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Review

# $Ca^{2+}$-dependent proteases in ischemic neuronal death A conserved 'calpain–cathepsin cascade' from nematodes to primates

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**Abstract**

From rodents to primates, transient global brain ischemia is a well known cause of delayed neuronal death of the vulnerable neurons including cornu *Ammonis* 1 (CA1) pyramidal cells of the hippocampus. Previous reports using the rodent experimental paradigm indicated that apoptosis is a main contributor to such ischemic neuronal death. In primates, however, the detailed molecular mechanism of ischemic neuronal death still remains obscure. Recent data suggest that necrosis rather than apoptosis appear to be the crucial component of the damage to the nervous system during human ischemic injuries and neurodegenerative diseases. Currently, necrotic neuronal death mediated by $Ca^{2+}$-dependent cysteine proteases, is becoming accepted to underlie the pathology of neurodegenerative conditions from the nematode *Caenorhabditis elegans* to primates. This paper reviews the role of cysteine proteases such as caspase, calpain and cathepsin in order to clarify the mechanism of ischemic neuronal death being triggered by the unspecific digestion of lysosomal proteases.


*Keywords:* Hippocampus; Ischemic neuronal death; Caspase; Calpain; Cathepsin; Calpain–cathepsin hypothesis

## 1. Introduction

The human brain is a sophisticated and complex organ in which about 100 billion neurons are assembled in circuits working together. When a nerve impulse arrives in the presynaptic axon terminal, neurotransmitters are released from the synaptic vesicles into the synaptic cleft. Neurotransmitters then bind to specific receptors in the postsynaptic neuron, causing influx of extracellular $Na^+$ through membrane ion channels. The action potential (nerve impulse) is simply a brief reversal of the resting potential; the inside of the membrane becomes positively charged with respect to the outside within a thousandth of second.

The $Ca^{2+}$ concentration is approximately 1–2 mM at the synaptic cleft whereas and ~100 nM in the cytosol, thereby generating a 10,000–20,000-fold gradient between outside and inside of the synapse. In the hippocampal neurons, for instance, the presynaptic activation causes a release of glutamate into the synaptic cleft, which acts on the postsynaptic AMPA or NMDA receptors. At resting potential, $Na^+$ passes through AMPA receptor while NMDA receptor is blocked with $Mg^{2+}$ without $Ca^{2+}$ influx. At depolarized potentials the block is removed and $Ca^{2+}$ enters the postsynaptic neurone.

Under physiological conditions, the intracellular $Ca^{2+}$ concentration is tightly regulated. In contrast, under pathological conditions, regulatory mechanisms are overwhelmed and the intracellular $Ca^{2+}$ concentration increases abnormally via two main routes: influx from extracellular pools through various channels and release from endoplasmic reticulum stores. Disruption of intracellular $Ca^{2+}$ homeostasis has been implicated in various forms of neuronal death and neurodegeneration from nematodes to mammals [1–4].

Various proteins in the neuronal membrane including receptors and channels, give rise to the unique capabilities of neurons to transmit, receive and store information. The rough ER synthesizes such membrane proteins, neurotransmitters, cytoskeleton proteins and other constituent proteins. For the post-translational processing, degradation, recycling

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or turnover of the abundant proteins, lysosomal hydrolytic proteases are indispensable.

The lysosomal membrane is a physical barrier that prevents hydrolytic enzymes from digesting the cell's own cytoplasm, but its severe damage can cause cell necrosis. In 1966, de Duve and Wattiaux [5] reported that the lethal cell injury occurs by the release of hydrolytic enzymes from the damaged lysosomes. In the execution of neuronal necrosis, the mechanisms of abnormal protein degradation mediated by lysosomal proteases could conceivably play an important role. Cysteine proteases, a set of lysosomal enzymes, represent a broad class of proteolytic enzymes widely distributed among living organisms. Cysteine proteases are actually recognized as multi-function enzymes, being involved in the processing and presentation of antigens, cleavage of membrane-bound proteins, degradation of the cellular matrix, and in the processes of tissue remodeling. Here, the role of $Ca^{2+}$-dependent cysteine proteases in the execution of neuronal necrosis will be reviewed.

## 2. Different mode of neuronal death

Necrosis and apoptosis are two distinct forms of cell death which have, essentially, different implications for the surrounding tissue. Necrosis occurs ATP-independently in the process of neurodegeneration, provoking damage to the tissue with spillage of the intracellular contents into the extracellular milieu. In contrast, apoptosis occurs ATP-dependently, without provoking inflammation and damage to the tissue. Further, apoptosis is programmed and essential for the normal development, shaping of organs and tissues, and homeostatic mechanisms [6–8]. In contrast, necrosis is unanticipated and inappropriate destruction of a cell, that is caused by certain stressful or abnormal conditions exceeding a certain threshold such as stroke, excessive mechanical strain (trauma) and genetic abnormalities underlying neurodegenerative diseases [9–13].

During development of the brain, neuronal death occurs mainly by caspase-dependent apoptosis [2,14] or autophagy, morphologically and mechanistically distinct [15]. In the adult brain, however, caspase-dependent apoptosis should be essentially minor [2]. In the animal models of neurodegeneration, the dominant forms of neuronal death are dark neuronal death in Huntington's disease [16] or paraptosis in amyotrophic lateral sclerosis [17]. Paraptosis is characterized by the extensive cytoplasmic vacuolation without prominent chromatin condensation. In this process, the morphology is similar to necrosis while the cascade of de novo protein synthesis is similar to apoptosis. Excitotoxic neuronal death due to cerebral ischemia or traumatic brain injury, may show many shapes and activate different cell death programmes. Necrotic rather than apoptotic cascade [18,19] or mixed apoptotic–necrotic cascades may be involved, depending on the intensity of insult, the age of subjects, and the brain region affected [20,21]. For instance, in the ischemic core, caspases are inactivated because of the rapid ATP depletion, impairment of the intracellular ion composition, massive production of nitric oxide or superoxide radicals as well as calpain activation [22–28]. The sustained calpain activation in the postischemic CA1 neurons caused long-standing lysosomal membrane disruption with the resultant leakage of various hydrolytic enzymes including cathepsins B, L and DNase II, processes that start immediately after ischemia and last until day 5 [19].

Although there are many reports of apoptotic death of neurons in the ischemic stroke models using adult animals, relatively few of them have been based on detection of the characteristic morphological features of apoptosis. Histological examination of hippocampus in a 63-year-old female dying 9 days after heart surgery undergoing transient cardiac arrest, showed an almost complete neuronal death specifically in the CA1 sector [28]. Here, all of the dying CA1 neurons showed a typical eosinophilic degeneration which was characterized by the shrunken eosinophilic cytoplasm and the nucleus with a coarse tigroid and thin chromatin distribution. However, there was no morphological evidence of apoptosis. In the postischemic CA1 neuronal death of monkeys [18,29–31], light microscopy showed similar eosinophilic coagulation necrosis, while electron microscopy showed frank membrane disruptions with punctuated chromatin condensation. Furthermore, DNA gel electrophoresis showed not ladder but smear pattern [18,19]. Accordingly, it is likely that even if the apoptosis cascade was actually activated, the final neuronal death pattern was that of necrosis.

Despite the significant impact of necrotic neuronal death in human brain injury and neurodegenerative diseases, the mechanism of neuronal necrosis remained poorly understood until recently. As models of neuronal necrosis and neurodegeneration became available, from *C. elegans* [32] to monkeys [28,29,33], a common denominator becomes increasingly clear. In contrast to the events taking place during apoptosis, in necrosis it is not the mobilization of molecular mechanisms but the excessive operation of the physiological cellular mechanisms that appear to execute neuronal necrosis process under the exceptional cell conditions that lead to extensive damage.

## 3. Three cysteine proteases

### 3.1. Caspase

In animal models of neuronal death, apoptosis of neurons has been often demonstrated only by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labelling (TUNEL) without detecting the characteristic morphological features of apoptosis. Accordingly, there are many reports that conclude that neuronal apoptosis after ischemia is mediated by cysteine-

requiring aspartate-directed proteases, caspases. However, the TUNEL technique was reported to label also cells undergoing necrosis [34–36]. Thus, even if caspases are activated, it is probable that they contribute more to neuronal necrosis than to neuronal apoptosis.

Caspases are mammalian homologues of the pro-apoptotic *C. elegans* CED-3 protein. In their catalytic site caspases contain the cysteine-containing pentapeptide motif QACXG (X being R, Q or G) and require an aspartate residue at the N-terminal end of the substrate cleavage site [37–41]. Caspases are synthesized as inactive proenzymes (procaspases) that comprise an N-terminal prodomain, a large subunit, and a small subunit. Activation results from the proteolytic cleavage of procaspases into its three component parts through the action of other activated caspases. Subsequently, two large and two small subunits associate to form the heterotetrameric active enzyme. The substrates cleaved by caspases include cytoskeletal and associated proteins, kinases, members of the Bcl-2 family of apoptosis-related proteins, presenilins and amyloid precursor protein, and DNA-modulating enzymes. Many of the substrates of caspases are localized in the pre- and/or post-synaptic compartments of neurons.

Caspases are believed to be central components for the implementation of neuronal apoptosis [42]. Caspase-3 was demonstrated to be overexpressed in CA1 after transient ischemia, and its specific inhibitor could attenuate ischemic neuronal death [43,44]. In the monkey experimental paradigm of cerebral ischemia also [18,19], caspase-3 activation occurred a few hours after ischemia, but its activation became negligible despite up-regulation of pro-caspase-3 on days 3–5. The caspase-activated DNase (CAD)/inhibitor of CAD (ICAD) complex was identified to be a substrate of caspase-3 [45–47]. When the catalytically-active caspase-3 cleaves ICAD, the final effector CAD transfers into the nucleus to cause DNA degradation [46–48]. Cao et al. [49] reported that transient global ischaemia in rats caused caspase-3-mediated cleavage of ICAD, resulting in the apoptotic degradation of DNA by CAD. Although expression of CAD was slightly up-regulated on days 1 and/or 2 with translocation of activated CAD on days 2–3 in the monkey experimental paradigm [18,19], the extent of CAD expression was actually much less compared to lymph node or intestine tissues. It is likely that CAD has partially participated in DNA degradation of the postischemic CA1 neurons in monkeys. However, as CAD up-regulation was mild and occurred transiently on days 1–2 in monkeys, calpain activation lasting as long as 5 days appears to be a rather critical factor for the CA1 neuronal death being completed on days 3–5 [19].

### 3.2. Calpain

The calcium-dependent neutral cysteine protease, calpain, is present in virtually all vertebrate cells [50,51]. Two isozymes: μ- and m-calpains show similar biochemical features, except for the $Ca^{2+}$ concentration necessary for activation in vitro: μ- or m-calpains require micromolar or milimolar levels of $Ca^{2+}$, respectively. Calpain is a heterodimer comprising a 30-kDa regulatory subunit and another 80-kDa catalytic subunit. During activation, the 30-kDa subunit is cleaved to yield a final 17-kDa form, while the 80-kDa subunit is converted to 76-kDa enzymatically active form. Calpain is activated both in physiological states and also during various pathological conditions such as phosphorylation [52], free radicals [53,54], brain ischemia-reperfusion [19,29], apoptosis [53,55], cataractogenesis [56], muscular dystrophy [57], and Alzheimer's [58] and Parkinson's [59] diseases. The substrates cleaved by μ-calpain include cytoskeletal and associated proteins, kinases and phosphatases, membrane receptors and transporters, and steroid receptors. Calpains are located throughout the neuron, both in the somatodendritic regions and in the axons. Excessive activation of calpain due to an increase in free $Ca^{2+}$ leads to cytoskeletal protein breakdown, subsequent loss of structural integrity and disturbances of axonal transport, and finally to neuronal death.

What is the in vivo substrate of activated μ-calpain in the postischemic CA1 neurons? As calpain cleaves the substrate protein without binding, one cannot detect the in vivo substrate of activated calpain, for example, by means of immunoprecipitation. Further, regarding the cleavage site specificity of calpain, there are suggestions that the PEST sequence is preferred, as generally indicated, but this issue is not firmly confirmed. It was recently speculated that calpain recognizes the conformational state rather than the protein sequence; relatively unstructured inter-domain sequences without α-helix or β-sheet seem to be good targets [60]. Therefore, as for most proteases, it is either impossible or not relevant to predict a calpain cleavage site in a given protein only based on the sequence information. Accordingly, instead of the use of biochemical procedures, we have demonstrated, by means of immunoelectron microscopy, the localization of activated μ-calpain at the lysosomal membrane [28,29]. Recently, we also reported sustained (i.e., from immediately after the ischemic challenge until day 5) activation of μ-calpain in the postischemic CA1 neurons [19]: the immunoreactivity of activated μ-calpain became maximal on days 2–3, being remarkable in lysosomes on day 2 while in the cytoplasm on day 3 (Fig. 1). The subsequent translocation of lysosomal cathepsins [28,33] as well as lysosome-associated membrane protein-1 (LAMP-1) (Fig. 2) indicated that activated μ-calpain caused spillage of hydrolytic enzyme cathepsins from lysosomes [19].

In neurological events associated with cerebral ischemia, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, a potential role of reactive oxygen species has been reported [61,62]. Another possible mechanism of lysosomal membrane rupture might be the damage induced

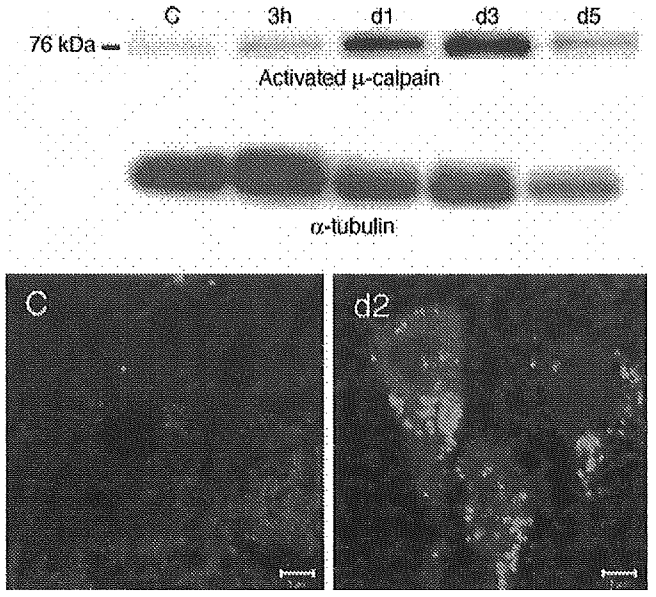


Fig. 1. Immunoblot analysis (upper) and immunofluorescent confocal images (lower), using antibody recognizing selectively activated μ-calpain. Upper: The expression of the internal control protein α-tubulin shows a gradual decrease on days 1–5 (d1, d3, d5) with neuronal degeneration. In contrast, activated μ-calpain is up-regulated from 3 h after ischemia until day 5 (d5), and is maximal on day 3 (d3) in the postischemic CA1 sectors. Lower: In the control CA1 neurons (C), the perikarya shows negligible immunostaining of activated μ-calpain. In contrast, on day 2 (d2) activated μ-calpain is immunostained as coarse granules with FITC in the perikarya. Scale bar = 5 μm.

by free radicals that are generated during the oxidative stress. Then, as a consequence of exposure to reactive oxygen species, oxidative stress may induce further calpain activation through rapid $Ca^{2+}$ mobilization either by stimulating $Ca^{2+}$ influx from outside or by increasing $Ca^{2+}$ release from the internal stores [63,64].

### 3.3. Cathepsin

Brunk et al. [65] suggested a quantitative relationship between the amount of lysosomal rupture and the mode of cell death: low intensity stresses would trigger a limited release of lysosomal enzymes to the cytoplasm followed by apoptosis, whereas high intensity stresses would provoke a generalized lysosomal rupture followed by necrosis. The lysosomal membrane is a physical barrier preventing hydrolytic enzymes from digesting the cell constituent proteins, but its severe disruption can cause cell necrosis in the pathologic states. The spreading of hydrolytic enzymes into the cytoplasm through the lysosomal membrane injury or rupture, was confirmed in both heart [66–68] and brain [25,33,69] ischemic injuries. It is most likely that the sustained calpain activation in the postischemic CA1 neurons, presumably with the aid of reactive oxygen species, may cause lysosomal membrane disruption with the resultant leakage of various hydrolytic enzymes (Fig. 3) [19,28].

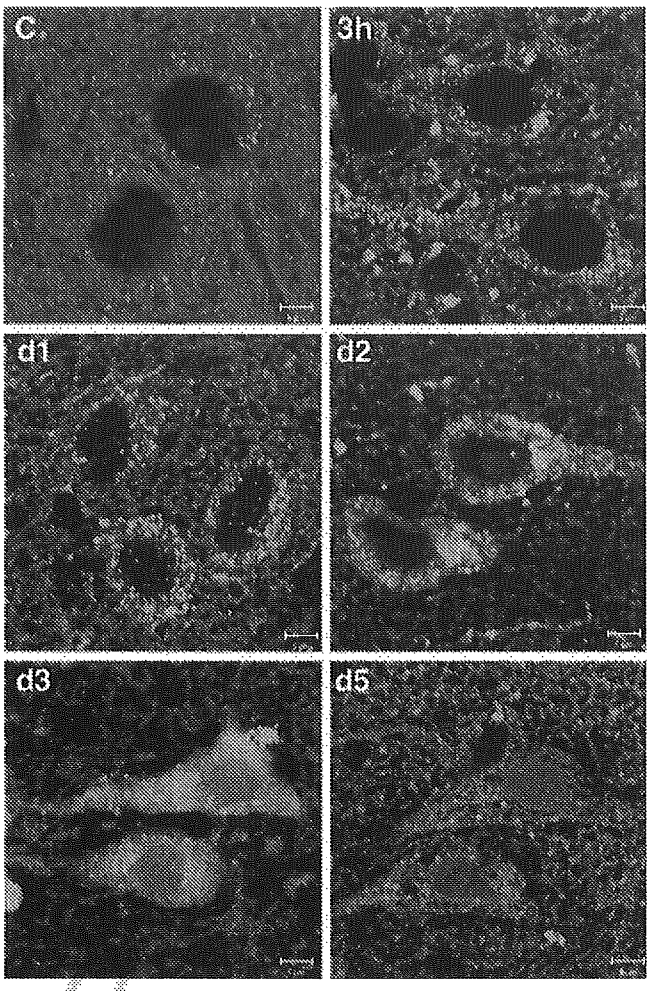


Fig. 2. Immunofluorescent confocal images of the lysosome-associated membrane protein (LAMP)-1 in the representative CA1 neurons of the control (C), immediate after ischemia (3 h), days 1 (d1), 2 (d2), 3 (d3) and 5 (d5). The LAMP-1 immunoreactivity is up-regulated in the perikarya from 3 h until day 5 (d5). Coarse granular staining becomes maximal on day 2 (d2), while the perikarya and the nucleus are diffusely stained on day 3 (d3). On day 5 (d5), the immunoreactivity decreases with neuronal degeneration. Scale bar = 5 μm

Lysosomes contain over 80 types of hydrolytic enzymes. Two classes of lysosomal hydrolytic enzymes appear to be most active in executing neuronal death: aspartyl (cathepsin D) and cysteine (cathepsins B, H, L) proteases. The former are characterized by the presence of a catalytic aspartic amino acid residue at their active site, while the latter are characterized by a catalytic cysteine residue at their active site. Cathepsin D mediates execution of neuronal death induced by ageing, transient forebrain ischemia and excitotoxicity [70] while cathepsins B and L execute hippocampal neuronal death after global ischemia [28].

A new L-*trans*-epoxysuccinyl peptide, CA-074 (*N*-(L-3-trans-carboxyoxirane-2-cabonyl)-L-isoleucyl-L-proline) was shown to inhibit cathepsin B 10,000–30,000-fold stronger than cathepsins H or L [71,72]. E-64c (*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucine-3-methylbutylamide),

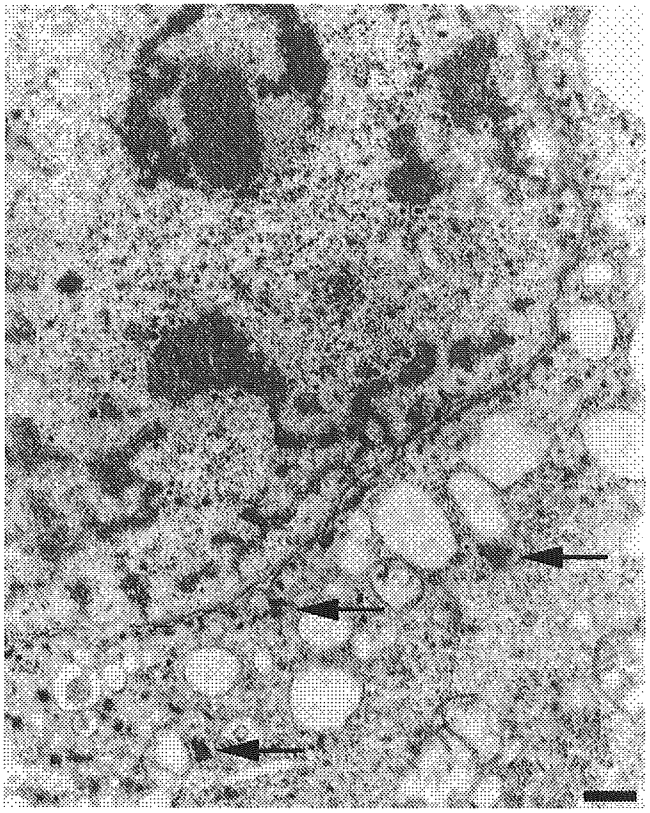

Fig. 3. Electronmicrograph of a CA1 neuron 2 days after 20 min whole brain complete ischemia. In the cytoplasm close to the nuclear membrane, there are numerous electron-dense granules (arrows) that were released from the lysosomes. Among the punctuated chromatin condensation are similar electron-dense granules. Scale bar = 1 μm.

the terminal agmatine of E-64 being replaced by isoalrylamide, shows strong inhibitory activity in vivo. E-64c has been demonstrated to inhibit cathepsins H and L as well as cathepsin B [73,74]. Concerning the kinetics [71–75] of each inhibitors, the $K_i$ of CA-074 for cathepsin B, as estimated by Dixon plots, was $2.0 \times 10^{-9}$ M, whereas the $K_i$ for cathepsins H and L was 75,000 and $233{,}000 \times 10^{-9}$ M, respectively. In contrast, the $K_i$ of E-64c for cathepsins B, H and L was, as estimated by Dixon plots, 8.7, 111 and $3.5 \times 10^{-9}$ M, respectively. The inhibitory effect of delayed neuronal death by E-64c was overall more remarkable than that of CA-074. This is probably because E-64c can inhibit not only cathepsins B and L but also calpains.

## 4. The calpain–cathepsin hypothesis

The neuroprotective effects of certain caspase inhibitors conceivably depend on mechanisms other than the inhibition of caspases, because the tetrapeptide inhibitor tyrosine–valine–alanine–aspartate–chloromethyl ketone (Ac-YVAD-cmk) was reported to rescue cultured neurons from cell death due to oxygen/glucose deprivation, by targeting lysosomal enzyme 'cathepsin B' [76]. Accordingly, to clarify the exact molecular mechanism of ischemic neuronal death, the contribution of cathepsin-mediated necrotic cascade should be studied in detail with particular attentions to the role of lysosomes.

'Calpain–cathepsin hypothesis' [33] was formulated on the basis of the experimental paradigm of global brain ischemia in the monkeys, and encompases two major players, calpain and cathepsin, as the key mediators (Fig. 4). First, an increase in the intracellular $Ca^{2+}$ mobilization occurs in response to the ischemic insult. Second, μ-calpain is activated as long as $Ca^{2+}$ concentration is elevated. Third, as activated μ-calpain compromises the integrity of lysosomal membranes, cathepsin proteases are liberated into the cytoplasm to induce breakdown of the cell constituent proteins. This process is reminiscent of autophagy, and supports de Duve's concept [77] of lysosomes as the cell's 'suicide bag'. Kitao et al. [78] confirmed the involvement of a similar cascade in the cultured hippocampal neurones, in which both kainate and glutamate induced the activation of μ-calpain and cathepsin B.

In addition to the ischemic injuries in the heart [66–68] and brain [25,69], the translocation of cathepsin B from lysosomes into the cytosol and nucleus was reported also for the bile salt-induced and TNF-triggered hepatic apoptosis. Similarly, Foghsgaard et al. [81] found that cathepsin B, which disappeared from the perinuclear granules (colocalizing with lysosomal markers) and distributed diffusely throughout the cell, is capable of acting as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. Using the monkey experimental paradigm, translocations of cathepsins B and L [28,33] as well as DNase II [18], were already suggested by immunohistochemistry in the postischemic CA1 neurons. It is suggested from these data that the sustained calpain activation in the postischemic CA1 neurons may cause long-standing lysosomal membrane disruption with the resultant consecutive leakage of lysosomal enzymes including cathepsins B, L and DNase II from immediately after ischemia until day 5. Based on the calpain–cathepsin hypothesis, Yoshida et al. [31] demonstrated in the monkey brain other than CA1 that 89.8% of caudate nucleus neurons were free from postischemic neuronal death on day 5 with 4 mg/kg of CA-074 treatment, while 75.0% of the cortical V layer neurons and 91.6% of the cerebellar neurons survived with 4 mg/kg of E-64c treatment.

Recently, Syntichaki et al. [32] reported that neuronal degeneration induced by various genetic lesions in *C. elegans* required the activity of the calcium-regulated CLP-1 and TRA-3 proteases (similar to calpains) as well as aspartyl proteases ASP-3 and ASP-4 (similar to cathepsins). The genes for calpain and lysosomal proteases have been detected in genetic screens for suppressors of neurodegeneration in *C. elegans*. This is encouraging, considering that the sophisticated genetics and molecular biology of the *C. elegans* neurodegeneration models have confirmed the ba-

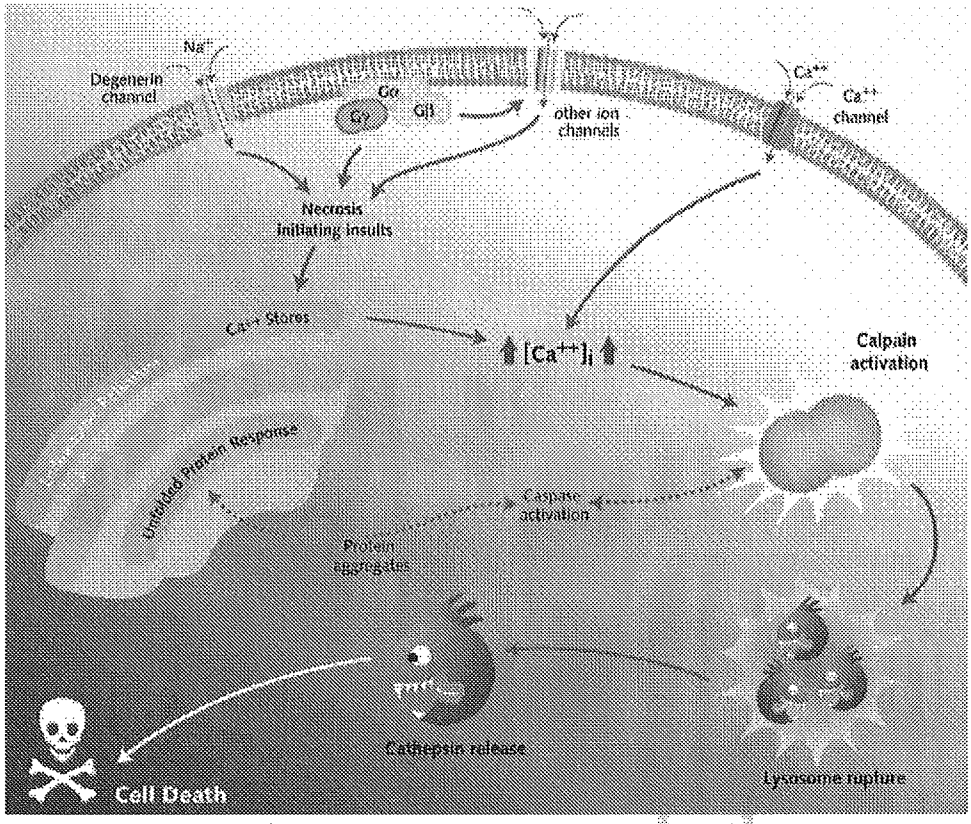


Fig. 4. Calcium-induced calpain–cathepsin cascade as a mechanism of ischemic neuronal death ('calpain–cathepsin hypothesis' formulated in [33]). The necrosis initiating insults may provoke intracellular $Ca^{2+}$ mobilization through uptake of extracellular $Ca^{2+}$ and/or release from internal stores. $Ca^{2+}$ mobilization subsequently induces lysosomal rupture presumably with the aid of reactive oxygen species. The released cathepsin proteases degrade cell constitutive proteins leading to the neuronal death (cited from EMBO Rep. 3 (2002) 604–609).

sic concept of the calpain–cathepsin hypothesis, originally proposed for the monkey experimental paradigm [33].

## 5. Cross-talks between apoptosis and necrosis, and between ischemia and Alzheimer

The postischemic neuronal death may involve a combination of apoptotic and necrotic processes even at the level of the individual neuron [12,82,83]. Although paradoxically, it is likely that caspase-mediated proteolysis contributes to neuronal necrosis by the cleavage and inactivation of the plasma membrane $Ca^{2+}$ pump [84]. This, in turn, disrupts intracellular $Ca^{2+}$ homeostasis with the resultant $Ca^{2+}$ overload, and further stimulates the calpain–cathepsin cascade through the sustained calpain activation [19]. Cleavage of the calpain inhibitor, calpastain by caspase-3 [85] might also stimulate calpain activation. Furthermore, cathepsins have been reported to activate caspase-3, either directly or indirectly [28,86]. Then, it is possible that caspases can be inappropriately activated, and participate to the initial phases of necrotic cascade, being isolated from the final phase of the apoptotic cascade. Accordingly, neither any discernible apoptotic morphology nor DNA ladder were seen in the postischemic CA1 neurons of monkeys despite cleavage and translocation of CAD [18,19].

Interestingly, in the pathogenesis of Alzheimer's disease, several reports pointed to an important role of calpains [87–92]. Widespread activation of μ-calpain in the Alzheimer brain has been demonstrated previously by biochemical methods [89]. Taniguchi et al. [91] analyzed expression of activated μ-calpain in human brain extracts by comparing eight Alzheimer patients (M:F = 2:6, mean 82 years old) with nine age-matched controls (M:F = 5:4, mean 77 years old). Intense bands of activated μ-calpain were consistently seen in the Alzheimer brain. Further, the band intensities of activated μ-calpain were about sevenfold ($P < 0.05$) in the Alzheimer brains compared to the control brains (Fig. 5) [91]. Previous studies showed that the populations of degenerating neurons in Alzheimer's disease exhibit robust up-regulation of the lysosomal system [92]. Then, it is probable that the calpain-mediated lysosomal spillage of hydrolytic enzymes might occur also in the Alzheimer neurons, and can explain the mechanisms of neuronal degeneration in Alzheimer's disease. It is probable that the mechanism of necrotic neuronal death should be conserved in spite of the diversity of pathologic conditions that initiate cell death.

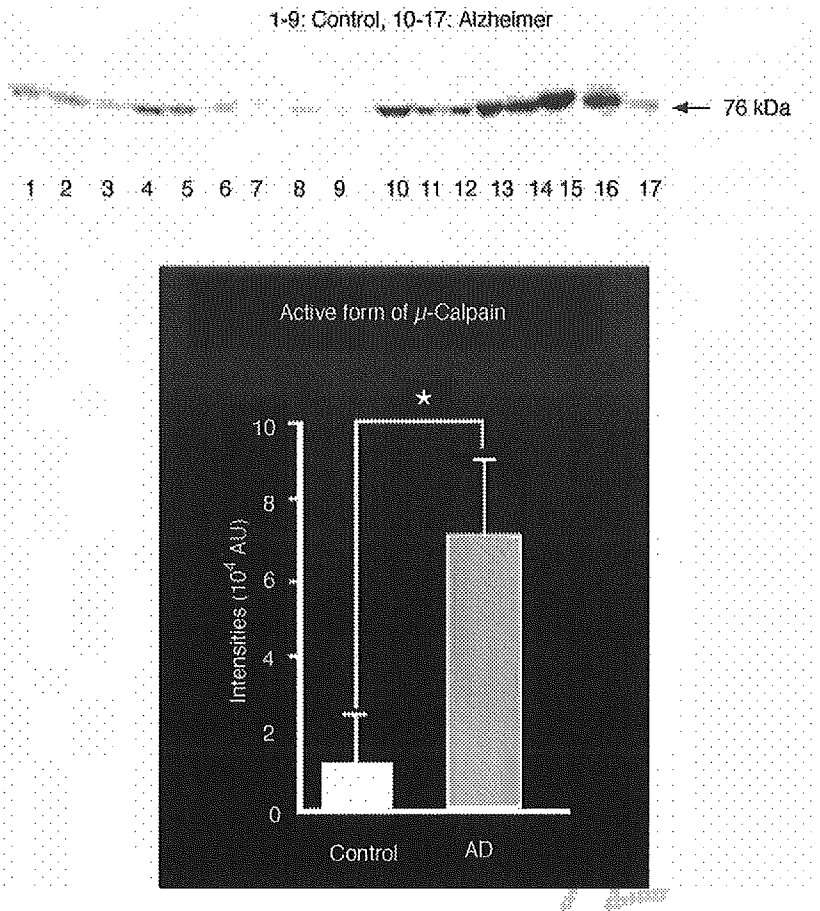


Fig. 5. Expression of activated μ-calpain in human brain extracts by comparing eight (10–17) Alzheimer patients with nine (1–9) age-matched controls. Intense bands of activated μ-calpain were consistently seen in the Alzheimer brain (upper), and its band intensities (AD) were about seven-fold ($^{*}P < 0.05$) compared to the control brains (lower) (cited from [91]).

## 6. Conclusion

Now, it is becoming widely accepted that the lysosomal system emerges as one of the main players during the final stage of neuronal necrosis. Spillage of hydrolytic enzymes from lysosomes into the cytoplasm due to the activated μ-calpain-mediated lysosomal membrane disruption, presumably with the aid of reactive oxygen species, executes neuronal necrosis or degeneration from *C. elegans* to primates.

## Uncited references

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