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Alteration of the nuclear pore complex in Ca^{2+}-mediated cell death

D Bano*[,1,4], D Dinsdale[1], A Cabrera-Socorro[1,6], S Maida[1,6], N Lambacher[1], B Mccoll[2], E Ferrando-May[3], MO Hengartner[4] and P Nicotera*[,1,5]

Cell death requires coordinated intracellular signalling before disassembly of cell architecture by degradative enzymes. Although the death signalling cascades that involve the mitochondria, the ER and the plasma membrane have been extensively characterized, only a handful of studies have examined the functional and structural alterations of the nuclear pore complex (NPC) during neuronal death. Here, we show that during excitotoxic neuronal degeneration calpains redistributed across the nuclear envelope and mediated the degradation of NPC components causing altered permeability of the nuclear membrane. In primary dissociated neurons, simultaneous recording of cytosolic [Ca^{2+}] and localization of fluorescent proteins showed that the onset of Ca^{2+} overload signalled a progressive increase in the diffusion of small reporter molecules across the nuclear envelope. Later, calpain-mediated changes in nuclear pore permeability allowed accumulation of large proteins in the nucleus. Further, in a model of excitotoxic neuronal degeneration in *Caenorhabditis elegans*, we found similar nuclear changes and redistribution of fluorescent probes across the nuclear membrane in dying neurons. Our findings strongly suggest that increased leakiness of the nuclear barrier affects nucleocytoplasmic transport, alters the localization of proteins across the nuclear envelope and it is likely to be involved in Ca^{2+}-dependent cell death, including ischemic neuronal demise.
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The execution of cell death requires coordinated signalling between intracellular compartments and the plasma membrane.[1] Death-initiating signals can be generated at the plasma membrane,[2] or as a result of intracellular stress.[3–5] As part of this signalling process, the redistribution of molecules across cellular compartments occurs in different cell death paradigms. Examples include poly-ADP-ribose polymers[6] and p53,[7] which translocate from the nucleus to the mitochondria, glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[8] which redistributes from the cytosol to the nucleus, and the pro-apoptotic protein apoptosis-inducing factor (AIF),[9,10] which diffuses from the mitochondria to the nucleus. As part of the death program, molecular redistribution is also required to process intracellular structures. Thus, the targets for the proteolytic systems involved in cell death include membranes that form selective permeability barriers to molecular trafficking.

Selective loss of nuclear permeability has been associated with virus-mediated and caspase-dependent cell death. In apoptosis, processing of several components of the envelope is downstream of caspase activation and thereby downstream of the mitochondrial signals for caspase activation. Disassembly of the nuclear envelope,[11] intranuclear proteins[12–15] and chromosomal DNA[16,17] are likely to have an important function in promoting disposal of the nucleus and its content. Probably because nuclear permeabilization is thought to be a late event in death programs, only a handful of studies have examined functional and structural alterations of nucleocytoplasmic transport during cell injury and death. The nature of the signals that cause early nuclear changes in cell demise is still unclear, although some reports have shown that during apoptosis degradation of nuclear pore complex (NPC) components increases leakiness of the nuclear barrier and facilitate the redistribution of proteins across the nuclear envelope.[18–21] It is not known, however, whether nuclear function is compromised at the signalling stage in non-apoptotic, caspase-independent excitotoxicity. Hence, we asked whether the death signals in the early phases of Ca^{2+}-mediated neurotoxicity trigger nuclear changes that participate in cell demise.

Here, we examined the deregulation of the NPC during calcium-mediated cell death. We have shown that Ca^{2+} overload and Ca^{2+}-dependent proteases (calpains) impaired the machinery responsible for macromolecular partitioning across the nuclear membrane. We established the order of the events that promote the redistribution of proteins across the nuclear envelope in primary neurons, in which Ca^{2+} overload was elicited by glutamate,[22,23] and in *Caenorhabditis elegans*,

[1]MRC Toxicology Unit, Hodgkin Building, University of Leicester, Leicester LE1 9HN, UK; [2]Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK; [3]Faculty of Biology, University of Konstanz, Konstanz D-78457, Germany; [4]Institute of Molecular Biology, University of Zurich, Zurich CH-8057, Switzerland and [5]German Centre for Neurodegenerative Diseases, DZNE, Bonn 57135, Germany
*Corresponding authors: D Bano, MRC Toxicology Unit, Hodgkin Building, University of Leicester, Lancaster Road LE1 9HN, Leicester, UK.
Tel: +44 0116 252 5527; Fax: +44 0116 252 5616; E-mail: db81@le.ac.uk
or P Nicotera, German Centre for Neurodegenerative Diseases, DZNE, Ludwig-Erhard-Alle 2, 57135 Bonn, Germany. E-mail: pierluigi.nicotera@dzne.de
[6]These authors contributed equally to this work
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Abbreviations: NPC, nuclear pore complex; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AIF, apoptotic-inducing factor; CGNs, cerebellar granule neurons; CNs, cortical neurons; YFP, yellow fluorescent protein; NES, nuclear export sequence
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where neurodegeneration is associated with a gain-of-function mutation in the degenerin plasma membrane channel DEG-3 acetylcholine receptor. We unveil a new mechanism that shows changes in NPC function and we propose that the signalling between the nucleus and the rest of the cell may have a relevant function in the early phases as well as in the execution of caspase-independent cell death.

Results

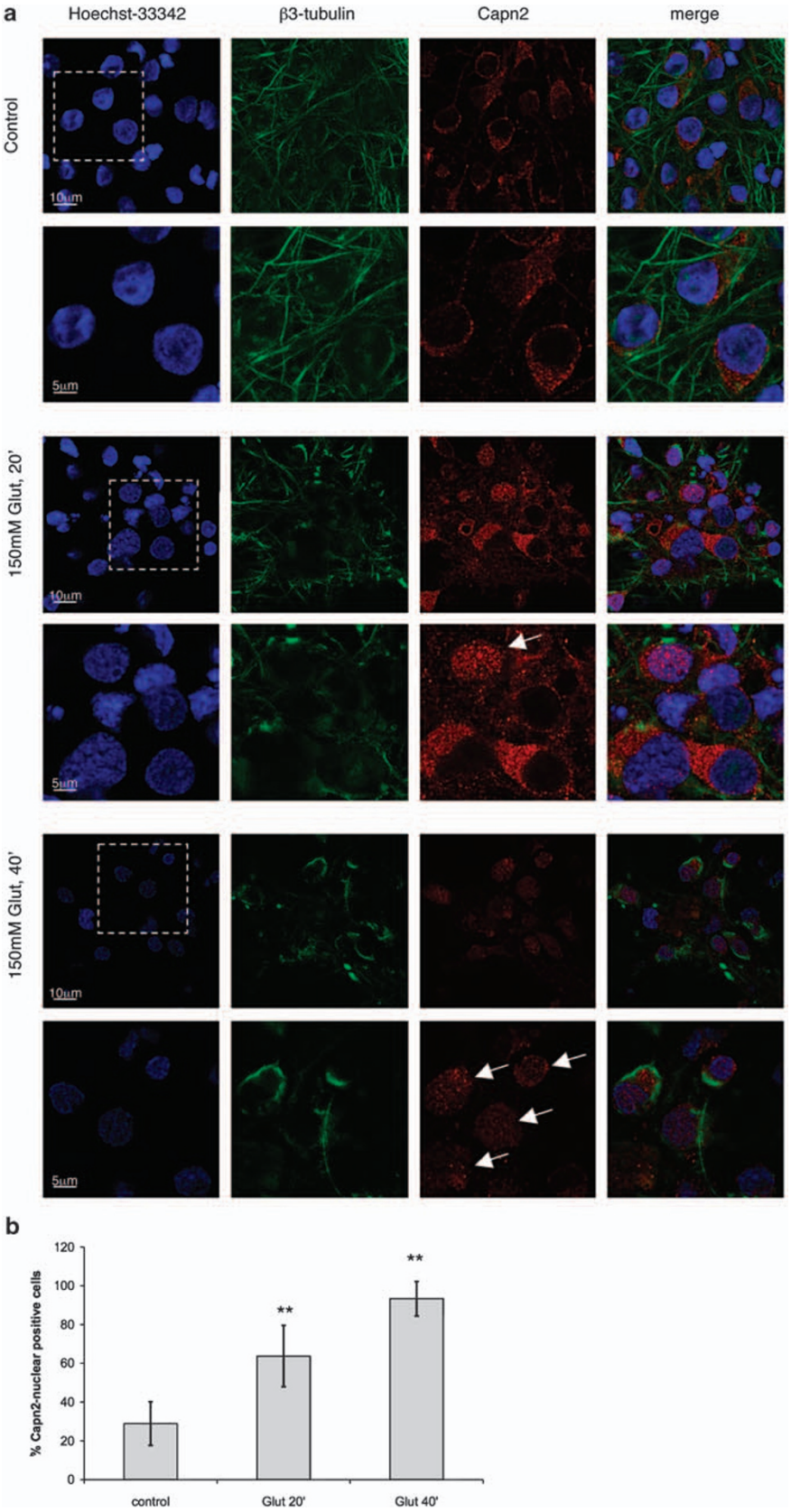
Changes of calpain localization and loss of NPC components on prolonged glutamate stimulation in primary neurons. Intracellular Ca^{2+} homeostasis is tightly regulated by a series of enzymes localized at the plasma membrane and intracellular organelles. However, sustained synaptic activity, such as during pathological conditions, leads to massive Ca^{2+} entry that activates calcium-dependent proteases, calpains, which are highly expressed in the nervous system. It has been widely shown that calpains have a pivotal role during excitotoxicity by selective cleavage of scaffolding proteins, receptors and enzymes.[24] Previously, we reported that calpains cleave the Na^{+}/Ca^{2+}-exchanger,[22] which is a plasma membrane-embedded enzyme involved in calcium homeostasis. We wanted to gain further insights into the role of calpains in neuronal demise and therefore we studied the localization of one of the ubiquitous calpains, calpain II, using a specific antibody, which does not recognize calpain I (data not shown). In healthy primary cortical neurons (CNs), calpain II was mainly localized in the cellular body and in the projections (Figure 1a), as shown in earlier reports in which calpains were demonstrated to localize in the cytosol or in association with intracellular membranes.[25,26] However, prolonged glutamate stimulation caused a time-dependent nuclear accumulation of calpain II (Figure 1a and b). Previously, it has been shown that during apoptosis caspases can accumulate in the nucleus and affect the composition of the NPC.[27] Therefore, we tested whether NPC components could be affected during excitotoxicity in the same time scale as calpain II nuclear accumulation. Immunocytochemical analysis was performed using Mab414, an antibody that recognizes most of the FXFG-containing nucleoporins.[28,29] Untreated cells showed punctuate staining around the nuclear rim, whereas, in glutamate-treated cells, we detected a time-dependent loss of this NPC staining (Figure 2a and b). We tested the loss of nucleoporins by immunoblot analysis and we observed that most of the FXFG-containing nucleoporins were degraded on excitotoxic glutamate treatment (Figure 2c). Our findings suggest that during glutamate-mediated neuronal death there is the loss of NPC components concomitant with the nuclear accumulation of calpains.

Calpains mediate degradation of NPC components during excitotoxicity. In eukaryotic cells, both the diffusion and the receptor-associated transport of molecules are mediated by the NPC,[30] which is a complex of multiple copies of about 30 different protein subunits, named nucleoporins.[31] Among the NPC components, nucleoporins containing hydrophobic peptide repeats FXFG have a major role in the docking and translocation of cargoes through the NPC channel. To better understand the changes in the NPC and in the nuclear compartments, we used cerebellar granule neurons (CGNs) and CNs as two separate cellular model systems. In CGNs treated with glutamate or deprived of oxygen and glucose a number of FXFG-containing nucleoporins underwent proteolysis in a time-dependent manner (Figure 3a; Supplementary figure S1A). As excitotoxic insults cause activation of calpains,[22] we inhibited calpain activity by expressing the endogenous inhibitor protein, calpastatin, or pharamacologically by using calpeptin. Both calpastatin and calpeptin inhibited calpain activity and prevented nucleoporin degradation (Figure 3b; Supplementary Figure S1B), whereas proteasome and cathepsin inhibitors could not block the degradation (data not shown). To confirm further that calpains could cleave nucleoporins, we performed a cell-free assay using recombinant purified calpain II. At all concentrations used, active calpains could decrease the amount of detectable full-length nucleoporins detected by the Mab414 (Figure 3c). In addition, we tested whether Nup93, which does not belong to the FXFG-containing nucleoporins family and is cleaved during caspase-dependent cell death,[21] was cleaved during excitotoxicity in primary dissociated murine CNs. We could not detect Nup93 fragmentation in neurons undergoing excitotoxicity (Supplementary Figure S1B), although recombinant calpains cleaved the protein in cell-free assays at the N-terminus generating two fragments (Figure 3c; Supplementary Figure S1C). We also tested whether calpains could affect the basal level of FXFG-containing nucleoporins. HeLa cells were transfected with RNAi against calpain small subunit 1 (Capn4S1), which stabilizes and regulates the protease activity in a Ca^{2+}-dependent manner.[32] Downregulation of Capn4S1 caused a loss of calpain activity, as tested by casein zymograph (data not shown). However, loss of Capn4S1 did not affect the nucleoporin protein level (Supplementary Figure S1D), which can rule out that a decrease in calpain activity can affect the expression and the composition of NPC components.

Taken together, these findings suggest that activation of calpains during excitotoxicity results in the degradation of most of the FXFG-containing nucleoporins.

Contrary to what is observed in apoptosis, we did not see degradation products in our immunoblot analyses but only in cell-free experiments. We therefore tested for the presence of calpain cleavage sites in nucleoporins. We selected Nup62, which is one of the components involved in the formation of the NPC central core, and overexpressed Nup62 fused at the C-terminus with yellow fluorescent protein (YFP) in 293T cells. Immunoblot analysis of calpain cell-free assay showed

Figure 1 Excitotoxic glutamate stimulation causes nuclear accumulation of calpain II. (**a**) Rat cortical neurons exposed to glutamate at the indicated time were fixed and immunostained with anti-β-3 tubulin (green) and calpain II (red) antibodies. Nuclei were visualized by Hoechst-33342 (blue). (**b**) Statistical analysis of time-dependent calpain II nuclear accumulation following excitotoxic insult. Bar graph represent the mean of three independent experiments and error bars indicate ± S.E.M. Significant differences are indicated by symbol **($P<0.01$; one-way ANOVA)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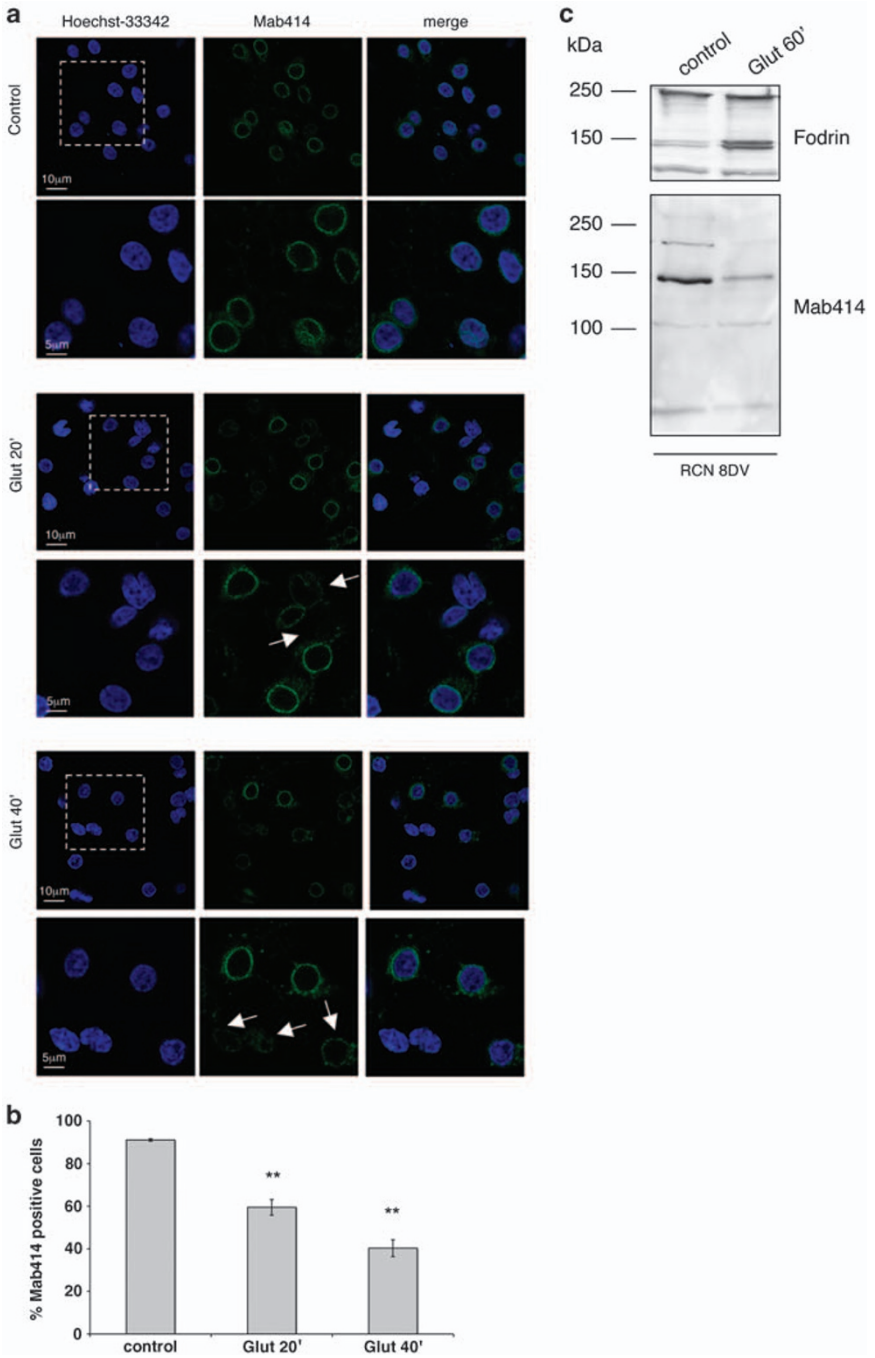

Figure 2 Prolonged glutamate treatment causes loss of NPC components. (**a**) Rat cortical neurons untreated or exposed to excitotoxic glutamate concentration for 20 and 40 min were fixed and stained with Mab414 (green) and Hoechst-33342 (blue). White arrows indicate cells with loss of Mab414 staining. (**b**) Statistical analysis of time-dependent loss of Mab414 staining (less then 50% compare with untreated cells) on glutamate treatment. Bar graph represent the mean of three independent experiments and error bars indicate ± S.E.M. Significant differences are indicated by symbol **($P<0.01$; one-way ANOVA). (**c**) Immunoblot analysis using Mab414 and fodrin of rat cortical neurons exposed to glutamate for 60 min

that an antibody specific for the GFP moiety recognized several proteolytic fragments, whereas Mab414 detected a loss of the signal corresponding to the full-length fusion protein and the endogenous Nup62 (Figure 3d). In addition, we generated an N-terminal and a C-terminal HA-containing Nup62 and we overexpressed each construct in 293T cells. As shown in Figure 3e, active recombinant calpains mediated the loss of the HA-tags, without generating any detectable proteolytic fragments. This strongly suggests the presence of calpain cleavage sites both at the N- and C-terminus of Nup62. We generated an HA-containing construct in which the first 100 amino acids of Nup62 were fused to YFP (HA-Nup62(1–100)-YFP). Homogenates of 293T cells over-expressing the fusion proteins were incubated with recombinant

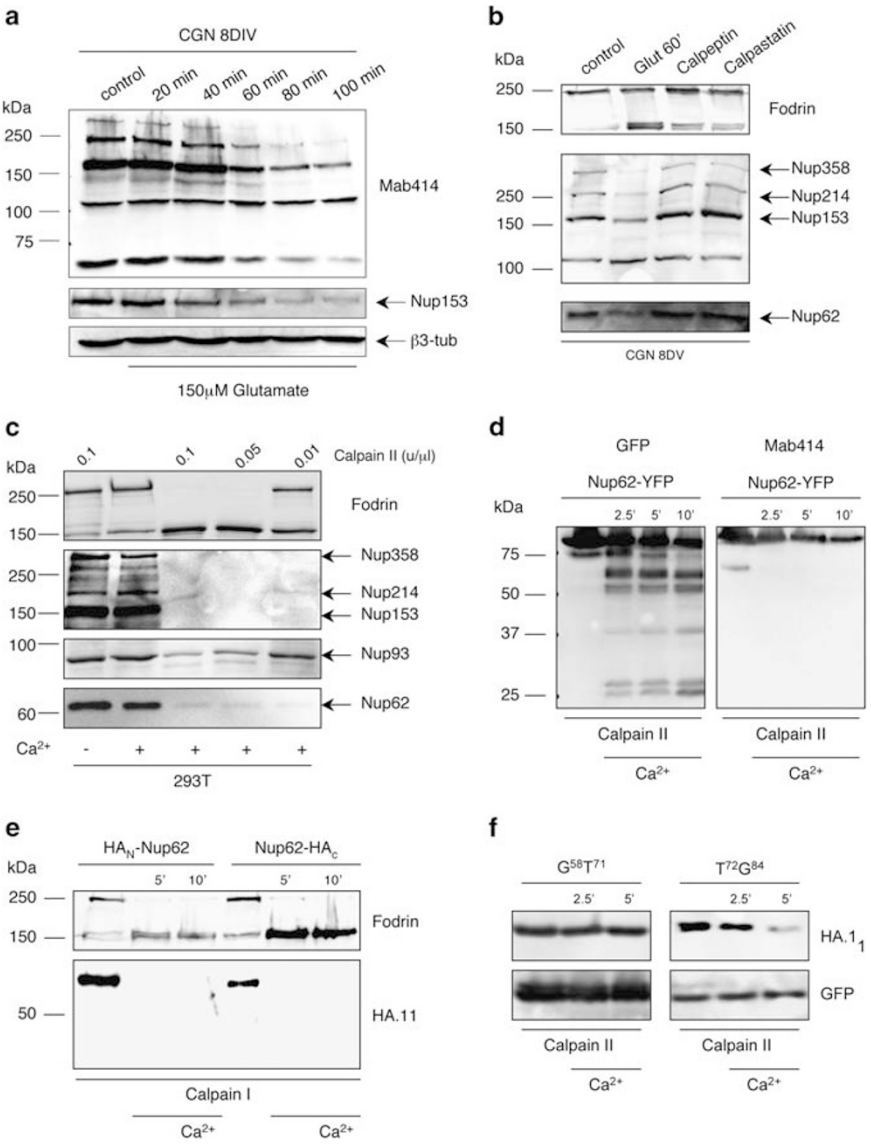

Figure 3 Cleavage of nuclear pore complex proteins is mediated by calpains. (**a**) Immunoblot analysis of CGNs exposed to glutamate at the indicated time points. Loss of nucleoporins was detected using Mab414 and anti-Nup153 antibody. β-3 tubulin was used as gel loading control. (**b**) Wild type or calpastatin overexpressing CGNs were treated with glutamate for 1 h, in presence or absence of calpeptin. The total cell lysate was subjected to western blotting analysis using antibodies to Mab414 and fodrin. (**c**) Homogenate of 293T was incubated with different concentrations of recombinant calpain II in the presence or absence of Ca^{2+}. Nitrocellulose filter was developed using Mab414, anti-fodrin and anti-Nup93 antibodies. (**d**) Homogenates of 293T cells overexpressing Nup62-YFP were incubated with recombinant calpain II in the presence or absence of calcium. Immunoblot analysis was performed using antibody to nucleoporins (Mab414) and GFP. (**e**) Homogenates of 293T cells overexpressing HA_N-Nup62 or HA_C-Nup62 were incubated with recombinant calpain I, in the presence or absence of Ca^{2+}. Nitrocellulose filter was developed using antibody to HA-tag, whereas anti-fodrin antibody was used as control for calpain activity. (**f**) Whole-cell extracts of 293T cells overexpressing HA-$G^{58}T^{71}$-YFP or HA-$T^{72}G^{84}$-YFP were incubated with recombinant calpain II, in presence or absence of calcium. Western blot was probed with antibody to HA-tag and GFP

calpains. Immunoblot analysis using an anti-HA antibody confirmed that calpains mediated the loss of the HA-tag, and it was possible to detect four fragments when the same filter was re-probed with an antibody against YFP (Supplementary Figure S1E). On the basis of molecular weight of the fragments, it was unlikely that calpain cleavage occurred within the first 50 amino acids. We narrowed down the region in which calpains cleave Nup62 using a series of constructs in which portions of Nup62 were fused to YFP. Homogenates of 293T cells overexpressing the fusion proteins were incubated with active recombinant calpains in a cell-free assay. Immunoblot analysis using antibodies against HA and GFP confirmed the presence of a calpain cleavage site within the region that spans from Thr 72 to Gly 84 (Figure 3f).

Taken together, these results suggest the presence of multiple calpain cleavage sites in Nup62 and probably also in other NPC components.

Redistribution of proteins between the nucleus and cytoplasm during neuronal excitotoxicity. NPCs control the active transport and limit the diffusion of RNA, ribosomes and proteins through the nuclear channels. To test whether

we could observe an increase in nuclear permeability, we measured the redistribution of GAPDH across the nuclear envelope in CGNs after treatment with exitotoxic glutamate concentrations. In healthy CGNs, GAPDH was found exclusively within the cytosol whereas on excitotoxic glutamate stimulation GAPDH accumulated in the nucleus in 25.6 ± 3.8%, of the neurons within 1 h (number of experiments = 2) (Figure 4a). More importantly, pretreatment with calpeptin caused a partial but statistically inhibition of the nuclear accumulation of GAPDH following glutamate treatment (Figure 4b), which indicates that calpains are responsible for the redistribution of cytosolic proteins across the nuclear envelope. Along with GAPDH, other cytosolic proteins, such as APAF-1[33] and HDAC4,[34] also accumulated in the nucleus on excitotoxic glutamate stimulation (data not shown).

To monitor whether protein redistribution in CGNs was linked to modifications of the nuclear transport and permeability barrier, we expressed a fusion protein between YFP and a nuclear export sequence (NES-2). Control neurons expressing the reporter only displayed fluorescence in the cytoplasm, whereas YFP accumulated in the nucleus 30 min after exposure to excitotoxic glutamate concentrations (Figure 5a). We investigated whether redistribution of the YFP reporter was caused by proteolytic removal of the NES. Immunoblot analysis showed that the fusion protein was neither cleaved in a cell-free assay by recombinant calpain II (Supplementary Figure S2B), nor in CGNs challenged with glutamate (Supplementary Figure S2C). To examine the temporal dynamics of NES-2 redistribution and nuclear translocation during the early stage of the excitotoxic process,

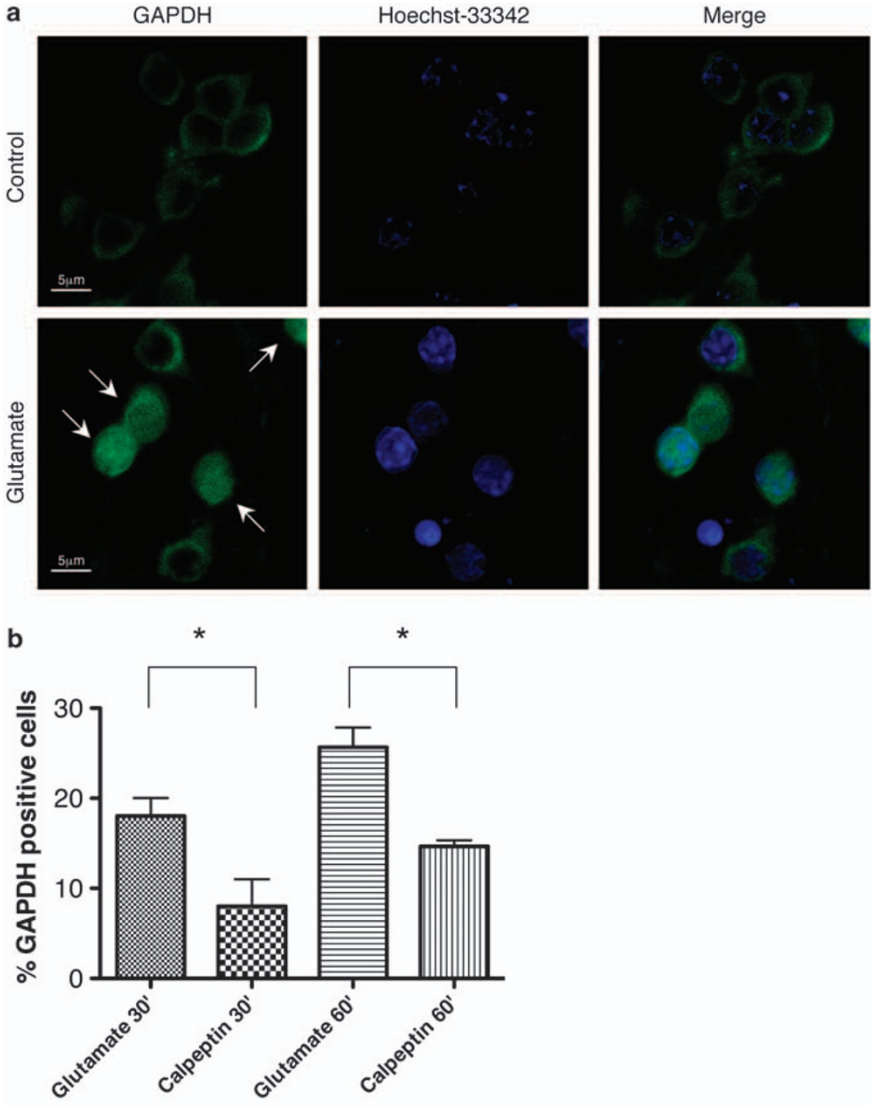

Figure 4 GAPDH accumulates in the nucleus during excitotoxicity. (**a**) Immunofluorescent localization of GAPDH (green) in healthy neurons and during excitotoxicity. Nuclei were visualized by Hoechst-33342 (blue), arrows indicate cell with nuclear GAPDH. (**b**) Statistical analysis of time-dependent GAPDH nuclear accumulation following glutamate treatment, in presence or absence of calpeptin. Bar graph represents the mean of three independent experiments and error bars indicate ± S.E.M. Significant differences are indicated by symbol *($P < 0.05$; one-way ANOVA)

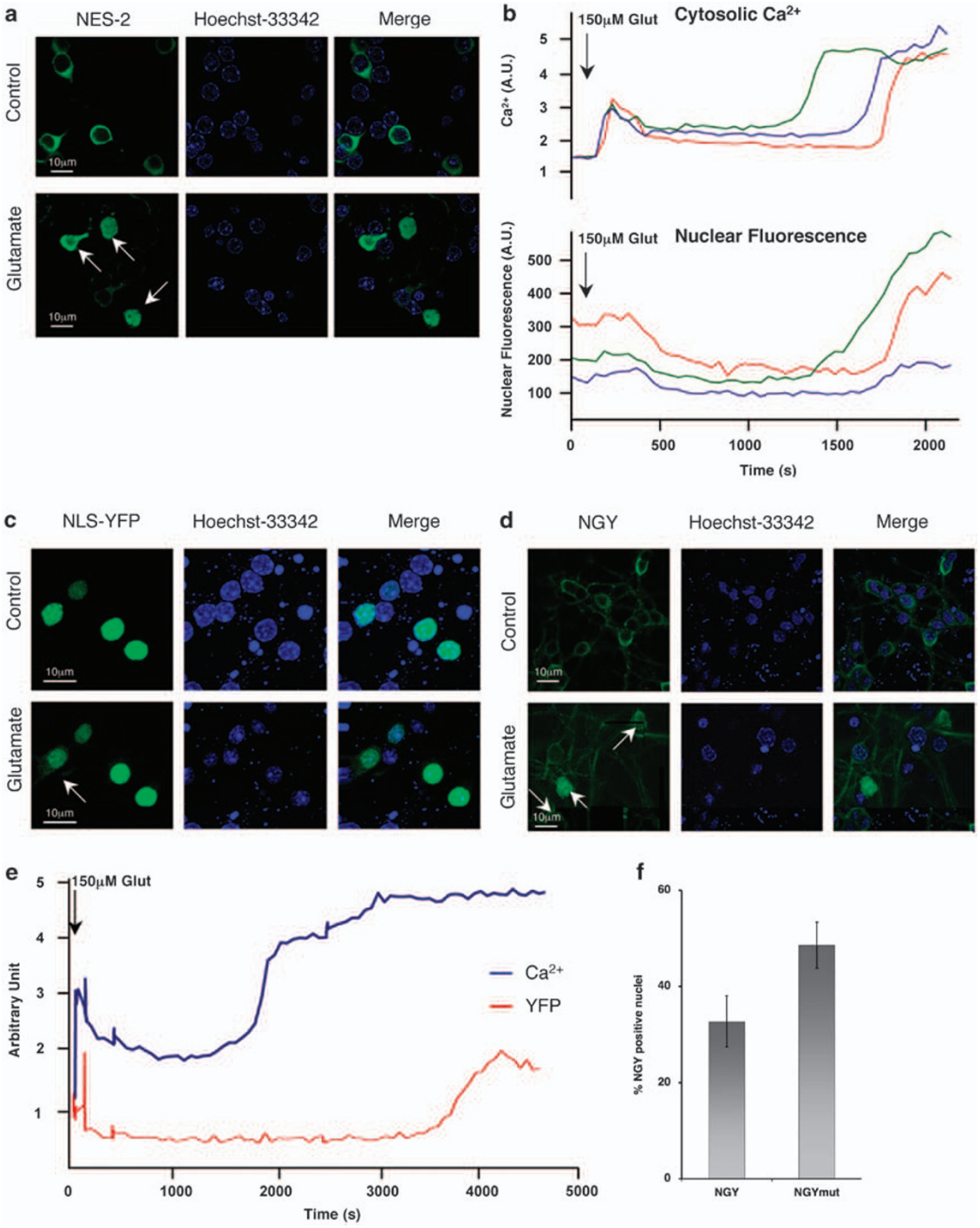

Figure 5 Increase of nuclear permeability during glutamate-mediated cell death. (**a**) Confocal analysis of NES-2 (green) in CGNs exposed to glutamate. Nuclei were labelled with Hoechst-33342 (blue). (**b**) Time course confocal microscopy of CGNs overexpressing NES-2 and loaded with Fura-Red (number of experiments = 5, number of cells per experiments > 10). Each color represents a single neuron. (**c**) Time lapse confocal microscopy analysis of CGNs overexpressing NLS-YFP (green) and treated with glutamate for 40 min. Nuclei were labelled with Hoechst-33342 (blue). (**d**) Confocal images of CGNs overexpressing NGY (green) exposed to glutamate for 30 min. Nuclei were visualized using Hoechst-33342 (blue). (**e**) Time course confocal microscopy of CGNs overexpressing NGY and loaded with Fura-Red. Re-localization of the fluorescent protein and Ca^{2+} homeostasis was simultaneously recorded by confocal microscopy after glutamate treatment (number of experiments = 5, number of cells for each experiments > 15). (**f**) CGNs were transduced with lentiviral particles coding NGY or NGYmut and treated with glutamate for 1 h. Accumulation of fluorescent reporter in the nucleus was scored and statistical analysis performed

we monitored simultaneously the nuclear accumulation of the NES-containing YFP and intracellular Ca^{2+} levels using real-time confocal microscopy. To this end, neurons overexpressing NES-2 were loaded with the Ca^{2+}-sensitive dye Fura-Red, which was used as a non-ratiometric dye for qualitative analysis. Interestingly, nuclear accumulation of

NES-2 began at the time when the irreversible secondary Ca^{2+} deregulation occurred (Figure 5b). To monitor calpain activation we also generated a fluorescent calpain reporter containing the cleavage site of NCX3[22] downstream of an NES (NES-3). In this case calpains cleaved the substrate, which accumulated in the nucleus with kinetics similar to those of NES-2 (Supplementary Figure S2A, S2D, S2E, S2F). Thus, calpain cleavage of the NES was not a prerequisite for their redistribution, because both cleavable and non-cleavable substrates were translocated across the nuclear envelope with similar kinetics. Therefore, Ca^{2+} overload can affect the localization of NES-containing small-size molecules across the nuclear envelope.

To examine whether nucleocytoplasmic traffic in the opposite direction, that is from the nucleus to the cytosol was also affected, we targeted YFP to the nucleus by fusing it to a nuclear localization sequence (NLS-YFP). In control CGNs, fluorescence was confined exclusively to the nucleus, whereas in neurons treated with glutamate, the nuclear marker was detected also in the axo-dendritic projections (Figure 5c). The redistribution was not because of cleavage of the NLS, as recombinant calpain did not alter the NLS-containing protein (Supplementary Figure S2G). These findings show that nucleocytoplasmic trafficking in both directions is affected at an early stage following exposure of CGNs to glutamate.

The NPC limits the passive diffusion of non-nuclear macromolecules across the nuclear envelope with an exclusion limit of approximately 50–60 kDa. To determine whether cytosolic proteins with a molecular mass exceeding the size exclusion limit could also accumulate into the nucleus during excitotoxicity, we generated a 72 kDa fusion protein of GAPDH with a NES-containing YFP (NGY). This protein was efficiently excluded from the nucleus of healthy neurons (Figure 5d) and was neither cleaved in CGNs treated with glutamate nor in cell-free assays by recombinant calpains (Supplementary Figure S2H–J). Notably, prolonged glutamate exposure caused redistribution of the fluorescent marker to the nucleus of neurons undergoing excitotoxicity (Figure 5d). Thus, we studied the kinetics of NGY diffusion as a marker of NPC permeability. CGNs overexpressing the large-size fluorescent protein were loaded with Fura-Red and both NGY redistribution and Ca^{2+} levels were measured simultaneously. NGY accumulated in the nucleus between 40 and 80 min after glutamate stimulation in the majority of neurons undergoing excitotoxicity, consistent with the time-frame of the loss of NPC components. In 36% of the cells NGY nuclear translocation occurred between 15 and 30 min after the irreversible secondary Ca^{2+} deregulation and the initial redistribution of the lower molecular mass fluorescent marker. In 54% of the cells, the redistribution of the probe occurred 30 min after the secondary Ca^{2+} overshoot (Figure 5e).

Nitric oxide (NO)-mediated neurotoxicity is known to trigger *S*-nitrosylation of GAPDH.[8] Furthermore, calpains modulate intracellular redistribution of NO.[35] To examine whether NO might contribute to the nuclear accumulation of our GAPDH fluorescent reporter, we mutated the cysteine at position 152 on GAPDH (which can be nitrosylated) and the lysine at position 227 (which interacts with Siah1) (NGYmut) and we monitored the localization of our fluorescent reporter on glutamate stimulation. As shown in Figure 5f, NGYmut accumulated in the nucleus in a manner similar to NGY (number of experiments = 3, number of cells for each experiment >50). Thus, the observed redistribution of NGY in our experimental system was not mediated by NO-dependent mechanisms.

All together, our data suggest that during excitotoxicity calcium and calpains affect the localization of proteins across the nuclear envelope. Moreover, as the kinetics of nuclear accumulation of NES-containing 70 kDa protein was different from the NES-GFP, we can exclude that the redistribution of the protein across the nuclear envelope is caused by an impairment of the transport machinery but rather by an increase in the leakiness of the NPC.

Calpeptin-sensitive proteases mediate the disassembly of NPC structure. Alteration of nuclear morphology is a common feature observed during cell death and it has been attributed to the detachment of the heterochromatin from the nuclear envelope following degradation of scaffolding proteins. We tested whether the loss of NPC components and the consequent increase of leakiness were an early event compared with the disassembly of the nuclear structure. Transmission electron microscopy showed that nuclear pores were retained on excitotoxic insult in the time range of 20–60 min, with no apparent differences from those seen in untreated cells (Figure 6a–d). Longer glutamate treatment (90 min) caused shrinkage of nuclei and condensation of heterochromatin into sharply defined electron-dense clumps, often abutting the inner membrane of the nuclear envelope. These clumps resembled those observed during the early stages of apoptosis but did not coalesce into the crescents of heterochromatin, often associated with that mode of cell death. However, nuclear pores were still retained between these clumps and their morphology was no different from that seen in untreated cells (Figure 6e). Notably, at a later stage (120 min), when heterochromatin clumps separated from the nuclear membrane of the majority of neurons (82%), clustering/opening of nuclear pores was observed in about one third of the cells (Figure 6f). Abnormal nuclear pores were not discernable in nuclei in which the separation of the heterochromatin from the nuclear membrane did not occur. In the presence of calpeptin, though all the early morphological changes associated with glutamate treatment were still observed, there was no separation of heterochromatin from the nuclear membrane or clustering/opening of nuclear pores (Figure 6g). This strongly supports the idea that calpains are involved in the alteration of NPC function and mediate the disassembly of the nuclear envelope, although this event occurs later and is not the cause of the altered protein distribution across the nuclear envelope.

Downregulation of certain nucleoporins by RNAi affects the localization of fluorescent reporters in HeLa cells. To mimic the loss of FXFG-containing nucleoporins and test whether this could affect nuclear permeability, we downregulated the expression of selected nucleoporins, which have been shown to be involved in nucleocytoplasmic transport[36,37] in HeLa cells. Specific siRNA synthetic duplexes against Nup214, Nup205, Nup153 and Nup62 were used to decrease the levels of these proteins in

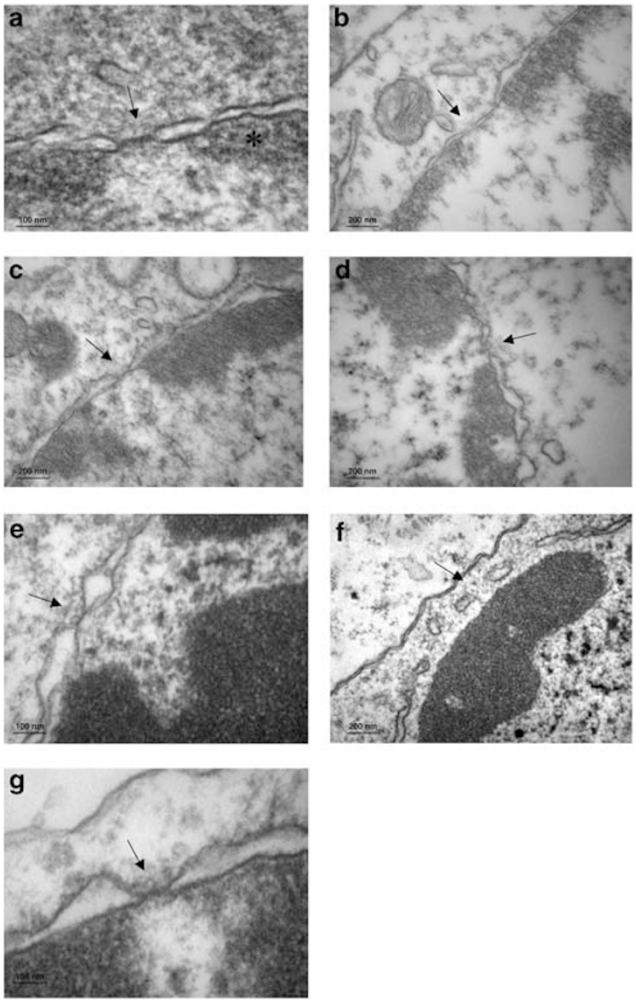

Figure 6 Transmission electron microscopy of CGNs. (**a**–**d**) Untreated cells (**a**) and neurons exposed to 150 μM glutamate for 20 (**b**), 40 (**c**) and 60 (**d**) min with normal distribution of heterochromatin (*) and presence of nuclear pores (arrows). (**e**) Longer (90 min) glutamate treatment caused condensation of heterochromatin as discrete electron-dense clumps (*), often abutting the inner membrane of the nuclear envelope. Nuclear pores are present between these clumps (arrow) and they retain a normal appearance despite mild swelling of the nuclear envelope (arrowheads). (**f**) Further (120 min) treatment with glutamate resulted in separation of the heterochromatin from the nuclear membrane and clustering/opening of nuclear pores. (**g**) Cells pretreated with calpeptin and exposed to glutamate showed all the early morphological changes associated with excitotoxic demise but no separation of the heterochromatin from the nuclear membrane or clustering/opening of nuclear pores (arrow)

HeLa cells. To determine the efficiency of the designed oligonucleotides, downregulation of Nup153 and Nup62 was confirmed by western blot analysis (Supplementary Figure S3A). Electron microscopy confirmed that none of the hairpins caused morphological changes of the nuclear pore (data not shown). Interestingly, downregulation by RNAi of Nup205 and Nup62, but not Nup214 and Nup153, caused nuclear accumulation of exogenous tetrameric Cherry (4xCherry; Supplementary Figure S3B), confirming that loss of the selected nucleoporins influences the size limit of the NPC. We tested whether the loss of a single nucleoporin could affect the localization of NES-containing protein. Thus, we overexpressed in HeLa cells NGY together with 4xCherry and then downregulated the expression of Nup153 and Nup62. Loss of Nup153 did not affect either the localization of 4xCherry or NGY, whereas RNAi against Nup62 caused nuclear accumulation of 4xCherry in most of the cells (Figure 7a). However, loss of Nup62 caused the accumulation of NGY in only 20% of 4xCherry nuclear-positive cells, which can be explained by the fact that Nup62 is involved in transport across the nuclear envelope.

Taken together, our data indicate that downregulation of one single NPC component can affect the permeability of the NPC's central transport channel, but it is not sufficient to induce massive changes in protein localization as observed during excitotoxicity-mediated nucleoporin degradation.

Hyperactive degenerative channels cause nuclear changes and relocation of exogenous fluorescent probes in dying neurons of *C. elegans*. In the nematode *C. elegans*, a gain-of-function mutation of the gene encoding for the α subunit of a nicotinic acetylcholine receptor *deg-3*[38] evokes an increase of intracellular Ca^{2+} concentration that causes neurodegeneration.[39] To gain insight into the molecular events following calcium deregulation, we tested nuclear morphological changes in dying neurons. We generated a strain of animals in which the promoter *unc-119* was used to drive the expression of mitochondria-targeted GFP and human histone, H2B, fused to Cherry (H2B-Cherry), which localized in the mitochondria and in the nucleus, respectively, of most of the neurons. As shown earlier,[40] in *deg-3(u662)* mutant larvae, dying neurons displayed large vacuoles with shrunken nuclei and condensed mitochondria clustered to one side of the swelling cell (Figure 8a). We tested the alteration of NPC functions during calcium-mediated cell death in living animals using a strain of animals carrying GFP fused to the *C. elegans* nucleoporin NPP-1 (NPP-1::GFP) expressed by the promoter *npp-1* (kindly provided by Dr. Jun Kelly Liu).[41] We introduced the integrated P_{npp-1}::*npp-1*::*GFP* transgene into the mutant *deg-3(u662)* and by confocal analysis we studied nuclear changes in degenerating neurons. As expected, GFP localization was limited at the nuclear envelope in healthy cells, whereas we could observe a partial (Figure 8b) or complete (Figure 8c) loss of NPP-1::GFP signal in degenerating neurons. Therefore, in nematodes calcium overload triggered by degenerin channels can affect nuclear morphology and induce the loss of NPC components, echoing our observation in primary neurons undergoing excitotoxicity.

To confirm further the general impairment of nuclear activity during calcium-mediated cell death in *C. elegans*, we generated a strain of animals overexpressing a Cherry construct targeted to the nucleus (NLS-Cherry) in the nervous system (Supplementary Figure S4A). We introduced the extrachromosomal array into the mutant *deg-3(u662)* carrying P_{npp-1}::*npp-1*::*GFP* transgene. In wild-type animals, NLS-Cherry was restricted to the nucleoplasm, whereas NPP-1::GFP was localized at the nuclear envelope (Figure 9a). In contrast, in *deg-3(u662)* animals confocal analysis clearly showed partial loss of NPP-1::GFP and redistribution of NLS-Cherry across the nuclear envelope (Figure 9a;

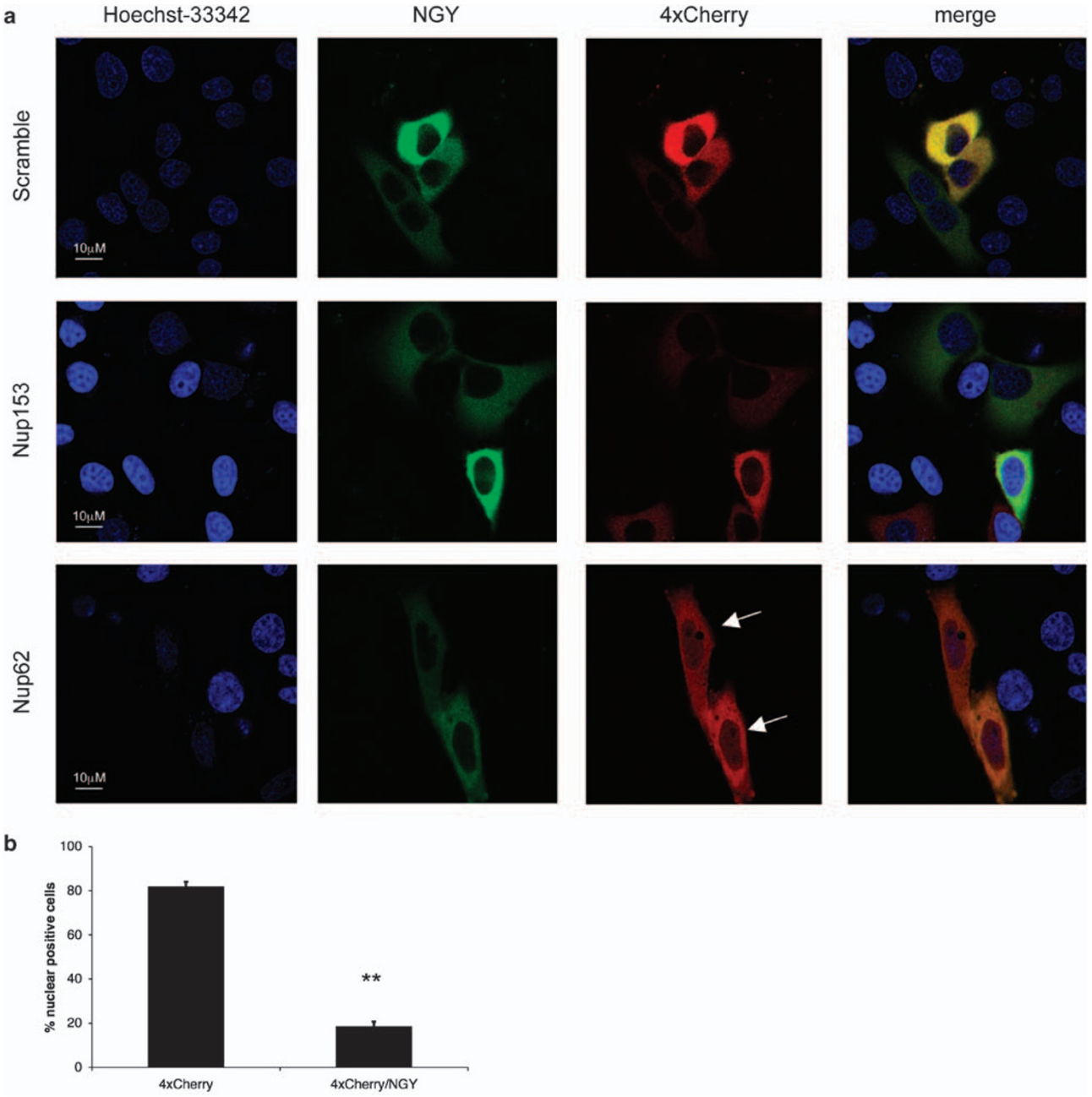

Figure 7 Nuclear accumulation of large fluorescent proteins in HeLa cells following downregulation of selected nucleoporins. (**a**) Confocal images of HeLa cells overexpressing NGY and 4xCherry and transfected with RNAi oligos against Nup153 and Nup62. As control, scramble RNAi was used. (**b**) Statistical analysis of 4xCherry and NGY nuclear accumulation following transfection of RNAi oligos against Nup62. Bar graph represents the mean of three independent experiments and error bars indicate ± S.E.M. Significant differences are indicated by symbol **($P < 0.01$; *t*-test)

Supplementary Figure S4B), which suggests the increase in NPC leakiness during calcium-mediated cell death in worms.

We studied whether a cytosolic fluorescent reporter could accumulate in the nucleus of dying neurons. We used the neuronal promoter *unc-119* to drive the expression of NGY in the majority of the neurons and analyzed the localization of the exogenous proteins in wild type and in *deg-3* mutant nematodes. In larval and adult wild-type animals, the fluorescent probe was localized in the cytosol and excluded from the nucleus, whereas in *deg-3(u662)* worms, swollen and vacuolated neurons exhibited a diffuse fluorescence (Figure 9b). As expected, in double mutants *deg-3(u662); clp-1(tm858)*, in which the activity of one of the calpains is suppressed,[39,42] the number of degenerating cells was reduced and nuclear accumulation of NGY was completely abolished (Figure 9b and c). This provides strong support for the hypothesis that calpains are involved in the increase of nuclear barrier leakiness in worms. Finally, we generated a strain of animals expressing NGY and NLS-Cherry under the control of the *unc-119* neuronal promoter. As expected, in

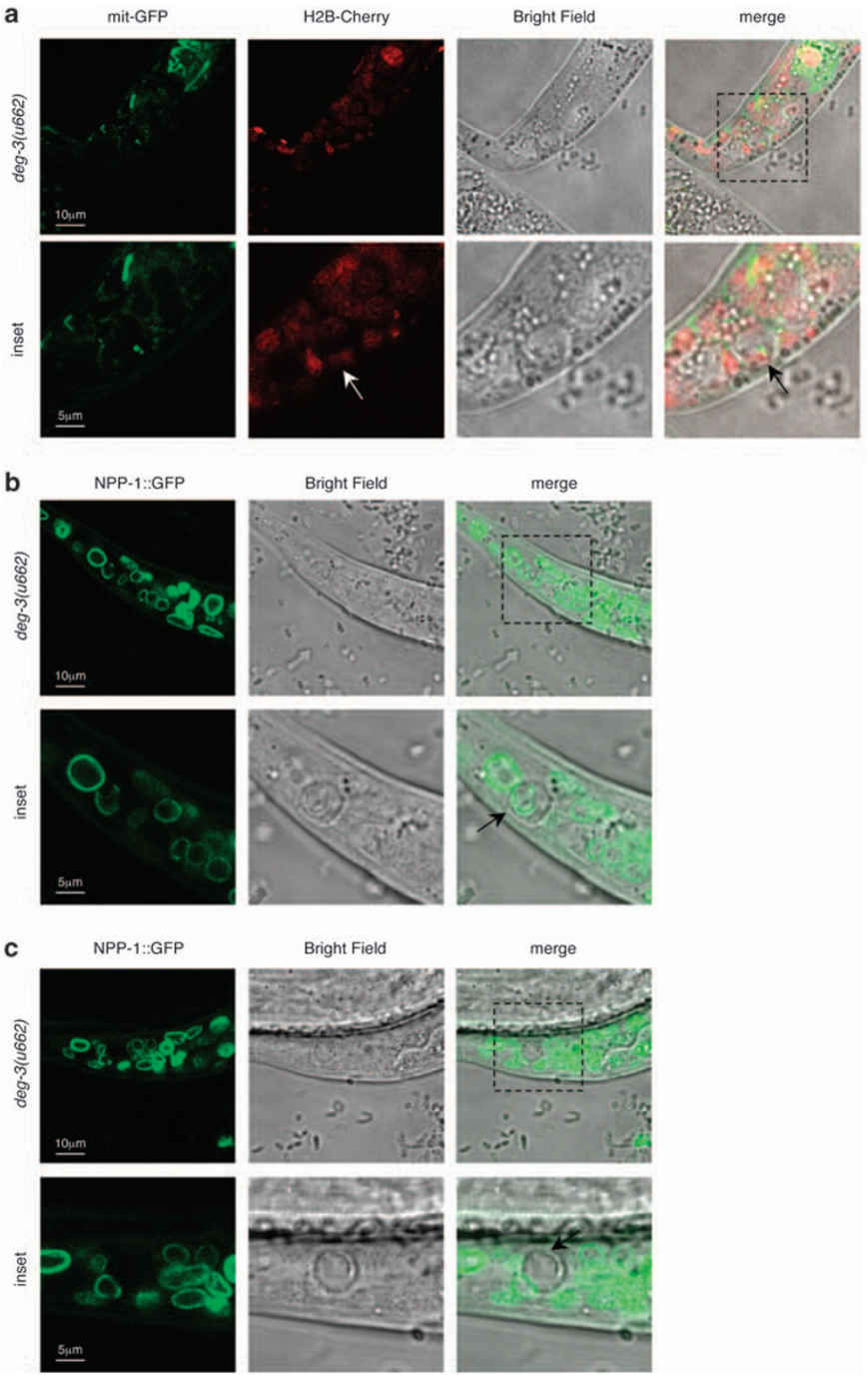

Figure 8 *C. elegans deg-3* gain of function mutation causes nuclear alteration. (**a**) Confocal analysis of *deg-3(u662)* animals overexpressing mitochondria-targeted GFP and nuclear H2B-Cherry. Degenerating neurons presented condensed nuclei close to the plasma membrane (arrow). (**b**, **c**) Confocal analysis of *deg-3* mutant worms carrying the P_{npp-1}**::** *npp-1***::** *GFP* transgene. Vacuolated neuron (arrow) presented partial or complete loss of GFP signal at the nuclear envelope

neurons of wild-type animals, NGY and NLS-Cherry were confined to the cytosol and the nucleus, respectively (Supplementary Figure S4C), whereas in degenerating cells of *deg-3(u662)* worms, we could observe redistribution of the two reporters across the nuclear envelope (Figure 9d).

Collectively, these studies in *C. elegans*, which are in line with our data on primary dissociated neurons, show an earlier unknown process: the evolutionarily conserved alteration of nucleocytoplasmic transport during Ca^{2+}-mediated neuronal death mediated by calcium and calpains.

Discussion

Although differences between cell death paradigms have been emphasized over the past 10 years, there is now an accumulating body of evidence that death programs share

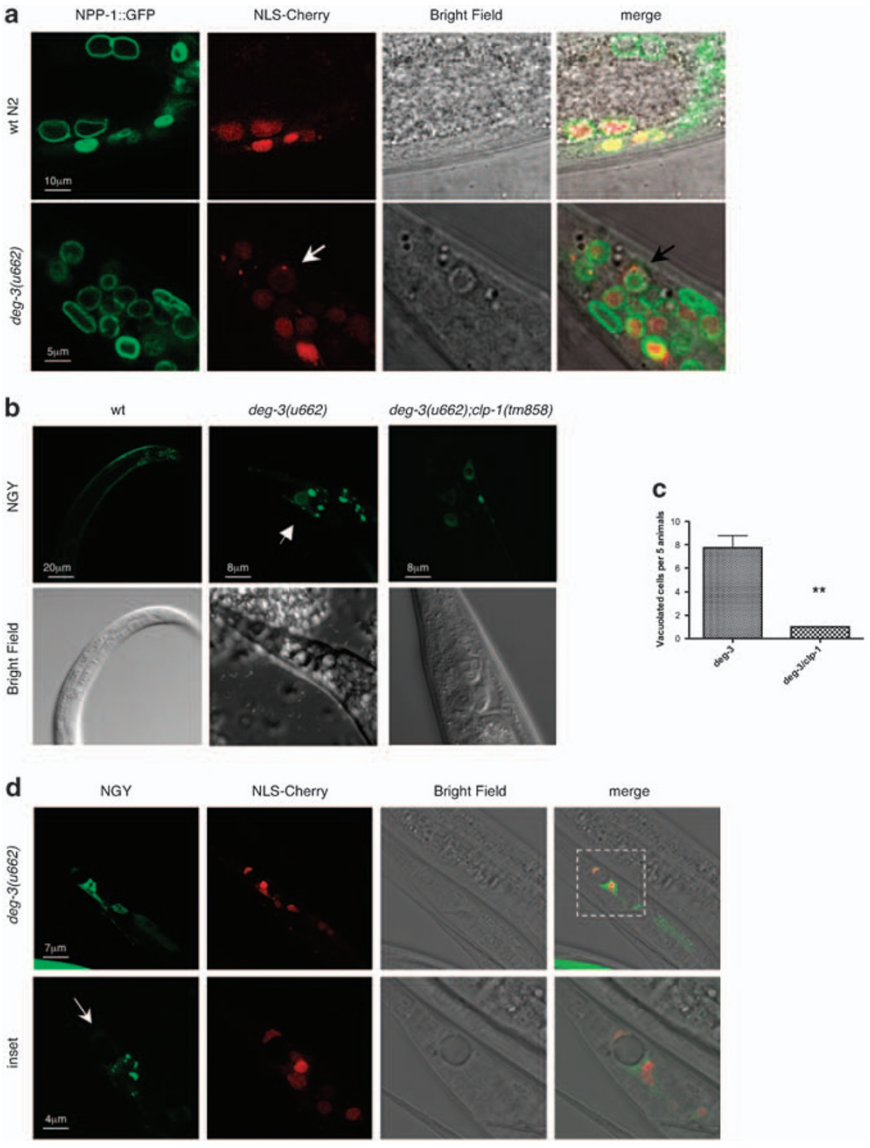

Figure 9 *C. elegans deg-3(u662)* presents redistribution of fluorescent probes across the nuclear envelope. (**a**) Confocal analysis of *deg-3(u662)* animals overexpressing NLS-Cherry and carrying the P_{npp-1}**::** *npp-1***::** *GFP* transgene. Vacuolated cell (arrow) showed redistribution of NLS-Cherry across the nuclear envelope. (**b**) Localization of NGY in neurons of wild-type N2 nematodes (left panel), in degenerating cells of *deg-3(u662)* (arrow, central panel) and *deg-3(u662);clp-1(tm858)* animals (right panel). (**c**) Statistical analysis of the number of vacuolated neurons in *deg-3(u662)* and *deg-3(u662);clp-1(tm858)* animals. Significant differences are indicated by symbol **($P<0.01$; *t*-test). (**d**) Colocalization of NGY (green) and NLS-Cherry (red) in degenerating cells of *deg-3(u662)* mutant (arrow)

very strong similarities, with distinct predominant mechanisms. In fact, a fundamentally conserved death program may have evolved multiple execution subroutines to evade takeover by opportunistic organisms and to accommodate differentiation. Engaging death programs involves an exchange of information between several cellular compartments regardless of the source of the death trigger. This may serve to amplify death signals or simply to prepare for cell disassembly. Nevertheless, there is a remarkable similarity between the events that lead to nuclear permeabilization and disassembly in death paradigms as different as excitotoxicity, caspase-independent cell death and classical apoptosis.

Here, we describe an earlier unknown sequence of events during calcium-dependent cell death. We show evidence that in primary dissociated neurons exposed to glutamate redistribution of small-sized cytosolic fluorescent reporters occurs immediately after the secondary calcium overload and the deregulation of the plasma membrane Ca^{2+} extrusion system.[22,43] Later proteolysis of nuclear pore elements causes redistribution of larger molecules across the nuclear membrane. Similarly, we observed a similar series of events occurring in *C. elegans, deg-3* mutants.

It has been recently shown that a $[Ca^{2+}]$ rise can alter the nuclear cytoplasmic shuttling by facilitating the formation of an importin cargo complex or by inhibiting nuclear export.[44] Although we cannot rule out the possibility that Ca^{2+} signals might affect the normal rate of import/export and so facilitate the diffusion of reporter proteins across the nuclear envelope,

the finding that different NES-containing proteins accumulate in the nucleus with different kinetics and the fact that an NES is not critical for the accumulation of the small-size fluorescent probes makes this unlikely.

Nuclear accumulation of fluorescent proteins larger than 70 kDa was detected after the delayed calcium overshoot, which suggests that a second mechanism was triggered to alter nuclear pore selectivity. On the basis of confocal analysis of CGNs overexpressing larger-size molecules, the increased leakiness of the nuclear barrier was linked to degradation of NPC components. Despite nuclear pore elements being degraded, the nuclear envelope appeared remarkably intact. This suggests that loss of the nuclear permeability barrier favored penetration of proteolytic enzymes, which then executed nuclear disassembly. Calpain-dependent disassembly of the envelope was indeed observed well after nucleoporin cleavage.

Nucleoporin loss seems to be an obligatory step shared by death pathways and some stress responses. Alteration of several nuclear–cytoplasmic pathways has been documented during viral infection and an increase in nuclear permeability is a general strategy adopted by several viruses such as poliovirus,[19,45] rhinovirus[46] and cardiovirus[47] to gain access to the cell genetic information. In this case, the increased redistribution of NLS and NES-containing EGFP has been attributed to a perturbation of the docking of receptor–cargo complexes to the NPC. Several nucleoporins are involved in nuclear export and import, especially FXFG repeat-containing proteins, which can interact with transport factors–cargo complex and mediate transport across the nuclear pore.[37] Notably, virus-infected cells presented a decrease in the levels of Nup153 and Nup62, which occurred at the same time as the relocalization of fluorescent reporters. Similarly, redistribution of proteins involved in nuclear transport, such as the nuclear protein Ran or nucleoporin-binding factors importin α and β, occurs during STS-mediated apoptosis in HeLa cells, before caspase activation and chromatin condensation.[20] In addition, translocation of cytosolic proteins such as GAPDH to the nucleus may occur in the signalling phase of cell death.[8,34]

Alterations in NPC functional size are considered relevant when proteins larger than 60 kDa diffuse.[48] In our settings, nuclear diffusion of large proteins correlated with a time-dependent decrease of FXFG repeats-containing nucleoporins. The observation that pharmacological inhibition of calpains or overexpression of calpastatin prevented both protein redistribution and nucleoporin cleavage strongly suggests that the loss of nucleoporins is due to their calpain-mediated degradation. The presence of calpain cleavage sites in Nup62-YFP fusion proteins supports this view. Notably, calpain inhibition in neurons and *C. elegans* delays cell death triggered by excitotoxic glutamate stimulation[22] and degenerin channels,[39] respectively. Thus, it is conceivable that blocking the degradation of NPC components would delay cell death. To definitively prove this contention, it would be necessary to generate nucleoporins that are not degraded by calpains. However, when considering that the NPC is a macromolecule of 125 MDa composed of approximately 30 different proteins most of which are degraded during calcium-mediated cell death, the generation of a calpain-insensitive NPC is not technically feasible at this point in time. Notably, the sole loss of individual nucleoporins, as modelled by the siRNA experiments, was not sufficient to affect the trafficking of NES-containing proteins, but it did inhibit nuclear exclusion of large-size fluorescent macromolecules. Although we cannot exclude the possibility that impaired nucleocytoplasmic trafficking is simply a consequence of the detrimental cascades triggered by calcium overload, preliminary data in our lab suggest that it occurs before the release of mitochondrial pro-apoptotic factors, including the AIF. Therefore, alteration of NPC functions may have a key role in the redistribution of death-inducing factors, in a positive amplification loop that would contribute to cell disassembly.

Finally, our studies in the *C. elegans* strain expressing mutated degenerin channels *deg-3* suggest that alteration of protein localization during cell death occurs in living animals and it is evolutionarily conserved. Notably, genetic deletion of *clp-1*, one of the calpains involved in the degenerative program, prevents the redistribution of a cytosolic reporter in worms. This supports the hypothesis that Ca^{2+} overload and calpain activation are sufficient to alter nuclear activity. More importantly, this also implies that alterations in Ca^{2+} signalling, leading to non-apoptotic demise trigger evolutionarily well-conserved death execution routines.

In conclusion, our results highlight what seems to be a common and evolutionarily conserved routine in cell death: a defect in nucleocytoplasmic transport and proteolysis of nucleoporins by different death-executing protease families.

Materials and Methods

Reagents and antibodies. The following regents were obtainded from commercial sources as indicated: monoclonal antibodies against GAPDH (Abcam, Cambridge, UK, clone 6C5; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, clone 6C5), APAF-1 (R&D systems, Abingdon, UK, clone 94408), HDAC4 (Abcam, HDAC-144), Mab414 (Covance, Harrogate, UK), Nup153 (Progen, London, UK), β-3 tubulin (Promega, Southampton, UK, clone 5G8), α II-spectrin (Chemicon, Chandlers Ford, UK, AA6), β-actin (Sigma, Poole, UK, Ac-15), α-tubulin (Sigma), Myc-Tag (Cell Signaling Technology, Beverly, MA, USA, clone 9B11), HA.11 (Covance), polyclonal antibodies against β-3 tubulin (Covance), APAF-1 (Abcam), Nup93 (BD Pharmingen, Oxford, UK), GFP (Molecular Probes-Invitrogen, Paisley, UK), anti-calpain small subunit (Chemicon, MAB3083), calpain 2 large subunit (Cell Signaling, 2539), secondary goat antibodies HRP-conjugated (PIERCE), secondary antibodies Alexa 488 and Alexa 546 (Molecular Probes), recombinant calpains I and II and calpains inhibitor Calpeptin (Calbiochem, Nottingham, UK).

Cell cultures and viral preparation. Cultures of rat CGNs were obtained as described earlier.[49] Briefly, primary cells were dissociated by trypsin digestion of cerebellum from 7-days-old animals. Cells were plated on poly-L-lysine (MW $>$ 300 kDa, Sigma)-coated plates or coverslips at the density of 1.2 million cells/ml. After 24–48 h, cytosine arabinoside (10 μM, Sigma) was added to inhibit cell proliferation. Neurons were used between 7 and 8 DIV. Murine and rat CNs were prepared from E17 embryos. Meninges were removed from the cortex and cells dissociated by gentle enzymatic digestion, plated on poly-L-lysine-coated dishes at a density of 0.6 million cells/ml and cultured in neurobasal medium (Invitrogen-Gibco, Paisley, UK) supplemented with 2 mM Glutamax (Invitrogen-Gibco), B27 supplement (Invitrogen-Gibco) and 1% penicillin–streptomycin (Invitrogen-Gibco). Cytosine arabinoside was added 7 days after plating and neurons were used after 8–10 DIV. Excitotoxicity was triggered by addition of 150 μM glutamate to cells bathed in $MgCl_2$-free/Gly-containing CSS5 (120 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 15 mM glucose, 25 mM HEPES, pH 7.4). For control experiments, cells were incubated in $MgCl_2$-containing CSS5. For hypoxic experiments, cells were incubated in $MgCl_2$ and glucose-free/Gly-containing CSS5 plus 10–30 μM glutamate. The plates were placed in a chamber in which oxygen

was replaced by N_2 and kept at 37°C for 30 min. Cells were collected at the indicated time. As a control, oxygen deprivation was performed with cells incubated in CSS5 containing 15 mM glucose and 2 mM magnesium. HEK 293T cells were cultured in Iscove's modified Dulbecco medium (IMDM, Sigma) supplemented with 10% dialyzed fetal calf serum (Invitrogen-Gibco), 2 mM glutamax, 100 units/ml penicillin–streptomycin. Viral particles were prepared as described by Follenzi and Naldini[50] and used as described earlier.[22]

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine and 100 U/ml penicillin/streptomycin (Invitrogen) and maintained at 37°C in a 5% CO_2 atmosphere. Calcium phosphate was used to transfect HeLa cells with mammalian expression vectors, whereas lipofectamine-2000 (Invitrogen) was used to transfect double-stranded oligonucleotides (Ambion, Applied Biosystem, Warrington, UK) designed against Nup 214 (ID 139480), Nup 205 (ID 261158), Nup 153 (ID 137888), Nup 62 (ID 133921 and 133922), calpain small subunit 1 (ID s2385). Scrambled siRNA was used as control. Analyses were performed 48 h after the siRNA transfection.

Immunofluorescent staining. After the appropriate experimental treatment, primary dissociated neurons or HeLa cells were rinsed with PBS, fixed for 20 min with 4% paraformaldehyde and permeabilized for 5 min with Triton X-100. Samples were then blocked with goat serum/1% BSA/0.01% Tween-20 and incubated for 1 h or overnight with primary antibodies, washed with blocking solution, incubated with the appropriate secondary antibody for 1 h, washed again, stained with Hoechst-33342, washed and then mounted with 5% *n*-propylgallate in absolute glycerol.

SDS gel electrophoresis and immunoblotting. Cells were collected in PBS and re-suspended in buffer (10 mM Tris-Hcl pH 7.4, 1 mM EDTA, 0.1% Triton X-100, complete mini EDTA-free proteases inhibitor (Roche, Burgess Hill, UK)) followed by protein quantification with the Bradford Reagent. In the case of the *in vitro* calpain assay, samples were re-suspended in calpain buffer (50 mM NaCl, 0.1% Triton, 10 mM EGTA, 100 mM HEPES pH 7.5). Recombinant calpain I or II (0.05units/μl unless indicated) was added at the concentration and for the time indicated, in the presence or absence of 20 mM $CaCl_2$. Loading buffer (3.3 × concentrate: 16.7% glycerol, 5% SDS, 1.3% Tris-HCl pH 6.8, bromophenol) was added in the ratio 1 : 3 to the samples. Proteins were boiled at 95°C for 5 min, separated by electrophoresis and transferred onto nitrocellulose filter using a semi-dry or wet blot chamber (Bio-Rad, Hemel Hempstead, UK). The membranes were saturated in PBS with 5% milk powder as blocking solution at room temperature for 2 h. Primary and secondary antibodies were incubated for 1 h at room temperature in blocking solution. Immunoblots were visualized with the chemiluminescent analyzer Kodak Station 440.

Real-time confocal microscopy of single cells. Primary dissociated neurons were loaded with 4 μM Fura-Red for 20–30 min at 37°C. Cells were then bathed in Mg^{2+}-free/Gly-containing CSS5 and treated with 150 μM glutamate. Simultaneous measurements of Ca^{2+} and nuclear translocation of fluorescent probes were performed at about 37°C using a Zeiss Axiovert 200 microscope with a × 63 water immersion lens.

Electron microscopy. After the appropriate experimental treatment, primary dissociated neurons were rinsed with PBS, fixed overnight, at 4°C with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and processed as described earlier.[51] The same treatment was used for HeLa cells.

Plasmid constructs. All the details of the constructs used in the manuscript are reported as Supplementary information.

Strains, genetics and confocal microscopy of *C. elegans*. Strains were maintained on NGM plates carrying a lawn of OP50 and were kept at 20°C. The following strains were used: N2 Bristol, LW0271,[41] *deg-3(u662)* and *clp-1(tm858)* (kindly provided by Dr. S Mitani, Tokyo Women's Medical University School of Medicine, Tokyo, Japan). $P_{unc-119}$::*NGY* or $P_{unc-119}$::*NLS-Cherry* plasmid was co-injected with a GFP marker DNA (P_{myo-2}::*GFP* plasmid) and pGEM (respectively at the concentration of 10, 2 and 80 ng/μl), into the gonads of gravid hermaphrodites. Transgenic animals were selected by expression of GFP in the pharynx. Confocal analysis was performed on larvae nematodes, paralyzed with 10 mM levamisole in M9 buffer, mounted on agarose pad slides and analyzed by confocal microscopy.

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