.M., Perry, V.H. and Anthony, D.C. (2004) Reduction of excitotoxicity and associated leukocyte recruitment by a broad-spectrum matrix metalloproteinase inhibitor. J. Neurochem. 89, 1378–1386.
[128] Yamashima, T. (2004) Ca^{2+}-dependent proteases in ischemic neuronal death: a conserved 'calpain–cathepsin cascade' from nematodes to primates. Cell Calcium 36, 285–293.