with secondary antibodies from various sources (Molecular Probes, Chemicon, Vector Laboratories).

**Pharmacological reagents**

Treatment with pharmacological reagents occurred 30–60 min before growth factor or cytokine administration. The reagents were either present throughout the experiment or alternatively the medium was replaced after 6–12 h incubation. Reagents in this study were: LY294002 (10–50 μM; Calbiochem) and okadaic acid (1 nM to 1 μM; Calbiochem and Alomone Laboratories).

**In vivo and in vitro kinase assays**

The in vivo kinase assay was performed largely as recommended by the supplier (Akt Kinase Assay Kit, Cell Signaling Technology). In vitro kinase assays were performed using 1 X buffer (Akt Kinase Assay Kit, Cell Signaling Technology), 100 μM ATP, 0.5 mM peptide (ENNEKMBQRQVYPPMFMDA), 300 U ml⁻¹ Akt protein kinase. For radioactivity incorporation assay, 250 μCi ml⁻¹ [γ³²P]ATP were included in the reaction. The reactions were quenched by addition of SDS gel buffer, and were either spotted on nitrocellulose membranes for immunoblotting, or were analysed by PAGE using 18% acrylamide gels.

**Transfections and constructs**

Transfections were performed with lipofection reagents (Effectene, Qiagen) largely as described. Neural stem cells grown on 150-mm dishes were collected and crosslinked by 1,000 mOsm sucrose, 1% formaldehyde, and 1% Triton X-100 for 10 min. The cells were washed twice with PBS and resuspended in 1 ml of PBS. The cell suspension was mixed with DNA transfection mix and vortexed. The mixture was added to the cells and incubated for 35 min. The DNA–cell mixture was transfected by gentle rocking for 1 h and the cells were washed with DMEM and incubated with DMEM containing 10% FCS and 0.1% Geneticin for 24 h.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation was performed on neural stem cells largely as described. Neural stem cells grown on 150-mm dishes were collected and crosslinked using 1% formalin for 10 min at room temperature, and the extracts were sonicated until the DNA fragments were 500–800 bp in size. Cell extracts were subsequently incubated with 5 μg IgG or antibodies against N-CoR or CBF1 overnight at 4°C. The extracts were incubated with protein A-sepharose beads (Sigma) for 1 h. After extensive washing of the beads, proteins were eluted and reversed by crosslinking for 6 h at 65°C. After DNA purification, PCR was performed at 29–35 cycles. Primers were: GFAP, 5’-GACGAATTCTTCCGTTTCTCCG-3’ (sense), 5’-CAAGGTCACTGTACCCAGAG-3’ (antisense); HES5, 5’-CGTGTCCTTCTCCTGAC-3’ (sense), 5’-GATCCAGTGTGATCCGCAGG-3’ (antisense). These primers were designed to amplify DNA fragments of approximately 200 base pairs.

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**Specific aspartyl and calpain proteases are required for neurodegeneration in C. elegans**

Poli Syntichaki*, Keli Xu†, Monica Driscoll ‡ & Nektarios Tavernarakis*

* Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion 71110, Crete, Greece
† Department of Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, 604 Allison Road, Piscataway, New Jersey 08855, USA

Necrotic cell death underlies the pathology of numerous human neurodegenerative conditions. In the nematode Caenorhabditis elegans, gain-of-function mutations in specific ion channel genes such as the degenerin genes deg-1 and mec-4, the acetylcholine receptor channel subunit gene deg-3 and the G protein α-subunit gene gsa-1 evoke an analogous pattern of degenerative (necrotic-like) cell death in neurons that express the mutant proteins. An increase in concentrations of cytoplasmic calcium in dying cells, elicited either by extracellular calcium influx or by release of endoplasmic reticulum stores, is thought to comprise a major death-signalling event. But the biochemical mechanisms by which calcium triggers cellular demise remain largely unknown. Here we report that neuronal degeneration inflicted by various genetic lesions in C. elegans requires the activity of the calcium-regulated CLP-1 and TRA-3 calpain proteases and aspartyl protease ASP-3 and ASP-4. Our findings show that two

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distinct classes of proteases are involved in necrotic cell death and suggest that perturbation of intracellular concentrations of calcium may initiate neuronal degeneration by deregulating proteolysis. Similar proteases may mediate necrotic cell death in humans.

Neuronal degeneration initiated by hyperactive MEC-4, an ion channel subunit that is normally required for mechanotransduction in the six touch receptor neurons of *C. elegans*, is reminiscent of excitotoxic cell death in mammals. Electron microscopy studies of dying neurons in animals expressing a gain-of-function *mec-4* allele (n231 or d; dominant) have shown extensive degradation of cellular contents during mid to late stages of cell death. This ultrastructural feature suggests that proteolysis may be central to the biochemical mechanism underlying neuronal degeneration. The main executioner protease, caspase CED-3, which mediates programmed cell death (apoptosis) in *C. elegans*, and three additional CED-3-related proteases (CSP-1, CSP-2 and CSP-3) that are encoded in the nematode genome are not required for cell death induced by *mec-4(d)* (ref. 11; P.S. and N.T., unpublished data). This indicates that a distinct, non-apoptotic mechanism, which probably involves different proteases, functions in neurodegeneration in the nematode.

Aspartyl proteases are a class of catabolic hydrolases that, among others, includes lysosomal (cathepsin D) and non-lysosomal (cathepsin E) enzymes. We tested the requirement for aspartyl protease activity in neurodegeneration inflicted by hyperactive MEC-4 in touch receptor neurons in three ways. First, we used genetic backgrounds that have diminished levels of aspartyl protease activity. Nematode strains carrying mutations in three genes, *cad-1, daf-4* and *unc-52*, that encode otherwise unrelated proteins have been shown to maintain aspartyl protease activity that is 90% lower than in wild-type animals. We found that neurodegeneration induced by *mec-4(d)* was suppressed in *cad-1; mec-4(d), daf-4; mec-4(d)* or *unc-52; mec-4(d)* mutant strains (Fig. 1a and Supplementary Information).

Second, we treated *mec-4(d)* animals with peptatin A, an inhibitor of aspartyl proteases. Treatment ameliorated necrosis of the six touch receptor neurons (Fig. 1a). Third, we subjected *mec-4(d)* animals to starvation conditions under which aspartyl protease activity has been shown to drop to 5–10% that of well-fed nematodes. We observed a reduction in the number of dying neurons in the progeny of starved animals (Fig. 1a). Survival of touch receptor neurons was confirmed by the presence of cells expressing green fluorescent protein (GFP) from the *mec-4* promoter in adult animals.

Suppression of *mec-4(d)*-induced cell death in the genetic backgrounds and under the conditions examined was not a consequence of a reduction in the quantities of toxic MEC-4(d) protein. We used the *mec-4* promoter to drive expression of both lacZ and GFP reporter genes in *cad-1, daf-4* or *unc-52* mutant strains, as well as in animals treated with peptatin A and starved animals. We did not detect any difference in reporter gene expression between these animals and well-fed wild-type animals (Fig. 1b, left, *cad-1(j1)* is shown as an example). Similarly, we assessed the relative stability of MEC-4 by using reporter genes with either lacZ or GFP fused at the carboxy terminus of the full-length MEC-4 protein. Quantities of protein were either not affected or even slightly increased by manipulations that reduced aspartyl protease activity (Fig. 1b, right, *cad-1(j1)* is shown).

We next determined whether aspartyl protease deficiency is generally protective against necrotic cell death. Gain-of-function (d) mutations in three otherwise unrelated genes, the degenerin *deg-1*, the α-7 nicotinic acetylcholine receptor *Ca*α7 channel subunit *deg-3*, and *gsa-1*, which encodes the Gαs subunit, trigger degeneration of specific sets of neurons expressing the toxic variant *α*gf. We tested the general requirement for aspartyl protease in neurodegeneration by introducing various death-inducing mutations into aspartyl-protease-deficient *cad-1, daf-4* and *unc-52* mutant strains. Cell death inflicted by toxic *deg-1(d)* and *deg-3(d)* alleles and by overexpressing the hyperactivated Gαs(Q227L) variant (*α*gf) was suppressed in genetic backgrounds deficient in aspartyl proteases. Starvation, which results in diminished aspartyl protease activity, also ameliorated neurodegeneration (Fig. 1c–e). Neuron survival was confirmed in adult animals by scoring expression of GFP. Similar to expression of *mec-4*, expression of *deg-1, deg-3* and *α*gf was not reduced in protease-deficient genetic backgrounds or under starvation conditions. We conclude that aspartyl protease activity is required generally for neurodegeneration caused by deleterious mutations in aspartyl proteases.

Figure 1 Aspartyl protease deficiency suppresses neurodegeneration in *C. elegans*. a, Number of vacuolated touch receptors at the L1 stage per 100 animals carrying the *mec-4(d)* allele in genetic backgrounds with reduced aspartyl protease activity, after treatment with peptatin A and under conditions of starvation (n = 250, P < 0.0001, unpaired t-test). b, Expression of lacZ in touch receptor neurons driven solely by the *mec-4* promoter (left) or fused at the end of the full-length *mec-4* gene (right). Aspartyl protease deficiency in *cad-1(j1)* (bottom) does not affect mec-4 expression or stability, as compared with the wild-type background (top). c, Vacuolated PVC interneurons at the L2 stage per 100 *deg-1(d)* animals in genetic backgrounds with reduced aspartyl protease activity and under conditions of starvation (n = 250, P < 0.0001, unpaired t-test). d, Vacuolated IL1 sensory neurons and PVC interneurons per 100 L1 *deg-3(gf)* mutant larvae in genetic backgrounds with reduced aspartyl protease activity and under conditions of starvation (n = 250, P < 0.001, unpaired t-test). e, Vacuolated PVC interneurons at the L1 stage per 100 *α*gf animals in genetic backgrounds with reduced aspartyl protease activity and under conditions of starvation (n = 250, P < 0.0001, unpaired t-test).
many different C. elegans genes.

There are at least six expressed aspartyl protease genes (asp-1 to asp-6) encoded in the nematode genome (ref. 16; see Supplementary Information for multiple sequence alignment and phylogenetic tree). To identify those that contribute to the protease activity required for neurodegeneration, we systematically knocked down the expression of each asp gene by RNA interference (RNAi) in mec-4(d), deg-3(d) and α(gf) genetic backgrounds. As a positive control in these experiments, we knocked down crt-1, which encodes calreticulin, an endoplasmic reticulum (ER) chaperone required for neurodegeneration induced by mec-4(d) and α(gf), but not deg-3(d) (ref. 8). Although RNAi is relatively ineffective for genes expressed in mature C. elegans neurons (ref. 17; N.T. and P.S., unpublished data), we observed suppression of neurodegeneration triggered by mec-4(d) in crt-1(RNAi) animals (Fig. 2a, c). It seems likely that RNAi is more effective with genes that are required at early developmental stages in the nervous system (degeneration occurs soon after the touch receptor neurons are born during late embryogenesis and the first larval stage in mec-4(d) C. elegans mutants).

Of the six aspartyl protease genes examined, asp-3 and asp-4 were specifically required for neurodegeneration (Fig. 2a–c). asp-1 knockdown also detectably reduced neurodegeneration but to a much lower extent. In a reciprocal approach, we introduced each asp gene into cad-1(j1);mec-4(d) double mutant animals.

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**Figure 2** Specific aspartyl proteases are required for neurodegeneration in C. elegans. a–c, Effect of RNAi with the indicated asp genes in mec-4(d), deg-3(d) and α(gf) mutants. RNAi with crt-1 was used as a positive control; RNAi with gfp was used as a negative control. Interference with asp-3 and asp-4 results in significant suppression in all three genetic backgrounds (n = 200, P < 0.0001, unpaired t-test). Efficacy of RNAi was assessed as described in Methods. d, Degenerating touch receptors in transgenic cad-1(f1); mec-4(d) animals carrying each of the indicated asp genes on extrachromosomal arrays. Ex[asp-3] and Ex[asp-4] restore cell death in cad-1(f1); mec-4(d) double mutants (n = 150, P < 0.0001, unpaired t-test). e, Top and middle, images of animals expressing ASP-3::GFP and ASP-4::GFP. Bottom, confocal images of the subcellular localization of ASP-3::GFP and ASP-4::GFP (see Supplementary Information for details).
neuronal degeneration caused by mec-4(d) is suppressed owing to the aspartyl protease deficiency of cad-1(j1). We observed that degeneration was restored in animals carrying the asp-3 and asp-4 transgenes and to a much lower extent in animals carrying the asp-1 transgene (Fig. 2d). Similarly, overexpression of asp-3 and asp-4 restored degeneration in daf-4(e1364);mec-4(d) and unc-52(su250);mec-4(d) double mutants (see Supplementary Information). Together, our results indicate that ASP-3 and ASP-4 aspartyl proteases are required for neurodegeneration inflicted by diverse genetic insults in C. elegans, but that ASP-1 contributes only marginally.

ASP-1 contains a conserved lysosome-targeting, N-glycosylation site (Asp 71) that is typical of cathepsin D lysosomal proteases that are predominantly localized to lysosomes. Notably, ASP-3 and ASP-4 do not contain this N-glycosylation site but have another potential N-glycosylation site that is common in non-lysosomal cathepsin E proteases (ref. 16 and see Supplementary Information). We examined the expression and subcellular localization of ASP-3 and ASP-4 by fusing GFP at the C termini of both proteins. Strong expression was observed in intestinal cells and to a much lesser extent in other types of cell, including muscle cells, the hypodermis and neurons (Fig. 2e). Both fusion proteins were localized mainly in the cytoplasm, but were also found in lysosomes that appear as distinct autofluorescent puncta (Fig. 2f).

Overexpression of caspase CED-3, the protease that mediates execution of programmed cell death, is sufficient to induce apoptosis in the absence of upstream death initiator signals^14. We examined whether, by analogy, increased expression of the aspartyl proteases ASP-1, ASP-3 and ASP-4 is sufficient to inflict degeneration of specific neurons in C. elegans. We used the mec-4 promoter to overexpress asp-1, asp-3 and asp-4 in the touch receptor neurons, and the motor neuron-specific unc-8 promoter^15 to drive overexpression in the ventral nerve cord motor neurons. A low percentage of transgenic animals expressing asp-3 and asp-4, but not asp-1, in touch receptor neurons showed spontaneous vacuolation of these neurons during late embryogenesis and the early L1 larval stage and failed to respond normally to gentle touch as adults (for asp-3, 12.3 ± 0.8%; for asp-4, 13.1 ± 1.2%; n = 250, background is zero). Similarly, increased expression of asp-3 and asp-4 in motor neurons resulted in variably uncoordinated animals with vacuolated cells in the ventral nerve cord (for asp-3, 9.2 ± 0.5%; for asp-4, 11.6 ± 0.7%; n = 250, background is zero). Staining of cell nuclei with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) in affected adults revealed missing neurons, indicating that overexpression of aspartyl protease caused neuronal death rather than mere malfunction (data not shown).

What is the mechanism that relays signals generated by upstream death initiating events to executioner aspartyl proteases? Perturbation of cytosolic calcium ([Ca2+]i) homeostasis has been implicated in necrotic cell death both in mammals and in C. elegans. But the mechanism by which Ca2+ contributes to cell death remains unclear. Calpains are diverse intracellular papain-like cysteine proteases that require Ca2+ for activation^20. In primate hippocampal neurons, degeneration after acute ischaemia is accompanied by an increase in [Ca2+]i, and concomitant activation of calpain. In addition, activated calpain seems to be localized to disrupted lysosomal membranes (reviewed in ref. 21). These findings have culminated in formulation of the ‘calpain–cathepsin’ hypothesis, whereby an increase in [Ca2+]i activates calpains, which in turn mediate rupture of lysosomes and leakage of killer cathepsins that dismantle the cell^21.

To elucidate the role of calpain activity in C. elegans neurodegeneration, we treated mec-4(d), deg-1(d), deg-3(d) and alpha(gf) mutant animals with Z-Val-Phe-CHO (MDL-28170), a potent calpain inhibitor. Treatment markedly reduced the number of degenerating neurons in all four mutants without reducing the expression of mec-4, deg-1, deg-3 or alpha(gf) (Fig. 3a). This observation suggested that calpain proteases are involved in the cell death process.

The C. elegans genome encodes 17 genes with similarity to calpain, 7 of which show significant identity to mammalian calpains over their whole length (clp-1 to clp-7, see WormBase (http://www.wormbase.org) and Supplementary Information for multiple sequence alignment and phylogenetic tree). clp-5 corresponds to trv-3, a previously characterized gene that is involved in C. elegans sex determination^22. The TRA-3 protease is regulated by Ca2+ but it lacks a typical calmodulin-like Ca2+-binding domain. In TRA-3, a C2-like domain and two Ca2+-binding sites in the protease core are probably Ca2+ sensors that activate this enzyme (see Supplementary Information). Examination of the other CLP sequences showed that CLP-1, CLP-2, CLP-6 and CLP-7 contain motifs that are typical of calpains, including a thiol (cysteine) protease active site and a Ca2+-binding domain, whereas the other two lack either or both
We investigated the role of calpain proteases in neurodegeneration by RNAi-mediated knockdown of clp-1, clp-3, clp-4, clp-6, clp-7 and tra-3 in mec-4(d), deg-3(d) and \( \alpha_c(gf) \) mutant strains. Cell death was suppressed in all three strains when animals were subjected to RNAi with clp-1 and tra-3 but not clp-3, clp-4, clp-6 or clp-7 (Fig. 3b–d). We obtained similar results with tra-3;mec-4(d), tra-3;deg-1(d), tra-3;deg-3(d) and tra-3;\( \alpha_c(gf) \) double mutants (Fig. 3e), further confirming the requirement for TRA-3 in neurodegeneration. Expression of mec-4, deg-3 and \( \alpha_c(gf) \) genes was not detectably reduced in these experiments.

Methods

Strains and genetics

We used standard procedures for C. elegans maintenance, crosses and other genetic manipulations. The cultivation temperature was kept at 20°C, unless noted otherwise. We used the following strains: wild-type N2 Bristol isolate, cad-1(i) II, cad-1(i) IV, unc-232, unc-295, dpy-6, unc-86, tra-3(e1107)/MI, and ASP-4 from the C. elegans database.

Plasmid constructs and RNA interference

We generated reporter constructs by fusing GFP at the C terminus of ASP-3 and ASP-4. We found that calpain activity is required for thapsigargin-induced cell death: treatment with thapsigargin was not effective in animals subjected to RNAi with clp-1, or in tra-3 mutants (Fig. 3f). We also examined the requirement for aspartyl protease activity in thapsigargin-induced cell death. cad-1 mutants and animals subjected to RNAi with asp-3 or asp-4 were resistant to the toxic effects of thapsigargin, but RNAi with asp-1 slightly ameliorated cell death. Observation that calreticulin function in ctp-1 (h229) null mutants, which blocks neurodegeneration induced by mec-4(d) and \( \alpha_c(gf) \), but not deg-3(d), did not suppress thapsigargin toxicity (Fig. 3f). These observations indicate that although CRT-1 is required for the build-up of noxious \([Ca^{2+}]_i\), calpain and aspartyl proteases function downstream of \([Ca^{2+}]_i\) signalling to facilitate death.

clp-1 is expressed strongly in many types of cell and tissue, including muscle cells and neurons (Fig. 4a and Supplementary Information). tra-3 is also expressed in the nervous system of the animal (S. Sokol and P. Kuwabara, personal communication). We examined synergy between proteases of the same type, as well as between aspartyl proteases and calpains, in neurodegeneration. We observed that simultaneous RNAi with both asp-3 and asp-4 resulted in an enhanced suppression of neurodegeneration induced by mec-4(d). Similarly, RNAi with both calpains further increased suppression. But we did not observe synergy between aspartyl proteases and calpains (Fig. 4b). Therefore, aspartyl and calpain proteases function in the same pathway that facilitates neurodegeneration in C. elegans. We did not achieve complete or near-complete blockage of neurodegeneration in these experiments. Incomplete suppression of cell death by aspartyl or calpain protease deficiency in our trials could be due to the limited capacity of RNAi to knockdown genes efficiently in the nematode nervous system, the contribution of other additional biochemical pathways and protease activities, or both. A comprehensive study of the remaining calpain proteases including CLP-2, which contains all of the catalytic residues that are typical of calpains (Supplementary Table 1), may illuminate this issue.

We propose that diverse death-initiating conditions converge, in part, to increase \([Ca^{2+}]_i\), which signals the activation of calpain proteases that subsequently engage executioner lysosomal and cytoplasmic aspartyl proteases, leading to cell destruction (Fig. 4c). Consistent with this model, neurodegeneration inflicted by cell-specific overexpression of asp-3 and asp-4 cannot be bypassed by a deficiency in either or both calpains (Supplementary Table 2). The identification of two specific classes of protease that are required for neurodegeneration in C. elegans may provide insight into similar pathologies in mammals. The lysosomal degradation system has been found to be upregulated in neurons of individuals affected with Alzheimer’s disease, and cathepsin D expression is induced under conditions of excitotoxic cell death (reviewed in ref. 25). In addition, calpain inhibitors can be protective in certain cases of nerve or muscle degeneration after ischemic episodes26,27. These findings suggest that, similar to apoptosis, necrotic cell death mechanisms are conserved from nematodes to humans, and they highlight specific executioner proteases as potential targets for therapeutic intervention in an effort to battle neurodegenerative disorders.
Somatic hypermutation of immunoglobulin genes is a unique, targeted, adaptive process. While B cells are engaged in germinal centres in T-dependent responses, single base substitutions are introduced in the expressed V(H)/V(D)J genes to allow the selection of mutants with a higher affinity for the immunizing antigen. Almost every possible DNA transaction has been proposed to explain this process, but each of these models includes an error-prone DNA synthesis step that introduces the mutations. The Y family of DNA polymerases is specialized for copying DNA lesions and have high rates of error when copying a normal DNA template. By performing gene inactivation in a Burkitt's lymphoma cell line inducible for hypermutation, we show here that somatic hypermutation is dependent on DNA polymerase iota.