45 Murphy, L.L. *et al.* (1990) Psychoactive and non-psychoactive cannabinoids and their effects on reproductive neuroendocrine parameters. In *Biochemistry and Physiology of Substance Abuse* (Vol. 2) (Watson, R.R., ed.), pp. 73–94, CRC Press
46 Specht, L.A. *et al.* (1981) Light microscopic immunocytochemical localization of tyrosine hydroxylase in prenatal rat brain. I. Early ontogeny. *J. Comp. Neurol.* 199, 233–245
47 Insel, T.R. (1995) The development of brain and behavior. In *Psychopharmacology: The Fourth Generation of Progress* (Bloom F.E. and Kupfer D.J., eds), pp. 683–694, Raven Press
48 Levitt, P. *et al.* (1997) New evidence for neurotransmitter influences on brain development. *Trends Neurosci.* 20, 269–274
49 Berger, B. *et al.* (1987) Transient expression of tyrosine hydroxylase immunoreactivity in some neurons of the rat neocortex during postnatal development. *Dev. Brain Res.* 23, 141–147
50 Pares-Herbuté, N. *et al.* (1989) Ontogeny of the metencephalic, mesencephalic and diencephalic content of catecholamines as measured by high performance liquid chromatography with electrochemical detection. *Int. J. Dev. Neurosci.* 7, 73–79
51 Fride, E. and Mechoulam, R. (1996) Developmental aspects of anandamide: ontogeny of response and prenatal exposure. *Psychoneuroendocrinol.* 21, 157–172
52 Fride, E. and Mechoulam, R. (1996) Ontogenic development of the response to anandamide and Δ^9-tetrahydrocannabinol in mice. *Dev. Brain Res.* 95, 131–134
53 Goldman, S.A. and Luskin, M.B. (1998) Strategies utilized by migrating neurons of the postnatal vertebrate forebrain. *Trends Neurosci.* 21, 107–113
54 Jacobson, M. (1993) *Developmental Neurobiology*, Plenum Press
55 Howlett, A.C. *et al.* (1990) The cannabinoid receptor: biochemical, anatomical and behavioral characterization. *Trends Neurosci.* 13, 420–423
56 Venance, L. *et al.* (1995) Inhibition by anandamide of gap junctions and intercellular calcium signalling in striatal astrocytes. *Nature* 376, 590–594
57 Sagan, S. *et al.* (1999) Anandamide and WIN-55,212-2 inhibit cyclic AMP formation through G-protein-coupled receptors distinct from CB_1 cannabinoid receptors in cultured astrocytes. *Eur. J. Neurosci.* 11, 691–699
58 Di Marzo, V. *et al.* (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* 372, 686–691
59 Guthrie, S.C. and Gilula, N.B. (1989) Gap junctional communication and development. *Trends Neurosci.* 12, 12–16

Calpain and caspase: can you tell the difference?

Kevin K.W. Wang

Both necrotic and apoptotic neuronal death are observed in various neurological and neurodegenerative disorders. Calpain is activated in various necrotic and apoptotic conditions, while caspase 3 is only activated in neuronal apoptosis. Despite the difference in cleavage-site specificity, an increasing number of cellular proteins are found to be dually susceptible to these cysteine proteases. These include α- and β-fodrin, calmodulin-dependent protein kinases, ADP-ribosyltransferase (ADPRT/PARP) and tau. Intriguingly, calpastatin is susceptible to caspase-mediated fragmentation. Neurotoxic challenges such as hypoxia–hypoglycemia, excitotoxin treatment or metabolic inhibition of cultured neurons result in activation of both proteases. Calpain inhibitors can protect against necrotic neuronal death and, to a lesser extent, apoptotic death. Caspase inhibitors strongly suppress apoptotic neuronal death. Thus, both protease families might contribute to structural derangement and functional loss in neurons under degenerative conditions.

Trends Neurosci. (2000) 23, 20–26

Kevin K.W. Wang is at the Dept of Neuroscience Therapeutics, Parke-Davis Pharmaceutical Research, Warner-Lambert Company, Ann Arbor, MI 48105, USA. (E-mail: kevin.wang@wl.com)

APOPTOSIS, or programmed cell death, is a form of physiological cell death characterized usually, but not always, by the presence of DNA condensation in the nuclei, DNA fragmentation at the nucleosome linkage regions, cell shrinkage and the ultimate formation of apoptotic bodies[1]. It is used by the organism during development and at other stages to eliminate unwanted cells. Necrosis usually occurs when cells are injured by extreme physical stress or chemical challenges to the point where they are beyond repair, and is characterized by the presence of massive ion influx, mitochrondrial swelling, cell swelling, nonspecific DNA breakage in the nuclei and, ultimately, plasma-membrane rupture[1]. Unscheduled apoptosis might also be triggered when cells face some certain physical (for example, hypoxia) or chemical challenges (for example, toxins). It is also conceivable that other forms of cell death exist but have not yet been identified.

Apoptosis and necrosis in neurological and neurodegenerative disorders

About five years ago, it was generally accepted that acute degenerative neuronal death, such as that seen in cerebral ischemia, traumatic brain injury (TBI) and spinal-cord injury (SCI) were necrotic in nature. Linnik and colleagues were the first to challenge this idea by showing evidence for apoptosis in a rat focal-ischemia model (defined by the presence of DNA laddering)[2]. Since then, numerous reports have documented similar findings in either global ischemia or excitotoxicity models[3,4]. With respect to excitotoxicity, Bonfoco *et al.* reported that high and low levels of excitotoxin challenge to neuronal cultures resulted in necrosis and apoptosis, respectively (on the basis of nuclear morphology)[5]. Subsequently, evidence for apoptotic neuronal death was confirmed in experimental TBI (Ref. 6) as well as in SCI (Ref. 7). In parallel with this, apoptotic

0166-2236/99/$ – see front matter © 2000 Elsevier Science Ltd. All rights reserved. PII: S0166-2236(99)01479-4

neuronal death has also been reported in various chronic neurodegenerative conditions, such as Huntington's disease (HD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and AIDS-associated dementia[8]. Yet, other researchers have evidence that does not support apoptotic neuronal death as having a major role in models of cerebral ischemia and glutamate toxicity[9], and therefore apoptotic and necrotic cell death are both probably occurring in these disorders. Recent studies have confirmed that in cultured neurons, neurotoxic challenges, such as hypoxia or hypoglycemia, neurotoxin administration [excitotoxin, 1-methyl-4-phenylpyridinium (MPP+)], metabolic inhibition (for example, 3-nitropropionic acid), and β-amyloid peptide or NO treatment could also result in both neuronal necrosis and apoptosis[5,8,10–12].

Calpain in necrotic death

Over the past decade, the significant focus of research was on calpain-mediated proteolysis, and its contribution to necrotic neuronal death in ischemic and excitotoxic neuronal injury[13]. The two ubiquitous calpains m- and μ-calpain) exist as a pro-enzyme heterodimer (80 kDa–29 kDa) in resting cells but this is activated by Ca^{2+} and autolytic processing (to produce a heterodimer 78 kDa–18 kDa; Table 1). Physiologically, the activity of these calpains might also be regulated by the endogenous protein inhibitor calpastatin (Table 1). Calpains could become over-activated under extreme conditions that result in sustained elevation of cytosolic Ca^{2+} levels, which is generally associated with necrosis. For example, in Ca^{2+}-ionophore-treated cells (such as A23187-treated Molt 4 cells), Ca^{2+} rushes into the cells through the pore-forming compound, A23187. In glutamate-treated rat cerebrocortical neurons, Ca^{2+} enters through the ionotropic glutamate receptors. Similarly, maitotoxin (MTX), a potent marine toxin that opens both voltage- and ligand-gated Ca^{2+} channels, also induces rapid and massive Ca^{2+} influx and subsequent calpain activation[14]. The mode of cell death induced by MTX has been investigated, but no DNA laddering or condensation has been found. General DNA degradation and dissolution, rather than condensation, were observed: properties that are associated with necrosis.

Calpain cleaves preferentially at Val, Leu or Ile residues in the P2 position of its target proteins, whereas the amino acids at the P1 site of target proteins are rather diverse (for example, Tyr, Gly, Arg) (Table 1). As expected, most proteins contain sites that are susceptible to the actions of calpain, yet only a small subset of cellular proteins have been reported to be vulnerable, while the majority of cellular proteins are resistant. In addition, unlike digestive proteases (such as trypsin), calpain substrates are cleaved into 'limited fragments' without further degradation[15] (Table 1). Calpain substrates include cytoskeletal, plasma-membrane-associated proteins (such as epidermal growth-factor receptor, platelet-derived growth-factor receptor), and signal transduction and calmodulin-dependent proteins and transcription factors (for a review, see Refs 13,16). Under limited calpain activation, the proteolytic modification of one or more of these substrate proteins might transduce a cellular signal or trigger cellular functions, such as membrane fusion or cell spreading. However, owing to the combined loss or alternation of function of these substrates, overactivation of calpain can be detrimental.

TABLE 1. Comparing and contrasting the properties of caspase 3 and calpain

	Caspase 3	Calpain
Protease class	Cysteine protease	Cysteine protease
Endogenous inhibitor(s)	IAP1, IAP2, NAIP	Calpastatin
Resting mode	Inactive pro-enzyme (32 kDa)	Inactive pro-enzyme (80 kDa + 29 kDa)
Activation mode	Proteolytic processing (to 17 kDa + 12 kDa)	Ca^{2+}, then autolytic processing (to 78 kDa + 18 kDa)
Preferred cleavage site (*)	AspxxAsp*x	(Leu, Val, Ile)x*x
Endogenous substrates	Subset of cytoskeletal, cytosolic and nuclear proteins or enzymes	Subset of cytoskeletal, cytosolic and nuclear proteins or enzymes
Consequence of substrate proteolysis	Produces limited fragment(s), sometime proteolytic activation	Produces limited fragment(s) sometimes with proteolytic activation
Cell-death involvement	Most forms of apoptosis	Most forms of necrosis, some forms of apoptosis
Inhibitors as neuroprotectants	Yes	Yes

Abbreviations: IAP, inhibitor of apoptosis protein; NAIP, neuronal apoptosis inhibitory protein.

Caspases in apoptosis

The discovery that a protein required for apoptosis in the nematode, *Caenorhabditis elegans*, CED-3 is homologous to the mammalian interleukin-1β-converting enzyme (ICE) led to the discovery of a large number of ICE-like proteases (renamed caspases) and their roles as mediators of apoptosis in a wide range of the cell types[17]. Caspase 3 (previously called CPP32) is of particular interest as it appears to be a common downstream apoptosis effector. It exists as proenzyme (pro-caspase 3) in most cells, including neurons, and is processed and activated by caspase 9 or caspase 8 to the heterodimeric form (17 kDa–12 kDa) by two distinct pathways[17]. First, in a mitochrondria-dependent pathway, the complexing of mitochrondrially released cytochrome C and apoptotic protease-activating factor 1 (APAF1) to caspase 9 leads to its dimerization and autolytic activation, which in turn processes and activates pro-caspase 3 (32 kDa). Second, through activation of death-domain containing receptors [such as tumor necrosis factor α receptor 1 (TNFαR1), FAS], the receptors recruit adapter protein(s), which also contain death domains [FADD (FAS-associated death-domain protein) and TNFαR1-associated death-domain protein (TRADD)]. The TRADD–FADD complex or receptor-associated FADD then induces the autolytic activation of the associated caspase 8, which activates caspase 3 proteolytically. In resting cells, caspase 3 might be suppressed directly by inhibitor proteins, such as inhibitor of apoptosis proteins 1 and 2 (IAP1 and IAP2) or neuronal apoptosis inhibitory protein (NAIP)[18] (Table 1). Interestingly, caspase 3, like calpain, is a cytosolic cysteine protease, but does not require Ca^{2+} for activity (Table 1). In fact, direct treatment of cells with high concentrations of Ca^{2+} chelator (for example, 2–5 mM EGTA) can lead caspase-mediated apoptosis[19]. It is worth noting that caspase 7, which is highly homologous to caspase 3 and has very similar substrate specificity, might substitute for caspase 3 in most cell types and tissues. The exception is the CNS, where no mRNA for caspase 7 has been detected[20]. Interestingly, a recent study of caspase-3 knockout mice showed normal development except in the CNS, where an excessive number of neurons was found[21]. For simplicity, this discussion will focus on caspase 3 (Table 1).

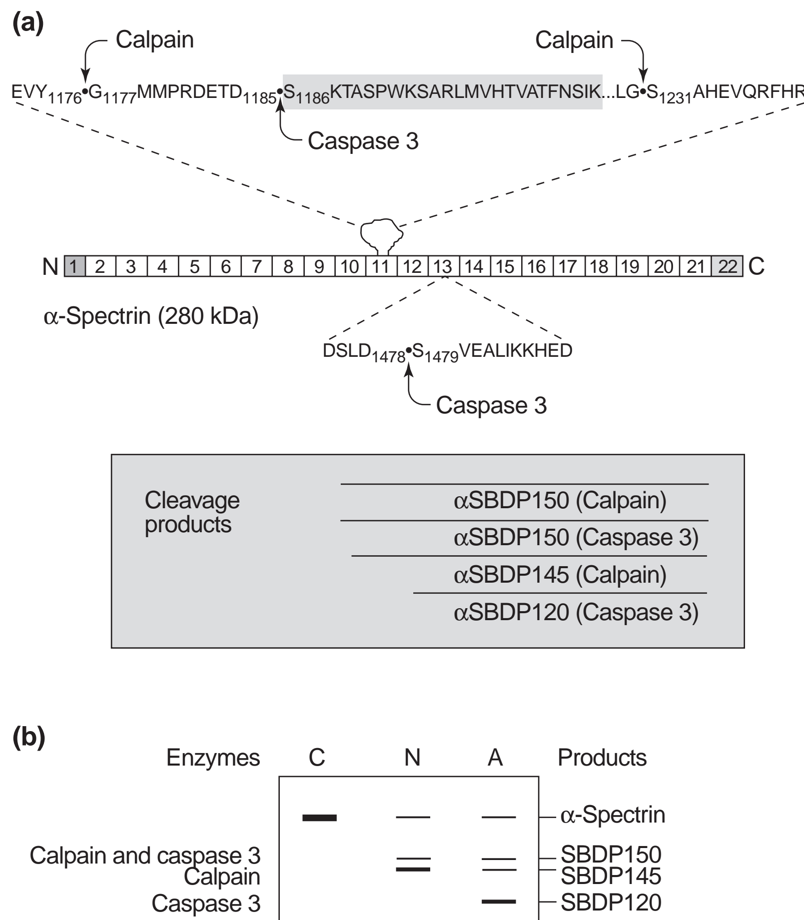

Fig. 1. ***αII-spectrin (α-fodrin) breakdown pattern as a marker for both caspase and calpain activation in neuronal death.*** **(a)** *Alpha-spectrin is alternatively cleaved by calpain and caspase 3. Both proteases cleave at adjacent sites in repeat 11, near the calmodulin-binding domain (gray) to produce two non-identical SBDP150s (with different N termini). Calpain subsequently cleaves this region again, producing a slightly smaller SBDP145. In addition, caspase 3 cleaves in repeat 13, producing the apoptosis-specific SBDP120.* **(b)** *an immunoblot for α-spectrin with samples from control neurons (C) and neurons undergoing either necrosis (N; with only calpain-generated SBDP150 and SBDP145) or apoptosis. (A; with both caspase and calpain generated SBDPs). The distinct fragment sizes and patterns of α-spectrin fragmentation are illustrated. Abbreviation: SBDP, αII-spectrin breakdown product.*

Like calpain, caspase 3 also has a finite number of cellular protein substrates, including cytoskeletal proteins, enzymes involved in signal transduction, cell-cycle proteins and nuclear DNA-repairing proteins (for review, see Ref. 18) (Table 1). The most-important specificity determinant is the Asp in both the P1 and P4 positions (Table 1). Again, caspase 3 tends to produce 'limited fragments' of its substrates, leaving them as fingerprints for caspase-3 activation. Recently, caspase-3 processing and caspase-3-mediated proteolysis have been reported in several neuronal apoptosis models, such as cultured neuroblastoma or neuronal cells subjected to staurosporine, as well as cerebellar granule neurons subjected to K^+ deprivation and hypoxia–hypoglycemia or excitotoxicity in cerebrocortical neurons[10,14,22]. There is now much literature that reports caspase-3 activation and proteolytic activity in *in vivo* models for cerebral ischemia, TBI and SCI (Refs 7,23,24). Caspases appear to be involved centrally in any apoptotic cascade in various cell types, as pan caspase inhibitors, such as Z-Asp-CH_2OC(0)-2,6-dichlorobenzene, Z-VAD(O-Me)-fluoromethylketone and the more-caspase-3-selective inhibitors (Ac-DEVD-CHO and acetyl-DEVD-fluoromethylketone) almost universally protect against any forms of apoptosis (Table 1). Similarly, these agents are found to be excellent neuronal apoptosis inhibitors *in situ*[14,22,25], but they are ineffective against necrosis[26]. Recently, several inhibitors of this class were used to suppress the 'apoptotic' component in cerebral ischemia[23].

Calpain is also activated in some apoptosis systems

Calpain activation in any form of apoptosis was first demonstrated in thymocytes, as measured by calpain autolysis[27]. In addition, various calpain inhibitors were found to protect against apoptosis in immune cells[27,28]. This finding was then extended to show that calpain was indeed activated in staurosporine-treated neuroblastoma SH-SY5Y cells, in NGF-deprived rat PC12 cells and in low-K^+-treated rat cerebellar granule neurons[14,29]. These data were obtained primarily by detection of calpain autolysis and specific α-spectrin II fragments that are generated by calpain (calpain-specific SBDP150 and SBDP145; see Fig. 1). Calpain inhibitors (for example, calpain inhibitor 1 and PD150606) appear to inhibit apoptosis only in specific systems (cerebellar granule neurons, neutrophils, etc.)[29,30–32] (Table 1). Most recently, Knepper-Nicolai showed that α-actinin and ezrin degradation was mediated by calpain in constitutive apoptosis in aged human neutrophils, and that a calpain inhibitor (calpeptin), in combination with a proteasome inhibitor (lactocystatin), suppressed this form of apoptosis[33].

Distinct α-spectrin breakdown patterns generated by calpain and caspase: diagnostic markers for neuronal apoptosis versus necrosis

Non-erythroid α-spectrin (also called α-fodrin) is degraded to a 120 kDa fragment, spectrin breakdown product 120 (SBDP120) in apoptotic neurons but not in necrotic neurons[14]. Yet, calpain-mediated α-spectrin breakdown to a 150 kDa and 145 kDa doublet is not only present in necrotic neuronal death, but also in most forms of neuronal apoptosis[14,29,34] (Fig. 1). In contrast, caspase-mediated formation of SBDP120 (and also caspase-specific SBDP150) occurs exclusively in neuronal apoptosis[25,34,35] (Fig. 1). The SBDP145 and SBDP120 can be easily distinguished by SDS–PAGE. Both SBDP150 fragments are in fact generated by calpain- and caspase-3-specific cleavages that are within a nine-residue span[34]. Indeed, using SBDP120 as a marker for apoptosis has the added advantage that commercial anti-α-spectrin antibodies react with rat (for example, cerebellar granule neurons) and human α-spectrin (SH-SY5Y cells)[14,34]. In fact, caspase 3, but not other caspases, is solely responsible for the production of SBDP120 (Refs 34,36). Subsequently, the presence of SBDP150, SBDP145 and, to a lesser degree, SBDP120 were found in both NMDA, kainate and glucose–oxygen-deprivation challenged cerebrocortical neurons[10]. This suggests, therefore, the presence of activation of both proteases in these neuronal injury models. Similarly, in an *in vivo* impact model of TBI, SBDP150 and SBDP145 were found in all affected areas of the brain, while the striatum and thalamus showed a twofold increase in SBDP120 levels[37,38].

Other common or related substrates for caspase 3 and calpain

The vulnerability of non-erythroid α-spectrin to proteolysis in both necrosis and apoptosis suggests that the proteolysis mediated by the caspase and calpain systems might have some common roles in mediating

cell death. In apoptosis, proteolysis is likely to be important in suspending cell function by disabling a number of enzymes involved in signal transduction, which disables the mechanisms that allow the cell to repair its DNA or to go through the cell cycle, and degrades its cytoskeleton network, causing membrane blebbing to occur and the subsequent phagocytosis of the apoptotic bodies by macrophages. In necrosis, although not necessarily by design, calpain-mediated proteolysis undoubtedly has a similar role in disabling signal transduction, membrane and cytoskeleton integrity, and the nuclear functioning of the cell. Furthermore, calpain might facilitate the apoptotic cell death by aiding caspase 3 in the proteolysis of cellular proteins. It seems that calpain and caspase indeed share a number of dual substrates and a few related substrates (Table 2).

The nonerythroid β-spectrin, in addition to α-spectrin is also susceptible to both calpain and caspase 3 (Ref. 34). Again, adjacent calpain and caspase-3 cleavage sites lead to 110 kDa β-SBDPs, while caspase cleavage at another major site produces an 85 kDa fragment. The simultaneous degradation of α- and β-spectrin in apoptosis (and necrosis) might compromise the cytoskeletal integrity, and possibly membrane permeability, significantly. Vimentin is an intermediate filament of the cytoskeleton. It was previously found to be degraded by calpain in lens-cell network[39] and its cleavage sites have been mapped out. Recently, its degradation was also observed in tamoxifen-treated apoptotic skin fibroblasts and in ionizing-radiation-induced apoptosis in prostate epithelial tumor cells[40,41]. On the basis of its sensitivity to a caspase inhibitor, it appears that vimentin is also degraded by caspase. Keratin is also susceptible to degradation by both calpain[42] and caspase[43], and similarly, actin, a major spectrin-binding protein, is degraded by caspase 3 both *in vitro* and in apoptotic cells[44], including neurons[45]. However, it seems to be degraded only partially, leading to the failure of some researchers to observe its cleavage in apoptosis. Interestingly, in neutrophils undergoing constitutive apoptosis, actin is also found to be in a calpain-inhibitor-I-sensitive state[34]. In lens cells, m-calpain also degrades actin into smaller fragment[46]. Tau, a microtubule (MT)-binding protein that stabilizes the assembly of MTs, is of extreme importance in neurons, as the transport of protein and other biomolecules in neurites (axons and dendrites) is supported by MTs. It is composed of up to six isoforms in adult human neurons and is sensitive to calpain proteolysis in neurons[47]. In fact, multiple fragments are generated: the major fragment is 42 kDa. Recently, Canu *et al.* have extended this finding by showing that in cerebellar granule-neuron apoptosis, tau is also degraded and that it is partially sensitive to caspase and calpain inhibitors[48]. In fact, the combination of inhibitors of both proteases provides the best protection.

ADP-ribosyltransferase [ADPRT or poly(ADP) ribose polymerase (PARP)] is the most-well-known substrate for caspase 3. During apoptosis, the 113 kDa ADPRT/PARP is degraded by caspase to a distinct 89 kDa fragment and 24 kDa fragment[49]. However, recently, it has been found to be cleaved at alternative site(s), generating fragments from 70 kDa to 40 kDa in size during necrosis[50,51]. Another recent study has described the purification of a non-caspase protease that cleaves bovine ADPRT/PARP (Ref. 52). This protease was identified as bovine m-calpain. The fragmentation of ADPRT/PARP by calpain *in vitro*[52] again yields fragments that range from 70 kDa to 40 kDa in size, similar to the ADPRT/PARP cleavage pattern obtained in necrotic cells[50]; when MTX is used, ADPRT/PARP is cleaved into 70 kDa and 40 kDa fragments that are sensitive to calpain-inhibitor I in necrosis, but the extent of calpain-mediated proteolysis in necrosis is much less than that observed by caspase-3-mediated apoptosis[53].

TABLE 2. Common or related substrates for caspase 3 and calpain

Substrate protein	Major fragments produced by caspase 3	Major fragments produced by calpain
αII-spectrin (280 kDa)	150 kDa, 120 kDa	150 kDa, 145 kDa
βII-spectrin (260 kDa)	110 kDa, 85 kDa	110 kDa
Actin (43 kDa)	35 kDa	40–42 kDa
Tau (55 kDa)	45 kDa	42 kDa and others
Vimentin (54 kDa)	52–64 kDa	44 kDa and others
Keratins (18 kDa, 8 kDa)	Multiple	Multiple
CaMKIV (55 kDa)	38 kDa	40 kDa
CaMKII (50 kDa)	35 kDa	35 kDa
FAK (125 kDa)	85 kDa	90 kDa
ADPRT/PARP (113 kDa)	89 kDa and 24 kDa	70 kDa and 40 kDa
Calpastatin (105 kDa)	75 kDa	Multiple
PKCα, β, γ (75 kDa)	–	46 kDa and 36 kDa
PKCδ and θ (75 kDa)	40 kDa	Not degraded
Phospholipase Cβ3 (155 kDa; membrane)	Not degraded	100 kDa
Phospholipase A_2 (100 kDa; cytosol)	70 kDa	Not degraded
Amyloid precursor protein (80 kDa)	Multiple	Multiple
BCL2 (24 kDa)	22 kDa	Not degraded
BAX (21 kDa)	Not degraded	18 kDa
IL1α (30 kDa)	Not degraded	18 kDa
IL1β (32 kDa)	17 kDa[a]	Not degraded

[a]Fragment produced by caspase 1.

Abbreviations: ADPRT, ADP-ribosyltransferase; BAX, BCL2-associated X protein; BCL2, B-cell lymphoma 2; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; FAK, focal-adhesion kinase; IL, interleukin; PKC, protein kinase C.

It has also been established that calmodulin-dependent protein kinase IV (CaMKIV) is fragmented by caspase 3 and calpain in staurosporine-induced apoptosis, but cleaved by calpain only in MTX-induced necrosis[54]. Here, caspase 3 produces an immunoreactive 38 kDa N-terminal fragment, while calpain produces a 35 kDa N-terminal fragment. In parallel with its proteolysis during apoptosis, a corresponding decrease in CaMK activity is seen in these cells. It has previously been shown that CaMKII is sensitive to calpain proteolysis[55,56]. In a recent study, CaMKII was also found to be sensitive to caspase-3-mediated proteolysis in apoptotic cells[54] (Table 2). In platelets, focal-adhesion kinase (FAK) is a 125 kDa non-receptor tyrosine kinase that is implicated in integrin-mediated signal transduction. It is degraded by calpain during platelet activation to 90 kDa, 45 kDa and 40 kDa fragments[57]. Recently, FAK was also found to be degraded to an 85 kDa fragment by either caspase 3 or caspase 7 during apoptosis in Jurkat cells[58]. However, changes in FAK activity following degradation have not been reported. Calpain cleaves amyloid precursor protein (APP) at three different sites, all located in the extracellular N-terminal domain[59]. It is therefore likely that internalized APP might be cleaved. The C-terminal cleavage of APP could indeed produce a fragment that contains the whole of the β-amyloid peptides 1–40 and 1–42 (amyloidogenic), which can be further processed by another protease. APP is also cleaved

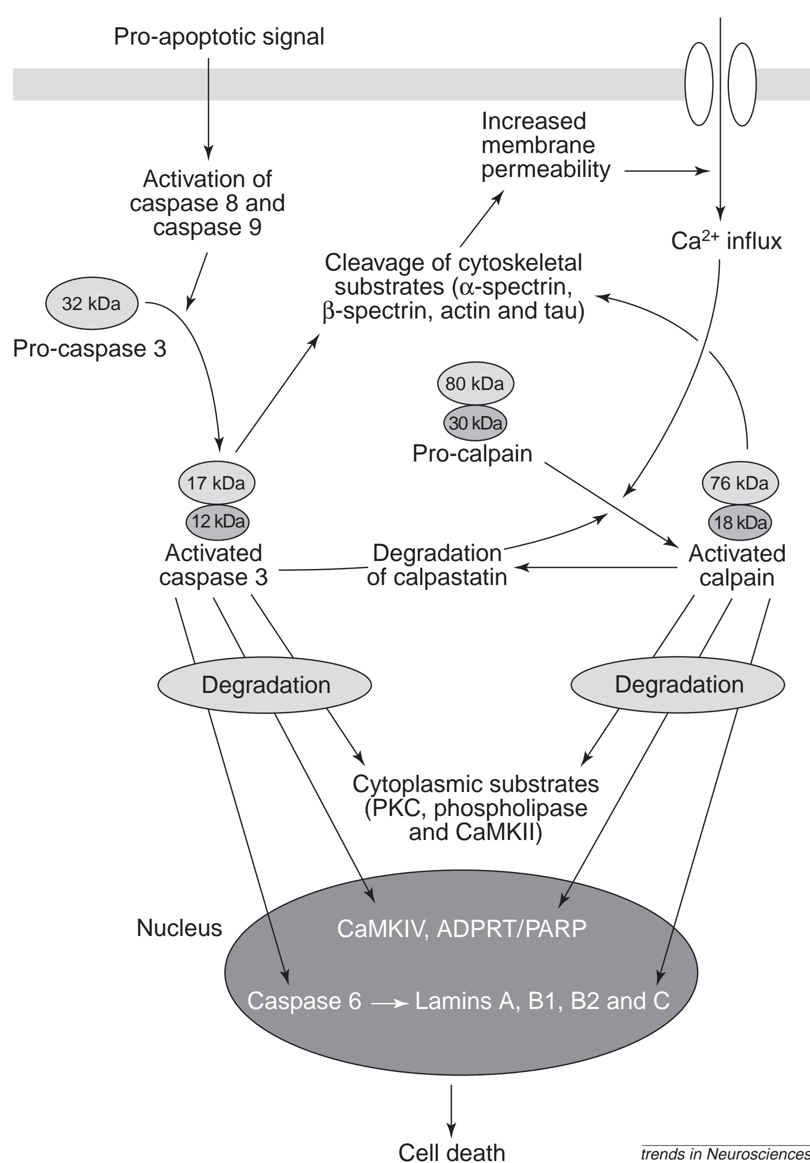

Fig. 2. ***The roles of calpain and caspase in protein degradation in neuronal death and their interactions.*** *A pro-apoptotic signal, via the mitochrondrial caspase-9 pathway or receptor-linked caspase-8 pathway leads to processing and activation of caspase 3. The action of caspase 3 on cortical cytoskeletal and plasma membrane integral proteins probably compromises the membrane permeability to* Ca^{2+}*, leading to elevated intracellular* Ca^{2+} *levels. Caspase 3 also degrades calpastatin, which, in combination with these events, facilitates calpain activation. Hence, caspase 3 and calpains further degrade a subset of important cytosolic, cytoskeletal and nuclear substrates, resulting in functional and structural destruction of the neuronal cell. Abbreviations: ADPRT, ADP-ribosyltransferase (also known as PARP); CaMKII,* Ca^{2+}*/calmodulin-dependent protein kinase II; PKC; protein kinase C.*

by caspase 3 at two sites in the extracellular N-terminal domain and at a third site near the C terminus. Again, the cleavage in the C-terminal region, although not at the exact β and γ cleavage sites, helps to facilitate the production of β-amyloid peptides[60,61].

The Ca^{2+}-dependent α-, β- and γ-isoforms of protein kinase C (PKC) have been found to be degraded by calpain *in vitro* and under a variety of conditions in cell culture[62]. In fact, calpain is suspected to have a role in the downregulation of PKC activity. Interestingly, two Ca^{2+}-independent PKC isoforms (δ and θ) have now been shown to be degraded by caspase 3, but not the Ca^{2+}-dependent isoforms[63,64]. The PtdIns-specific phospholipase Cβ3 is activated proteolytically by calpain[65], whereas phospholipase A2 (PLA_2)[66] is activated proteolytically by caspase 3. However, Adam-Klages reported that cytosolic PLA_2 was actually inactivated by caspase cleavage[67]. Both phospholipid hydrolases are thought to be involved in transmembrane signal transduction. While the anti-apoptotic protein BCL2 is cleaved by caspases to produce a truncated form with pro-apoptotic properties[68], BAX is also found to be truncated by calpain (not caspase) in drug-induced apoptosis of HL-60 cells[69] and in staurosporine-treated SH-SY5Y cells[70].

It has been reported previously that calpastatin (CAST) is degraded by calpain in A23187-treated cells[71]. More recently, the 105 kDa high-molecular-weight form of CAST was also found to be very sensitive to caspase proteolysis during early apoptosis (staurosporine-treated SY5Y cells, anti-FAS treated Jurkat cells and TNFα-treated U937 monocytic leukemic cells)[72,73] (Fig. 2). This proteolysis, which is thought to involve caspases 1 and 3, produces a major 75 kDa fragment of CAST and several low-molecular-weight fragments. One can envision that, in early apoptosis, caspase 3 begins to degrade CAST and compromise plasma-membrane and cortical cytoskeleton integrity simultaneously, which results in the elevation of intracellular Ca^{2+} levels. The overall effects form a trigger for increasing calpain activity. Hence, calpain furthers the proteolytic destruction initiated by caspase 3. While it might not be directly related to cell death, it is worth noting that caspase 1 (ICE) is the key enzyme that processes pro-interleukin 1β (Ref. 74), while calpain is a major protease for processing pro-interleukin-1α (Ref. 75; Table 2).

Most recently, pro-caspase 3 was found to be a calpain substrate[75]. Instead of processing pro-caspase 3 (32 kDa) to its activated form (17 kDa–12 kDa), calpain truncated it in the N-terminal region to a 30 kDa, which appeared to be less vulnerable to being activated by caspase 8 or caspase 9. Thus, these findings add to the complex interaction between the two protease systems.

Two proteases: two forms of neuronal death

In this article, the role of two cytosolic cysteine proteases (calpain and caspase 3) in two forms of neuronal death (necrosis and apoptosis) has been highlighted (Table 1). On the basis of genetic data and the elucidation of the apoptosis cascade, it is apparent that caspase has a central role in transducing the apoptosis signal. Caspase-3 activation is a unique feature of apoptosis. By contrast, necrotic cell death is, almost without exception, associated with massive Na^+ and Ca^{2+} influxes and, thus, calpain activation. Conversely, apoptosis might or might not be associated with Ca^{2+} overload. In anti-FAS-treated Jurkat T cells and other cells, little evidence of calpain activation is seen. The difference could be the rapid onset of apoptosis and the resultant cell death induced by anti-FAS (which takes 3–4 h). It has also been found that EGTA treatment (2 mM) leads to calpain-independent but caspase-3 dependent apoptosis[19]. In this case, the presence of excess extracellular EGTA sequesters intracellular Ca^{2+} from the cells, and therefore prevents calpain activation. Yet, it has been demonstrated repeatedly that both caspase and calpain are activated in apoptotic neurons (as determined from the spectrin breakdown pattern). Given the importance of Ca^{2+} homeostasis and the abundance of calpains in neurons, calpain activation can be expected in these extraneous conditions. Thus, in the case of neuronal apoptosis, in addition to caspase 3, calpain appears to assist in the degradation of key cellular proteins and, thus, can be viewed as having an auxiliary and augmentative role in the transduction of neuronal apoptosis (Fig. 2).

The striking similarity between the identified substrates for calpain and caspases raise the possibility that both protease families are active participants in the irreversible destruction or functional alteration of a subset of important cellular enzymes or structural proteins, and, thus, contribute to structural derangement and functional loss in neurons under acute neurodegenerative conditions (Table 1). A number of earlier and recent publications have illustrated that calpain and caspase inhibitors both have impressive neuroprotective effects in *in vivo* models of cerebral ischemia[23,76] (Table 1). Interestingly, calpain and caspase inhibitors appear to have additive neuroprotective effects against neuronal apoptosis[29,38]; it will be of great interest to explore the benefit of combination-therapy approach that involves both a calpain inhibitor and a caspase inhibitor in *in vivo* models of neurodegenerative diseases where both necrosis and apoptosis have been implicated.

Selected references

1 Majno, G. and Joris, I. (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* 146, 3–15
2 Linnik, M.D. *et al.* (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 24, 2002–2008
3 MacManus, J.P. *et al.* (1993) Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci. Lett.* 164, 89–92
4 Filipkowski, R.K. *et al.* (1994) DNA fragmentation in rat brain after intraperitoneal administration of kainate. *NeuroReport* 5, 1538–1540
5 Bonfoco, E. *et al.* (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. U. S. A.* 92, 7162–7166
6 Rink, A. *et al.* (1995) Evidence of apoptotic cell death after experimental traumatic brain injury in the rat. *Am. J. Pathol.* 147, 1575–1583
7 Emery, E. *et al.* (1998) Apoptosis after traumatic human spinal cord injury. *J. Neurosurg.* 89, 911–920
8 Nicotera, P. *et al.* (1999) Neuronal cell death, a demise with different shapes. *Trends Pharmacol. Sci.* 20, 46–51
9 Heron, A. *et al.* (1993) Regional variability in DNA fragmentation after global ischemia evidenced by combined histological and gel electrophoresis observations in the rat brain. *J. Neurochem.* 61, 1973–1976
10 Nath, R. *et al.* (1998) Evidence for activation of caspase 3-like protease in excitotoxin- and hypoxia/hypoglycemia-injured neurons. *J. Neurochem.* 71, 186–195
11 Pang, Z. and Geddes, J.W. (1997) Mechanisms of cell death induced by the mitochondrial toxin 3-nitropropionic acid, acute excitotoxic necrosis and delayed apoptosis. *J. Neurosci.* 17, 3064–3073
12 Bonfoco, E. *et al.* (1996) Cytoskeletal breakdown and apoptosis elicited by NO donors in cerebellar granule cells require NMDA receptor activation. *J. Neurochem.* 67, 2484–2493
13 Yuen, P-W. and Wang, K.K.W. (1998) Calpain inhibitors, novel neuroprotectants and potential anticataractic agents. *Drugs Future* 23, 741–749
14 Nath, R. *et al.* (1996) Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1β-converting-enzyme-like protease(s) in apoptotic cells, contributory roles of both protease families in neuronal apoptosis. *Biochem. J.* 319, 683–690
15 Wang, K.K. *et al.* (1989) Calmodulin-binding proteins as calpain substrates. *Biochem. J.* 262, 693–706
16 Carafoli, E. and Molinari, M. (1998) Calpain, a protease in search of a function? *Biochem. Biophys. Res. Commun.* 2457, 193–203
17 Nicholson, D.W. and Thornberry, N.A. (1997) Caspases, killer proteases. *Trends Biochem. Sci.* 22, 299–306
18 Deveraux, Q.L. and Reed, J.C. (1999) IAP family proteins – suppressors of apoptosis. *Genes Dev.* 13, 239–252
19 McGinnis, K.M. *et al.* (1999) Alternations of extracellular calcium elicit selective modes of cell death and protease activation in SH-SY5Y human neuroblastoma cells. *J. Neurochem.* 72, 1853–1863
20 Juan, T.S. *et al.* (1997) Identification and mapping of Casp7, a cysteine protease resembling CPP32 beta, interleukin-1β converting enzyme, and CED 3. *Genomics* 40, 86–93
21 Kuida, K. *et al.* (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* 384, 368–372
22 Posmantur, R. *et al.* (1997) Characterization of CPP32-like protease activity following apoptotic challenge in SH-SY5Y neuroblastoma cells. *J. Neurochem.* 68, 2328–2337
23 Endres, M. *et al.* (1998) Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family. *J. Cereb. Blood Flow Metab.* 18, 238–247
24 Yakovlev, A.G. *et al.* (1997) Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. *J. Neurosci.* 17, 7415–7424
25 Schulz, J.B. *et al.* (1996) Potassium deprivation-induced apoptosis of cerebellar granule neurons, a sequential requirement for new mRNA and protein synthesis, ICE-like protease activity, and reactive oxygen species. *J. Neurosci.* 16, 4696–4706
26 Armstrong, R.C. *et al.* (1997) Activation of the CED3/ICE-related protease CPP32 in cerebellar granule neurons undergoing apoptosis but not necrosis. *J. Neurosci.* 17, 553–562
27 Squier, M.K. *et al.* (1994) Calpain activation in apoptosis. *J. Cell Physiol.* 159, 229–237
28 Sarin, A. *et al.* (1995) A protease-dependent TCR-induced death pathway in mature lymphocytes. *J. Immunol.* 154, 5806–5812
29 Nath, R. *et al.* (1996) Effects of calpain and ICE-like protease inhibitors on neuronal apoptosis. *NeuroReport* 8, 249–256
30 Vanags, D.M. *et al.* (1996) Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J. Biol. Chem.* 271, 31075–31085
31 Squier, M.K. *et al.* (1999) Calpain and calpastatin regulate neutrophil apoptosis. *J. Cell Physiol.* 178, 311–319
32 Brown, S.B. *et al.* (1997) Actin is cleaved during constitutive apoptosis. *Biochem. J.* 323, 233–237
33 Knepper-Nicolai, B. *et al.* (1998) Constitutive apoptosis in human neutrophils requires synergy between calpains and the proteasome downstream of caspases. *J. Biol. Chem.* 273, 30530–30536
34 Wang, K.K.W. (1998) Simultaneous degradation of αII- and βII-spectrin by caspase 3 (CPP32) in apoptotic cells. *J. Biol. Chem.* 273, 22490–22497
35 Martin, S.J. *et al.* (1995) Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. *J. Biol. Chem.* 270, 6425–6428
36 Janicke, R.U. *et al.* (1998) Caspase 3 is required for α-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. *J. Biol. Chem.* 273, 15540–15545
37 Pike, B.R. *et al.* (1998) Regional calpain and caspase 3 proteolysis of alpha-spectrin after traumatic brain injury. *NeuroReport* 9, 2437–2442
38 Pike, B.R. *et al.* (1998) Temporal relationships between *de novo* protein synthesis, calpain and caspase 3-like protease activation, and DNA fragmentation during apoptosis in septo-hippocampal cultures. *J. Neurosci. Res.* 52, 505–520
39 Roy, D. *et al.* (1983) Lens Ca^{2+} activated proteinase, degradation of vimentin. *Biochem. Biophys. Res. Commun.* 116, 204–209
40 Hashimoto, M. *et al.* (1998) Rapid fragmentation of vimentin in human skin fibroblasts exposed to tamoxifen, a possible involvement of caspase 3. *Biochem. Biophys. Res. Commun.* 247, 401–406
41 Prasad, S.C. *et al.* (1998) Apoptosis-associated proteolysis of vimentin in human prostate epithelial tumor cells. *Biochem. Biophys. Res. Commun.* 249, 332–338
42 Ando, Y. *et al.* (1988) Purification and characterization of calpains from pig epidermis and their action on epidermal keratin. *J. Invest. Dermatol.* 90, 26–30
43 Ku, N.O. *et al.* (1997) Apoptosis generates stable fragments of human type I keratins. *J. Biol. Chem.* 272, 33197–33203
44 Mashima, T. *et al.* (1995) Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. *Biochem. Biophys. Res. Commun.* 217, 1185–1192
45 Yang, F. *et al.* (1998) Antibody to caspase-cleaved actin detects apoptosis in differentiated neuroblastoma and plaque-associated neurons and microglia in Alzheimer's disease. *Am. J. Pathol.* 152, 379–389
46 Yoshida, H. *et al.* (1984) Degradation of actin and vimentin by calpain II, a Ca^{2+}-dependent cysteine proteinase, in bovine lens. *FEBS Lett.* 170, 259–262
47 Litersky, J.M. and Johnson, G.V. (1995) Phosphorylation of tau *in situ*, inhibition of Ca^{2+}-dependent proteolysis. *J. Neurochem.* 65, 903–911
48 Canu, N. *et al.* (1998) Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis. *J. Neurosci.* 18, 7061–7074
49 Lazebnik, Y.A. *et al.* (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371, 346–347
50 Shah, G.M. *et al.* (1996) Different cleavage pattern for poly(ADP-ribose) polymerase during necrosis and apoptosis in HL-60 cells. *Biochem. Biophys. Res. Commun.* 229, 838–844
51 Sallmann, F.R. *et al.* (1997) Characterization of antibodies specific for the caspase cleavage site on poly(ADP-ribose) polymerase, specific detection of apoptotic fragments and mapping of the necrotic fragments of poly(ADP-ribose) polymerase. *Biochem. Cell Biol.* 75, 451–456
52 Buki, K.G. *et al.* (1997) Isolation and identification of a proteinase from calf thymus that cleaves poly(ADP-ribose) polymerase and histone H1. *Biochim. Biophys. Acta.* 1338, 100–106

53 McGinnis, K.M. *et al.* (1999) Pro-caspase-3 and poly(ADP)ribose polymerase (PARP) are calpain substrates. *Biochem. Biophys Res. Commun.* 263, 94–99
54 McGinnis, K.M. *et al.* (1998) Ca^{2+}/calmodulin-dependent protein kinase IV is cleaved by caspase 3 and calpain in SH-SY5Y human neuroblastoma cells undergoing apoptosis. *J. Biol. Chem.* 273, 19993–20000
55 Yoshimura, Y. *et al.* (1996) Purification and characterization of active fragment of Ca^{2+}/calmodulin-dependent protein kinase II from the post-synaptic density in the rat forebrain. *J. Biochem.* 119, 268–273
56 Hajimohammadreza, I. *et al.* (1997) Neuronal nitric oxide synthase and calmodulin-dependent protein kinase II α undergo neurotoxin-induced proteolysis. *J. Neurochem.* 69, 1006–1013
57 Cooray, P. *et al.* (1996) Focal adhesion kinase (pp125FAK) cleavage and regulation by calpain. *Biochem. J.* 318, 41–47
58 Wen, L.P. *et al.* (1997) Cleavage of focal adhesion kinase by caspases during apoptosis. *J. Biol. Chem.* 272, 26056–26061
59 Siman, R. *et al.* (1990) Proteolytic processing of beta amyloid precursor by calpain I. *J. Neurosci.* 10, 2400–2411
60 Barnes, N.Y. *et al.* (1998) Increased production of amyloid precursor protein provides a substrate for caspase-3 in dying motoneurons *J. Neurosci.* 18, 5869–5880
61 Gervais, F.G. *et al.* (1999) Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-β precursor protein and amyloidogenic Aβ peptide formation. *Cell* 97, 395–406
62 Kishimoto, A. *et al.* (1989) Limited proteolysis of protein kinase C subspecies by Ca^{2+}-dependent neutral protease (calpain). *J. Biol. Chem.* 264, 4088–4092
63 Ghayur, T. *et al.* (1996) Proteolytic activation of protein kinase C delta by an ICE/CED 3-like protease induces characteristics of apoptosis. *J. Exp. Med.* 184, 2399–2404
64 Datta, R. *et al.* (1997) Caspase 3-mediated cleavage of protein kinase C theta in induction of apoptosis. *J. Biol. Chem.* 272, 20317–20320
65 Banno, Y. *et al.* (1995) Endogenous cleavage of phospholipase Cβ3 by agonist-induced activation of calpain in human platelets. *J. Biol. Chem.* 270, 4318–4324
66 Wissing, D. *et al.* (1997) Involvement of caspase-dependent activation of cytosolic phospholipase A2 in tumor necrosis factor-induced apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* 94, 5073–5077
67 Adam-Klages, S. *et al.* (1998) Caspase-mediated inhibition of human cytosolic phospholipase A2 during apoptosis. *J. Immunol.* 161, 5687–5694
68 Cheng, E.H. *et al.* (1997) Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science* 278, 1966–1968
69 Wood, D.E. *et al.* (1998) Bax cleavage is mediated by calpain during drug-induced apoptosis. *Oncogene* 17, 1069–1078
70 McGinnis, K.M. *et al.* (1999) Endogenous Bax translocation in SH-SY5Y human neuroblastoma cells and cerebellar granule neurons undergoing apoptosis. *J. Neurochem.* 72, 1899–1906
71 Mohan, P.S. and Nixon, R.A. (1996) Calpain activation and calpastatin processing in cultured human retinoblastoma (Y–79) cells. *Soc. Neurosci. Abstr.* 22, 1918
72 Wang, K.K.W. *et al.* (1998) Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. *Arch. Biochem. Biophys.* 356, 187–196
73 Porn-Ares, M.I., Samali, A. and Orrenius, S. (1998) Cleavage of the calpain inhibition, calpastatin, during apotosis. *Cell Death Differ.* 5, 1028–1033
74 Li, P. *et al.* (1995) Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell* 80, 401–411
75 Kobayashi, Y. *et al.* (1990) Identification of Ca^{2+}-activated neutral protease as a processing enzyme of human interleukin 1 alpha. *Proc. Natl. Acad. Sci. U. S. A.* 87, 5548–5552
76 Markgraf, C.G. *et al.* (1998) Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. *Stroke* 29, 152–158

Acknowledgements
The author thanks his co-workers, past and present (including Kim McGinnis, Rathna Nath, Rand Posmantur and Iradj Hajimohammadreza) and his collaborators Ronald Hayes and Margaret Gnegy for their original contributions. With apologies to the many researchers whose work was not cited owing to limited space.

Retinal mosaics: new insights into an old concept

Jeremy E. Cook and Leo M. Chalupa

It has been known since the middle of the 19th century that different neuronal types are distributed across the retinal surface in non-random arrays: indeed, these arrays, called 'mosaics', have long been considered to be a fundamental feature of retinal organization. However, until recently, little was known about how such mosaics are established during development. In the hope of stimulating further research, this article reviews the current status of three very different approaches to this intriguing general problem. The first postulates arrays of molecular markers, which are produced by specific cell types shortly after their final mitotic divisions and could be influential in the differentiation of other cell types. The second invokes a tangential dispersion of differentiating cells to generate spatial order, either while these cells are still migrating or soon after they reach their laminar destinations. The third involves the elimination of wrongly positioned cells through the process of naturally occurring cell death.

Trends Neurosci. (2000) 23, 26–34

Jeremy Cook is in the Dept of Anatomy and Developmental Biology, University College London, Gower Street, London, UK WC1 6BT, and Leo Chalupa is in the Section of Neurobiology, Physiology and Behavior, Division of Biological Sciences, University of California at Davis, CA 95616, USA.

ALL PROGRESS in science depends on the recognition of patterns and relationships, but by no means all of these are as tangible and aesthetically pleasing as the regular patterns that were first noted in the retina over 150 years ago[1]. Retinal mosaics in adult animals have been studied in some detail and offer insights into fundamental issues of organization and function (Box 1). But we know very little about their developmental origins – and what we do know has been learned piecemeal from several model systems. The available evidence suggests three distinct mechanisms by which mosaics can be established, not only in the retina but also in other parts of the brain (Box 2). This article considers the evidence for each and indicates the gaps in our knowledge that should be addressed by future studies.

From a functional perspective, the presence of regular neuronal distributions across the retina is intimately related to the degree of dendritic overlap exhibited by different neuronal types. There is a substantial literature that deals with the growth and remodelling of dendritic processes in the developing retina. This aspect will be largely ignored, partly because of the limited space available and also because the mechanisms that underlie the elaboration of dendrites are widely

 0166-2236/99/$ – see front matter © 2000 Elsevier Science Ltd. All rights reserved. PII: S0166-2236(99)01487-3