It is not apparent at this stage of molecular cloning how many receptors constitute this family. The diversity of sequences seen with the presently discovered members suggest that the receptor subtype yet to be cloned might have a role in appetite regulation[20,28,35]. This could be addressed by the development of mutant animals that lack both the Y_1 and Y_5 receptors. The identification of NPY feeding receptors and their complimentary and/or overlapping functions are obviously of great importance to the molecular basis of the hypothalamic regulation of feeding and body weight. The development of potent and fully selective receptor antagonists could lead to the determination of their pathophysiological significance in humans, the species for eventual study.

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Chemical names

BIBO3304: (R)-*N*-[[4-(aminocarbonylaminomethyl)phenyl]-methyl]-N^2-(diphenylacetyl)-argininamide-trifluoroacetate

BIBP3226: R-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide

CGP71683A: [(4-{[(4-aminoquinazolin-2-yl)amino]methyl}-cyclohexyl)methyl](naphthylsulphonyl)amine

LY357897: 1-{1-[3-((3s)(3-piperidyl))-propyl]-2-[(4-chlorophenoxy)-methyl]indol-3-yl]-2-(4-piperidylpiperidyl)ethan-1-one

1229U91 (GR231118, BW1229): [(Ile-Glu-Pro-(2,3-diaminopropionic acid)-Tyr-Arg-Leu-Arg-Tyr-$NH_2)_2$, cyclic(2,4′), (2′,4)-diamide)-amide]

Neuronal cell death: a demise with different shapes

Pierluigi Nicotera, Marcel Leist and Luigi Manzo

Severe neuronal loss is common to many neurodegenerative diseases. Although necrotic features are often prevalent in neuropathological conditions, there is now increasing evidence to show that apoptosis can significantly contribute to neuronal demise in neurodegenerative diseases, including Huntington's and Alzheimer's diseases and HIV-associated dementia. Furthermore, a role in other disorders such as stroke, trauma, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis has been suggested from animal studies[1]. Nevertheless, it is unclear which of the two types of demise, apoptosis or necrosis, prevail in acute and slowly developing neurogenerative disorders, and whether the mode of cell death is relevant for the ultimate progression of the disease.

The debate on the occurrence and prevalence of either type of death in pathological conditions such as stroke or neurotoxic injury could be resolved in part by considering that different types of cell death within a tissue reflect either the complete or the partial execution of a common

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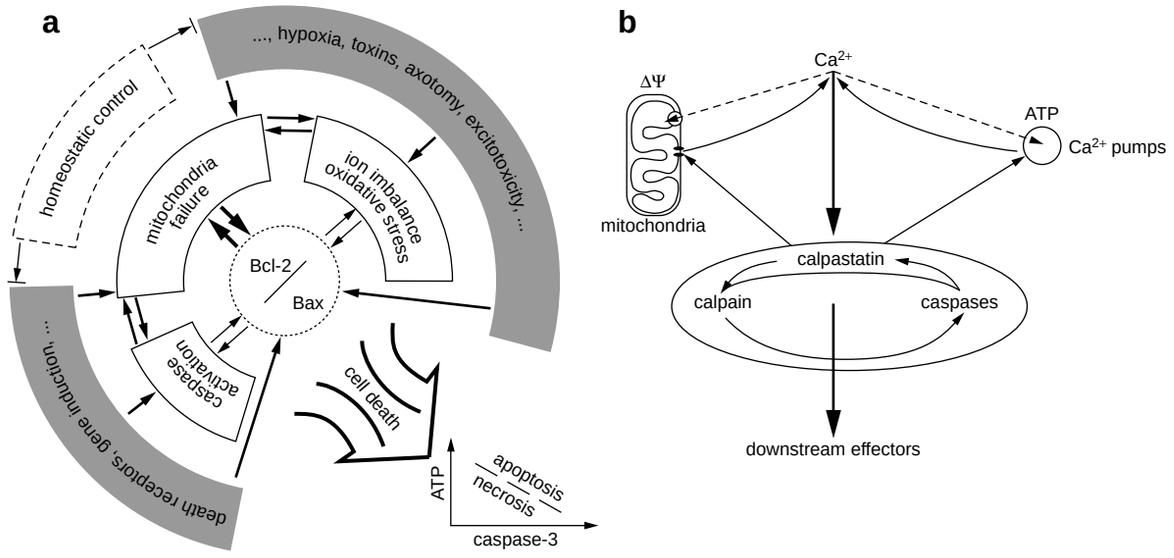

Fig. 1. Control of life and death in neurones. **a:** Outer circle: neuronal death can be triggered by stimuli as diverse as trauma, hypoxia, metabolic deficiency or receptor activation. All death stimuli are initially balanced or compensated for by homeostatic mechanisms. Inner circle: once a threshold of compensatory capacity and plasticity is passed, mitochondria fail, caspases are activated, or the loss of ion and redox control is beyond the cell's compensatory ability. Any of these mechanisms could lead to cell death independently or in cooperation with each other. Often, different sets of proteases are activated. The mode and shape of cell death is decided by the contribution of the individual mechanisms, and possibly by the set of downstream degradative mechanisms activated or inhibited in each particular case. Self-perpetuating feedback loops can be established when the balance between negative and positive controllers of apoptosis shifts towards proapoptotic mechanisms (i.e. caspase-mediated cleavage of Bcl-2, or release of Bax from its complex with Bcl-2). Most of the control function of Bcl-2-like proteins seems to be exerted at the level of mitochondria, although Bcl-2 might also act in various other cellular compartments. The shape of cell death (apoptosis or necrosis) is then frequently decided by the availability of sufficient ATP/dATP to promote activation of execution caspases, and by the relative activity of, for example, caspase 3, which is responsible for certain apoptotic features such as oligonucleosomal DNA fragmentation. **b:** Excitotoxic neuronal death can be facilitated or triggered by mitochondrial failure and ATP-depletion without caspase 3 activation: functioning mitochondria normally protect cells from Ca^{2+} overload by sequestering the ion in a membrane potential ($\Delta\Psi$)-dependent manner and by providing ATP for its sequestration across the plasma membrane or into the endoplasmic reticulum (Ca^{2+} pumps). Inhibition of mitochondrial respiration and ATP-depletion would facilitate or aggravate an increase of $[Ca^{2+}]_i$ (bold arrows), and activation of Ca^{2+}-dependent proteolytic systems (e.g. calpains). Caspases might be activated by calpains[24]. Conversely, caspases could activate calpains by cleaving the endogenous protease inhibitor, calpastatin[40]. Both classes of proteases can cause mitochondrial permeability transition and inactivation of Ca^{2+} sequestering transport systems. This would further increase $[Ca^{2+}]_i$ and Ca^{2+}-dependent protease activity in a cycle that eventually leads to cell death.

death programme. Some endogenous mediators might modulate the shape (morphological appearance) of cells in the death process; such modulation could have implications for the neighbouring tissue by interfering with the execution of apoptosis-specific subroutines, thereby changing apoptosis to necrosis.

Cell death: a core programme with different execution subroutines

The mode of cell death by either apoptosis or necrosis is frequently discerned by morphological criteria[2]. Programmed cell death, which also occurs during development, often results in morphology associated with apoptosis[2]. However, the concept of a death programme is not necessarily linked to morphological appearance. For example, developmental cell death does not always display apoptotic-like characteristics[3]. A controlled (programmed) series of events can also give rise to death with necrotic appearance. Fibroblasts[4], oligodendrocytes[5], neurones[6] or hepatocytes[7] can all die in an organized manner (involving signalling, control and execution) that results in a cell morphology associated with necrosis. The apoptotic programme can also be interrupted before the formation of the characteristic morphology is complete. Cells are then terminated either by an overwhelming accumulation of uncontrolled lethal reactions[8], or by the progression of the cell-death programme via pathways that are not specifically associated with apoptosis[9]. Also, the triggering signals for cell death and the time course of execution are not discriminating. For example, a large number of 'accidental' traumatic or toxic insults leads to a rapid form of cell demise characterized by an apoptotic morphology[1,2].

Thus, the impression that several, entirely distinct types of cell demise take place under a variety of conditions could be deceiving. Cell death might be accomplished following a core programme, with a choice of different execution routines, which often produce dissimilar morphologies. The prevalence of certain subroutines might reflect the functional or structural differentiation of a given cell type. For example, neuronal projections might be eliminated prior to nuclear condensation and mitochondrial damage in the cell soma during some axonopathies[10],

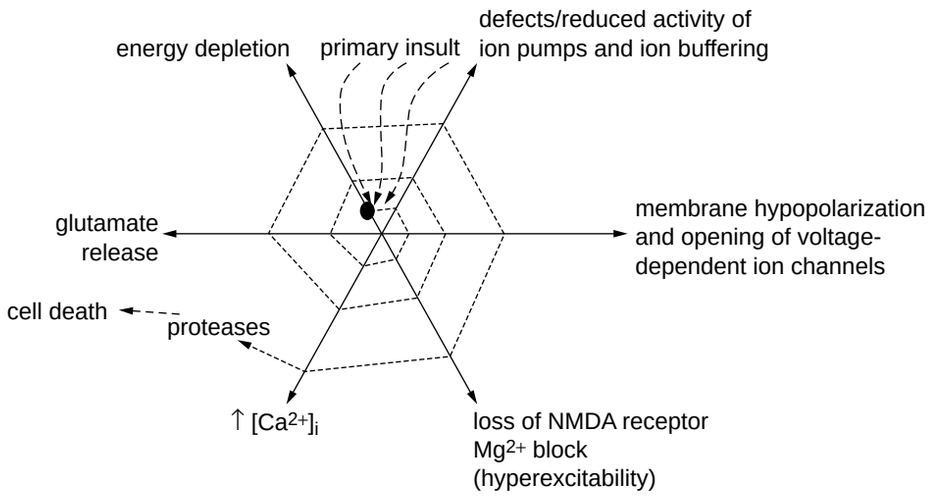

Fig. 2. Accelerating loops in indirect excitotoxicity. An initial insult could cause a limited disturbance of cellular homeostasis, for example, the starting point is shown for mitochondrial toxins (represented by a black dot), such as methylphenylpyridinium (MPP^+). Looping mechanisms would cause an ever increasing mitochondrial dysfunction, increase in $[Ca^{2+}]_i$, glutamate release, membrane depolarization and *N*-methyl-D-aspartate (NMDA) receptor desensitization. Beyond a certain threshold, the activation of proteases would further accelerate the cycles that could finally result in excitotoxic cell death.

whereas an early, axon-sparing loss of somata is observed in excitotoxicity[11]. Of the several possible execution subroutines, some are closely associated with the issue of life or death, whereas others might only confer the morphology of cell demise (Fig. 1a). Thus, inhibition of elements of the death programme that are mainly responsible for the apoptotic morphology does not necessarily result in cell survival; rather, it changes the nature of cell death from apoptosis to necrosis[9,12–14].

All of these considerations seem to be at odds with the concept that a simple linear cascade of events might be sufficient in all cases to promote cell death and confer its shape. Multiple pathways could interact to form a complex system of positive-feedback loops to execute cell death, whereas the resulting morphological appearance of death would be decided by the metabolic situation, the activation or suppression of individual subroutines or the relative speed of their execution in each cell[8,9,12–15] (Figs 1, 2).

Ischaemia and excitotoxicity: death comes in many shapes

Excitotoxicity is a pathological condition that occurs in a variety of neurological disorders such as hypoxia, hypoglycaemia or toxic conditions[16,17], and describes the supraphysiological stimulation of glutamate receptor subtypes such as the NMDA receptor. These ionotropic receptors act as ligand-gated Ca^{2+} channels and their prolonged activation results in intracellular Ca^{2+} overload and cell death[18]. Until recently, developmental neuronal death or neuronal demise resulting from excitotoxicity or exposure to neurotoxicants have been considered as conceptually and biochemically different types of death. It had been thought that injury by neurotoxins or glutamate lacked the regulated series of events involved in a death programme that lead invariably to necrosis[19]. However, recent evidence suggests that key regulators of apoptosis, i.e. p53 (Ref. 20), Bax (Ref. 21), Bcl-2 (Ref. 11) and caspases are also involved in excitotoxic–ischaemic neuronal injury. For example, prevention of neuronal death in cerebral ischaemia by caspase inhibitors[22,23] suggests that at least some mechanisms of this death are similar to those of other forms of programmed cell death[1]. This concept is directly corroborated in *in vitro* experiments showing that excitotoxicity can cause both apoptosis and necrosis[8], and that caspase inhibitors significantly reduce the extent of excitotoxic damage[24–26].

Excitotoxicity is further complicated by the prospect that execution of alternative subroutines might differ in different neuronal subpopulations. For example, the contribution of individual receptors could be relevant in the 'decision' of the mode of cell death[27]. The degree of Ca^{2+} overload might also convert the mode of cell death in some cases from apoptosis to necrosis[19]. Autocrine neuronal apoptosis elicited by synaptic release of excitotoxins seems to favour apoptosis[25], whereas massive glutamate receptor stimulation by exogenous glutamate can additionally elicit rapid necrosis[8].

Part of the problem in determining the relevance of apoptosis in neurodegeneration has originated from the assumption that inhibitors of protein synthesis protect cells from the apoptotic, but not necrotic, demise. However, cell death associated with typical apoptotic morphology can occur in the presence of protein synthesis inhibitors[28], whereas neither neuronal apoptosis triggered by autocrine excitotoxicity, NO donors, nor Hg^{2+} are modified by agents that inhibit protein synthesis[29,30].

The shaping of neuronal death: recruitment of different subroutines

Contrasting interpretations of the mode of excitotoxic death have originated from the significance attributed to single apoptosis-linked alterations in dying cells. In cerebellar granule cells, glutamate induces typical signs of apoptosis, such as chromatin condensation with the biochemical correlate of high-molecular-weight DNA fragmentation. However, oligonucleosomal DNA laddering and nuclear fragmentation, which are also indicative of apoptosis, are less apparent[8]. On the other hand, cerebellar granule cells exposed to other apoptotic stimuli (for example, staurosporine or 4-hydroxynonenal) exhibit the typical DNA laddering, chromatin

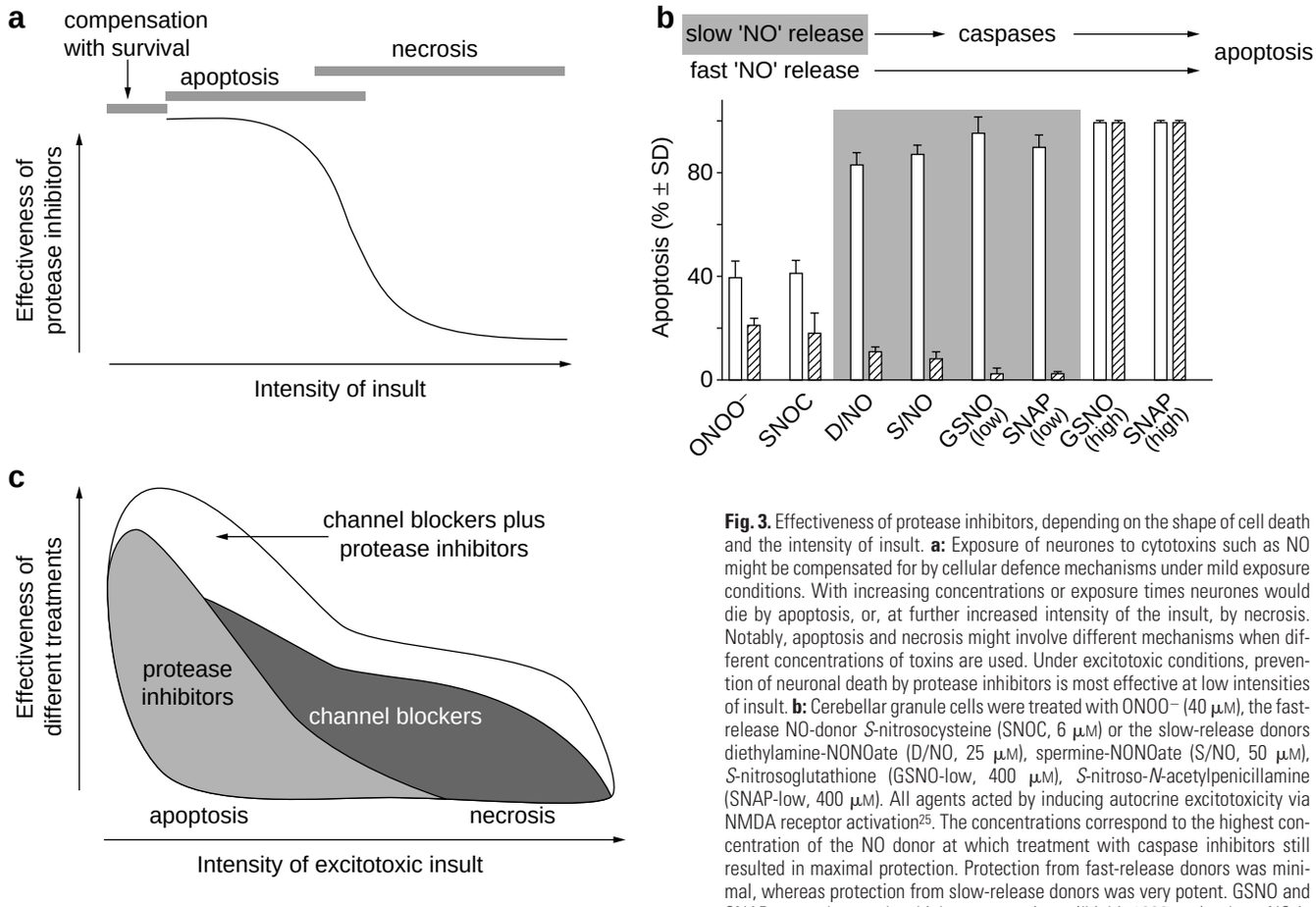

Fig. 3. Effectiveness of protease inhibitors, depending on the shape of cell death and the intensity of insult. **a:** Exposure of neurones to cytotoxins such as NO might be compensated for by cellular defence mechanisms under mild exposure conditions. With increasing concentrations or exposure times neurones would die by apoptosis, or, at further increased intensity of the insult, by necrosis. Notably, apoptosis and necrosis might involve different mechanisms when different concentrations of toxins are used. Under excitotoxic conditions, prevention of neuronal death by protease inhibitors is most effective at low intensities of insult. **b:** Cerebellar granule cells were treated with $ONOO^-$ (40 μM), the fast-release NO-donor *S*-nitrosocysteine (SNOC, 6 μM) or the slow-release donors diethylamine-NONOate (D/NO, 25 μM), spermine-NONOate (S/NO, 50 μM), *S*-nitrosoglutathione (GSNO-low, 400 μM), *S*-nitroso-*N*-acetylpenicillamine (SNAP-low, 400 μM). All agents acted by inducing autocrine excitotoxicity via NMDA receptor activation[25]. The concentrations correspond to the highest concentration of the NO donor at which treatment with caspase inhibitors still resulted in maximal protection. Protection from fast-release donors was minimal, whereas protection from slow-release donors was very potent. GSNO and SNAP were also used at high concentrations ('high', 1000 μM), where NO is released rapidly and, in this case, caspase inhibitors were no longer protective. Under these conditions neurones still died by apoptosis. Notably, NMDA-receptor block completely protected neurones in all conditions shown. White bars, NO donor; hatched bars, NO donors plus caspase inhibitors. **c:** *In vivo*, protease inhibitors could protect neurones from low-level, excitotoxic insult and, in addition, from apoptosis triggered by oxygen radicals, lipid peroxidation products and other endogenous mediators generated under ischaemic conditions. Channel blockers (for example NMDA receptor antagonists) or other inhibitors acting downstream of an initial Ca^{2+} influx (for example, NO synthase inhibitors) might strongly lower the intensity of the primary excitotoxic insult. Under these conditions, inhibitors of caspases or other proteases might be more effective, and combined treatment could lead to increased neuroprotection.

condensation and nuclear fragmentation[31,32]. Because DNA laddering seems to depend on nucleases activated by caspase 3, it is not surprising that, in some forms of cell death where this caspase is not active, its downstream effects are also lacking. Caspase 3-independent apoptosis is well documented in neurones[21,24,25,33,34] and other cells[34,35]. Thus, the existence of alternative execution pathways, which become active in certain neuronal populations but not in others, could in part account for the multiple shapes of excitotoxic demise (Fig. 1a).

Execution patterns and the resulting morphology can also depend on the combination of death stimuli or on selective interference of each stimulus with individual subroutines. For example, caspase 3 processing and the downstream subroutines can be impaired by concomitant intracellular ion influx[36]. Apoptosis triggered by primary cytoskeletal damage, for example that elicited by colchicine[37], obviously gives rise to a different shape of demise than that observed in developmental or receptor-triggered apoptosis, although they could have some execution subroutines in common. Toxic reactions such as those elicited by oxidative or nitrosative stress could at the same time trigger the core death programme and inhibit some of its shape-determining pathways[25].

There is evidence that the intensity of the insult also confers the shape of neuronal demise. On exposure to mild excitotoxic insults, neurones die exhibiting the typical apoptotic features. With stronger insults, the degradative processes responsible for apoptotic morphology are often prevented and neurones die by necrosis[8,38]. This could be due to untimely energy dissipation[8]; in fact, recent evidence has revealed that, when energy levels are rapidly compromised, cells triggered to undergo apoptosis are instead forced to die by necrosis (see below)[9,39].

It is conceivable that different execution patterns could involve distinct proteolytic families, where the

predominance of a given protease family would depend on the stimulus or intensity of insult. Moreover, different proteases could interact with each other in self-amplifying loops (Fig. 2). For example, there is evidence that calpains, inhibitors of Ca^{2+}-activated proteases, are as effective as caspase inhibitors in preventing neuronal apoptosis following excitotoxicity[24]. The available data suggest that calpains might process pro-caspases, whereas active caspases cleave Ca^{2+} transport proteins or the endogenous calpain inhibitor protein calpastatin[40] (Fig. 1b). Caspase activation seems to be the predominant execution pathway in physiological cell death and death associated with moderate insults. Accordingly, brain development is severely disturbed in animals with a targeted deletion of the apoptosis-executing caspases 3 and 9 (Refs 34, 41 and 42). However, thymocytes from caspase 9 (−/−) embryos are still sensitive to apoptosis elicited by UV radiation or anti-CD95 antibodies[41], which suggests a differential requirement for this execution caspase in different types of apoptotic cell death. In adult neurones dying after a severe insult, caspase 3- or caspase 9-dependent pathways might be inhibited, whereas other proteolytic systems can still execute cell death[25]. This becomes particularly relevant when considering possible therapeutical approaches to conditions where excitotoxicity plays a role (Fig. 3). Observations from stroke models suggest that apoptosis occurs mainly in the border regions of the affected region (penumbra)[43], while necrosis dominates in the more severely stressed areas of the ischaemic core[44]. The aim of intervention a few hours after the ischaemic insults is normally to reduce the spread of the lesion and to inhibit delayed cell death in the border areas. Assuming that the activation of different pathways for the execution of cell death is determined by the level of injury, it is apparent that caspase inhibitors, for example, might be most effective in areas where the intensity of the excitotoxic insult is low, and ineffective in regions where the stress is more intense. Thus, therapeutic strategies that combine agents to reduce the overall intensity of the insult and the overall lesion size (i.e. NMDA receptor antagonists or selective bNOS inhibitors) with agents that block execution of apoptosis (caspase inhibitors) might prove more successful than individual treatments[45] (Fig. 3).

ATP and different shapes of cell death

To examine the events that determine the mode of execution of cell death (apoptosis or necrosis) following exposure to a single insult, individual parts of the death programme can be blocked by manipulating the intracellular ATP level. When ATP levels are reduced, typical apoptotic stimuli cause necrosis instead of the expected apoptosis[9]. If the intracellular ATP concentration is markedly reduced during a crucial time window, activation of downstream caspases and all or most typical apoptotic changes are prevented. Stimulated cells die nonetheless. However, the demise has necrotic features. These findings provide direct evidence that the complete apoptotic programme involves energy-requiring steps, at least in some cells. More recent work suggests that one of the ATP-requiring steps is at the level of the formation of the protein complex between Apaf-1, procaspases and cyt-c released from damaged mitochondria[46,47]. Blocking the default programme at this stage prevents the resulting downstream degradative processes including caspase 3 activation, poly-(ADP-ribose)-polymerase cleavage and lamin cleavage, and exposure of PS on the outer membrane[9,39]. Cells dying with prevalent necrotic appearance do not exhibit these features.

Intracellular protein localization or transport might also be relevant in determining the shape of cell death. Some death signals (for example those activated after irradiation or treatment with topoisomerase inhibitors) are predominantly generated within the nucleus. Because controllers and execution systems are believed to be located in the cytoplasm or mitochondria, the yet-unknown death signals have to be transmitted from the nucleus to these compartments. Also, some caspases seem to be transported from the cytoplasm into the mitochondria or nucleus[48]. Thus, the permeability of the nuclear pore and the accessibility of mitochondrial sites, or both, might be relevant factors in deciding execution of individual subroutines in apoptosis[49].

Intracellular mediators control the occurrence and shape of cell death: nitric oxide, Dr Jekyll or Mr Hyde?

Experimental manipulation of intracellular ATP levels shows that apoptosis can be switched to necrosis. Is there a mediator responsible for such conversion during pathological conditions? It is already known that the same stimulus can trigger apoptosis in some systems but prevent it in others. Endogenous mediators such as NO can, for example, trigger pro-apoptotic mechanisms[25,30,38,50] (Fig. 3), but can also prevent apoptosis in neurones[51,52] and other cells[53–55]. The factors determining the killing or protective actions of NO could be related to the cell type, the pathway of execution of cell death and perhaps the chemical forms of NO. In several cell types, NO can prevent the execution of apoptosis, partly by inhibiting active caspases, via *S*-nitrosylation[51,55], and partly by preventing caspase activation via cGMP-dependent pathways[52,55].

Our recent experiments have shown that NO prevents caspase activation by inhibiting mitochondrial respiration, and thereby lowering intracellular ATP levels. By this mechanism, NO prevented DNA fragmentation, chromatin condensation and the translocation of phosphatidylserine, which is a marker for apoptotic cells to be phagocytosed[56]. However, as already discussed[12], the prevention of cell death was only ephemeral. Cell death was delayed and doomed

cells died eventually by necrosis. When non-mitochondrial, glycolytic ATP generation was supported via glucose supplementation to the culture medium, death restored its apoptotic appearance. *In vivo*, halting the apoptotic programme would have two possible implications: first, cells protected by NO via a stop of the apoptotic execution cascade would have time to recover from a transient or mild insult, and thus survive; second, cells hit by a lethal, normally apoptotic insult would eventually lyse without having been removed by phagocytosis. Thus, depending on the situation, endogenous mediators such as NO could either prevent cell demise or convert apoptosis to necrosis. In the latter, the release of factors from dead cells and the ensuing inflammation would further aggravate tissue damage.

Concluding remarks

It is not surprising that initially simple death programmes, developed early during phylogeny, undergo complex modifications in mammalian cells. A further consequence of the increased complexity could be that an increasing number of feedback loops gives rise to many possibilities of initiation, control and execution (Figs 1, 2).

Multiple pathways probably cooperate to ensure the removal of injured cells, and positive feedback loops amplify death signals to prevent survival of 'undead' cells. In complex cellular systems such as neuronal networks, one can even speculate that execution of the death programme is predominantly apoptotic in certain subcellular regions, whereas other subroutines prevail in other cellular domains. The main implication of this standpoint is the exclusion of a single, predominant and molecularly defined commitment step. It seems likely that accumulation of damage incompatible with cell survival would require disruption of several vital functions. Once such a threshold is trespassed, other positive feedback loops would ensure the progression of the death programme and the safe disposal of the injured cell. Also, it is apparent that the morphological appearance of cell death (apoptosis or necrosis) is not linked to a putative commitment point, but it is rather the result of a more-or-less complete execution of subroutines of the death programme. Overall, the arguments presented here support the view that in neuronal death, individual, intricately interconnected pathways self-amplify or delete each other in an inflationary process, which results in the many shapes of cell death.

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