As published in BTi April/May 2006

Proteolytic pathways in necrotic cell death

by Dr Nektarios Tavernarakis

The proper development and precise wiring of the nervous system depends critically on programmed apoptotic cell death. However, genetic or accidental factors can lead to the premature, non-programmed death of neurons during adult life. This inappropriate death of cells in the nervous system is the cause of multiple neurodegenerative disorders. Adult 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 ischaemia, stroke or trauma. Elevated intracellular calcium is the most ubiquitous feature of neuronal death and is accompanied by concomitant activation of cysteine calcium-dependent proteases, calpains, which, together with the lysosomal aspartyl proteases, cathepsins, have emerged as key mediators of neuronal necrotic death. Interfering with the activity of these enzymes may be an effective strategy to combat neurodegenerative disorders in humans.

Neurodegeneration involves multiple mechanisms of cell death, as well as crosstalk between different death pathways. It is likely that, depending on the nature and severity of the insult as well as on the cellular context, specific mechanisms dictate cell death in specific neurodegenerative diseases. Acute and chronic neurodegenerative conditions have been associated with both apoptotic and necrotic cell death. While necrosis and apoptosis can be distinguished in some situations, in others the distinction is not so clear and certain dying cells can show distinctive apoptotic and necrotic features. The idea is emerging of a continuum of responses ranging from apoptosis to necrosis, with the relative contribution of each process varying in individual situations.

Apoptosis is a genetically-regulated process of cell suicide that is made up of a variety of cellular signalling pathways that include common death effectors with a range of different stimuli [1]. The morphological features of apoptosis include nuclear and cytoplasmic condensation, internucleosomal DNA cleavage and the packaging of the cell into apoptotic bodies that are engulfed by phagocytes, preventing release of intracellular components. Although carried out through diverse mechanisms, apoptosis always requires the proteolytic activation of effector caspases such as caspase-3 and caspase-7 [2]. Canonical caspase-dependent neuronal apoptosis shapes the nervous system during development. However, things get more complicated in mature neuronal death. Neurons often show caspase-independent apoptotic features and in some cases, co-activation of several parallel death pathways are required before cell death can take place. As well as caspases, calpains and cathepsins have also been implicated in apoptotic neuronal death.

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

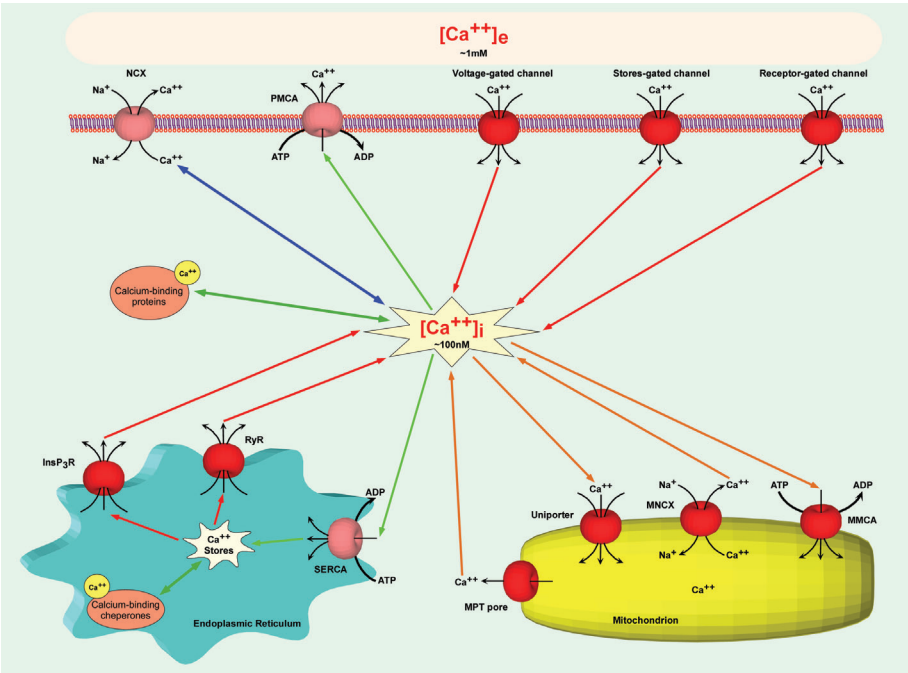

Figure 1. Cellular calcium homeostasis. Multiple mechanisms operate in a concerted manner to maintain low cytoplasmic calcium concentrations against steep electrochemical gradients. NCX: Na^+/Ca^{2+} exchanger, PMCA: plasma membrane Ca^{2+} ATPase, InsP3R: inosytoltriphospate receptor Ca^{2+} channel, RyR: ryanodine receptor Ca^{2+} channel, SERCA: sarcoplasmic-endoplasmic reticulumn Ca^{2+} ATPase, MPT: membrane permeability transition, MNCX: mitochondrial NCX, MMCA: mitochondrial membrane Ca^{2+} ATPase.

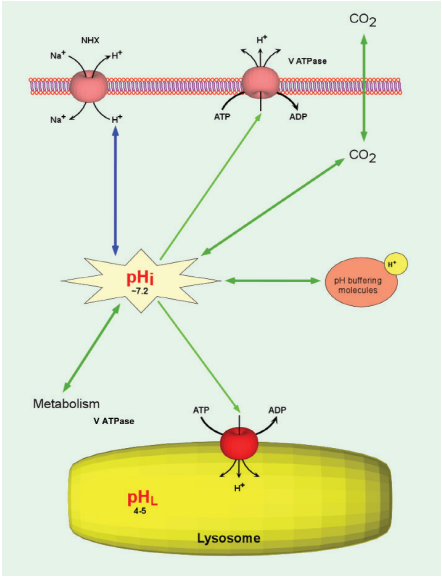

Figure 2. Cellular pH homeostasis. Cytoplasmic pH is maintained around 7.2, while in lysosomes and other acidic organelles it reaches values below 4.0. NHX: Na^+/H^+ exchanger, V- ATPase: vacuolar H^+ ATPase.

plasma membrane rupture. The cell lyses without formation of vesicles. As a consequence, cellular contents are liberated into the intracellular space and damage neighbouring cells to evoke an inflammatory response [3]. Necrosis typically occurs following ischaemia, hypoxia, stroke or trauma and has also been reported in Alzheimer's, Huntington's and Parkinson's diseases, as well as in amyotrophic lateral sclerosis (ALS) [4, 5]. Although the molecular mechanisms that bring about necrotic cell death have not been fully elucidated, experimental evidence indicates that necrosis of adult neurons is mediated by increases of intracellular Ca^{2+} and is independent of caspases. Instead, cytosolic calpains and spilled lysosomal cathepsins are the major players in necrotic neuronal death.

Energy depletion is a potent trigger of necrosis. During ischaemic and hypoglycaemic episodes, lack of oxygen or essential nutrients leads to a shortage of energy needed to sustain the membrane potential. As a consequence depolarisation occurs, which results in massive release of the excitatory neurotransmitter glutamate at the synaptic clefts. This phenomenon is known in mammals as excitotoxicity and primarily involves the increase of intraneuronal calcium concentration. Excitotoxicity is not only related to acute neurodegenerative conditions such as ischaemia/anoxia, epilepsy, brain trauma or spinal cord injury, but also to chronic neurodegenerative conditions such us Alzheimer's disease [6].

Apart from excitotoxicity, other neurotoxic processes have been identified in mammalian systems that are involved in neuronal death. Acidification, which is a consequence of oxygen depletion, also plays an important role in necrotic neuronal death. Acidosis activates Ca^{2+}-permeable acid sensing ion channels (ASICs) resulting in glutamate receptor-independent neuronal injury due to Ca^{2+} toxicity [7]. Injection of ASIC1a blockers or knockout of the ASIC1a gene protects the brain from ischaemic injury more potently that glutamate antagonism.

Elevated intracellular Ca^{2+} is the most constant feature of neuronal death and can result in both apoptotic- and necrotic-like types of cell death. The contribution of each type of cell death seems to correlate with the severity of the insult. Calcium overload triggers lethal downstream reactions, including oxidative stress, mitochondrial dysfunction and calcium-dependent protease activation. Proteases can be considered as the executioners of cell death, and when a 'suicide' protease-cascade is set in motion, cell demise is the outcome. A wide variety of proteases are engaged in cell death processes through both non-specific and limited proteolysis. In the following sections we mainly focus on cytosolic cysteine-proteases (caspases and calpains) and lysosomal aspartyl-proteases (cathepsins) involved in necrotic cell death and neurodegeneration.

Caspases

Constituting a family of cysteine proteases which specifically cleave after aspartic residues in target proteins, caspases are the main executioners of apoptotic cell death. Apoptotic caspases are classified either as initiator caspases (caspase-2, -8, -9, and -10) or effector (or executioner) caspases, which include caspase-3, -6, and -7. Caspases are produced as inactive zymogens and must undergo activation during apoptosis. Whereas the activation of effector caspases is carried out through cleavage at specific internal Asp residues by an activated initiator caspase, the activation mechanism of initiator caspases is autocatalytic.

This activation triggers the apoptotic caspase cascade and is therefore tightly regulated, often requiring 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. Once activated, the executioner caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular targets, 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 signal transduction enzymes.

Caspase-mediated proteolysis appears to also play a role in necrosis. Caspases can activate calpain proteases by mediating degradation of calpastatin, an endogenous inhibitor of calpain and caspases may become activated by calpain proteases. Excess intracellular calcium results in mitochondrial damage and cytochrome c release, which might ultimately lead to caspase activation. Caspase-3 has been involved in neuronal death after transient cerebral ischaemia and the plasma-membrane Ca^{2+} pump in neurons is a substrate for caspase-mediated cleavage and inactivation. In addition, caspases might also contribute to the neuronal death associated with the formation of intracellular polypeptide aggregates.

Calpains

These are a family of calcium-dependent cysteine proteases that perform limited proteolytic cleavage of a variety of cellular substrates [8]. There are two ubiquitous isoforms of calpains, namely μ-calpain (calpain I) and m-calpain (calpain II) which are

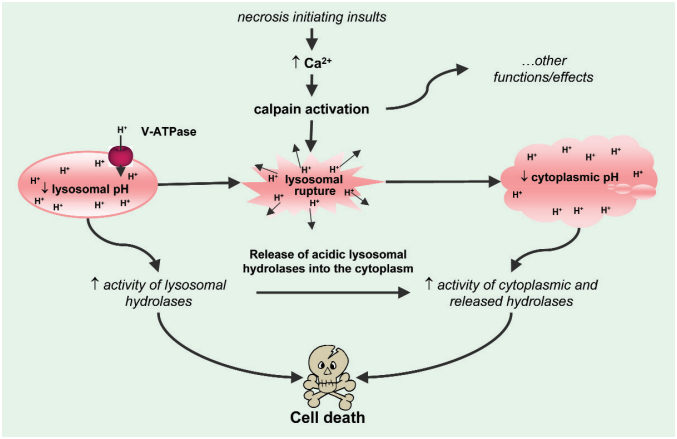

Figure 3. The calpain-cathepsin axis in necrotic cell death. Calpain mediated proteolysis may compromise the integrity of lysosomal membranes, inducing leakage of acidic contents and lysosomal proteases. These proteases coupled with concomitant cytoplasmic acidification contribute to cellular destruction during necrosis.

respectively activated *in vitro* by micro- and milli-molar concentrations of Ca^{2+} [9]. These isoforms are heterodimers composed of a distinct 80 kDa catalytic subunit and a common 30 kDa regulatory subunit with both the large and the small subunit containing multiple calcium-binding sites. Genetic deletion of the shared 30 kDa subunit results in embryonic lethality whereas deletion of the 80 kDa subunit of μ-calpain does not lead to severe phenotypes. This suggests that m-calpain plays an important role during early development. Additional functions have been ascribed to calpains in cell and growth cone motility and guidance. Mutations in Calpain III, a skeletal muscle-specific calpain, results in a recessive form of limb-girdle muscular dystrophy.

The role of calpains in neuronal cell death has been reported in a number of neuropathological conditions. Calpain activation has also been reported in a number of *in vivo* and cell culture models of apoptosis. For example, there is evidence of caspase-independent contribution of calpains to apoptotic events related to excitotoxicity [10].

Multiple studies have established the importance of calpain activation in acute cell injury and necrotic cell death triggered by calcium influx. 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. Genetic studies in *C. elegans* demonstrate the requirement of calpain activation for the execution of neurodegenerative cell death inflicted by various genetic lesions [11].

Cathepsins

The neuronal lysosomal system plays a key role in cell destruction. Two classes of lysosomal proteolytic enzymes seem to be the most active in neurodegeneration - aspartyl (cathepsin D) and cysteine (cathepsin B, H and L) proteases. Cathepsin proteases have been implicated in both intracellular proteolysis and extracellular matrix remodeling and play important roles in cellular processes by exerting degradation and regulatory functions. Lysosomal cathepsins play a role in both apoptosis and necrosis [12].

Several studies report differential expression of lysosomal cysteine proteases in models of neurological disorders. For example, lysosomal cathepsins B and L have been implicated in delayed neuronal death after global and focal cerebral ischaemia. Cathepsin D is involved in the execution of neuronal death induced by ageing, transient forebrain ischaemia and excessive stimulation of glutamate receptors during excitotoxicity. The requirement for Cathepsin D aspartyl protease activity in necrotic cell death and neurodegeneration has been demonstrated in *C. elegans*. Reduction of aspartyl protease activity by specific mutations, chemicals or starvation, protected against neurodegeneration inflicted by various insults [11].

Protease activation in neurodegeneration

What signals the proteases to dismantle the cell? The obligatory activator of calpains is the elevation of intracellular calcium concentration. Since elevation of intracellular calcium is the most ubiquitous feature in neurodegeneration, calpain activation represents a critical step in both apoptotic and necrotic neuronal death. 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. However, cytotoxic influx of Ca^{2+} results in excess calpain activation and cellular degeneration. Increases in intracellular Ca^{2+} can occur in response to diverse necrosis-initiating stimuli such as excess glutamate, acidosis or reactive oxygen/nitrogen species (ROS). It is conceivable that the degree of Ca^{2+} insult and ensuing degree of calpain activation will determine whether cells die by apoptosis or necrosis. Mild Ca^{2+} elevation results in apoptosis whereas acute calpain activation induces necrosis by the catastrophic cleavage of regulatory and structural proteins.The same holds true for cathepsins. Cathepsins play important roles for the development and functioning of the nervous system through regulated intracellular and extracellular proteolysis. However, the inappropriate activation of cathepsins has devastating consequences for cell viability.

What activates cathepsins or provokes their leakage from lysosomes? In the case of necrotic cell death, studies in primates indicate that injury to the lysosomal membrane is inflicted enzymatically by the action of activated calpains. Calpains localise to lysosomal membranes after the onset of ischaemic episodes with the subsequent spillage of cathepsins to the cytoplasm [13]. These observations led 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 dismantle the cell. Permeabilisation of lysosomal membranes has also been reported in apoptotic processes [3].

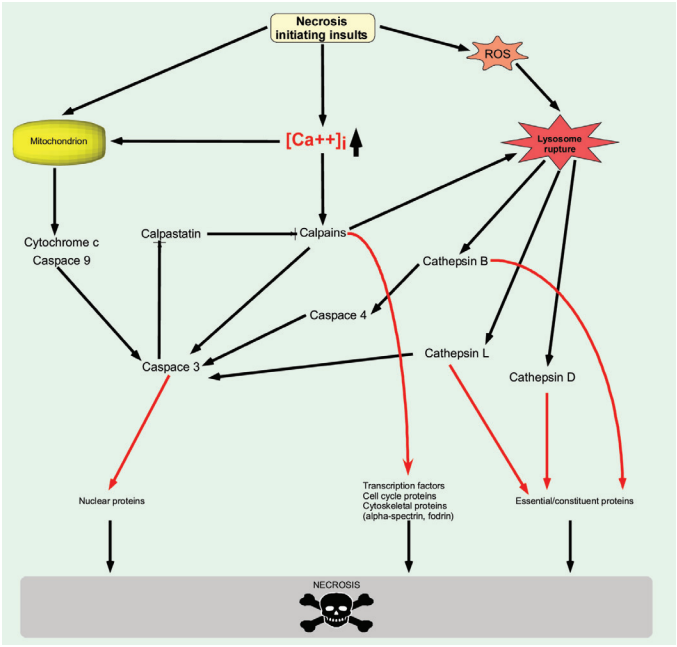

Figure 4. Proteolytic cascades mediating necrosis. Proteolytic pathways implicated in necrotic cell death. Necrotic insults converge to increase cytoplasmic calcium concentration ($[Ca^{2+}]i$). Calpain proteases play a central role in sensing excessive calcium levels and in turn initiating the death response by activating several classes of effector proteases such as cathepsins. In addition, calpains contribute directly to cell destruction by degrading essential structural components of the cell. ROS: reactive oxygen species.

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

Perspectives

Enzymatic proteolysis is involved in diverse neuropathological conditions including acute and chronic neurodegenerative diseases. Numerous investigations have revealed the participation of caspases, calpains, cathepsins and other proteases in both apoptotic and necrotic neuronal death. However, the molecular mechanisms governed by the activation of different proteases in neurodegenerative processes remain elusive. Several factors have hampered progress in the elucidation of the molecular pathways of enzymatic proteolysis during neurodegeneration, among them the fact that caspases and calpains cleave many common substrates including cytoskeletal and regulatory proteins, and also the extensive crosstalk among proteolytic systems. The ability of proteases to modulate signal transduction pathways further complicates the identification of their roles *in vivo* due to amplification of the effect. Another important issue is that synthetic inhibitors thought to be specific for a given protease have shown a broader inhibition range. For example, calpain-specific synthetic inhibitors act on other cysteine proteases, such as cathepsin B 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 [14]. Care should therefore be taken when drawing conclusions from experiments using synthetic inhibitors.

Disruption of calcium homeostasis plays a key role in neuronal injuries. It is therefore of great importance to elucidate the mechanisms of action of calpains, which are strictly regulated by intracellular calcium. The recent development of genetically engineered mice overexpressing the endogenous calpain inhibitor calpastatin, as well as the recent progress in imaging calpain protease activity in living mice will certainly contribute to increasing our understanding of the significance of calpain activation in different neurodegenerative processes *in vivo*.

As research in the field of neurodegeneration moves forward, the intricate network of signalling pathways and the variety of cell death mechanisms that are involved in neuronal degeneration is becoming apparent. However, growing knowledge about proteolytic events mediated by cytosolic, lysosomal and microglial proteases will not only contribute to better understanding the molecular pathways leading to neurodegeneration, but also help in the development of protease inhibitors as novel neuroprotective agents in our effort to battle neurodegenerative disorders.

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The author

Nektarios Tavernarakis, Ph.D.,
Institute of Molecular Biology and Biotechnology,
Foundation for Research and Technology,
Vassilika Vouton, PO Box 1385,
Heraklion 71110, Crete, GREECE
Tel +30 2810 39 1066 Fax +30 2810 39 1067
e-mail: tavernarakis@imbb.forth.gr