Title: Multiple interacting cell death mechanisms in the mediation of excitotoxicity and ischemic brain damage: a challenge for neuroprotection.

Authors: Puyal J, Ginet V, Clarke PG

Journal: Progress in neurobiology

Year: 2013 Jun

Volume: 105

Pages: 24-48

DOI: 10.1016/j.pneurobio.2013.03.002

Multiple interacting cell death mechanisms in the mediation of excitotoxicity and ischemic brain damage: a challenge for neuroprotection

Julien Puyal[&]*, Vanessa Ginet[&], Peter G.H. Clarke

Département des Neurosciences Fondamentales, Université de Lausanne, Rue du Bugnon 9, 1005 Lausanne, Switzerland

[&] equal contributions

* To whom correspondence should be addressed:

Total word count: 28'802

Key words: neuronal death, apoptosis, necrosis, autophagy, stroke, hypoxia-ischemia

Abbreviations:

AIF, Apoptosis inducing factor; Ambra1, activating molecule in beclin1-regulated autophagy; Apaf-1, adapter protein apoptotic protease-activating factor-1; ASIC, acid-sensing ion channel; atg, autophagic related genes; Bad, Bcl-2-associated death promoter; BAF, boc-Asp-fmk; Bax, Bcl-2–associated X protein; Bak, Bcl-2 homologous antagonist killer; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; BH, Bcl-2 homology domains; Bim, Bcl-2-interacting mediator of cell death; ER, endoplasmic reticulum; FADD, Fas-Associated Death Domain; FasL, Fas ligand; HI, hypoxia-ischemia; IAP, Inhibitor of Apoptosis Protein; KA, kainate; LAMP, lysosomal-associated membrane protein; LC3, microtubule-associated protein 1 light chain 3; MCAo, middle cerebral artery occlusion; mTor, mammalian target of

rapamycin; NMDA, N-methyl-D-aspartate; PARP, Poly(ADP-Ribose) Polymerase; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol-3 kinase; PUMA, p53 upregulated modulator of apoptosis; Q-VD-OPH, (Quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxy]-methyl ketone); RIP1, receptor-interacting protein 1; Smac/Diablo, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI; tBid, truncated BH3 interacting-domain death agonist; TNF, Tumor Necrosis Factor; TRADD, TNF Receptor-Associated Death Domain; TRAIL, TNF-Related Apoptosis-Inducing Ligand; ULK1/2, UNC-51-like kinase 1/2; UVRAG, UV irradiation resistance-associated gene; 3-MA, 3-methyladenine.

Abstract

There is currently no approved neuroprotective pharmacotherapy for acute conditions such as stroke and cerebral asphyxia. One of the reasons for this may be the multiplicity of cell death mechanisms, because inhibition of a particular mechanism leaves the brain vulnerable to alternative ones. It is therefore essential to understand the different cell death mechanisms and their interactions. We here review the multiple signaling pathways underlying each of the three main morphological types of cell death – apoptosis, autophagic cell death, and necrosis – emphasizing their importance in the neuronal death that occurs during cerebral ischemia and hypoxia-ischemia, and we analyze the interactions between the different mechanisms. Finally, we discuss the implications of the multiplicity of cell death mechanisms for the design of neuroprotective strategies.

Contents

1. Introduction

Despite a considerable research effort, there is still no approved neuroprotective pharmacotherapy for acute neurological conditions such as stroke (Stankowski and Gupta, 2011; Yuan, 2009) and neonatal cerebral asphyxia (van Bel and Groenendaal, 2008). The reasons for this are multiple, but one of the problems appears to be the multiplicity of cell death mechanisms, because inhibition of a particular death mechanism will be ineffective if alternative mechanisms are able to kill the cell. It is therefore essential to understand the different cell death mechanisms and their interactions.

Morphological studies indicate that there are three main types of cell death (Fig. 1): type 1 or apoptosis, type 2 or autophagic death, and type 3 or necrotic/cytoplasmic death. Initial arguments for this trichotomy focused on cell death during normal development (Clarke, 1990), but a similar classification appears to be valid in both adult and neonatal pathological situations including ischemic brain damage (Bredesen, 2008; Clarke, 1999). The coverage of neonatal ischemic (including hypoxic-ischemic) models in the present review is limited to ages at which the brain damage is mainly in the gray matter - after postnatal day (P) 7. Very early cerebral ischemia (P2-P4) causes primarily white matter lesions, by entirely different mechanisms, which is beyond our present concern.

We here review critically, in the context of cerebral ischemia and hypoxia-ischemia, the available evidence on the multiple mechanisms underlying the three types of cell death. In particular, we focus on four problem areas. 1. The factors that determine whether a given type predominates. 2. The interactions between the multiple mechanisms and the hybrid forms of cell death that can result. 3. The current controversy about whether autophagy is a death mediator in type 2 cell death (Clarke and Puyal, 2012; Shen *et al.*, 2012; Yuan and Kroemer, 2010). 4. The challenge that the multiple mechanisms constitute for the design of neuroprotective strategies.

1.1. Excitotoxicity

The multiplicity of cell death mechanisms in excitotoxicity and cerebral ischemia results from the fact that these events trigger multiple pathways. Excitotoxicity, defined as toxicity due to the excessive activation of ionotropic and metabotropic glutamate receptors, is well known to be the main cell death mechanism in cerebral ischemia and hypoxia-ischemia. Glutamate receptor activation depolarizes the neuron and raises the level of intracellular calcium due to its influx through N-methyl-D-aspartate (NMDA) channels and other voltage-sensitive channels and its release from intracellular stores, which triggers a variety of death pathways and leads organelle dysfunction, the production of free radicals and nitric oxide, and the activation of proteases, lipases and kinases). Excitotoxicity is not the only mechanism – transient receptor potential channels, acid-sensing channels, pannexins and hemichannels can all play a role as well (Tymianski, 2011) – but we here focus on excitotoxicity since it is the main toxic mechanism in hypoxic and hypoxic-ischemic brain injury. Moreover, the best understood influences of the nonexcitotoxic mechanisms converge on the same intracellular pathways as excitotoxicity; for example, transient receptor potential channels and acid-sensing channels mediate the entry of calcium and sodium (Tymianski, 2011).

2. Type 1 cell death: apoptosis

2.1 Morphological definition of apoptosis

Type 1 cell death, or apoptosis, has also been called the nuclear type of cell death because it is characterized by striking morphological changes in the nucleus: compaction of chromatin and nuclear condensation leading to its pyknosis and then its fragmentation (Fig. 1). These modifications of the nuclear morphology are accompanied by shrinkage of the

cytoplasm and a distorsion of the plasma membrane into folds that break off to form apoptotic bodies. These portions of the cell will be degraded by heterophagy after phagocytosis, by the lysosomes of a phagocyte. There can also be a loss of ribosomes from the rough endoplasmic reticulum (ER) and from the polysomes, but most of the other organelles appear morphologically normal, at least initially (Clarke, 1990) (Fig. 1).

Historically, apoptosis is the best known and best characterized form of programmed cell death, occurring naturally in most tissues during embryonic and postnatal development. In the first phase of neuronal death, during neurogenesis, the typology of cell death has not been studied in depth, but the main type seems to be apoptotic (Valenciano *et al.*, 2009). The second phase of neuronal death, which occurs while the neuronal connections are being formed, involves all three types of cell death. Like the other two types, apoptosis has been well demonstrated ultrastructurally in numerous situations (Clarke, 1990).

Apoptosis is the type of cell death whose molecular mechanisms have been the most intensely studied. It can be activated by two main pathways: an intrinsic pathway that depends on the integrity of the mitochondria, and an extrinsic one that is activated by extracellular signals resulting from the binding of extracellular proteins to so called “death receptors” on the cell membrane (Fig. 2).

2.2. Brief summary of the intrinsic apoptotic pathway

The intrinsic pathway of apoptosis depends on a set of proteins belonging to the Bcl-2 family (which takes its name from “B-cell lymphoma 2”, founding member of the Bcl-2 family of apoptosis regulators). These proteins are sensitive to intracellular stress originating from the ER, the cytoskeleton, the nucleus and the mitochondria. The classification of these proteins is based mainly on the type and number of Bcl-2 homology domains (BH) that they possess. The induction of apoptosis depends on the balance between the two main classes

of Bcl-2 family proteins, which are as follows (Antonsson and Martinou, 2000; Bredesen, 2007; Llambi *et al.*, 2011) (Fig. 2): 1) The anti-apoptotic members such as Bcl-2, Bcl-xL (B-cell lymphoma-extra large) and Mcl-1 (Myeloid cell leukemia-1) possess 3 to 4 BH domains (BH1-4) and prevent apoptosis by sequestering the pro-apoptotic members. 2) The pro-apoptotic members are divided into two sub-groups: a) members such as Bax (Bcl-2–associated X protein) and Bak (Bcl-2 homologous antagonist killer), with multiple BH domains (BH1-3), contribute directly to the permeabilization of the mitochondrion whereas b) some "BH3-only" members, such as PUMA (p53 upregulated modulator of apoptosis) and Bad (Bcl-2-associated death promoter), are sensitizers, inhibiting anti-apoptotic proteins by sequestering them and thus allowing the liberation of the pro-apoptotic BH1-3 members. Other members of the "BH3-only" category, such as Bim (Bcl-2-interacting mediator of cell death) and tBid (truncated BH3 interacting-domain death agonist), are termed activators, playing more active roles in the permeabilization of the mitochondrion.

The mitochondrion is a reservoir of apoptotic factors: cytochrome c (12kDa), Smac/Diablo (second mitochondria-derived activator of caspases/direct IAP binding protein with low pI) (27kDa), AIF (Apoptosis inducing factor) (67kDa), endonucleaseG (~34kDa), Omi/HtrA2 (High temperature requirement protein A2) (48kDa). These are all located in the intermembrane space. Even in healthy mitochondria, the outer mitochondrial membrane (OMM) is permeable to very small molecules (less than 5kDa), but under the influence of apoptotic signals, the proteins Bax and Bak make the OMM permeable to larger proteins causing various apoptotic factors to be released into the cytosol. Thus Bax and Bak are major effectors of apoptosis as their absence protects cells from many apoptotic stimuli (Wei *et al.*, 2001). The three BH3-only proteins tBid, Bim and PUMA are essential activators of Bax and Bak (Ren *et al.*, 2010). While Bak is constitutively present in the OMM, Bax is mainly cytosolic in healthy cells, and Bax recruitment to the OMM is dependent on tBid (Martinou

and Youle, 2011). How the OMM permeabilization is achieved remains unclear (reviewed in (Garcia-Saez, 2012; Martinou and Youle, 2011; Tait and Green, 2010). The most commonly established model is the formation of a large pore in the OMM upon the activation and oligomerization of Bax and Bak (Galluzzi *et al.*, 2007; Korsmeyer *et al.*, 2000). Another emerging hypothesis is the formation of a "toroidal or lipidic pore" following interaction of activated Bax or Bak with OMM lipids (Hardwick and Polster, 2002). Alternatively, but less likely, Bax and Bak may act on the opening of other channels such as the mitochondrial permeability transition pore (mPTP) located at the junctions between the inner and outer membranes inducing the entry of water and hence mitochondrial swelling and ultimately rupture of the outer membrane. Whatever the way of mitochondrion permeabilization, the different factors released from the mitochondrion into the cytosol can activate a caspase-dependent apoptotic pathway and a different, caspase-independent pathway (Fig. 2).

2.2.1. Caspase-dependent intrinsic pathway

Cytochrome c, which is an essential element in the respiratory chain, is released into the cytosol and can play a pro-apoptotic role by interacting with Apaf-1 (adapter protein apoptotic protease-activating factor-1). This interaction induces a conformational change of Apaf-1 enabling it to form heptameters and to expose its CARD domains (caspase recruitment domains) whose role is to recruit the caspase-9 initiator in its inactive form (zymogen) into a complex (apoptosome) that promotes a process of auto-catalytic activation. An exchange of dADP with exogenous dATP on Apaf-1 is necessary to form an active apoptosome (Kim *et al.*, 2005), however the origin of the dADP is still a subject of debate (Reubold and Eschenburg, 2012). The active form of caspase-9 then cleaves the effector caspases 3, 6 and 7 which leads in turn to their activation (Boatright *et al.*, 2003; Bredesen, 2007).

Caspases-3, -7 and -9 are inhibited by the IAP (Inhibitor of Apoptosis Protein) family of proteins whose members include XIAP (X-linked inhibitor of apoptosis protein) and survivin. When the mitochondrial proteins Smac/Diablo or Omi/HtrA2 are released into the cytoplasm during the apoptotic process, they inhibit the IAP members and hence disinhibit the caspases (Bredesen, 2007; Du *et al.*, 2000; Martins *et al.*, 2002). Among the targets of caspases are the Inhibitor of Caspase-Activated Deoxyribonuclease (ICAD), the Poly(ADP-Ribose) Polymerase (PARP) family members, lamin A and alpha-fodrin; their caspase-mediated cleavage leads to cell shrinkage, membrane blebbing, inhibition of DNA repair, chromatin condensation and DNA fragmentation (Earnshaw *et al.*, 1999; Fan *et al.*, 2005).

Activation of the transcription factor p53 can trigger apoptosis under several stress conditions such as DNA damage, Ca^{2+} overload, hypoxia and oxidative stress. The apoptotic mitochondrial pathway is induced by p53 activation and p53-dependent enhanced expression of proapoptotic proteins like Bax, Bid, PUMA and Noxa (Culmsee and Mattson, 2005) . However p53 has also been demonstrated to have a transcription-independent apoptotic effect of due to a direct action on mitochondria leading to a substantial increase in their permeability (Moll *et al.*, 2005; Speidel, 2010).

The ER can also be involved in caspase activation. In conditions of stress (oxidative stress, or accumulation of proteins with a conformational abnormality) caspase-12, bound to the cytosolic side of the ER membrane, is activated by cleavage (by m-calpain) and can then activate the effector caspases (Groenendyk and Michalak, 2005).

2.2.2. Caspase-independent intrinsic pathway

A caspase-independent apoptotic pathway can be activated by some of the factors released by the mitochondria. These include AIF (an oxidoreductase) and endonuclease G (endoG), which both migrate into the nucleus, where they play a role in the fragmentation of

DNA (Joza *et al.*, 2001; Susin *et al.*, 1999). AIF is a flavoprotein that, in healthy cells, plays an important role in oxidative phosphorylation as a NADH oxidase. It is located in the mitochondrial inner membrane but during apoptosis AIF is cleaved to produce a soluble protein released from mitochondria into the cytosol from where it is translocated to the nucleus causing large-scale DNA fragmentation (Susin *et al.*, 1999; Wang and Youle, 2009). EndoG, is a DNA/RNA nonspecific nuclease. In healthy cells, endoG located in mitochondria intermembrane space has been involved in some cellular functions such as in mitochondrial DNA replication (Cote and Ruizcarrillo, 1993). EndoG has by itself a DNase function (Li *et al.*, 2001). When released from mitochondria and in the presence of interacting partners endoG efficiently cleaves double-stranded DNA (Kalinowska *et al.*, 2005; Varecha *et al.*, 2012).

2.3 Brief summary of the extrinsic apoptotic pathway

Apoptosis can also be induced by extracellular signals which activate “death” receptors, including the TNF (Tumor Necrosis Factor) receptor, FasL (Fas ligand) and TRAIL (TNF-Related Apoptosis-Inducing Ligand) receptors. Their ligand-binding leads to the formation of homotrimeric proteins and to the recruitment via their cytoplasmic domain (called the death domain – DD) of adaptor proteins such as TRADD (TNF Receptor-Associated Death Domain) and FADD (Fas-Associated Death Domain) (Chinnaiyan *et al.*, 1995; Kaufmann *et al.*, 2012; Kischkel *et al.*, 1995). The resulting complex, called DISC (Death Inducing Signaling Complex), recruits and assembles caspase-8 zymogens via the so called Death Effector Domain (DED), leading to their activation either through autoproteolytic cleavage or through trans-cleavage by other caspases (Schmitz *et al.*, 2000; Schulze-Osthoff *et al.*, 1998). The cFLIPs (cellular FADD-like IL-1β-converting enzyme-inhibitory proteins)) are inhibitors of the extrinsic apoptotic pathway since they contain two DED domains which compete with caspase-8 for binding to FADD (Irmler *et al.*, 1997).

Thus, the extrinsic pathway converges with the intrinsic one to activate effector caspases 3, 6 and 7. However, there is a cross talk between the pathways at an earlier level, because caspase 8, once activated, cleaves Bid into tBid and thus contributes to permeabilization of the mitochondrion and hence activation of the intrinsic pathway at an early level (Li *et al.*, 1998; Luo *et al.*, 1998).

2.4. Apoptosis in excitotoxicity and cerebral hypoxia/ischemia

Neuronal death with apoptotic features and caspase activation occurs frequently in excitotoxicity and cerebral hypoxia/ischemia, although pure canonical apoptosis is rare in these situations.

Studies on human brains indicated the presence of apoptosis (activation of caspases 3- and -9, cytochrome c release, TUNEL staining) in ischemic neurons mainly in the peri-infarct region (Duan *et al.*, 2010; Mitsios *et al.*, 2007; Rami *et al.*, 2003; Sairanen *et al.*, 2009). In experimental models apoptosis has been reported to be an important neuronal death mechanism in acute neurodegenerative conditions such as head trauma or cerebral ischemia (Li *et al.*, 1995; Rink *et al.*, 1995). In focal cerebral ischemia, apoptosis is involved mainly in the ischemic penumbra, the border of the ischemic region where the levels of oxygen and energy are sufficient to allow apoptosis to occur (Benchoua *et al.*, 2001; Broughton *et al.*, 2009; Ferrer *et al.*, 2003) (Benchoua et al., 2001; Ferrer et al., 2003; Broughton et al., 2009). This penumbral cell death is delayed by several or many hours with respect to that occurring in the ischemic core, indicating that its mechanisms are potential targets for neuroprotection. In several animal models, the activation of caspases 1, 3, 8, 9 and 11, the release of cytochrome c from mitochondria (Benchoua *et al.*, 2001; Kang *et al.*, 2000) and the involvement of Bcl-2 family members (Martinou *et al.*, 1994; Plesnila *et al.*, 2001) have been reported in cerebral ischemia models. In mice exposed to cerebral

ischemia, TAT-mediated delivery of Bcl-X_L has been shown to decrease caspase activation and brain damage (Cao *et al.*, 2002; Kilic *et al.*, 2002). Caspase-3 deficiency in mice reduced the cortical infarct volume by more than by half following middle cerebral artery occlusion (MCAo) (Le *et al.*, 2002) Furthermore, pharmacological inhibition of caspases has been shown to protect the brain against cerebral ischemia and to reduce the behavioral consequences in rodents (Braun *et al.*, 2007; Fink *et al.*, 1998; Fink *et al.*, 1999; Hara *et al.*, 1997; Rabuffetti *et al.*, 2000). Molecular genetic inhibition of caspases by the overexpression of Apaf-1-interacting protein (AIP), inhibiting thereby apoptosome-induced caspase-3 activation, likewise enhanced neuronal survival (by 38%), in hippocampal CA1 after rat transient global cerebral ischemia (Cao *et al.*, 2004) and deletion of the (pro-apoptotic) Bid gene was strongly neuroprotective in transient MCAo (Plesnila *et al.*, 2001). However the severity and the type of ischemic insult (global ischemia, permanent or transient focal ischemia) influence the pattern of caspase activation and the neuroprotective efficiency of caspase inhibition (Li *et al.*, 2000; Manabat *et al.*, 2003).

Hypoxia-ischemia in rat pups leads to strong activation of apoptotic mechanisms (much more strongly than in adults), which develops progressively over several hours after hypoxia-ischemia (HI) and contributes to the hypoxia-ischemia induced “delayed” neuronal death (Nakajima *et al.*, 2000; Northington *et al.*, 2001b; Zhu *et al.*, 2005). The neuronal death resembles apoptosis morphologically, with condensation of chromatin (Fig. 3). The affected neurons express cleaved caspases such as caspase 3, and in rodent models caspase inhibition has been reported to be neuroprotective in some cases (Cheng *et al.*, 1998; Han *et al.*, 2002) but not in others (Joly *et al.*, 2004; Puyal *et al.*, 2009; Zhu *et al.*, 2003). As in adult models, Tat- Bcl-X_L has been shown to be neuroprotective, preventing both caspase-dependent and independent pathways (Yin *et al.*, 2006). In a moderate HI model in rat neonates, the intracerebroventricular injection of caspase inhibitor BAF (boc-Asp-fmk)

reduces the neuronal loss in the CA1 region of the hippocampus, provided the BAF is administered before the HI (Adachi *et al.*, 2001; Zhu *et al.*, 2005), and gives substantial protection in the cortex and striatum even when the BAF is given 3h after HI (Cheng *et al.*, 1998). In a more severe model, pretreatment with a specific inhibitor of caspase-3 (M826) likewise reduces the lesion size in the cortex, striatum and hippocampus (Han *et al.*, 2002). More recently, the genetic inhibition of caspase-2 (Carlsson *et al.*, 2011), and the inhibition of caspases by using a pentapeptide-based irreversible caspase inhibitor (Chauvier *et al.*, 2011), were shown to be strongly neuroprotective against both neonatal HI and excitototoxicity. In *bax*$^{-/-}$ mice there is a 36% reduction in the volume of damaged tissue in the hippocampus, but the activation of caspase-3 is not totally abolished in these mice because the extrinsic pathway of apoptosis persists and activates caspase-8 which in turn activates caspase-3 (Gibson *et al.*, 2001). Similarly, a Bax-inhibiting peptide was neuroprotective against hypoxic-ischemic neonatal brain injury, but did not totally eliminate caspase activation (Wang *et al.*, 2010). Another complication is that the elimination of caspase-3 activation throughout development, by its genetic deletion, exacerbates the vulnerability to hypoxia-ischemia and *increases* in the size of the lesion (West *et al.*, 2006).

Upregulation of p53 occurs rapidly following excitotoxic insults in both adult (Li *et al.*, 1994; Sakhi *et al.*, 1994) and neonatal models (Nijboer *et al.*, 2008). Deletion of the p53 gene (Crumrine *et al.*, 1994; Morrison *et al.*, 1996) or inhibition of p53's transcriptional effect by pifithrin-α both gave neuroprotection (Culmsee *et al.*, 2001; Leker *et al.*, 2004), and inhibition of the p53 pathway clearly decreased apoptotic processes (cytochrome c and Smac/Diablo release, caspase-3 activation, Noxa and PUMA expression, TUNEL staining) (Culmsee *et al.*, 2001; Leker *et al.*, 2004; Nijboer *et al.*, 2011). The pro-apoptotic effects of p53 are mediated in two ways, by its transcriptional effect (which is inhibited by pifithrin-α) and by its direct association with mitochondria (inhibited specifically by pifithrin-μ). The mitochondrial

association of p53 occurs very early, within 30min after HI (Nijboer *et al.*, 2008), and this effect of p53 seems to be the more important of the two in hypoxia-ischemia, because pifithrin-μ was even more neuroprotective than pifithrin-α and led to a lasting improvement in functional outcome (Nijboer *et al.*, 2011).

Caspase-independent apoptosis is also involved. The translocation of AIF into the nucleus occurs rapidly after adult MCAo (Culmsee *et al.*, 2005) and neonatal HI (Zhu *et al.*, 2003), even before the release of cytochrome c and the consequent caspase activation. Mice that express 60% less than normal AIF (Harlequin mice) are less sensitive than wild-type mice to adult MCAo (Culmsee *et al.*, 2005) and neonatal HI, and treatment of these mice with the broad-spectrum caspase inhibitor Q-VD-OPh (Quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxy]-methyl ketone) gives a further protection against neonatal HI; the effects of Harlequin and caspase inhibition are additive, suggesting that their effects are on independent mechanisms (Zhu *et al.*, 2007a). EndoG nuclear translocation also occurs rapidly in *in vitro* model of oxygen-glucose deprivation (Zhao *et al.*, 2009) or following MCAo in mice (Nielsen *et al.*, 2009; Wu *et al.*, 2004) and decreased expression of endoG in heterozygote mice protects the hippocampi to KA-induced excitotoxic death (Lee *et al.*, 2005).

The extrinsic apoptotic pathway is also triggered by cerebral ischemia. Fas, FasL and TRAIL expression, and caspase-8 activation, are increased in both adult cerebral ischemia (Benchoua *et al.*, 2001; Buttini *et al.*, 1996; Harrison *et al.*, 2001; Martin-Villalba *et al.*, 1999; Rosenbaum *et al.*, 2000) and neonatal cerebral HI (Feng *et al.*, 2003; Nijboer *et al.*, 2009; Northington *et al.*, 2001a; Szaflarski *et al.*, 1995). Mice lacking functional Fas or FasL (Martin-Villalba *et al.*, 1999; Martin-Villalba *et al.*, 2001; Rosenbaum *et al.*, 2000) or deficient for TNF (Martin-Villalba *et al.*, 2001) are significantly protected against focal cerebral

ischemia. Neuronal caspase-8 deletion reduced hippocampal cell death following kainate (KA) injection (Krajewska *et al.*, 2011) and caspase-8 inhibitor was neuroprotective in both adult MCAo (Inoue *et al.*, 2006) and neonatal HI (Feng *et al.*, 2003).

In conclusion, apoptosis involvement in excitotoxicity and cerebral hypoxia-ischemia has been deeply investigated and characterized over the past 20 years. Nevertheless, some discrepancies remain concerning its role and importance in term of neuroprotection. This may be due to differences between the experimental models with respect to the strength of stimulation, the timing of therapeutic intervention, gender, strains and species used, age, spectrum and properties of caspase inhibitors used. Moreover, besides the fact that the strength of the apoptotic response needed to be evaluated in each model of acute neuronal disorder, another emerging problem may be the multiplicity of the apoptotic pathways involved (and of nonapoptotic death pathways, as is discussed below), since cells appear to activate alternative pathways to counteract the protective effect of apoptosis inhibition. Furthermore, most of the therapeutic interventions against apoptosis are focused at the level of caspases, which may be too low in the apoptotic cascade at a stage when the cell is already seriously damaged.

3. Type 2 cell death – autophagic

Autophagy is a catabolic process that is complementary to the proteasome pathway. It degrades long-lived proteins and organelles (including mitochondria, portions of ER, and peroxisomes). Even though the role of autophagy in healthy cells is moderate and physiological, some dying cells exhibit grossly enhanced autophagy, which has led to the identification of “type 2” or “autophagic” cell death as a distinct type of cell death (Figs 1, 2). We first provide a brief review of the cell biology of autophagy and some technical difficulties

in demonstrating its enhancement, before discussing the somewhat controversial question of its role in cell death.

3.1. Different types of autophagy

The term autophagy includes three different catabolic processes that all lead to degradation in lysosomes (Cuervo, 2004) (Fig. 4). Microautophagy is the sequestration of a part of the cytosol by an inward budding of the lysosome's own membrane. There is only limited evidence for its occurrence in mammalian cells (Sahu *et al.*, 2011). Chaperone-mediated autophagy is specific for proteins carrying a particular sequence of 5 amino acids (KFERQ). This sequence is recognized by a chaperone protein (hsc73) and some co-chaperones that direct it to the lysosome whose membrane contains a protein receptor (LAMP-2A, lysosomal-associated membrane protein-2A) that recognizes it and mediates its translocation into the lysosome (Dice, 2007). Then, macroautophagy (henceforth called simply autophagy) is the main form of autophagy and the best studied (Puyal *et al.*, 2012). Originally, the term autophagy was coined by De Duve in 1963 to distinguish the heterophagic and autophagic functions of lysosomes (Klionsky, 2008) following Novikoff's observation of lysosome-like structures containing organelles in the process of being degraded. De Duve called these structures autophagic vacuoles. Autophagy requires an intermediate multimembranous compartment, the autophagosome, which engulfs then sequesters portions of cytosol containing the proteins and organelles that are to be degraded. The mature autophagosomes then fuse with acidic compartments (late endosomes, lysosomes etc.) to form autolysosomes, which contain the hydrolases necessary for the autophagic degradation.

Until recently, (macro)autophagy was thought to be a nonspecific mechanism, but evidence accumulated over the last 5-10 years indicates that autophagic adapters such as

p62 and NBR1 can act as cargo receptors for the selective autophagy of ubiquitinated substrates such as misfolded proteins, p62 bodies, aggresomes, peroxisomes, mitochondria and invading bacteria (Johansen and Lamark, 2011). Selective autophagy can also occur independently of ubiquitin (Johansen and Lamark, 2011). The selective autophagy of peroxisomes is called pexophagy and that of mitochondria is called mitophagy, and both of these events can play a role in cell death or its prevention, as is mentioned below.

3.2. Summary of the molecular mechanisms of autophagy

The progressive discovery of 31 autophagy genes (named atg: autophagic related genes) in yeast was crucial for the elucidation of the molecular mechanisms of autophagy. Several homologous genes were then identified in mammalian cells showing that autophagy was a strongly conserved process throughout evolution. The autophagic process can be divided into three main stages in which the different Atg proteins come into play with complex interrelationships (He and Klionsky, 2009; Simonsen and Tooze, 2009) : the induction of autophagy (nucleation), the formation of the autophagosome (elongation, incurvation, closure), and its maturation (formation of the autolysosome) (Fig. 5).

3.2.1. Phase of induction

Being an essential physiological process, autophagy is tightly regulated. It is maintained at a weak basal level in normal conditions, and is induced when necessary. Autophagy starts by the isolation of a membrane, called the pre-autophagosome, where all the proteins necessary for the formation of an autophagosome are recruited. In mammals, the origin of the pre-autophagosome has long been obscure, but recent studies showed that at least some autophagosomes are formed from membranes of pre-existing organelles such as the ER (an ER subdomain called the omegasome), the Golgi apparatus (Mari *et al.*, 2010a;

Ohashi and Munro, 2010) and/or mitochondria (Axe *et al.*, 2008; Hailey *et al.*, 2010; Hayashi-Nishino *et al.*, 2009). Plasma membrane can be also a source of membrane for autophagosome formation (Ravikumar *et al.*, 2010). The molecular regulators acting in this early phase of autophagy include a central player, the serine/threonine protein kinase mTor (mammalian target of rapamycin), which inhibits autophagy at two levels (Carrera, 2004). On the one hand it affects certain Atg proteins, such as mAtg13 (mammalian Atg13) and ULK1/2 (UNC-51-like kinase 1/2; mammalian homologues of yeast Atg1), and thus interferes directly with the formation of autophagosomes (cf. below). On the other hand, mTor acts indirectly on the expression level of certain proteins implicated in autophagy such as LC3 (microtubule-associated protein 1 light chain 3; homologue of yeast Atg8) and Atg14 (Reggiori and Klionsky, 2002). Unlike in yeast, the ULK1/2-mAtg13-FIP200 (focal adhesion kinase family-interacting protein of 200 kD) complex appears to be stable whatever the nutritive conditions, but the binding of mTor to the complex depends on its activation. In pro-autophagic conditions as nutrient deprivation, mTor is inhibited and does not bind to the complex, with the result that ULK1/2 can phosphorylate and activate mAtg13 and FIP200. The ULK1/2 complex is located at the level of the pre-autophagosome and is therefore important for the initial induction of the autophagy (Hosokawa *et al.*, 2009; Jung *et al.*, 2009). However an mTor-independent autophagy has also been described (Sarkar *et al.*, 2009; Tan *et al.*, 2012; Williams *et al.*, 2008).

In cerebral HI injury, the following deleterious events have been shown to be inducers of autophagy in different cell types: an increase in cytosolic calcium (Gao *et al.*, 2008; Hoyer-Hansen *et al.*, 2007; Wang *et al.*, 2008), hypoxia (Scherz-Shouval and Elazar, 2007) and reactive oxygen species (H_2O_2 and O_2^-) production (Chen *et al.*, 2009; He and Klionsky, 2009; Scherz-Shouval *et al.*, 2007).

3.2.2. Phase of autophagosome formation

During the phase of autophagosome formation, the pre-autophagosome undergoes a phase of elongation, followed by incurvation leading to end-to-end fusion to form a closed multi-membranous vesicle, the autophagosome, in which the cytoplasmic constituents to be degraded are sequestered. These stages depend on the interaction of different complexes in which the various Atg proteins play a variety of roles. The best known interactions are those formed by the Beclin1/PI3K-III (class III phosphatidylinositol-3 kinase) complex, the LC3 conjugation system, the Atg12-Atg5 conjugation complex and mAtg9 (Fig. 5).

The Beclin1/PI3K-III complex plays a role in both the induction of autophagy and in the formation of autophagosomes. While PI3K-I (class I phosphatidylinositol-3 kinase) is an inhibitor of autophagy in mammals, PI3K-III is an activator. Two Beclin1/PI3K-III complexes have been identified up till now in mammals (Itakura *et al.*, 2008). Both are composed of PI3K-III (homologue of Vps34, vacuolar protein sorting 34, in yeast), of p150 (homologue of Vps15) and of Beclin1 (homologue of Atg6). Beclin1 can bind either to Atg14L (yeast Atg14-like; also called Barkor (Sun *et al.*, 2008c)) or to UVRAG (UV irradiation resistance-associated gene) (Liang *et al.*, 2006; Takahashi *et al.*, 2009). The Beclin1/PI3K-III/Atg14L complex is important for the formation of the pre-autophagosome because it plays an essential role in the recruitment of the Atg12-Atg5 protein complex and of LC3 (Itakura *et al.*, 2008; Matsunaga *et al.*, 2009; Zhong *et al.*, 2009). Recently a domain on Atg14L has been identified that recruits Atg14L specifically to curved membranes enriched in PtdIns(3)P and involved in autophagosomes formation (Fan *et al.*, 2011). The Beclin1/PI3K-III/UVRAG complex not only promotes the activation of PI3K-III via UVRAG, but also plays a role in the deformation and curvation of the membrane of the pre-autophagosome via the interaction of UVRAG with Bif-1 (Bax-interacting factor-1 or endophilin B1). Bif-1 is bound to membranes of different organelles (Golgi, mitochondria) and plays a role in the regulation of their dynamics

by its capacity to generate curvature (Takahashi *et al.*, 2009). Furthermore, other protein interactions with Beclin1 have been revealed, suggesting the existence of additional Beclin1/PI3K-III complexes that are currently unknown. Indeed, it has been shown that Beclin1 can bind to Ambra1 (activating molecule in beclin1-regulated autophagy; (Fimia *et al.*, 2007)) and to VMP1 (vacuole membrane protein 1; (Vaccaro *et al.*, 2008)), two activator proteins of autophagy.

The LC3 (Atg8) conjugation system controls the size of the autophagosome by its capacity to determine the elongation, curvature and end-to-end fusion of the pre-autophagosome membrane (Fujita *et al.*, 2008a; Nakatogawa *et al.*, 2007; Sou *et al.*, 2008; Xie *et al.*, 2008). Several homologues of Atg8 exist in mammals, including the GABARAP (γ-aminobutyric acid type A receptor-associated protein) family, and the transport factor GATE-16 (Golgi-associated ATPase enhancer of 16kDa), but LC3 is the best studied and the most used for characterizing the presence of autophagy. In a manner similar to that of ubiquitination, LC3 is conjugated to phosphatidylethanolamine (PE), an abundant membrane phospholipid. LC3 first loses 22 amino acids due to the action of the cysteine protease Atg4, producing the cytosolic form of LC3 designated LC3-I (the first of two functions of Atg4 in this pathway). LC3-I is then activated by a thioester linkage to Atg7 (enzyme E1) and is then transferred to Atg3 (enzyme E2) by another thioester linkage. Finally, LC3-I becomes LC3-II through its conjugation to a phosphatidylethanolamine, causing a change of conformation that permits it to be incorporated in the pre-autophagosome membrane (Geng and Klionsky, 2008). Once the autophagosome has been formed, LC3-II is removed from the external membrane by Atg4 which hydrolyses the link between LC3-I and its phosphatidylethanolamine (second function of Atg14). LC3-II remains however in the internal membrane of the mature autophagosome.

The Atg12-Atg5-Atg16L conjugation system is essential for the elongation process, for the recruitment of LC3 at the pre-autophagosomal level and its conjugation to phosphatidylethanolamine (Fujita *et al.*, 2008b; Longatti and Tooze, 2009; Mizushima *et al.*, 2001). In a manner resembling that of the LC3 conjugation system, Atg12 is conjugated to Atg5. Atg12 is first activated by its binding to Atg7 (enzyme E1), is then transferred to Atg10 (enzyme E2), and is finally conjugated to Atg5 (Geng and Klionsky, 2008). Atg16L (homologue of Atg16) binds to the complex via Atg5, which then binds to the membrane of the pre-autophagosome (Mizushima *et al.*, 2003). The Atg16L complex determines where LC3 will be lipidated and consequently the membrane biogenesis site. Atg12-Atg5-Atg16L may have an E3-like activity in the LC3 conjugation system (Fujita *et al.*, 2008b; Romanov *et al.*, 2012).

Mammalian Atg9 (mAtg9) is an integral membrane protein required for the formation of the autophagosome. In mammals, mAtg9 is localized in the trans-Golgi network and in late endosomes but has not been observed in mitochondrial membranes as in yeast (Reggiori *et al.*, 2005). A function of mAtg9 in the elongation of the pre-autophagosome is suspected probably by the recruitment of membrane lipids or of trans-Golgi network proteins necessary for the elongation stage (Orsi *et al.*, 2012; Webber *et al.*, 2007). Bif-1 has been shown to be necessary for Atg9 trafficking from Golgi to early autophagosomes (Takahashi *et al.*, 2011).

It has recently been proposed that autophagy can occur by alternative pathways in addition to the conventional one, dependent on the Atg5-Atg12 and LC3 conjugation systems. One such pathway was reported in *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ MEF cells, where the formation of autophagosomes appears to occur by the fusion of unconventional pre-autophagosomes with vesicles from the trans-Golgi network and late endosomes (Nishida *et al.*, 2009). Like the conventional pathway, this alternative one is dependent on Beclin1/PI3K-III, but several studies have shown that Beclin1-independent autophagy can occur and can

moreover promote cell death in conditions of stress (Grishchuk *et al.*, 2011; Scarlatti *et al.*, 2008; Zhu *et al.*, 2007b).

Most proteins involved in the biogenesis of autophagosomes, except LC3-II in the internal membrane, are removed before the complete formation of the autophagosome. These proteins are apparently recycled for reuse (Reggiori *et al.*, 2004). This may explain why the synthesis of new proteins is not essential for the induction of autophagy and the formation of autophagosomes (Abeliovich *et al.*, 2000; Lawrence and Brown, 1993).

3.2.3. Phase of autophagosome maturation

Autophagosome maturation produces a functional autolysosome. This can happen only after the autophagosome has been completely formed and the Atg16L and LC3-II complexes have been removed from the external membrane. In mammals, the maturation of the autophagosome into a functional degradation compartment is complex. It leads to the acidification of the compartment and the presence of many different hydrolases (including cathepsins B, D and L). The formation of the autolysosome requires the intermediate stage of fusion with different endosomal compartments (early endosomes, multivesicular bodies, late endosomes) forming amphisomes before fusion with a lysosome (Simonsen and Tooze, 2009). Thus, proteins necessary for endocytosis play a direct or indirect role in the maturation of autophagosomes, including Rab7, COPI (coat protein complex I) and ESCRT (endosomal sorting complex required for transport) (Gutierrez *et al.*, 2004; Razi *et al.*, 2009; Rusten and Stenmark, 2009). LAMP proteins, especially LAMP-2, are also involved in the maturation of autophagosomes (Saftig *et al.*, 2008).

In addition to its function in the formation of the autophagosome, the complex Beclin1/PI3K-III/UVRAG may play a role in the maturation of endosomes and autophagosomes (Matsunaga *et al.*, 2009). The recently discovered autophagy-inhibiting protein Rubicon (RUN domain and cysteine-rich domain containing, Beclin1-interacting

protein) can bind to the Beclin1/PI3K-III/UVRAG complex and reduce the activity of the PI3K-III and hence the maturation of the autophagosomes and endosomes (Matsunaga *et al.*, 2009; Zhong *et al.*, 2009).

3.3. Difficulties concerning the demonstration of enhanced autophagy

Over the past ten years, there has been a great increase in the study of autophagy, in both physiological and pathological situations. Autophagy (stricto sensu) is due to an increase in both autophagosome formation and lysososomal clearing, but in numerous studies, the induction of autophagy was deduced on the sole grounds that the number of autophagosomes was increased. Such evidence is however ambiguous, because it could be due either to a real increase in autophagic activity (i.e. an increase in autophagic flux) or to a defect in lysosomal degradation, which would lead to autophagosome accumulation. Such incomplete analyses may explain some of the controversies concerning the roles of autophagy in different situations (Puyal *et al.*, 2012; Sridhar *et al.*, 2012). In the nervous system, an interesting example of such ambiguity comes from studies on chronic neurodegenerative diseases. In Alzheimer's disease, morphological studies had shown increased numbers of autophagosomes in affected neurons, and the initial conclusions were in favor of an increase in autophagy that could be involved in the development of the disease (Cataldo *et al.*, 1995; Cataldo *et al.*, 1996). But recent studies have clearly demonstrated that this autophagosome increase is not due to an increase in autophagy but to a decrease in lysosomal degradation (Lee *et al.*, 2010; Nixon and Yang, 2011), and the restoration of lysosomal function in animal models of Alzheimer's disease have been found to restore neuronal function and increase cognitive performance (Yang *et al.*, 2011).

For these reasons explained above, the involvement of autophagy stricto sensu in a particular situation needs to be demonstrated by monitoring not only autophagosome

formation but the whole process of autophagy, i.e. the autophagic flux (Klionsky and 1269 others, 2012).

3.4 The term “autophagic cell death”

There has been controversy, as is discussed below, concerning the importance, and even the existence, of “autophagic cell death” (Clarke and Puyal, 2012; Kroemer and Levine, 2008; Puyal *et al.*, 2012; Yuan and Kroemer, 2010). Part of the controversy has been due to confusion related to changes in the meaning of this term. It was coined in the 1970s in a purely morphological context to refer to dying cells that contained numerous autolysosomes (and sometimes autophagosomes) and lacked the characteristics of other types of cell death (Clarke, 1990). Based on this morphological observation, it was supposed that in some conditions autophagy could be implicated in promoting cell death, because the autolysosomes often contained more than half the cytoplasm, and sometimes even parts of the nucleus including some of its DNA (Clarke, 1990; Hornung *et al.*, 1989). Nevertheless, death-mediation was not strictly part of the original definition.

Confusingly, the term “autophagic cell death” is now used in at least three different ways: 1) autophagy-associated cell death (the original definition), 2) autophagy-mediated cell death (which might involve a standard mechanism of cell death triggered by autophagy, because strongly autophagic dying cells sometimes also show characteristics of apoptosis and/or necrosis), and 3) a distinct mechanism of cell death, independent of apoptosis or necrosis (Fig. 6). We shall use the term “autophagic cell death” in this third sense, in conformity with most (Clarke *et al.*, 2008; Elgendy *et al.*, 2011; Galluzzi *et al.*, 2012; Shen and Codogno, 2011) but not all (Uchiyama *et al.*, 2009) recent usage. Thus, to qualify as “autophagic” the cell death must show greatly enhanced autophagic flux, the autophagy must be death-promoting, and the cell death must not be apoptotic or necrotic. For in vivo studies

of mammalian neurons, it is hard to measure autophagic flux directly, but the presence of greatly increased numbers of autolysosomes (not just autophagosomes) would be a useful indication. To prove a death-mediating role of autophagy can be difficult, because cells (especially mammalian cells) often respond to the inhibition of one death pathway by activating an alternative one. Therefore, a cell that was genuinely dying by autophagic cell death might still die, but by apoptosis or necrosis, if tested with an inhibitor of autophagy. For this reason, we think that cell death showing strongly enhanced autophagy and no sign of apoptosis or necrosis should be considered "autophagic" if autophagy inhibition delays the death, even if the cells ultimately die by apoptosis or necrosis.

3.5. Autophagy in the mediation of cell death

As discussed above, two different questions have to be asked about situations where autophagy is enhanced in the presence of cell death: 1) Is the cell death autophagy-mediated, or is the induction of autophagy playing some other role, perhaps as an ultimate reaction to protect the cell? 2) If the cell death is indeed autophagy-mediated, does it qualify as true autophagic cell death in the strict sense described above.

To demonstrate a death-mediating role of autophagy, the universal strategy has been to show that its inhibition reduces (or at least delays) the cell death (Lenardo *et al.*, 2009). Early studies suggesting a death-mediating role of autophagy relied on pharmacological PI3K inhibitors, notably 3-methyladenine (3-MA) (Seglen and Gordon, 1982) and sometimes wortmaninn or LY294002, (Blommaart *et al.*, 1997). These inhibitors prevent autophagy, because they inhibit PI3K-III, which is essential for most autophagy. 3-MA and other PI3K inhibitors attenuate or prevent autophagy-associated cell death in numerous situations, e.g. following nerve growth factor deprivation of sympathetic neurons (Xue *et al.*, 1999), serum deprivation of PC12 cells (Guillon-Munos *et al.*, 2005) or hypoxia of different cancer cell lines

(Azad *et al.*, 2008). However, in view of the incomplete specificity of the inhibitors, notably by their probable pro-apoptotic action on PI3K-I, or by different temporal patterns of inhibition for PI3K-I and PI3K-III (Wu et al., 2010), it has been necessary to clarify the relationship between autophagy and cell death by more specific techniques, notably the genetic inhibition of Atg proteins.

The first molecular genetic evidence for a death-mediating role of autophagy came from two studies showing that cells incapable of activating the mechanisms of apoptosis could die by an autophagic form of death. In one of these studies, general inhibition of caspases or specific inhibition of caspase-8, in different cell lines or in macrophages, induced nonapoptotic cell death involving the presence of multiple autophagosomes. This death was blocked by the inhibition of Beclin1 or of Atg7 by RNA interference (Yu *et al.*, 2004). It was later shown that this type of death induced by caspase inhibition led to an accumulation of reactive oxygen species following the selective autophagic degradation of catalase, probably through pexophagy (Yu *et al.*, 2006a). In the other study, MEF cells from bax/bak deficient mice or MEF cells overexpressing Bcl2/BclXL contained numerous autophagosomes after treatment with certain apoptotic stimuli. Their death could be prevented not only by treatment with 3-MA or wortmaninn, but also by diminishing the expression of Beclin1 or of Atg5 by RNA interference (Shimizu *et al.*, 2004). These results provided strong support for a death-mediating role of autophagy, but other studies showed that when autophagy was induced in the very same $bax^{-/-}/bak^{-/-}$ MEF cells by amino acid deprivation, its inhibition by 3-MA was death-promoting rather than being protective (Tsujimoto and Shimizu, 2005). The role of autophagy is then highly dependent notably on the cell type and the nature of the stimulus. The studies of Yu et al. (Yu *et al.*, 2004) and Shimizu et al. (Shimizu *et al.*, 2004) showed that autophagy can be a mediator of cell death in cultured cells whose apoptotic machinery was inactivated, suggesting a death role for autophagy as an alternative to apoptosis. But there is

other evidence that autophagy can promote cell death by triggering apoptosis (see below) as when Atg1 is overexpressed in Drosophila inducing autophagy that leads to cell death with an apoptotic morphology and caspase activation (Scott *et al.*, 2007).

On the other hand, autophagy can mediate cell death independently of apoptosis in cells that are apoptosis-competent. In various cancer cell lines, hypoxia induces a death that is not affected by the presence of a caspase inhibitor (z-VAD-fmk) but is strongly reduced by RNA interference of Atg5 or of Beclin1 (Azad *et al.*, 2008). Similar results were obtained when the death was induced by oxidative stress due to the addition of H_2O_2 on cell lines (Chen *et al.*, 2008). Adult hippocampal neural stem cells that were dying because of insulin withdrawal showed morphological and biochemical characteristics of enhanced autophagy, leading to an apoptosis-independent cell death (Baek *et al.*, 2009; Yu *et al.*, 2008). In these cells, autophagy inhibition by Atg7 knockdown was demonstrated to be protective and autophagy stimulation using rapamycin increased cell death. This work demonstrated that autophagy-mediated cell death could occur in apoptosis-competent stressed neurons without the involvement of apoptosis.

Dictyostelium cells are an interesting in vitro model to study autophagic cell death because they lack apoptotic pathways (Giusti *et al.*, 2010), and show the complexity of the roles that enhanced autophagy could play depending on the conditions. When starved and in presence of cAMP, Dictyostelium cells induced autophagy as a survival response, but once they had been sensitized by this first stress, a second signal (the differentiation factor DIF-1) converted the enhanced autophagy into a destructive mechanism (Luciani *et al.*, 2011). This suggests that autophagic cell death may require two successive signals, of which the first enhances autophagy and sensitizes the cells to the second one.

Another excellent example is the cell death that occurs during the metamorphosis of Drosophila. Whereas the salivary glands disappear during this period by a combination of

autophagy and apoptosis (Berry and Baehrecke, 2007), the elimination of the midgut results from a caspase-independent cell death that requires the activation of autophagy (Denton *et al.*, 2009). Thus, RNA interference knockdown of caspases has no effect on the midgut elimination but knockdown of Atg1 or Atg18 greatly delays the cell death. It also tends to increase caspase activity, but the knockdown of both caspases and autophagy in parallel does not cause an additional effect, implying a primary role for autophagy but not caspases in the death process. This study is to date the most convincing line of evidence for the existence of autophagic cell death in a natural situation.

3.6. Autophagy in excitotoxicity and cerebral ischemia

Autophagy has been shown to be present in dying neurons in various models of excitotoxicity both *in vitro* and *in vivo*: hippocampal slices exposed to NMDA (Borsello *et al.*, 2003), the long term exposure of organotypic cultures of lumbar spinal cord to an inhibitor of glutamate transporters (Matyja *et al.*, 2005), the direct *in vivo* injection of kainate into the brains of mice (Shacka *et al.*, 2007) or into the spinal cord (Kanno *et al.*, 2009), and traumatic brain injuries (Luo *et al.*, 2011).

The first study showing an activation of autophagy after cerebral ischemia was that of Nitatori et al. in 1995 who demonstrated ultrastucturally the presence of numerous autophagic vacuoles and an increase in the size of cathepsin B positive vesicles in hippocampal CA1 neurons 3 days after ischemia (Nitatori *et al.*, 1995; Nitatori *et al.*, 1996). Later studies on adult cerebral ischemia and neonatal HI showed increases in the activation and expression of lysosomal proteases (Seyfried *et al.*, 1997; Wen *et al.*, 2008), found an increased expression of autophagosomal marker LC3-II by Western blot (Degterev *et al.*, 2005; Ginet *et al.*, 2009; Puyal *et al.*, 2009; Zhu *et al.*, 2005) and showed increased numbers of autophagosomes and autolysosomes by electron microscopy (Adhami *et al.*, 2006; Ginet

et al., 2009; Puyal *et al.*, 2009). Enhanced autophagy has also been demonstrated in a retinal ischemia model (Piras *et al.*, 2011). Recently, *in vivo* imaging through the skull of GFP-LC3 transgenic mice showed a peak in fluorescent signal in the ischemic hemisphere 24h after transient MCAO (Tian *et al.*, 2010).

The role of this induction of autophagy was first studied *in vivo* in a model involving the intra-striatal injection of kainate; injection of 3-MA or an inhibitor of cathepsin B was shown to reduce the size of the lesion, even when performed 3h post-kainate (Wang *et al.*, 2006; Wang *et al.*, 2008). Similar results were obtained in a model involving MCAo when similar inhibitors were injected at the beginning of ischemia (Wen *et al.*, 2008). In a severe neonatal (12 day old) model of cerebral ischemia, 3-MA was also strongly neuroprotective, reducing the lesion volume by almost 50% when given as late as 4.5 h after the onset of ischemia. In contrast, caspase inhibition (with Q-VD-OPh or Z-VAD-fmk) provided no protection (Puyal *et al.*, 2009). However, an opposite role for enhanced autophagy has been proposed by Carloni and colleagues in neonatal HI since in their model 3-MA had no effect on brain lesion whereas rapamycin was protective (Carloni *et al.*, 2008). However they used a very weak dose of 3-MA, and its effect on autophagy was checked only indirectly, by measuring Beclin1 expression, never by a direct marker such as LC3-II.

A problem with all the above studies is that 3-MA and rapamycin have limited specificity for the modulation of autophagy (Hughes and Kennedy, 2012; Wu *et al.*, 2010), so confirmation was needed with more specific, molecular methodologies. An important study (Koike *et al.*, 2008) in neonatal conditional knockout mice with neuron-specific deletion of Atg7 showed that the absence of autophagy led to resistance against HI in the hippocampus, providing the strongest evidence so far for a death-mediating role of autophagy in hypoxic-ischemic neurons. Such a study is impossible in adult models due to the essential role of basal autophagy and the neurodegeneration that occurs in the knockout mice from 3 weeks

of age (Komatsu *et al.*, 2006; Komatsu *et al.*, 2007). More recently, it has been shown that the downregulation of Beclin1 by lentiviral delivery of shRNA into the thalamus of adult rats decreased the neuronal death that occurs there secondarily to cerebral (mainly cortical) infarction following distal MCAo. In this experiment the MCAo affected the thalamic neurons only indirectly, because of their connections with the cortex (Xing *et al.*, 2012).

In conclusion, there is compelling evidence for a death-mediating role of enhanced autophagy in experimental excitotoxicity, cerebral hypoxia-ischemia and stroke, in both adult and immature brains (Puyal *et al.*, 2012). In many cases the autophagy triggers apoptosis, as is discussed further in section 6.2., but in some cases the cell death may be “autophagic” in the strict sense.

4. Cell death of type 3 - necrotic

4.1. Morphological definition of type 3 cell death

Type 3 cell death was initially defined in the context of normal development (Schweichel and Merker, 1973), whereas necrosis (also called coagulative necrosis by pathologists) was initially considered to occur only in severely damaged tissues (Wyllie *et al.*, 1980), but the two types resemble each other morphologically, and are now generally considered to be variants of a single type of cell death.

In the context of development, type 3 cell death was divided into two main variants, 3A and 3B (Clarke, 1990), but we here ignore type 3A because it is rare and has never been reported in the nervous system. Type 3B is also referred to as the *cytoplasmic type* of cell death (Pilar and Landmesser, 1976), or *paraptosis* (Sperandio *et al.*, 2000). Most morphological studies of type 3B cell death have been in developing neurons; it is characterized particularly by dilation of the organelles and of the perinuclear space and by a vacuolization of the cytoplasm. The nucleus is relatively unaffected, but finally swells and is

lysed, and the cell is ultimately phagocytozed (Clarke, 1990; Clarke, 1999) (Fig. 1). The biochemical pathways of this kind of cell death are not well understood. There has been some recent progress in cell lines, but the relevance of this our present focus on excitotoxicity in neurons is unclear (Sperandio *et al.*, 2010). We therefore focus the present discussion on necrosis.

Necrosis resembles types 3A and 3B to the extent that it is characterized by vacuolization (Fig. 2D), due primarily to organelle dilation, but the changes are more dramatic and necrotic cells are invariably very swollen, in both cytoplasm and nucleus. In the cytoplasm, there is initially a gross dilation of mitochondria, whose cristae break, and then of the ER, which fragments into vesicles. The nucleus also swells, and its heterochromatin becomes coarser, forming discrete masses on the nuclear membrane, before its dissolution, which leaves a nuclear "ghost" (Majno and Joris, 1995; Wyllie *et al.*, 1980). Many authors have claimed that the cellular swelling causes such tension in the plasma membrane that it breaks, and that this is the cause of the cell death (Majno and Joris, 1995; Rothman, 1985). This may be true in some situations, but this is no longer believed to be the main cause of necrotic cell death, at least not in vivo (Clarke, 1999). There are no specific biochemical markers of necrosis, although markers of calpain activation may be suggestive of it in certain contexts, as we shall discuss. Labeling by vital dyes to show permeabilization of the plasma membrane can likewise be suggestive of necrosis, but only electron microscopy can demonstrate this type of cell death definitively.

Necrosis was originally considered to be a purely passive and uncontrolled type of cell death, and it is true that it often occurs in situations of energy failure where energy-dependent mechanisms such as caspase-activation and autophagy cannot occur (Golstein and Kroemer, 2007; Kitanaka and Kuchino, 1999), but there is nevertheless evidence that necrosis can be regulated and for this reason some authors refer to "programmed necrosis"

to emphasize that signaling pathways are involved (Edinger and Thompson, 2004; Galluzzi *et al.*, 2011). Nevertheless, current understanding of these signaling pathways is rather limited. In many cases, including those of most relevance to this review, necrotic cell death is initiated by a substantial rise in cytosolic calcium, which then leads to cell death through various calcium-dependent pathways. Some of these involve the activation of calpains, as is discussed below. In other cases, (programmed) necrosis is initiated by the activation of death receptors, in which case it is usually termed *necroptosis* (Galluzzi *et al.*, 2011). Necroptosis is caspase-independent and can occur in apoptosis-incompetent cells. It is activated by the receptor-interacting protein 1 (RIP1), which is a kinase and sometimes called RIP1-kinase or RIP1K. Cells can be protected against necroptosis by necrostatin-1, a specific inhibitor of RIP1 (Galluzzi *et al.*, 2011). Other members of the RIP-kinase family may also be involved in necroptosis, and a whole family of necrostatins are currently being characterized (Degterev *et al.*, 2008; Smith and Yellon, 2011). The promise of this research for neuroprotection against cerebral ischemia seems considerable, as is discussed in the next section.

4.2. Neuronal necrosis in excitotoxicity and cerebral ischemia

Excitotoxicity and cerebral ischemia frequently involve necrosis, and a major cause of it is the massive influx of calcium that occurs mainly through voltage-dependent channels following cellular depolarization (Hou and MacManus, 2002). The intensity of glutamate exposure and the subsequent loss of membrane potential are key players in necrosis (Ankarcrona *et al.*, 1995). Thus, it is induced rapidly in both excitotoxicity (Arthur *et al.*, 2007) and in cerebral ischemia (Ueda, 2004). In focal cerebral ischemia, neuronal death occurs most massively and rapidly in the “core” of the ischemic region, where the ischemia is almost total and the energy deprivation severe, and this is the site where neuronal necrosis predominates (Hou and MacManus, 2002) (Fig. 3D). The duration of ischemia and the

amplitude of reperfusion are among the parameters determining the core area. Necrotic cell death mechanisms have a more significant role in adult models since immature neurons are very sensitive to apoptosis (Liu *et al.*, 2004; Zhu *et al.*, 2005; Zhu *et al.*, 2003). However, in neonatal cerebral hypoxia-ischemia necrosis likewise occurs rapidly (Nakajima *et al.*, 2000; Northington *et al.*, 2001a; Northington *et al.*, 2001b) and contributes importantly to the cerebral lesion (Carloni *et al.*, 2007; Towfighi *et al.*, 1995).

In most cases of excitotoxicity and in cerebral ischemia, the main stimulus to neuronal necrosis is a rise in intracellular calcium due largely to calcium entry through NMDA channels. In view of the importance of calcium in the mediation of necrotic cell death, several studies of cerebral ischemia and cerebral HI have investigated the roles of calpains, which are a class of cytosolic cystein proteases that are activated by raised calcium and are known to be involved in the mediation of necrotic cell death. Calpains degrade many essential proteins including α-fodrin, which is a cytoskeletal protein. They cleave it specifically to give a fragment of 150 kDa that can be identified in Western blots. Numerous authors have used this specific fodrin cleavage as a marker to evaluate the activation of calpains in response to an ischemic or HI insult (Zhu *et al.*, 2005), and have investigated the neuroprotective effects of calpain inhibitors. The interpretation of these results is complicated by the fact that calpains can also be activated in non-necrotic forms of cell death, for example by caspase-12 during apoptosis (Nakagawa and Yuan, 2000), or can mediate excitotoxic apoptosis (Orsi *et al.*, 2012).

Nevertheless, in view of the difficulty of analyzing necrotic death mechanisms by other biochemical approaches, the studies of calpains provide some of the most valuable evidence about necrotic mechanisms in the brain, especially when morphological studies confirm that necrotic cell death is indeed occurring. In adult focal cerebral ischemia and neonatal hypoxia-ischemia, calpain-specific cleavage of α-fodrin occurs (Han *et al.*, 2002; Zhu *et al.*, 2005), as

expected, rapidly in the core and somewhat later in the peri-infarct region, preceding the neuronal death (Kambe *et al.*, 2005). Furthermore, calpain inhibitors have been found in many studies to be neuroprotective against the ischemia (Kambe *et al.*, 2005), and in view of the long therapeutic window of up to six hours found in animal models (Markgraf *et al.*, 1998) they may have clinical potential.

Necroptosis is a form of programmed necrosis dependent on the serine–threonine kinase RIP1 and necrostatin-1 has been identified as a selective inhibitor of RIP1 which does not affect caspase activation (Degterev *et al.*, 2005; Degterev *et al.*, 2008). It has been shown that retinal and cerebral ischemia involves necroptosis (Degterev *et al.*, 2008; Rosenbaum *et al.*, 2010). Administration of necrostatin-1 afforded neuroprotection against MCAo in mice even when injected 6h after the insult, whereas zVAD-fmk was no longer efficient at this time point (Degterev *et al.*, 2008). In neonatal mice, post-treatment with necrostatin-1 also gave neuroprotection against HI (Northington *et al.*, 2011). These results showed that necroptosis is an important delayed mechanism of ischemic brain damage and could be an important therapeutic target.

In excitotoxicity and cerebral ischemia pathological activation of Poly(ADP-Ribose) Polymerase (PARP) occurs and contributes to necrotic cell death mainly via cytosolic NAD+ depletion (Alano *et al.*, 2010; Moroni, 2008). PARP inhibition or deletion of PARP-1, which is the member of PARP family responsible for more than 90% of cellular PARP activity, is then strongly neuroprotective (Endres *et al.*, 1997; Kaundal *et al.*, 2006; Nakajima *et al.*, 2005) – especially in males (see section 5.2.). PARP appears to be involved in both passive and active varieties of necrosis. Its primary role is to detect single-strand DNA breaks and initiate repair, but its excessive activation is a powerful stimulus for cell death. Extreme PARP-activation can deplete a cell's nicotinamide adenine dinucleotide and consequently its ATP to such an extent that it causes necrosis of the standard, passive kind, while inhibiting energy-

dependent types of cell death such as apoptosis (Ha and Snyder, 1999; Kaundal *et al.*, 2006). But PARP can also induce caspase-independent active cell death, via the production of poly(ADP-ribose) polymer (PAR), which translocates from the nucleus to the cytosol where it interacts with the mitochondrial outer surface and stimulates AIF release (Fig. 7) (Wang *et al.*, 2011; Yu *et al.*, 2006b). Even though most AIF-induced cell death is apoptotic (section 2.2.2.) in situations of strong PARP activation it takes on a different morphology and recent papers argue that it is a distinct form of cell death, termed parthanatos (Wang *et al.*, 2009b), which is arguably a form of programmed necrosis (Galluzzi *et al.*, 2012). The difference between AIF-induced apoptosis and parthanatos appears to result from a binding of PAR to AIF (Wang *et al.*, 2011).

5. Parameters influencing the cell death mechanisms involved in cerebral hypoxic-ischemic damage

5.1. Brain maturity

The age at which hypoxia-ischemia is performed is another important parameter affecting the severity of the lesion (Towfighi *et al.*, 1997). The conclusions of studies on neuroprotection in adult models of cerebral ischemia cannot be transposed to the neonatal situation without further confirmation, because the different physiological and metabolic properties of immature brains lead to different molecular consequences. For example, the basal activity of caspase-3 is much higher in immature brains than in those of adults, making them more sensitive to the induction of apoptosis (Zhu *et al.*, 2005; Zhu *et al.*, 2003). Hence, unlike in adults, HI in neonatal rats activates apoptotic mechanisms within only a few hours (Nakajima *et al.*, 2000; Northington *et al.*, 2001a; Zhu *et al.*, 2005).

Another age-related difference is that the subunits of NMDA receptors change during development and they become more heterogeneous. Immature brains express mainly subunits NR1, NR2B and NR2D, but very few NR2A in comparison with adults (Gurd *et al.*,

2002; Johnston, 2005; Mishra *et al.*, 2001; Wenzel *et al.*, 1997). Thus the immature NMDA receptors have different properties from those in adults. Their permeability to Ca^{2+} is higher, they are more readily activated by glycine, and they are less sensitive to blockade with Mg^{2+}. These properties all make immature NMDA receptors more readily activatable, which plays a positive role in many processes during brain development (establishment of neural connections, neuronal migration and long term potentiation) but increases the vulnerability to hypoxia-ischemia. AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors likewise change during the course of development, in both their composition and in their localization (Simeone *et al.*, 2004). Expression of the GluR2 subunit, which confers impermeability to Ca^{2+}, increases with age in rodents. For this reason, immature brains express a higher proportion of calcium-permeable AMPA receptors, enhancing their sensitivity to excitotoxicity. This may help to explain the switch in vulnerability to excitotoxicity, during the first week of life, from white matter to gray matter (Talos *et al.*, 2006). Thus, in 2-day old rats, calcium-permeable AMPA receptors are mainly expressed in the white matter (on radial glia and oligodendrocyte precursors), which is particularly vulnerable. But at 7 days, the calcium-permeable receptors are mainly on cortical neurons, and at this age the gray matter is more vulnerable (Talos *et al.*, 2006). Another factor that exacerbates the vulnerability of immature brains is that GABA receptors, which are inhibitory in adults, are excitatory (depolarising) during the first two weeks of life in rodents (Ben-Ari *et al.*, 1997; Jensen, 2002). Immature brains are also particularly vulnerable to oxidative stress because their antioxidant defences are week, and they contain high concentrations of free iron and unsaturated fatty acids (Vannucci and Hagberg, 2004).

5.2. Gender differences

Both human and animal studies have revealed a sexual dimorphism in adult and neonatal cerebral ischemia (Alkayed *et al.*, 1998; Hurn *et al.*, 2005; Turtzo *et al.*, 2011; Turtzo and McCullough, 2010; Vagnerova *et al.*, 2008). The overall incidence rate of stroke is higher in men than in women. However stroke risk depends on the age group studied since in some periods of life (20-35, 44-55, >85 years) women are more likely to suffer cerebral ischemia (Turtzo and McCullough, 2010). Sex hormone exposure is an important factor and female rats are more resistance to MCAo than males or ovariectomized females of the same age (Alkayed *et al.*, 1998). However, hormonal factors are not the only cause of sex differences; strictly genetic parameters also play a role (Siegel *et al.*, 2010; Turtzo *et al.*, 2011; Turtzo and McCullough, 2010) and sexual dimorphism could be observed even in *in vitro* models of excitotoxicity (Du *et al.*, 2004; Fairbanks *et al.*, 2012; Li *et al.*, 2005; Sharma *et al.*, 2011). Most studies indicate that cell death in females occurs mainly via a caspase-dependent pathway whereas in males AIF translocation and PARP-1 activation are more important players (Lang and McCullough, 2008; Renolleau *et al.*, 2008; Zhu *et al.*, 2006). Only females presented a rapid release of cytochrome c with enhanced caspases activation and were protected by caspase inhibition following Q-VD-OPh administration, in both adult and neonatal rodent models of cerebral ischemia (Liu *et al.*, 2009; Liu *et al.*, 2011; Renolleau *et al.*, 2007). Deletion of the gene coding for PARP-1 reduced the lesion size in neonatal and adult males, but had no effect in neonatal females after HI (Hagberg *et al.*, 2004) and even exacerbated stroke damage in adult females (Liu *et al.*, 2011; McCullough *et al.*, 2005). Other studies of neuroprotection have revealed additional differences between males and females in neonatal (Bona *et al.*, 1998; Hill *et al.*, 2011; Igarashi *et al.*, 2001; Nijboer *et al.*, 2007; Wen *et al.*, 2006) or adult (Li and McCullough, 2009; Siegel *et al.*, 2011) models, underlining the

importance of gender for the design of neuroprotective agents against hypoxic-ischemic brain damage in adults and infants.

6. Interconnections between the different types of cell death

6.1. Interconnections between necrosis and apoptosis

Necrosis and apoptosis can share common initial events such as Fas activation (Kawahara *et al.*, 1998) or mitochondrial permeabilization (Baines, 2011). A cell's decision to die by apoptosis or necrosis can be dependent on different factors including its capacity for caspase activation (Hartmann *et al.*, 2001; Lemaire *et al.*, 1998; Prabhakaran *et al.*, 2004), production level of reactive oxygen species (Kalai *et al.*, 2002) and the degree of lysosomal membrane disruption (Brunk *et al.*, 1997), but the level of ATP seems to be a crucial determinant since the induction of apoptosis requires energy, whereas necrosis often occurs in states of energy/ATP depletion (Leist *et al.*, 1997).

The existence of common mediators for apoptosis and necrosis can produce intermediate forms of cell death especially in neurons. In the context of excitotoxicity and cerebral ischemia, it was proposed, on the basis of ultrastructural evidence, that there is a continuum between apoptosis and necrosis in the brains of both immature and adult rats (Martin *et al.*, 1998; Portera-Cailliau *et al.*, 1997). Similar conclusions were reached at about the same time using different techniques on cultured neurons (Cheung *et al.*, 1998). The continuum view conceives of the pure forms of apoptosis and necrosis as being at the two extremes of a spectrum of hybrid morphologies. This implies that the pathways of apoptosis and necrosis are not mutually exclusive, but can be activated together, although the extent to which this occurs depends on the type and maturity of the neurons.

Hybrid morphologies have been reported in animals of all ages including adults (Wei *et al.*, 2004), but occur more readily during development, and it is difficult to find classic

ultrastructural morphologies of pure neuronal apoptosis or necrosis in neonatal models such as the HI model of Rice and Vannucci (Rice *et al.*, 1981). Using this model, several studies have shown the predominance of hybrid morphologies in various brain regions in neonatal rats and mice (Nakajima *et al.*, 2000; Sheldon *et al.*, 2001). It has been proposed that the presence of hybrid morphologies may result from the incomplete execution of apoptosis in neurons that therefore make an incomplete transition to necrosis (Northington *et al.*, 2007).

Why hybrid forms occur in some situations but pure apoptotic or necrotic forms in others is only partly understood. The situation is complicated, because there is evidence in a variety of neuronal and non-neuronal cell types for both positive and negative cross-talk between the apoptotic and necrotic pathways. We here limit discussion to neurons, emphasizing apoptotic-necrotic cross-talk in cerebral ischemia and excitotoxicity. For reasons discussed above, we take caspase activation as emblematic of apoptosis and calpain activation as emblematic of necrosis, although this is a simplification. But there is cross-talk between caspase and calpain signaling, and the two groups of proteases share many of the same substrates (Schwab *et al.*, 2002; Wang, 2000).

Caspase activation leads to the cleavage of many different proteins, in addition to those involved in the pro-apoptotic pathways, and it has both pro- and anti-necrotic effects. In some cases the pro-necrotic effects predominate, and, in a model of focal cerebral ischemia-reperfusion, inhibition of caspase-3 was found to diminish the ischemia-induced activation of mu- and m-calpains, possibly due, at least partly, to a concurrent increase in the level of the calpain inhibitor calpastatin (Sun *et al.*, 2008b). The mechanism of this positive cross-talk is only partly understood, but one of the direct pro-necrotic effects of caspase action is that they cleave plasma membrane calcium pumps leading to calcium overload, which promotes necrosis (Schwab *et al.*, 2002). But caspases also have anti-necrotic effects, and one of these is due to the caspase-mediated degradation of AMPA receptor subunits (Glazner *et al.*,

2000). AMPA receptor activation tends to induce necrosis rather than apoptosis, so the elimination of AMPA receptors tends to reduce necrosis and promote pure apoptosis.

Conversely, the activation of calpains has pro- and anti-apoptotic effects. In focal cerebral ischemia-reperfusion, calpain inhibition was found to diminish the ischemia-induced activation of caspase-3 in the core (but not in the penumbra) (Sun *et al.*, 2008b). It would be beyond our present scope to discuss in detail the numerous effects of calpains on apoptotic pathways, but these are summarized by Sun et al. (2008) who mention that calpains can cleave caspases 3, 7, 8, 9 and 12, as well as several apoptosis regulatory proteins including apoptosis protease-activating factor-1, Bcl-xL, Bax, Bid and p53, with both positive and negative effects on the apoptotic cascade (Sun *et al.*, 2008b). In neonatal hypoxia-ischemia, the same damaged neurons could express both cleaved caspase-3 and calpain-dependent fodrin breakdown product, and the early activation of m-calpains appears to have contributed to the subsequent activation of caspase-3 in the second phase of the neuronal death (Blomgren *et al.*, 2001).

6.2. Interconnections between autophagy and apoptosis

Some cases of autophagy-mediated cell death are caspase-independent and clearly different from apoptosis, but a mixed form with both autophagic and apoptotic characteristics is sometimes found (Zakeri *et al.*, 1995). This is not surprising, because the autophagic and apoptotic pathways share common mediators and are interconnected (Eisenberg-Lerner *et al.*, 2009; Maiuri *et al.*, 2007).

Several molecules known to belong to the apoptotic pathway may be also involved in autophagy regulation. Bcl2 is both an anti-apoptotic protein and an inhibitor of autophagy. In fact, the autophagy protein Beclin1 was initially identified as a protein interacting with Bcl2 via its BH3 domain. In conditions of stress such as lack of nutrients, Beclin1 is detached from

Bcl2 permitting the induction of autophagy (Pattingre *et al.*, 2005). Thus, Bcl2 can inhibit the pro-autophagic effect of Beclin1 by sequestering it. This effect is not, however, reciprocal, because the overexpression of Beclin1 does not modify the anti-apoptotic effect of Bcl2 (Ciechomska *et al.*, 2009). The dissociation of Beclin1 from Bcl2 or $BclX_L$ has been shown to be regulated proapoptotically by the death-associated protein kinase (DAPK) (Zalckvar *et al.*, 2009b; Zalckvar *et al.*, 2009a). The proapoptotic BH3-only protein Bim can also be an autophagy inhibitor by its interaction with Beclin1 (Luo *et al.*, 2012) (Luo et al., 2012). In some cases caspases activation can lead to Beclin1 cleavage and inhibition of Beclin-dependent autophagy (Djavaheri-Mergny *et al.*, 2010; Luo and Rubinsztein, 2010). Like Bcl2 and $BclX_{L,}$ MCL-1 has recently been shown to be an important regulator of both apoptosis and autophagy in cell lines and post-mitotic neurons (Germain *et al.*, 2011). FLIP proteins are also not only anti-apoptotic but are also anti-autophagic because they inhibit the action of Atg3 and prevent the lipidation of LC3 in different cell lines (Lee *et al.*, 2009). The overexpression of PUMA or Bax, two members of the Bcl2 family, in different cell lines can contribute to the induction of autophagy and particularly of mitophagy which, in turn, is associated with induction of the apoptosis (Yee *et al.*, 2009). However, mitophagy of *dysfunctional* mitochondria can *reduce* apoptosis, presumably by preventing the release of cytochrome c and other proapoptotic factors (Kim *et al.*, 2007). Overexpression of a nuclear FoxO1 (Forkhead box protein O1) can initiate caspase-dependent apoptosis, whereas cytosolic FoxO1 induces an autophagy-mediated cell death independently of caspases (Zhao *et al.*, 2010).

Conversely some autophagic proteins have been shown to interact with the apoptotic process. Atg5 can also induce apoptosis independently of its action on autophagy (Luo and Rubinsztein, 2007). First, in HeLa cells treated with interferon-γ (IFNγ), Atg5 can interact with FADD (Fas-Associated Protein with Death Domain) and thus activate caspase-dependent

cell death (Pyo *et al.*, 2005). Second, in response to various apoptotic stimuli, Atg5 can be cleaved by calpains in different cell lines and one of its cleavage fragments is translocated to mitochondria where it interacts with Bcl-xL, thus contributing to the initiation of the mitochondrial pathway of apoptosis (Yousefi *et al.*, 2006). More recently it has been proposed that autophagosomal membrane could be a platform to recruit, via Atg5, an intracellular DISC which induces caspase-8 activation (Young *et al.*, 2012). Atg4D can also be pro-apoptotic independently of its autophagy function; an Atg4D fragment is recruited to mitochondria and can induce the apoptotic mitochondrial pathway (Betin and Lane, 2009). Overexpression of Atg1 in Drosophila leads to a form of cell death that is both caspase and autophagy dependent (Scott *et al.*, 2007). Recently it has been shown that Atg7 can control p53 functions in both the cell cycle and in cell death (Lee *et al.*, 2012).

Links between apoptosis and autophagy have also been found in neurons. Activation of p53 probably participates in excitotoxic neuronal death through both apoptotic and autophagic pathways (Dong *et al.*, 2012; Wang *et al.*, 2009a; Zhang *et al.*, 2009). In a study of photoreceptors (*in vivo* and in a photoreceptor cell line), oxidative stress was shown to induce autophagy and caspase-dependent apoptosis. The contribution of autophagy to the apoptosis was shown in the cell line by the fact that 3-MA treatment or RNA-silencing of Beclin1 and Atg5 diminished the caspase activation and the cell death. A caspase inhibitor (zVAD-fmk) also gave partial protection, but the combination of 3-MA and z-VAD-fmk gave no additional protection, suggesting that both were acting on the same cell death pathway. Taken together, these results indicated that the oxidative stress first induced autophagy, which in turn triggered the caspase-dependent apoptosis (Kunchithapautham and Rohrer, 2007).

Autophagy can also be involved in the induction of a caspase-independent neuronal apoptosis. Deprivation of serum and potassium triggers the death of cultured cerebellar

granule neurons, which shows autophagic features and enhanced intralysosomal expression of cathepsin L. Inhibition of cathepsin L is more strongly protective (40%) than caspase-3 inhibition (31%), but combination of the two inhibitors is still more protective (80%). Moreover, treatment with 3-MA is strongly protective (60%) (Kaasik *et al.*, 2005). This cell death is thus mainly due to the activation of autophagy, and partly independent of caspase-3, (the effect of inhibiting autophagy on caspase activation was not studied). Nerve growth factor deprivation induces the apoptosis of sympathic neurons in culture (caspase activation, cytochrome c release and DNA fragmentation), but also induces autophagy. 3-MA prevents the appearance of apoptotic features and protects against the neuronal death (Xue *et al.*, 1999). However caspase inhibition (with boc-Asp-fmk) prevents neither the neuronal death nor the induction of autophagy. .

In primary cortical neurons treated with the apoptotic stimulus staurosporine, enhanced autophagy contributes to caspase-dependent and independent apoptosis since autophagy inhibition (3-MA, Atg5 and Atg7 downregulation) is strongly neuroprotective and decreases both caspase-3 activation and AIF nuclear translocation (Grishchuk *et al.*, 2011). Moreover, a combination of 3-MA and the pan-caspase inhibitor Q-VD-OPh is more protective than each inhibitor alone. Similar results are obtained with two other apoptotic stimuli, MK801 and etoposide.

In excitotoxic conditions, dying neurons often present both autophagic and apoptotic features (Adhami *et al.*, 2006; Ginet *et al.*, 2009; Koike *et al.*, 2008; Piras *et al.*, 2011; Rami *et al.*, 2008). The fact that inhibition of autophagy with 3-MA (Choi *et al.*, 2012; Luo *et al.*, 2011; Piras *et al.*, 2011; Puyal *et al.*, 2009; Wang *et al.*, 2008) or by knockout of atg7 (Koike *et al.*, 2008) reduces apoptotic markers (caspase-3 and -9 activation or AIF translocation), but that caspase inhibition fails to affect autophagy (Puyal *et al.*, 2009)(Puyal et al., 2009), suggests a role of autophagy in mediating apoptosis (Puyal and Clarke, 2009). In cerebral

ischemia, increased autophagy and apoptosis are detected in the same region (penumbra), suggesting that these two modes of neuronal death interact (Puyal *et al.*, 2009; Rami *et al.*, 2008). In neonatal HI likewise, the induction of autophagy seems to be an important mechanism in neuronal death, but its role may depend on the brain region involved (Ginet *et al.*, 2009). In the cortex, the classic morphologies of pure autophagic (type II) cell death or of pure apoptosis (type I) were never observed; the morphologies were always a mixture of the two suggesting that enhanced autophagy coexists with apoptosis and may trigger it. This is supported by results in neonatal focal cerebral ischemia where inhibition of autophagy with 3-MA reduced apoptotic features (Puyal *et al.*, 2009). But, in the hippocampus, cell death morphology tended to be purely apoptotic or purely autophagic (Ginet *et al.*, 2009). In CA3 the morphology of dying neurons was strongly autophagic and there was no activation of caspase 3 (Fig. 3C), whereas in CA1 cell death occurred more rapidly, had an apoptotic morphology, and was accompanied by caspase 3 activation.

Some studies have shown that lysosomal enzymes may be involved in excitotoxic neuronal death. Cathepsin B expression and activity increased following striatal injection of kainate (Wang *et al.*, 2006), traumatic brain injury (Luo *et al.*, 2010) or cerebral ischemia (Ginet *et al.*, 2009; Nitatori *et al.*, 1995; Puyal *et al.*, 2009; Seyfried *et al.*, 1997). Inhibition of this hydrolytic enzyme is neuroprotective and decreases apoptotic features (Luo *et al.*, 2010; Seyfried *et al.*, 1997; Wang *et al.*, 2006; Wen *et al.*, 2008). The death-mediating role of the cathepsin B activity is not understood, but one possibility is that it might be released from its normal lysosomal location into the cytosol, and kill by activating apoptosis through the cleavage of important protective proteins as Bid, Bcl-2, Bcl-xL or Mcl-1 (Repnik *et al.*, 2012). This would imply a destabilization of lysosomal membranes, but whether this occurs in excitotoxicity is unclear. Some studies did indeed suggest a release of lysosomal enzymes during cerebral ischemia (Hill *et al.*, 1997; Kilinc *et al.*, 2010; Yamashima *et al.*, 2003), but

others showed an increase in lysosomal activity (acid phosphatase, β-hexosaminidase) in apparently intact lysosomes or autolysosomes in hippocampal organotypic cultures exposed to NMDA (Borsello et al., 2003) and in models of cerebral ischemia (Puyal et al., 2009; Ginet et al., 2009).

6.3. Interconnections between autophagy and necrosis

Autophagy can also promote or inhibit necrosis, depending on the situation (Shen and Codogno, 2012). Autophagy promotes necrosis in MEF cells incapable of apoptosis (*bax*$^{-/-}$ */bak*$^{-/-}$ or overexpressing Bcl2) that are subjected to an ER stress for 2 or more days (Ullman *et al.*, 2008). Autophagy likewise plays a pro-necrotic role in a model of neuronal death in the nematode *C. elegans*, where excessive autophagosome formation is induced early in the cell death process, and genetic or pharmacological inhibition of autophagy inhibits the development of necrosis (Samara *et al.*, 2008). On the other hand, TNFα-induced autophagy in L929 cells appears to act as a *negative* feedback to necroptosis (Ye *et al.*, 2011), and necrotic cell death during starvation-induced *Dictyostelium discoideum* development is enhanced when autophagy *is inhibited* by knockdown of atg1 (Luciani *et al.*, 2009).

The relationship between autophagy and necrosis in cerebral ischemia has scarcely been studied, but 3-MA treatment was shown to protect CA1 neurons against necrotic death in a severe model of adult global cerebral ischemia (Wang et al., 2011).

7. Development of neuroprotective strategies in the light of the multiple cell death mechanisms

Despite the increasing knowledge on neuronal death and the important effort in research to identify neuroprotective agents, none have so far been translated to patients suffering from cerebral ischemia. The only pharmacological molecules approved in clinical

conditions are tissue-plasminogen activators (t-PA) to restore perfusion to adult ischemic brain. However t-PA has to be administrated within about 3h of stroke onset because of increased risk of intracranial hemorrhage and neurotoxic effects following delayed or prolonged use. For this reason very few patients (1-2%) can benefit from this treatment (Liberatore *et al.*, 2003; Lopez-Atalaya *et al.*, 2008; Stankowski and Gupta, 2011). For neonatal hypoxia-ischemia the only approved treatment is moderate hypothermia (Azzopardi *et al.*, 2009; Gluckman *et al.*, 2005; Shankaran *et al.*, 2005; Shankaran, 2009). The development of a clinically safe and effective neuroprotectant is thus still an unmet goal for both adults and newborns. Among the difficulties in clinical trials, the heterogeneity of the ischemic insults is one of the most important. To maximize the chances of confirming a drug's neuroprotective effect in humans, there is a need to design better-defined patient groups with more restricted conditions (Fisher, 2011; Gladstone *et al.*, 2002; Tymianski, 2010).

Even if animal (mainly rodent) models of stroke and neonatal HI are imperfect imitations of human pathologies, experimental research on excitotoxic neuronal death has clearly demonstrated the complexity of the cell death pathways. The fact that multiple interacting cell death mechanisms are activated in neurons exposed to excitotoxic/ischemic stress has important implications for the design of neuroprotective therapy. Indeed, the failure of attempts so far to develop neuroprotective pharmacotherapy for ischemic stroke (Stankowski and Gupta, 2011; Yuan, 2009) and neonatal cerebral asphyxia (van Bel and Groenendaal, 2008) is probably due in part to the fact that inhibition of a particular death mechanism is ineffective when alternative ones can kill the cell. The optimal way to solve this problem is unknown, but the multiplicity of the pathways is illustrated in Fig. 7, which is highly simplified. An early approach was to inhibit the excitotoxic pathway at its origin, using NMDA receptor antagonists to block the massive calcium entry through these receptors. Intervening

at this early level has the advantage of inhibiting virtually all the death-mediating pathways, but unfortunately affects also those necessary for normal neuronal function and survival, generating many adverse side effects and necessitating the use of doses below the optimal ones for protection (Dingledine *et al.*, 1999; Haberny *et al.*, 2002; Hardingham and Bading, 2003; Ikonomidou *et al.*, 1999). Another problem is that the protective time window for NMDA receptor antagonists is only 1-2 h (Gladstone *et al.*, 2002). Probably for these reasons, all clinical trials so far of NMDA receptor antagonists have failed (Ikonomidou and Turski, 2002). Alternative strategies have been developed to prevent complete inhibition of NMDA receptors and target downstream effectors. For example, promising results were obtained in preclinical studies with a peptide (NA-1 or Tat-NR2B9c) interrupting intracellular molecular pathway triggered by NMDA receptors activation in excitotoxic conditions by preventing interactions between postsynaptic density 95 (PSD95) and the NR2B subunits of the NMDA receptor (Aarts *et al.*, 2002; Cook *et al.*, 2012; Sun *et al.*, 2008a; Tymianski, 2010).

In the light of the multiple cell death mechanisms it may not be possible for a single neuroprotectant to have a long term protective effect. An alternative approach might be to intervene much further downstream, providing a cocktail of inhibitors against the separate death effector mechanisms (Culmsee *et al.*, 2004; Fagan *et al.*, 1999; Rogalewski *et al.*, 2006). The combination of two different agents might allow the doses of the individual drugs to be diminished, leading to reduced side effects (Park *et al.*, 2007; Wang *et al.*, 2012). Furthermore, there is evidence that early administration of one of the two drugs may delay deleterious events of the second one and extend the latter's therapeutic time window (Kim *et al.*, 2010; Ma *et al.*, 2001) such as in thrombolytic strategies (Zhang *et al.*, 2010; Zhang *et al.*, 2008). An application of such a window-extending strategy has been reported for inhibition of acid-sensing ion channel (ASIC) 1a, which contributes to neuronal calcium influx following cerebral ischemia and is involved in acidosis (Mari *et al.*, 2010b). Acidosis occurs early in

cerebral ischemia and gates NMDA receptors. Psalmotoxin (PcTX), a specific ASIC1a inhibitor that is neuroprotective in murine models with a large time window, has been shown to extend the therapeutic time window of NMDA receptor inhibition with an additive effect (Pignataro *et al.*, 2007; Xiong *et al.*, 2004). Combination of pharmacological treatment with hypothermia, should also be considered (Cilio and Ferriero, 2010; Froehler and Ovbiagele, 2010; Tang *et al.*, 2009). Another therapeutic target that needs to be considered is the epigenetic modifications such as methylation of DNA or histones that occur following cerebral ischemia. Recent studies have shown that preventing these can be neuroprotective against stroke (Hwang *et al.*, 2013; Noh *et al.*, 2012).

Such “polytherapy” could therefore allow, at least in theory, a longer therapeutic window. However intervening too late in the cell death cascades of events induced by a hypoxic/ischemic episode, at a downstream level where cellular damage has already begun, may be insufficient to prevent cell death. It may therefore be necessary to intervene at an intermediate level, downstream of calcium entry but upstream of particular death effector mechanisms. Before an optimal strategy can be devised, more research will be needed on the complex network of cascades linking excitotoxicity to apoptosis, autophagy and necrosis to identify the point of no return in the molecular cascades leading to neuronal death. However while anti-apoptotic agents such as caspase inhibitors are available for blocking most apoptosis, the specific blockade of autophagy requires RNA interference technology that would be difficult to use clinically; and, while necrosis can sometimes be prevented using calpain inhibition, this appears not to be a specific inhibitor of necrosis, but acts further upstream affecting various pathways. Recently it has been shown that the combination of necrostatin-1 (anti-necroptosis) (see above) with the peptide humanin (anti-apoptotic properties) (Hashimoto *et al.*, 2001; Xu *et al.*, 2006; Zapala *et al.*, 2010) synergistically reduced lesion size and improved neurological outcomes in mice when administrated 4h after

MCAO (Xu *et al.*, 2010). In general, preclinical studies of such combination treatments raise hope that clinically useful neuroprotection against ischemic brain damage may soon be achieved.

8. Conclusions

There are at least three main types of cell death, all of which are triggered by active signaling and involve multiple signaling pathways. All the main types of cell death occur in neurons exposed to ischemia or to hypoxia-ischemia, and the multiple pathways interact. In particular situations a single pathway may predominate, in which case inhibition of a single group of enzymes, such as caspases, may give neuroprotection. But, in most situations, multiple pathways are involved and the development of clinical neuroprotection strategies will require an understanding of all the pathways and of their interactions. The discovery of a successful therapy, using a single agent or a cocktail to interfere with the diverse cell death pathways remains a challenge for researchers and clinicians.

Acknowledgements: Our research and the writing of this article were supported by grants from the Swiss National Science Foundation (310030-130769) and from the Faculty of Biology and Medicine of the University of Lausanne.

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Figure legends

Fig. 1. Schematic description of the three main morphological types of cell death. (A) Healthy cell. (B) Type 1-Apoptosis. Nucleus: shrinkage, chromatin condensation, pyknosis, fragmentation. Plasma membrane: convolution, budding, formation of apoptotic bodies. Cytoplasm: shrinkage, organelles appear almost normal, but loss of ribosomes from the RER and polysomes. (C) Type 2-Autophagic cell death. Nucleus: sometimes shrinkage and moderate pyknosis. Plasma membrane: often intense endocytosis. Cytoplasm: numerous autophagosomes and autolysosomes, Golgi often enlarged. (D) Type 3– Necrosis. Nucleus: little change, but swelling. Plasma membrane: swelling and rounding up of cell, sometimes with rupture of plasma membrane. Cytoplasm: dilation of organelles, vacuolization. ER: endoplasmic reticulum. (Other type-3 subtypes exist). Inspired by Clarke (1990) and Clarke et al. (2008).

Fig. 2. Simplified scheme of the intrinsic and extrinsic molecular pathways of apoptosis.
The intrinsic (or mitochondrial) pathway is triggered by loss of integrity of the mitochondria and the release of proapoptotic molecules. The extrinsic pathway, also called the death-receptor pathway, is activated by extracellular signals that involve the binding of extracellular proteins to “death receptors” on the cell membrane. Both pathways lead to caspase activation, but caspase-independent apoptosis can be triggered by the release of AIF and EndoG. AIF: apoptosis inducing factor; Apaf-1: adapter protein apoptotic protease-activating factor-1; Bax: Bcl-2–associated X protein; Bad: Bcl-2-associated death promoter; Bcl-2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma-extra large; tBid: truncated BH3 interacting-domain death agonist; Bim: Bcl-2-interacting mediator of cell death; CAD: caspase-activated deoxyribonuclease; EndoG: endonuclease G; ER: endoplasmic reticulum; FADD: Fas-associated death domain; FasL: Fas ligand; IAP: Inhibitor of Apoptosis Protein; IMM: inner

mitochondrial membrane; OMM: outer mitochondrial membrane; mPTP: mitochondrial permeability transition pore; Omi/HtrA2: High temperature requirement protein A2; PUMA: p53 upregulated modulator of apoptosis; Smac/Diablo: second mitochondria-derived activator of caspases/direct IAP binding protein with low pI; TNF: Tumor Necrosis Factor; TRAIL: TNF-related apoptosis-inducing ligand.

Fig. 3. Examples from our own research of the different morphological types of neuronal death after neonatal cerebral hypoxia-ischemia. (A) Healthy neuron in cortex of P12 rat. (B) Dying cortical neuron displaying apoptotic-like features after hypoxia-ischemia in P7 rat. Nuclear chromatin is clumped; cytoplasm is shrunken but mitochondria (m) appear almost normal. (C) Dying neuron with strong autophagic features after neonatal cerebral hypoxia-ischemia at P7 (CA3 hippocampus). Nuclear surface is convoluted. Cytoplasm contains numerous autophagosomes and autolysosomes (see high magnification panel, top right) as well as empty vacuoles. (D) Dying neuron with necrotic features observed in the center of the cortical lesion after focal ischemia at P12. Contents of nucleus and cytoplasm are largely destroyed. Remaining organelles are dilated including mitochondria (m) and endoplasmic reticulum (ER). Plasma membrane appears ruptured (arrows). N: nucleus; Bars = 2µm.

Fig. 4. Different forms of autophagy. Autophagy is an important physiological process involving intracellular degradation by lysosomes, acting in parallel with the proteasome system. The three different forms of autophagy differ in the mode of delivery of material to the lysosome. Microautophagy involves direct delivery. Chaperone-mediated autophagy is involved in the degradation of specific cytosolic proteins (with a KFERQ sequence) that are recognized by chaperone/co-chaperone complexes that then transport these proteins to the lysosome by interacting with LAMP2A. Macroautophagy consists in the sequestering of long-

lived proteins and nonfunctional or damaged organelles to an intermediate compartment, the autophagosome, which will fuse with the lysosome to form an autolysosome in which the lysosomal hydrolases degrade the autophagosome content. LAMP: lysosomal associated membrane protein.

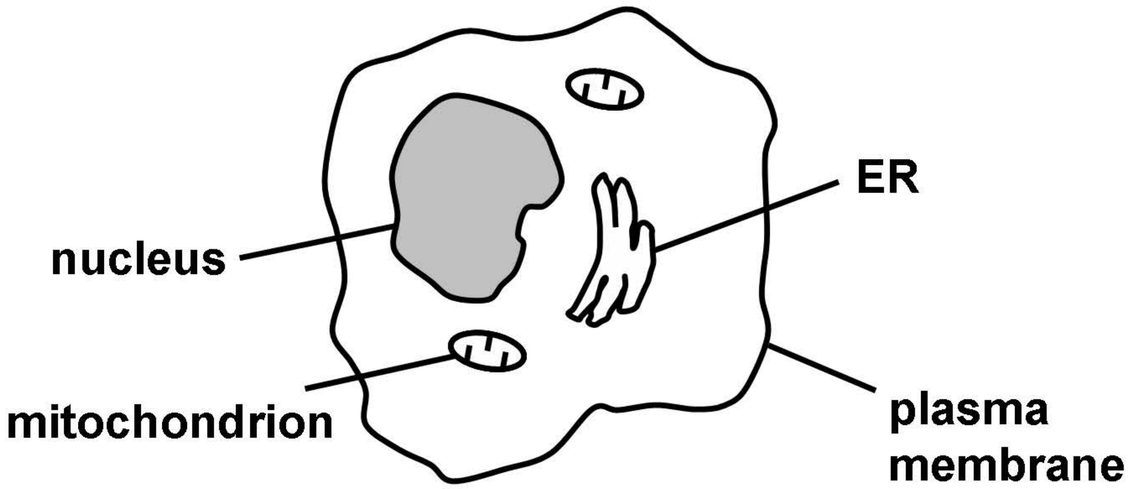
Fig. 5. The molecular pathways of macroautophagy. Macroautophagy is a complex process driven by numerous protein-protein interactions between autophagy-related gene (Atg) proteins. Autophagosome biogenesis (green arrows) includes the nucleation of the pre-autophagosome, its elongation, incurvation and closure. Source of membrane for autophagosome formation include a subdomain of ER called the omegasome, the Golgi apparatus, the outer membrane of mitochondria and the plasma membrane. Autophagosome maturation (blue arrows) involves fusion with a lysosome permitting the digestion of its content by lysosomal hydrolases at an acidic pH. The ULK1/2 complex acts in the initiation step. Beclin1 takes part in different complexes involved in autophagosome biogenesis: Beclin1/PI3K-III/Atg14L, Beclin1/PI3K-III/UVRAG. Bif-1 contributes to curvature of the membrane. LC3-II, the form of LC3 that is conjugated to a phosphatidylethanolamine (PE), plays an important role in autophagosome formation (elongation, curvature and end-to-end fusion). The Atg12-Atg5-Atg16L system and mAtg9 are essential for the elongation process. Atg7/Atg10 and Atg3/Atg4/Atg7 are enzymes implicated in conjugation of the Atg12-Atg5 and LC3 systems respectively. Proteins required for endocytosis such as Rab7 or ESCRT intervene also in the maturation of autophagosomes. Rubicon can bind to the Beclin1/PI3K-III/UVRAG complex and inhibits the role of this complex in autophagosome maturation. Bif-1: Bax-interacting factor-1 or endophilin B1; ESCRT: endosomal sorting complex required for transport; LAMP: lysosomal associated membrane protein; LC3: microtubule-associated protein 1 light chain 3; Rubicon: RUN domain as Beclin 1-interacting and cysteine-rich

containing; ULK1/2: UNC-51-like kinase 1/2; UVRAG: UV irradiation resistance-associated gene.

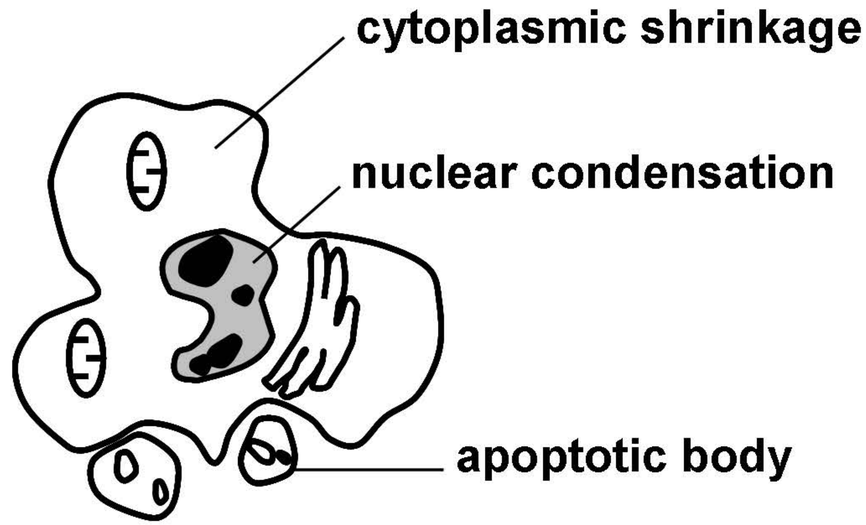
Fig. 6. The different involvements of autophagy in autophagy-associated cell death. Logically there are four possibilities. (A) *Autophagic cell death* can be defined as cell death mediated by autophagy without any sign of apoptosis or necrosis. Autophagy inhibition prevents or at least delays the cell death. (B) *Autophagy-mediated cell death* can be defined as apoptosis or necrosis mediated by autophagy. Cell death is prevented by inhibition of autophagy or of apoptosis/necrosis. (C) Autophagy may be epiphenomenal to the cell death, in which case inhibition of autophagy will not affect it. (D) Autophagy may play an anti-death role, in which case, inhibition of autophagy increases the cell death.

Fig. 7. The main interconnections involved in the mediation of excitotoxicity-induced neuronal death, leading to the different cell death phenotypes. The arrows indicate pathways that may be direct or indirect. The short downward arrows under “ATP depletion” indicate that mild depletion promotes mitochondrial permeabilization whereas strong depletion promotes necrotic cell death. TRPC: transient receptor potential channel; PARP: Poly(ADP-Ribose) Polymerase; NMDAr: N-methyl-D-aspartate receptor; ASICs: acid-sensing ion channels.

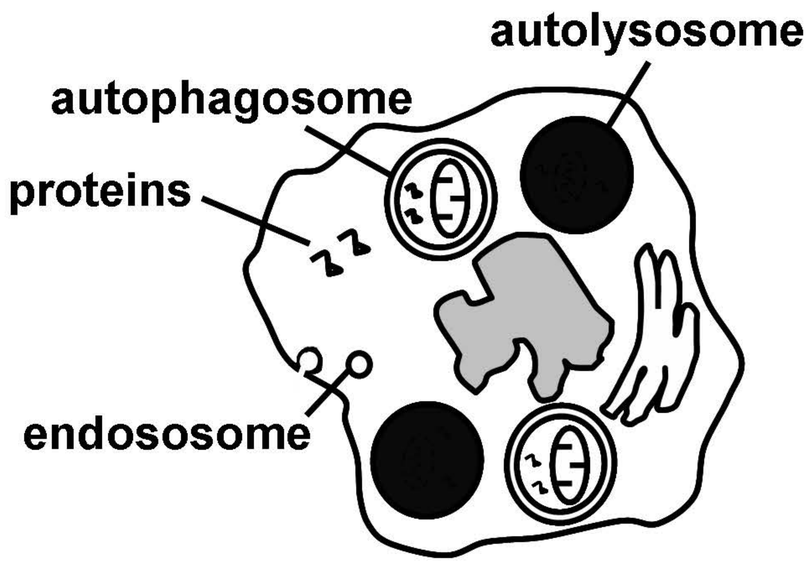
A. Healthy cell



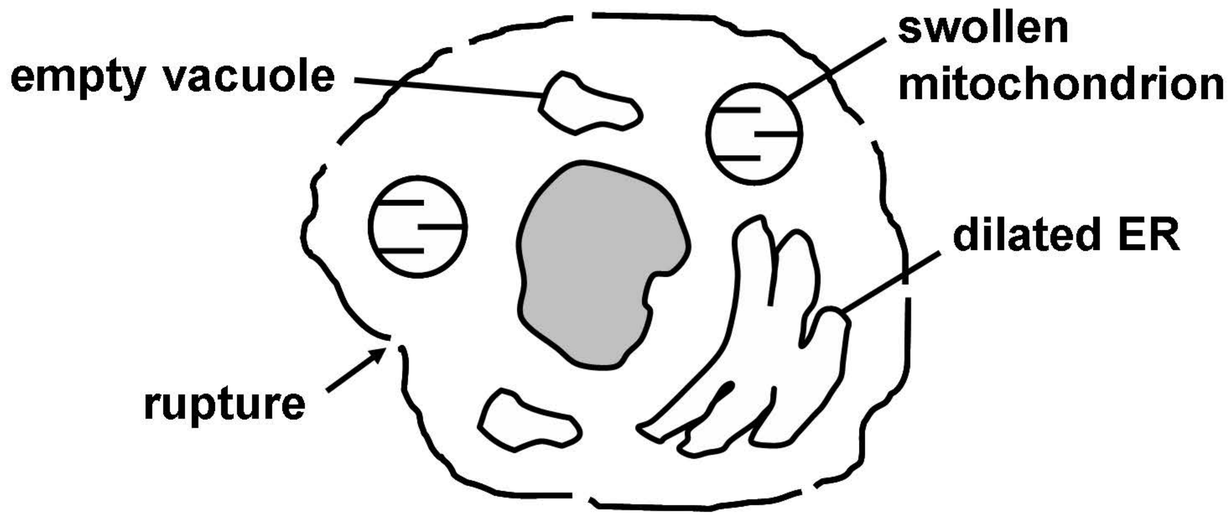
B. Type 1 – Apoptosis

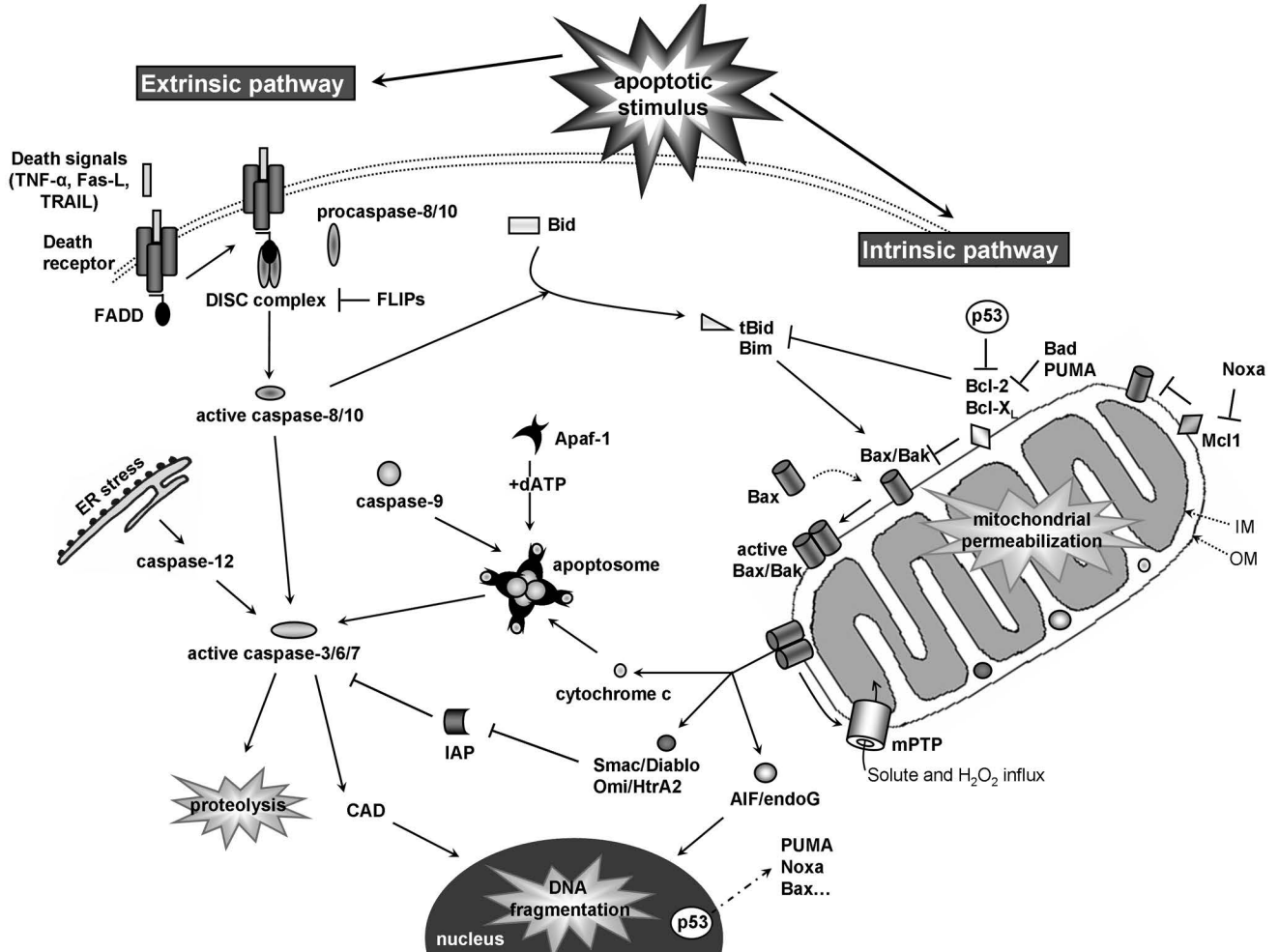


C. Type 2 – Autophagic cell death



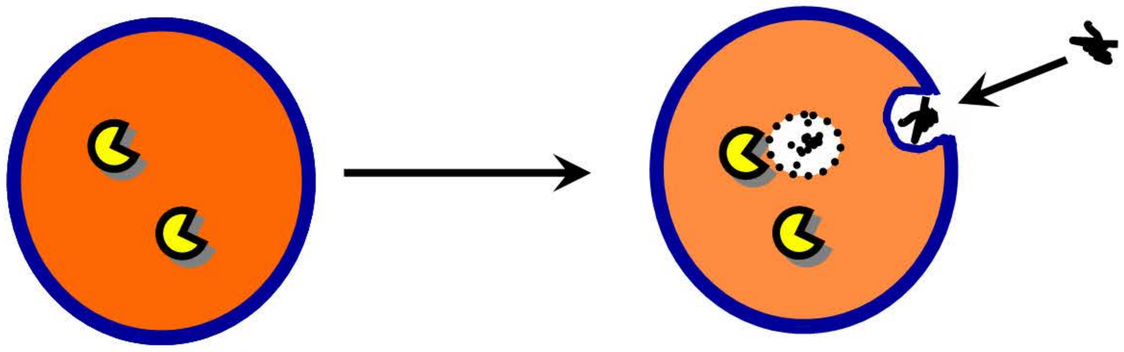
D. Type 3 – Necrosis




Extrinsic pathway
apoptotic stimulus
Intrinsic pathway
Death signals (TNF-α, Fas-L, TRAIL)
Death receptor
FADD
procaspase-8/10
DISC complex
FLIPs
Bid
tBid
Bim
p53
Bad
PUMA
Bcl-2
Bcl-X$_L$
Noxa
Mcl1
active caspase-8/10
Apaf-1
+dATP
caspase-9
Bax/Bak
Bax
active Bax/Bak
mitochondrial permeabilization
IM
OM
ER stress
caspase-12
apoptosome
active caspase-3/6/7
cytochrome c
IAP
Smac/Diablo Omi/HtrA2
AIF/endoG
mPTP
Solute and H_2O_2 influx
proteolysis
CAD
DNA fragmentation
nucleus
p53
PUMA
Noxa
Bax…

MICROAUTOPHAGY

Lysosome

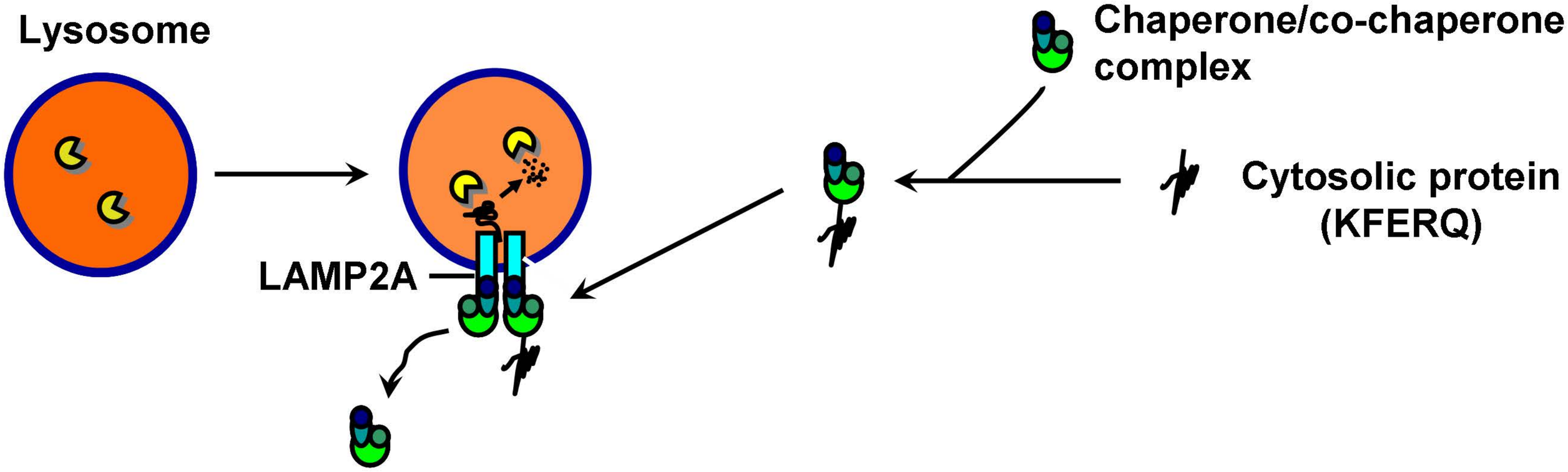


= Proteins

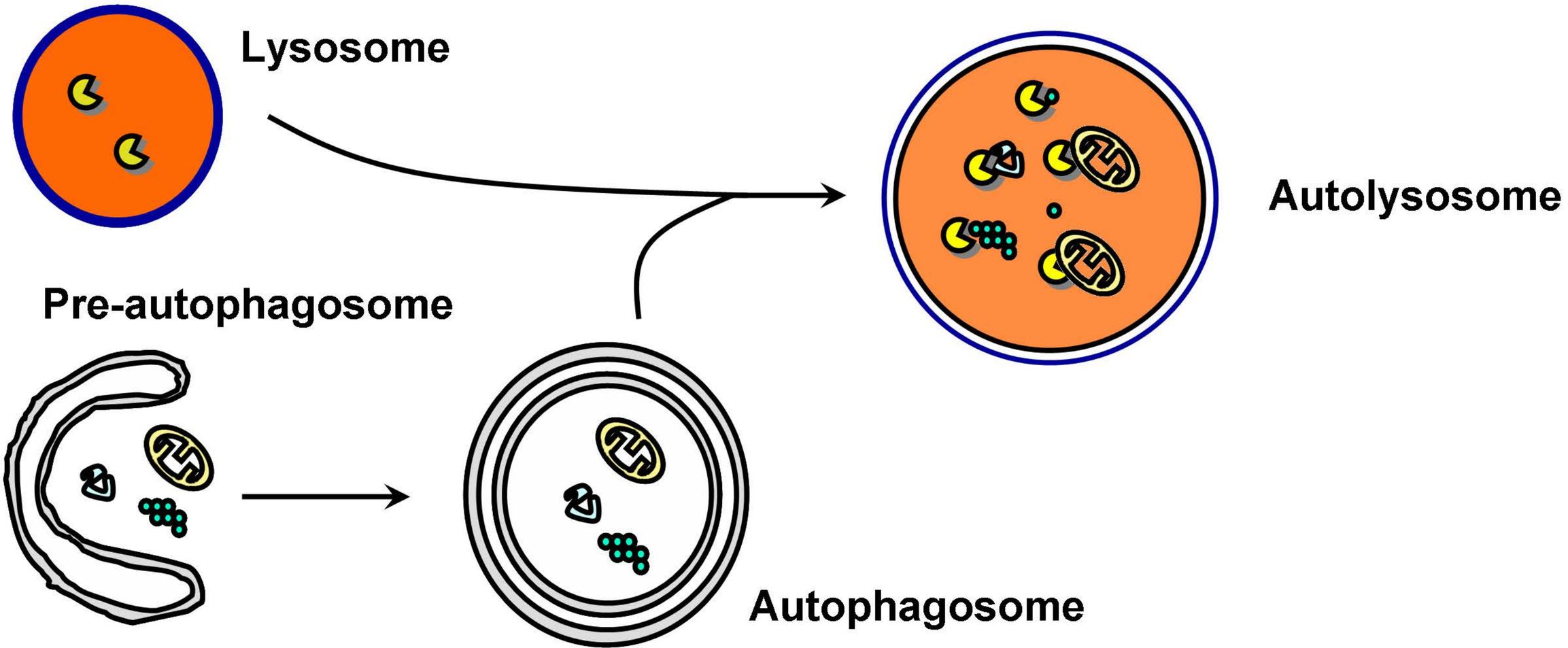
= Lysosomal hydrolases

= Organelles

CHAPERONE-MEDIATED AUTOPHAGY



MACROAUTOPHAGY



Endocytic vesicle

Recycling

Early endosome

Rab5

Rubicon

UVRAG complex

ESCRT

Late endosome or multivesicular body

Rab7

Plasma membrane

Trans-Golgi network

mAtg9

Bif-1

Lysosome

LAMP1/2

omegasome

?

nucleus

ER

ULK1/2 complex

Atg14L complex

Atg16L

1) Atg7
2) Atg10

Atg12

Atg12+Atg5

UVRAG complex

LC3-II

1) Atg4
2) Atg7
3) Atg3

LC3-I

Pre-autophagosome

mAtg9

Atg4

Autophagosome

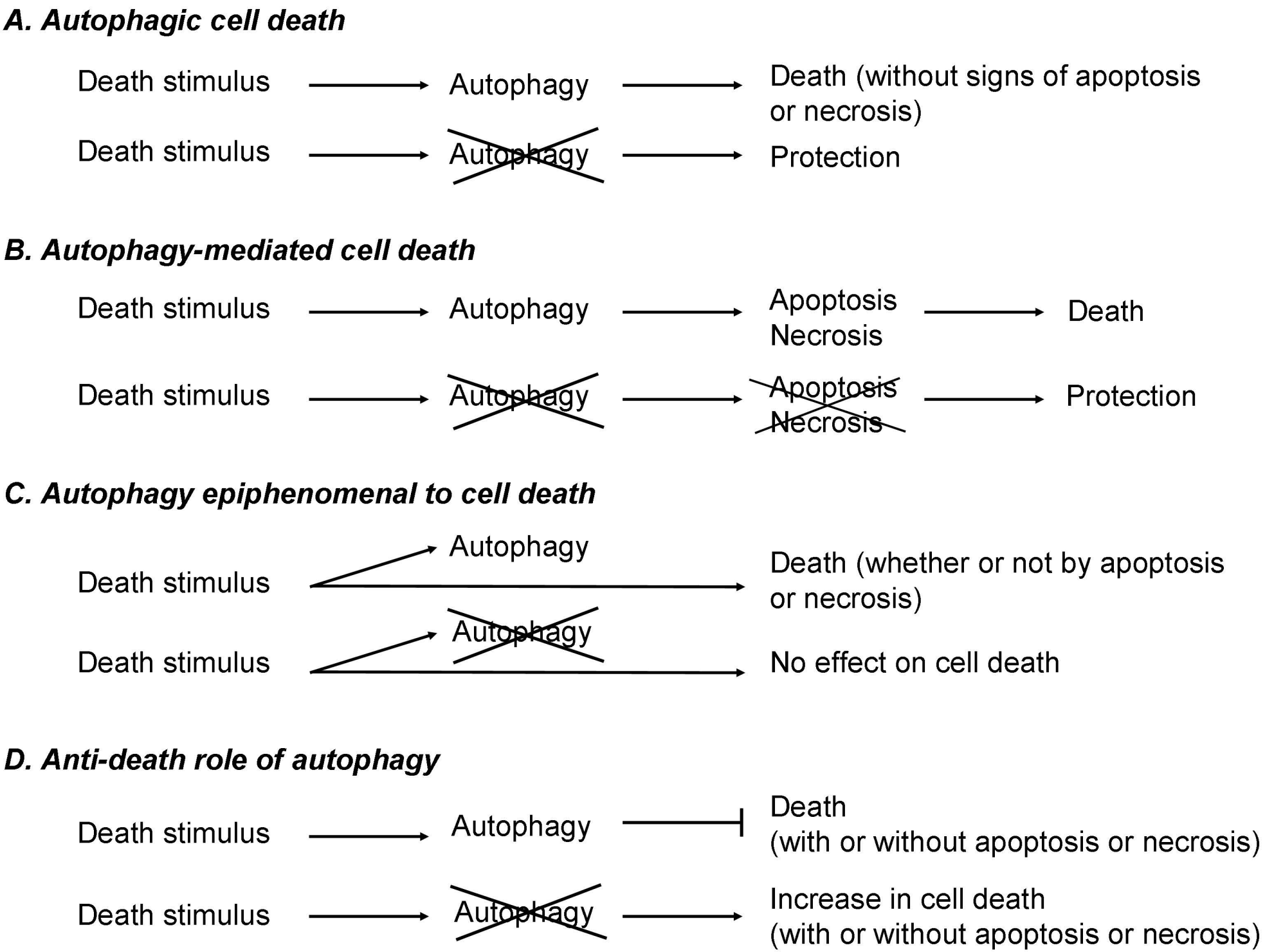
Amphisome

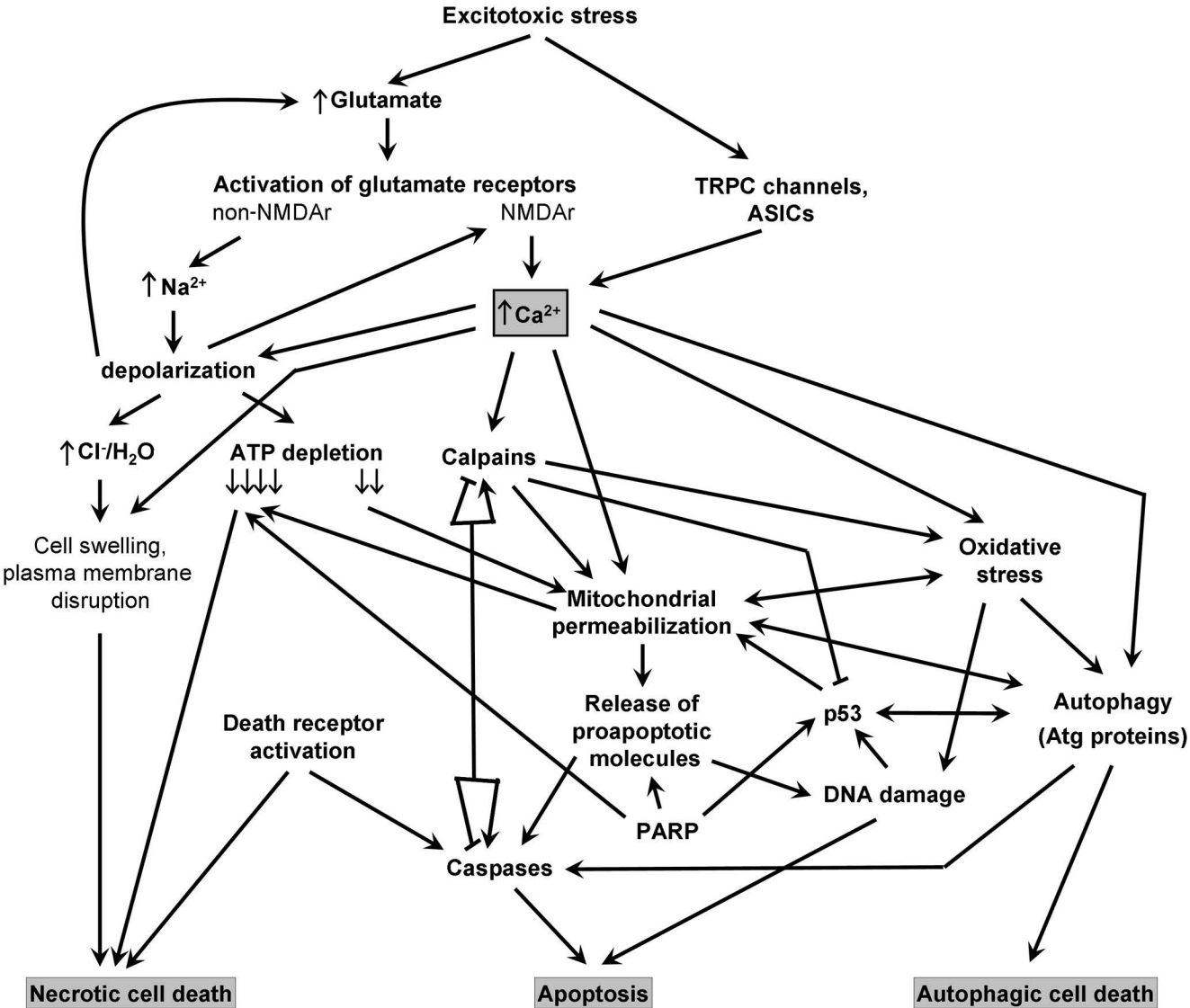
Autolysosome

Mitochondrion

Lysosome

= Atg12
= Beclin1
= Atg14L
= p150-PI3KIII
= LAMP1/2
= Acidic pH
= Atg5
= LC3-I
= UVRAG
= Atg14L complex
= Hydrolases
= Atg16L
= PE
= Rubicon
= UVRAG complex
Autophagosome biogenesis
= mAtg9
= LC3-II
= Bif1
= ULK1/2-mAtg13-FIP200
Autophagosome maturation


A. Autophagic cell death
Death stimulus
Autophagy
Death (without signs of apoptosis or necrosis)
Death stimulus
Autophagy
Protection
B. Autophagy-mediated cell death
Death stimulus
Autophagy
Apoptosis
Necrosis
Death
Death stimulus
Autophagy
Apoptosis
Necrosis
Protection
C. Autophagy epiphenomenal to cell death
Death stimulus
Autophagy
Death (whether or not by apoptosis or necrosis)
Death stimulus
Autophagy
No effect on cell death
D. Anti-death role of autophagy
Death stimulus
Autophagy
Death
(with or without apoptosis or necrosis)
Death stimulus
Autophagy
Increase in cell death
(with or without apoptosis or necrosis)


Excitotoxic stress
↑Glutamate
Activation of glutamate receptors
non-NMDAr
NMDAr
TRPC channels,
ASICs
↑Na2+
↑Ca2+
depolarization
↑Cl-/H2O
ATP depletion
Calpains
Cell swelling,
plasma membrane
disruption
Oxidative
stress
Mitochondrial
permeabilization
Release of
proapoptotic
molecules
p53
Autophagy
(Atg proteins)
Death receptor
activation
DNA damage
PARP
Caspases
Necrotic cell death
Apoptosis
Autophagic cell death