Pathological apoptosis in the developing brain

Klas Blomgren · Marcel Leist · Laurent Groc

Abstract More than half of the initially-formed neurons are deleted in certain brain regions during normal development. This process, whereby cells are discretely removed without interfering with the further development of remaining cells, is called programmed cell death (PCD). The term apoptosis is used to describe certain morphological manifestations of PCD. Many of the effectors of this developmental cell death program are highly expressed in the developing brain, making it more susceptible to accidental activation of the death machinery, e.g. following hypoxia-ischemia or irradiation. Recent evidence suggests, however, that activation and regulation of cell death mechanisms under pathological conditions do not exactly mirror physiological, developmentally regulated PCD. It may be argued that the conditions after e.g. ischemia are not even compatible with the execution of PCD as we know it. Under pathological conditions cells are exposed to various stressors, including energy failure, oxidative stress and unbalanced ion fluxes. This results in parallel triggering and potential overshooting of several different cell death pathways, which then interact with one another and result in complex patterns of biochemical manifestations and cellular morphological features. These types of cell death are here called "pathological apoptosis," where classical hallmarks of PCD, like pyknosis, nuclear condensation and caspase-3 activation, are combined with non-PCD features of cell death. Here we review our current knowledge of the mechanisms involved, with special focus on the potential for therapeutic intervention tailored to the needs of the developing brain.

Keywords Brain development · Hypoxia-ischemia · Irradiation

K. Blomgren (✉)
Center for Brain Repair and Rehabilitation, Institute of Neuroscience and Physiology, Göteborg University,
SE 405 30 Göteborg, Sweden
e-mail: klas.blomgren@neuro.gu.se

M. Leist
University of Konstanz,
Box M657, D-78457 Konstanz, Germany
e-mail: marcel.leist@uni-konstanz.de

L. Groc
CNRS-UMR 5091, Physiologie Cellulaire de la Synapse,
Université Bordeaux 2,
146 rue Léo Saignat,
33077 Bordeaux Cédex, France
e-mail: laurent.groc@u-bordeaux2.fr

Abbreviations

AIF	apoptosis-inducing factor
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APAF-1	apoptosis protease-activating factor-1
BDNF	brain-derived neurotrophic factor
CREB	cAMP-response element-binding protein
DG	dentate gyrus
EE	early elimination
ERK	extracellular signal-regulated protein kinase
HI	hypoxia-ischemia
IL	interleukin
JNK	jun N-terminal kinase
MAP kinase	mitogen-associated protein kinase
mtPT	mitochondrial permeability transition
NOS	nitrogen oxide synthase
NGF	nerve growth factor
NMDAR	N-methyl-D-aspartate receptor
P7	postnatal day 7
PCD	programmed cell death
ROS	reactive oxygen species

SGZ	subgranular zone (of the dentate gyrus)
SOD2	superoxide dismutase 2
SVZ	subventricular zone (of the lateral ventricular wall)
TRX 2	thioredoxin 2
XIAP	X-linked inhibitor of apoptosis protein

Physiological cell death of developing neurons

Strictly controlled cell death is an essential feature of normal development and homeostasis in multi-cellular organisms, in order to maintain the normal function of different organs and tissues, including the brain. A plethora of neurons is produced in the developing brain, many of which will be removed during embryonal and postnatal refinemen of the central nervous system. This process of physiological cell death, whereby cells are discretely removed without interfering with the further development of remaining cells, is called programmed cell death (PCD). The term apoptosis is used to describe certain morphological manifestations of PCD [1]. The masterful work of A. Glücksmann has provided one of the best descriptions of physiological cell death during development regarding morphogenesis, phylogenesis, and histiogenesis [2]. In the nervous system, Hamburger and Levi-Montalcini described more specificall the developmental cell death of motoneurons and its regulation by factors located within the innervating region [3]. This pioneering work laid the ground for the concept that neuron precursors are produced in excess and later selected for survival or cell death depending on extracellular ("the neurotrophic hypothesis") [4] and intracellular processes [5]. In some brain regions more than half of the initially-formed neurons are lost during normal development but the extent and characteristics of PCD of developing neurons varies greatly at different stages of development and among different neuronal populations [6–8]. In many different species, two waves of neuronal cell death have been described during development [9]. The firs wave consists of a large number of dividing neurons being eliminated during the peak of neurogenesis at mid-embryogenesis, here called early elimination (EE). The second wave consists of differentiated neurons dying while migrating toward their target locations or while connecting to target cells during the early postnatal period. The latter process is often referred as classical PCD [10]. These two waves likely serve two different purposes: to regulate the neuronal precursor pool size and to ensure the proper wiring of developing neuronal networks, respectively. In this firs section of the review we intend to schematically describe the main pathways leading to physiological cell death, from the extracellular effectors to the intracellulars actors. Recent finding that shed new light on the PCD process are particularly emphasized and obvious differences between the developmental physiological PCD and pathological cell death are pointed out.

Cell death during development: extracellular effectors

From the establishment of the neurotrophic hypothesis, several key additional extracellular effectors have been shown to be instrumental in developmental PCD. Among these are neurotransmission in general, including the activation of N-methyl-D-aspartate receptors (NMDAR), extracellular nucleotides, the glial sheet development, the guidance clues and the adhesive molecules (Fig. 1). It is then obvious that developmental PCD is a highly regulated process that depends on the input/output neuronal activity, controlled both by distant target cells and by local intercellular interactions (e.g. synapse formation).

Positive regulators—death by neglect

As described above, two waves of neuronal cell death have been described during development, EE and PCD. Schematically, it is proposed that both processes result in part from competition between neurons for a limited supply of neurotrophic factors, and PCD is considered to be the default pathway. Among the neurotrophic factors, the nerve growth factor (NGF) family (e.g. NGF itself, brain derived neurotrophic factor (BDNF) and neurotrophin 3), the glial cell-derived neurotrophic factor (GDNF), and the neurotrophic cytokines (e.g. ciliary neurotrophic factor (CNTF) and interleukin-6) have attracted the most attention [11, 12]. Although both EE and PCD are modulated by neurotrophic factors, the source of the factors involved seem to differ significantl , such that the factors are produced locally during early cell death and distally during PCD [13]. Moreover, the same neuronal population can respond differently to the same neurotrophic factors depending on its maturational stage. For instance, GDNF has been shown to rescue dopaminergic neurons against PCD *in vitro* [14] and *in vivo* [15], but GDNF-/-mice that die perinatally exhibit normal numbers of nigral dopaminergic neuron precursors [16], indicating that early cell death (throughout EE) is insensitive to GDNF. Ret, a member of the receptor tyrosine kinase superfamily that transduces GDNF signaling, is required *in vivo* for the survival of dopamine neurons [17]. Along the same lines, chick retinal ganglion cells require NGF and BDNF to survive during early neural development whereas neither of these factors have any effect later during PCD [18]. The proneurotrophins that bind with high affinit to the p75 neurotrophin receptor (p75NTR) but lack the capacity to bind Trk receptors (e.g. NGF family receptor) can elicit apoptosis via p75NTR in neurons expressing survival-promoting Trk receptors. However, this process seems to

Fig. 1 Schematic representation of the extracellular effectors involved in the physiological survival/cell death balance of developing neurons. See text for details
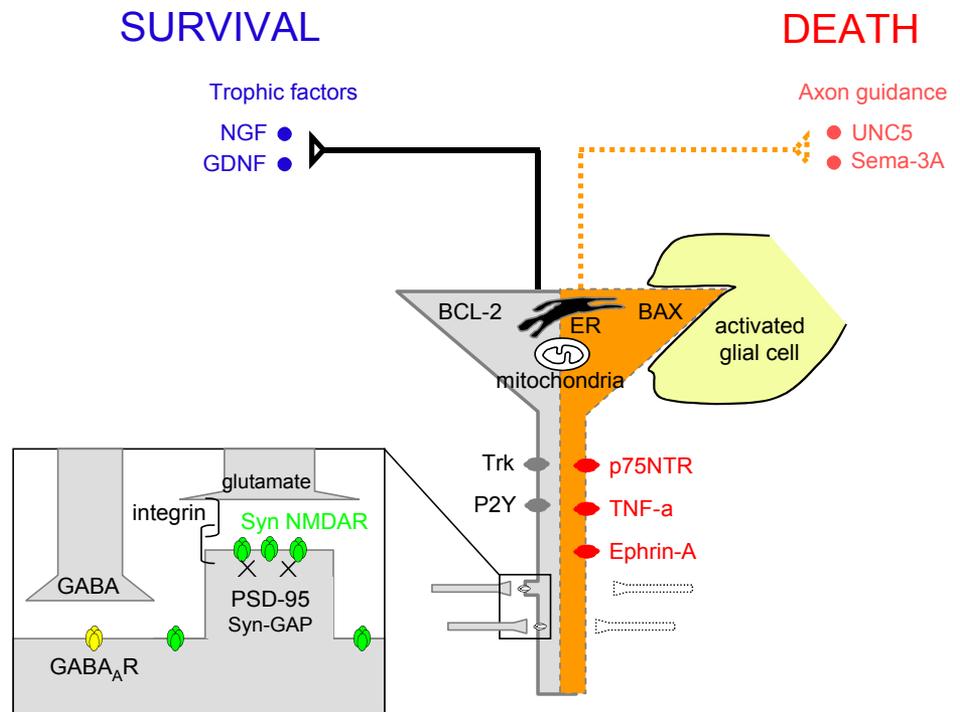
be mostly activated under pathological conditions [19]. In the retina, p75NTR and TrkA receptor regulate the number of retinal ganglion cell number in the EE phase but not at later stages, although their respective roles in triggering cell death remain unclear [20]. Moreover, during a define developmental period trophic factors can have antagonistic effects on neurons. For instance, transforming growth factor (TGF)-beta and insulin display opposite effects in regulating postnatal apoptosis since TGF-beta induces apoptosis whereas insulin has a well-described anti-apoptotic effect [21]. In all, the regulation of neuronal death during development by neurotrophic factors relies on a finel tuned balance between numerous partners, which can be disrupted under pathological conditions.

Recently, it has been shown that neurotransmission plays a key role in controlling developmental cell death (Fig. 1). An obvious difference between EE and PCD is the influenc of neurotransmission and synaptic activity [22]. Suppression of synaptic activity during embryonic development does not have any obvious effects on brain, neuronal or synaptic morphology [23]. In contrast, during the postnatal period, when synaptogenesis is at its peak, massive neuronal cell death can be triggered by an acute blockade of glutamate receptors [24–29]. Demonstrating the pivotal role of the glutamatergic NMDA receptor in controlling developmental cell death has been a major recent advance in our understanding of death induction in developing neurons. Blockade of NMDA receptors induces cell death during the early postnatal period in rodents and monkeys [24, 26, 30–32]. The localization of the NMDA receptors, i.e. in the synaptic or extrasynaptic membranes, influence the survival balance of neurons. Indeed, calcium influ through synaptic or extra-synaptic NMDA receptors produced opposite effects on CREB function, BDNF gene regulation, neuron survival [33] and Akt phosphorylation [34]. There are even situations where increased intracellular concentrations of calcium can foster neuronal survival [35] and calcium depletion may induce cell death [36]. Peripheral sympathetic and sensory neurons are strictly dependent on NGF for survival directly after isolation and have intracellular calcium levels of about 100 nM, rising to 250 nM after 3 weeks in culture, concomitantly losing their requirement for NGF to prevent apoptosis. Artificiall raising the intracellular calcium levels early during culture abolishes the NGF requirement, indicating that at least some neuronal populations have a developmentally regulated set-point, and that intracellular calcium concentrations determine their dependence on trophic factors and control neuronal survival [37, 38].

The activation of synaptic NMDARs that are present at the surface of neurons at early stages [39] has a trophic effect on neuronal survival. An elegant recent study has even shown that the survival of new neurons is competitively regulated by their own NMDA receptors during a short, critical period soon after birth [40]. Such a role for glutamate during physiological cell death stands in contrast to its potent cell death-inducing effect under pathological conditions, where massive release of glutamate induces spill-over from the synapse, activation of extrasynaptic NMDA receptors and cell death [41] and the NMDAR antagonist MK-801 is highly protective in models of ischemia in the developing

brain [42–44]. Furthermore, the AMPA type (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) glutamatergic receptor, does not appear to play a role in developmental cell death, whereas changes in AMPA signaling have been reported in pathological models of glutamate/NMDA-mediated cell death [24–26, 44, 45]. Hence, during neuronal network formation most neurotransmission has trophic effects and, conversely, insufficien activity is likely proapoptotic. Further investigations are clearly needed to clarify, for instance, the role of different neurotransmitters in this process [46], the role of synaptic components [28, 47] and whether neurotransmitter-induced neuronal survival simply requires depolarization [48], which can be achieved by both glutamate and GABA at early stages, or whether it is ligand specific Other extracellular molecules acting on gated channels, such as nucleotides (e.g. ATP), also modulate neuronal survival during development [49, 50], further suggesting that the neuronal activity status is a key control point for neuronal survival.

Negative regulators—death by murder

In contrast to the cell death mechanism induced by lack of survival signaling, some extracellular molecules have been shown to act as direct inducers of neuronal death during development. For instance, the proneurotrophins that bind with high affinit to the p75 neurotrophin receptor (p75NTR) can directly induce neuronal death. These receptors are often referred as "death receptors" and are coupled to specifi apoptotic effectors (e.g. caspase-8). Fas, a member of the TNF-alpha receptor family, is expressed during development in the spinal cord, hippocampus, and cortex [51] and is involved in the death of motoneurons [52]. However, convincing evidence from *in vivo* studies is still lacking [53, 54].

Interestingly, over the last years, several molecules involved in various steps of neuronal development were unexpectedly found to regulate physiological neuronal death. Glial cells have emerged as key regulators of neuronal development, regulating for instance the number of neurons at early stages by dynamically influencin neural precursors divisions, and at later stages by promoting neuronal cell death by engulfment [55]. Moreover, the chemorepellent netrin-1 receptors, UNC5 proteins and the axonal guidance molecule semaphorin 3A mediate neuronal cell death [56, 57]. Although it still needs to be firml established, it is possible that glial cells and the guidance molecules actively remove neurons that are mislocated, where cell density is too high, or neurons that send their axons to inappropriate target areas. Consistently, molecules from the extracellular matrix component family, integrin and ephrin, that ensure proper cell adhesion and cell recognition, play a role in developmental apoptosis [58, 59].

Cell death during development: intracellular effectors

Over the last decade, the number of molecules involved in cell death has increased dramatically. In this section, we mainly focus on the intracellular molecules that are activated during cell death of developing neurons, with special emphasis on the difference between EE and PCD and between physiological and pathological cell death. The molecular cascades will be discussed in the next sections. The morphological characteristics of dying immature neurons during development have revealed heterogeneity in the cell death process. Schematically, three types of cell death have been described [60]. The firs is characterized by shrinkage of the nucleus with condensed chromatin (internucleosomal DNA cleavage), cytoplasmic condensation and fragmentation of the neurons in "cellular bodies". This cell death type, called apoptosis [1], is by far the most abundant during PCD [60]. The second type is characterized by the appearance of autophagic vesicles within the cytoplasm. The third type exhibits swelling of cellular organelles, resembling a necrotic morphology. The significanc of this morphological heterogeneity is still unclear and apoptosis remains by far the most studied of these processes. Schematically the pathways that lead to apoptosis can be subdivided into external and internal signals, as described above. Studies from knockout mice have provided essential information on the effectors [6, 61, 62]. Activation of the caspase-9/APAF-1 complex, which in turn activates caspase-3, is a central pathway for PCD, and to some extent EE. The suppression of these effectors by genetic targeting produced severe brain malformations during brain development (including hyperplasia), indicating insufficien removal of neuronal precursors and postmitotic neurons [6, 63–68]. However, none of these mutations produced alterations in the spinal cord or brainstem structures [66], or in other organs where extensive PCD occurs, such as the thymus [63], indicating that involvement of specifi caspases may be brain region, neuronal type, and maturational stage dependent. Consistently, apoptosis of proliferating cerebellar granule cells and young pre-migratory cells occurs in the absence of caspase-3 cleavage, whereas cell death of post-mitotic migratory neurons is directly linked to caspase-3 activation [69]. Other caspases, such as caspase-2, which is an early checkpoint for apoptosis initiation in cortical neurons [70], have been involved and further investigations will shed light on additional apoptotic effectors during developmental cell death of neurons. Upstream of caspase activation, the Bcl-2 family constitutes a critical intracellular checkpoint in the intrinsic pathways of neuronal apoptosis during development [62]. Genetic mutations of the Bcl-2 family members do not produce hyperplasia or the massive brain malformations that were observed in, for instance, caspase-3-deficien mice, but neuronal cell death of postnatal motoneurons, sensory, retinal, cortical and cerebellar

neurons is affected [6, 71–78]. The commitment for developing neurons toward survival or cell death would then depend on the ratio between anti-apoptotic (e.g. Bcl-2 and Bcl-X$_L$) and pro-apoptotic (e.g. Bax) factors [62]. Upon receipt of a neuronal death signal Bax/Bak is a gateway to the intrinsic pathway operative at the mitochondrial level, which is a critical organelle for developmental apoptosis. In addition to mitochondria, proteins from the endoplasmic reticulum and the endosomal system also activate the apoptotic machinery during developmental cell death of neurons [79–81]. Furthermore, the PI3K-Akt signaling pathway plays a critical role in mediating survival signals in a wide range of neuronal cell types. The identificatio of a number of substrates for the serine/threonine kinase Akt suggests that it blocks cell death by both impinging on the cytoplasmic cell death machinery and by regulating the expression of genes involved in cell death and survival [82]. It should also be mentioned that additional molecules such as the kinesin superfamily protein 4 (KIF4) and poly (ADP-ribose) polymerase-1 (PARP-1) [83], the notch signaling pathway [84, 85], the p53 family member p63 [86], survivin, an inhibitor of apoptosis protein (IAP) [87], have been involved in EE and PCD. A distinct difference in the apoptotic mediators between EE and PCD is that EE involves a reentry into the cell cycle [88, 89]. For instance, the neurotrophin-3 withdrawal-induced developmental cell death of dorsal root ganglia neuron precursors was regulated by cell cycle molecules such as the cyclins D3 and E [90]. However, during PCD of postmitotic motoneurons [91], cerebellar [92, 93] and nigral dopamine neurons, cell cycle pathways are not activated, suggesting a specifi role for cell cycle regulation in EE of neuronal precursors.

Excessive oxidative stress in neurons, mainly mediated by reactive oxygen species (ROS) [94], has been highlighted as a candidate pathway to trigger PCD [95–101]. For instance, the apoptotic death of cultured sympathetic neurons or motoneurons can be blocked by ROS scavengers or overexpression of antioxidant enzymes [96, 102]. A particularly interesting model to study the role of oxidative stress during PCD is the dopaminergic system. Nigral dopamine neurons are highly sensitive to oxidative stress under pathological conditions, such as models of Parkinsons disease [98, 103]. Antioxidants protect cultured dopamine neurons from death due to serum deprivation [104–108], suggesting that developing dopamine neurons undergo oxidative stress and subsequently PCD. However, *in vitro* models are not always readily translated into physiological PCD since important differences between cultured and *in vivo* dopaminergic neurons have been observed. For instance, cultured nigral dopamine neurons do not express functional NMDA receptors [109] whereas nigral dopamine neurons from postnatal brains do display functional NMDA signaling [110]. It is unclear to what extent the *in vitro* mechanisms of oxidative stress can be applied to physiological conditions. Moreover, studies on the physiological cell death of postnatal nigral dopaminergic neurons [111, 112] clearly showed that the *in vivo* PCD is not dependent on oxidative stress [113–116]. Nitric oxide (NO) was found not to be involved in the PCD of dopamine neurons [108, 115], although there may exist differences between brain areas [117]. How can initial reports of high neuronal ROS contents during cell death be reconciled with a negative role for oxidative stress in PCD? A recent findin indicated that ROS accumulation in neurons accompanies the differentiation of progenitors into neurons, indicating that ROS play a role in the development of the neuronal phenotype and that high ROS contents for a neuron may not necessarily be a sign of cell death [118]. Based on these studies, it seems unlikely that the highly reactive and non-specifi ROS involved in pathological cell death would control the highly coordinated PCD under physiological conditions [119, 120].

In conclusion, depending on the trigger (see Fig. 1) several cell death cascades can lead developing neurons to apoptosis. The EE and PCD of neurons seem to have distinct main molecular pathways but common effectors are observed (e.g. caspases). Comparing the molecular pathways of physiological and pathological cell death of developing neurons, e.g. the oxidative stress, it appears that pathological cell death does not replicate the physiological one, although effectors may be shared.

Pathological apoptosis

Why "pathological" apoptosis?

There are reasons to consider PCD occurring after a pathological insult, such as hypoxia-ischemia (HI) or irradiation (IR), as different from the normal physiological, developmentally regulated PCD. The reasons for this will be discussed in this second part of the review. An ischemic insult will affect fully differentiated neurons more than immature precursors, whereas IR will affect predominantly precursor cells. Mature, post-mitotic neurons require much energy to maintain ionic gradients and cellular processes up to 40,000 times longer than their cell bodies and are therefore more susceptible to HI and subsequent energy depletion [121]. Neuronal precursors in the neurogenic regions of the postnatal brain, mainly the dentate gyrus subgranular zone (SGZ) in the hippocampus and the subventricular zone (SVZ) of the lateral ventricular wall, proliferate and are therefore more susceptible to the DNA damage caused by IR [122]. Morphological characteristics of both apoptosis and necrosis may be present after HI in the immature brain, even in the same cell [123, 124], and the relative appearance of these features is age-dependent [125].

Under pathological conditions several cell death pathways are activated simultaneously

A: The classical apoptosis pathway based on the specifi release of protein factors from the mitochondrial inter-membrane space. These factors include cytochrome *c* and AIF and their respective effectors can interact with one another and trigger either apoptosis (when caspases predominate) or apoptosis-like death (when AIF predom-inates) (Fig. 2).
B: Excessive elevation of intracellular calcium, which can lead to the activation of hydrolytic enzymes and trigger mitochondrial permeablity transition (Fig. 3).
C: Ionic imbalances and intracellular edema due to exces-sive Na and Cl influx
D: Oxidative stress resulting in depletion of glutathione and increased levels of unbuffered ROS (Fig. 3).

Interactions between pathways

Multiple interactions between these pathways take place. For example, caspase activation (pathway A) can be modulated by all the other three pathways in different ways. ATP is required for apoptosome formation and subsequent caspase activation, but within a couple of minutes after the onset of HI the tissue will suffer from complete loss of ATP, interfer-ing with caspase activation. Very soon after the drop in ATP, ionic gradients can no longer be maintained and intracellular Ca, Na and Cl concentrations increase, triggering mitochon-drial dysfunction and activation of many enzymes, including phospholipases and calpains. Formation of the apoptosome is also sensitive to the ionic composition of the cytosol and excessive influ of sodium would also block the process. Finally, cysteine proteases, including caspases are sensitive to the redox balance of the cell and can be inactivated un-der oxidative conditions. These are just a few examples out of many possible pathway interactions. Sometimes one of the pathways can be partially activated before another one takes over, explaining the mixed morphologies with features of both apoptosis and necrosis found under such conditions. For example, a cell may activate the apoptotic program and half way down the road experience a severe lack of ATP, resulting in disruption of membrane potential, influ of ions with subsequent swelling and protease activation, resulting in morphological signs of necrosis being added to the apop-totic morphology already partially established. The reverse may also occur. Neurons in the penumbral border zone of an infarct area will initially experience complete ATP deple-tion, activating mechanisms of necrosis and, upon restora-tion of blood fl w, energy levels will be restored, interrupting the necrotic pathways and enabling activation of apoptotic mechanisms.

Delayed, "secondary" energy failure

The onset of HI results in depletion of oxygen in the is-chemic tissue within seconds, resulting in impaired mito-chondrial oxidative phosphorylation. ATP levels are pre-served for a couple of minutes through anaerobic glycol-ysis and through reaction of ADP with phosphocreatine cat-alyzed by creatine kinase [126]. Restoration of blood fl w within 20–30 min in rodent models results in substantial recovery of metabolic activity of all cells within the firs 20 min and survival of most cells [127, 128]. The near complete restoration of ATP and phosphocreatine after is-chemia is not associated with restored mitochondrial oxida-tive metabolism to pre-ischemic levels. The mitochondrial respiration is depressed after neonatal HI and, after a par-tial transient recovery, a secondary decrease occurs [129] coinciding with decreased tissue utilization of glucose and activation of apoptosis-related mechanisms, including ac-tivation of caspase-3 and DNA fragmentation [130, 131]. This secondary energy failure develops in most brain re-gions 6–48 h after the insult in immature animal models [129–131].

Young brains have more apoptotic machinery and neurons often display mixed apoptotic/necrotic morphology after ischemic injury

Some of the apoptotic effectors, such as caspase-3 [132, 133], APAF-1 [134], Bcl-2 [135] and Bax [136], are expressed at higher levels in the developing brain and apoptotic mecha-nisms seem to be more important in the development of brain injury in the immature than in the adult brain [132, 133, 137–142]. Even AIF, where the expression level remains constant during postnatal brain development, is activated to a greater extent in the immature than in the juvenile and adult brain after HI [142, 143]. This seems reasonable in light of the extensive apoptosis taking place during brain development and the large numbers of cells that need to be discretely removed, supporting the notion that embryonic and early postnatal neurons have a greater capacity and readiness to undergo apoptosis. Based on morphological criteria, the ini-tial injury (within a few hours) in the striatum and cortex of neonatal rats was found to be necrosis, but in thalamus the delayed neurodegeneration (within hours to days) was primarily apoptosis, indicating that the morphology of neu-ronal death is determined by time, region, and potentially by patterns of neuronal connectivity [123]. There are several studies describing dying cells with a mixed morphology, dis-playing characteristics of both apoptosis and necrosis [144], including after neonatal HI [123–125, 145], further support-ing the concept of cross-talk between pathways. The concept of continuum cell death was coined to emphasize that the
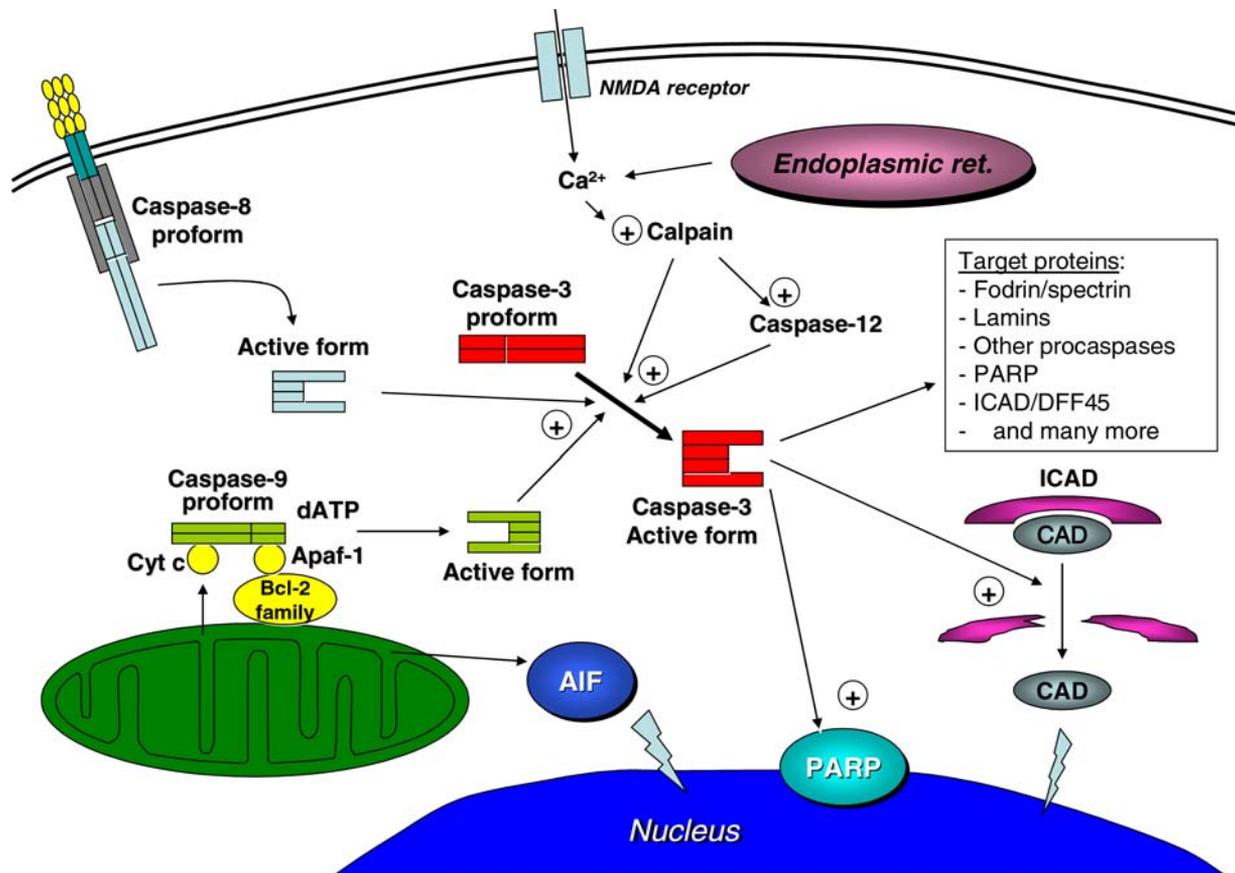
Fig. 2 Schematic representation of the three mechanisms converging on caspase-3, which is the major execution caspase in the brain, particularly in the developing brain. Activated caspase-3 will cleave a number of target proteins, including ICAD/DFF45 and PARP, as indicated in the figure Also indicated is release of the caspase-independent AIF from mitochondria

morphology of dying neurons in the immature brain after HI displayed the full range from necrotic to apoptotic [146]. In Fig. 4 cortical neurons displaying different morphologies after HI can be seen, including necrosis, apoptosis and the mixed pathological apoptosis (Fig. 4).

Extracellular effectors

Death receptor pathway

Evidence for involvement of the Fas/CD95 receptor and its ligand in ischemic brain injury has been obtained from both the adult and developing brain. After transient focal ischemia in adult rats Fas expression was increased and mice carrying a deficien Fas gene (*lpr* mice) were less injured than wild type animals (Martin-Villalba 1999). After neonatal rat HI both Fas (Felderhoff-Mueser et al., 2000) and its receptor were upregulated after the insult, in concert with cleavage of procaspase 8 to its active form [147]. Furthermore, mice lacking functional Fas death receptors were protected from

HI brain damage in cortex, striatum, and thalamus [148]. The hippocampus was the only region not protected in these mice, and the selective vulnerability of the hippocampus correlated with lower basal expression of [Fas-associated death-domain-like IL-1beta-converting enzyme]-inhibitory protein (FLIP), and indications of necrosis, as judged by calpain activation and a higher percentage of non-apoptotic morphology was present after HI [148].

Neurotrophins/Cytokines

Neurotrophins have attracted much attention as potential therapeutic agents after ischemia. BDNF, for example, provided impressive neuroprotection after neonatal (P7) HI [149, 150], but not in older (P21) animals. BDNF treatment produced robust phosphorylation of Trk receptors in P7 but not P21 rats, indicating either that the trkB receptors are downregulated with age, or that another factor is missing to mediate the BDNF effects [149]. From the wide range of cytokines only one will be mentioned here, erythropoietin.
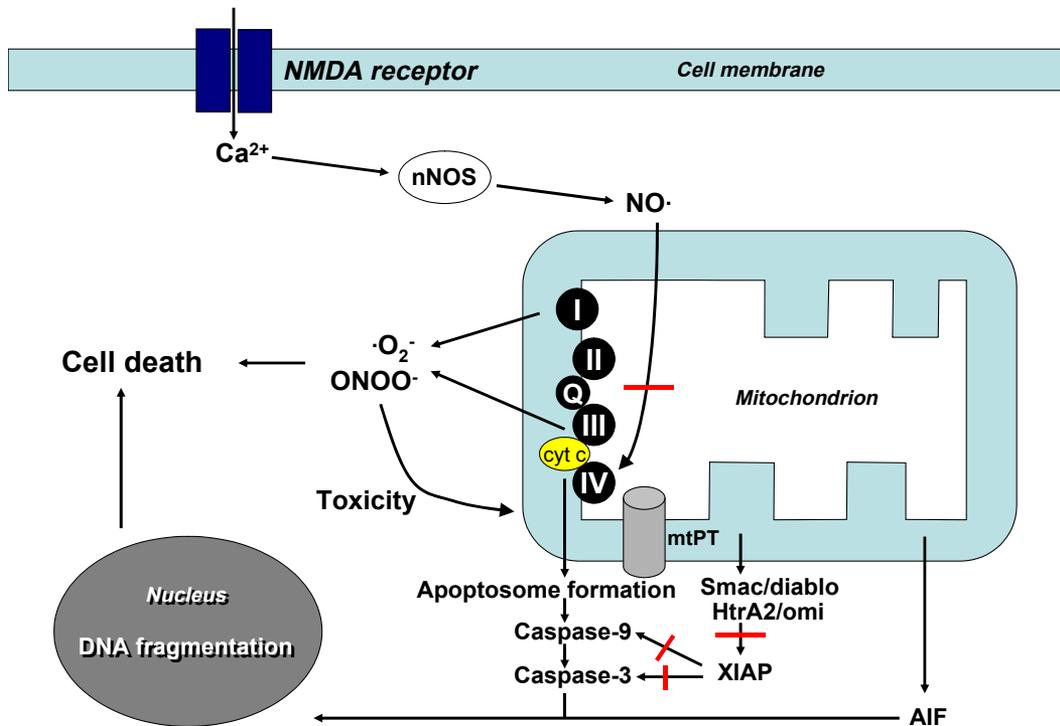
Fig. 3 Mitochondria are a major source of oxygen free radicals, but also a target for free radical attack, promoting mitochondrial permeability transition and release of pro-apoptotic proteins from mitochondria. Excitotoxic events, leading to calcium influ through NMDA receptors, activates neuronal nitric oxide synthase (nNOS) which may impair oxidative phosphorylation
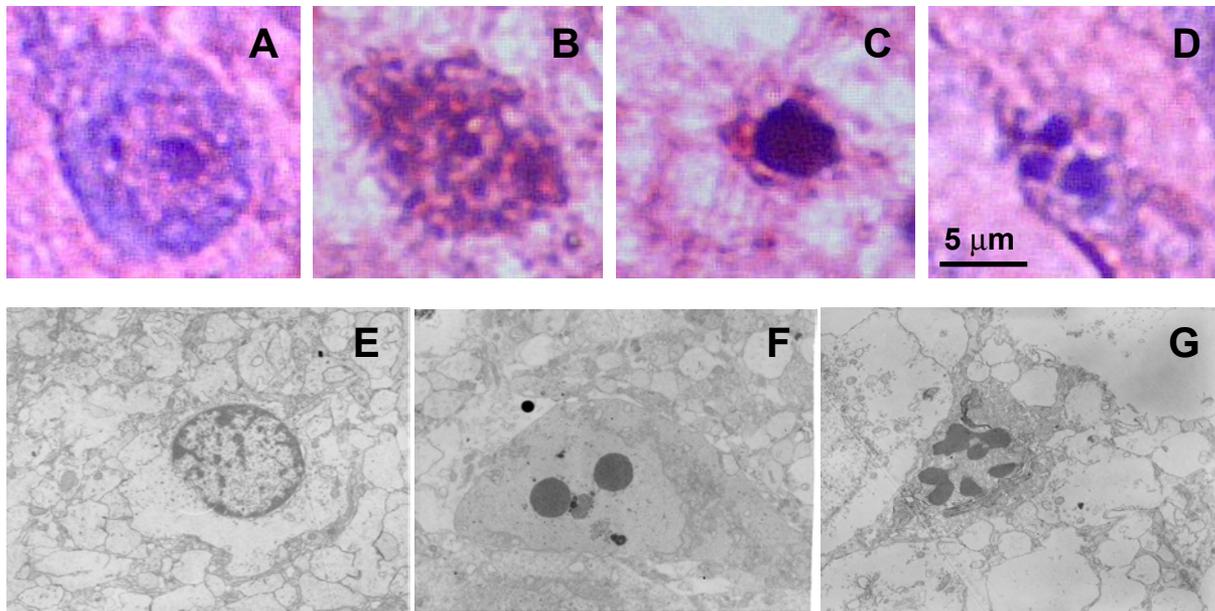

Fig. 4 Microphotographs from neonatal mouse brains after hypoxia-ischemia (HI), demonstrating different morphologies in cortical neurons. The upper panels show light microscopic pictures after thionin/fuchsin staining, demonstrating cortical neurons with a morphology typical for (A) a normal cell, (B) necrosis, (C) apoptosis and (D) pathological apoptosis. The lower panels show electron microscopic pictures, demonstrating neurons with a morphology typical for (E) necrosis, (F) apoptosis and (G) pathological apoptosis. Panels A–D: courtesy of Dr. Changlian Zhu, Göteborg University, Sweden. Panels E–G: courtesy of Dr. Frances Northington, Johns Hopkins University, Baltimore, MD, USA

It has been shown that erythropoietin and non-erythrogenic derivatives by signaling through a non-hematopoietic receptor in the central nervous system afford neuroprotection in various models of brain injury [151–153], including the developing brain [154–156]. Erythropoietin did not, however, prevent radiation-induced loss of precursor cells in the DG or SVZ [122].

Potassium and chloride ion homeostasis

A universal characteristic of apoptosis is the loss of cell volume or cell shrinkage. While cell shrinkage has traditionally been viewed as a passive event during apoptosis, recent work has shown that the flux of ions associated with the change in cell size plays a critical role in the regulation of the cell death machinery [157]. Apoptotic volume decrease (AVD) has even been shown to be an early prerequisite of apoptosis [158]. Physiological concentrations of potassium prevent death receptor activation, cytochrome c release, apoptosome formation, caspase activation and apoptotic nuclease activity [159–161], at least in lymphocytes, and conversely, loss of intracellular potassium provided conditions compatible with activation of caspases and apoptotic nucleases. Cortical neurons exposed to NMDA in medium containing reduced Na^+ and Ca^{2+} (resembling the conditions in ischemic brain tissue) lost substantial intracellular K^+ and underwent apoptosis. Both K^+ loss and apoptosis were attenuated by increasing extracellular K^+, indicating that NMDA receptor-mediated K^+ efflux may contribute to neuronal apoptosis after brain ischemia [162]. Furthermore, the K^+ channel blockers tetraethylammonium (TEA) and clofilium attenuated murine cortical neuronal apoptosis induced by hypoxia *in vitro* and infarct volume induced by focal ischemia *in vivo* [163]. In another study, however, neither increased extracellular K^+ nor the K^+ channel blocker could prevent staurosporine-induced apoptosis in cultured cortical neurons [164]. The main inhibitory amino acids gamma-aminobutyric acid (GABA) and glycine are excitatory in the developing brain by depolarizing developing neurons that have high intracellular Cl^- concentrations and a depolarized Cl^- equilibrium potential. GABA becomes inhibitory as net outward neuronal transport of Cl^- develops in a caudal-rostral progression. This seems to be of major importance for the wiring of neuronal circuits [165]. Prenatal or neonatal stress, for example hypoxia, can affect the programming of neurotransmitter and receptor expression, which can lead to long-term behavioral effects [166]. The Na^+-K^+-$2Cl^-$ co-transporter (NKCC1) facilitates the accumulation of Cl^- in neurons and therefore facilitates seizures in the developing brain, indicating that NKCC1 inhibitors like bumetanide could be useful in the treatment of neonatal seizures [167].

Intracellular effectors

Caspases

As mentioned above in the paragraph on secondary energy failure, the mitochondrial respiration is depressed after neonatal HI and, after a partial transient recovery, a secondary decrease occurs [129] parallel to the decrease of tissue utilization of glucose, loss of MAP-2 immunostaining, activation of caspase-3 and DNA fragmentation [130, 131]. This mitochondrial dysfunction may contribute to or facilitate the release of proapoptotic factors from the inter-membrane space of the mitochondria, such as cytochrome c, AIF, endonuclease G, SMAC/Diablo and HtrA2/Omi [168]. Subsequently, caspase-dependent and caspase-independent mechanisms will ensue. Release of Cytochrome c interacts with APAF-1 and dATP/ATP to form the apoptosome, leading to activation of procaspase-9 [169], which in turn cleaves and activates pro-caspase-3, the most abundant effector caspase in the brain. As mentioned above, caspase-3 expression is strongly regulated with age. Caspase-3 protein and mRNA in control rat brains decreased more than 80% from postnatal day 10 to 21 [132], corresponding to when brain growth levels out. The role of the inflammatory caspases (mainly caspase-1, also called IL-1 converting enzyme) in apoptosis is not clear, but they do seem to contribute to brain injury after ischemia through their pro-inflammatory actions. Adult mice lacking the caspase-1 gene (Schielke et al., 1998) or expressing a gene acting as a dominant negative caspase-1 inhibitor under a neuronal promoter (Friedlander et al., 1997) displayed reduced injury after focal ischemia, indicating that neuronal caspase-1 may participate in the cell death process. Similarly, neonatal caspase-1-deficient mice also displayed reduced injury compared with wild type animals, but only if the injury was mild (Liu et al., 1999). Caspase-1 cleaves and activates both IL-1 and IL-18, and neonatal IL-18-deficient mice also showed less brain injury after HI (Hedtjärn et al., 2002).

Differential effects of the endogenous caspase inhibitor XIAP under normal and pathological conditions

Another finding supporting the concept of pathological apoptosis is that the endogenous caspase inhibitor X-linked Inhibitor of Apoptotosis (XIAP) protein apparently has different effects under physiological and pathological conditions. For example, over-expression of XIAP in neurons did not interfere with physiological neuronal apoptosis, as judged by the normal brain morphology of these mice [122, 170, 171]. In the dentate gyrus (DG), where there is constant neurogenesis and turnover of neurons throughout life, the size and appearance of the DG was not different between wild type and XIAP over-expressing mice. However,

Fig. 5 A schematic diagram showing the balancing effects of X-linked inhibitor of apoptosis protein (XIAP) under pathological conditions. XIAP can inhibit caspase-3 and caspase-9 by direct binding to these proteases, but XIAP may also be cleaved by for example caspase-3. HtrA2 and Smac/diablo are released from mitochondria and inhibit XIAP, as does XAF-1. A recently discovered function of XIAP is down-regulation of oxidative stress through up-regulation of the anti-oxidant enzymes SOD2 and TRX2, via NF-κB. The fina outcome depends on the relative strength of the cell death mechanisms
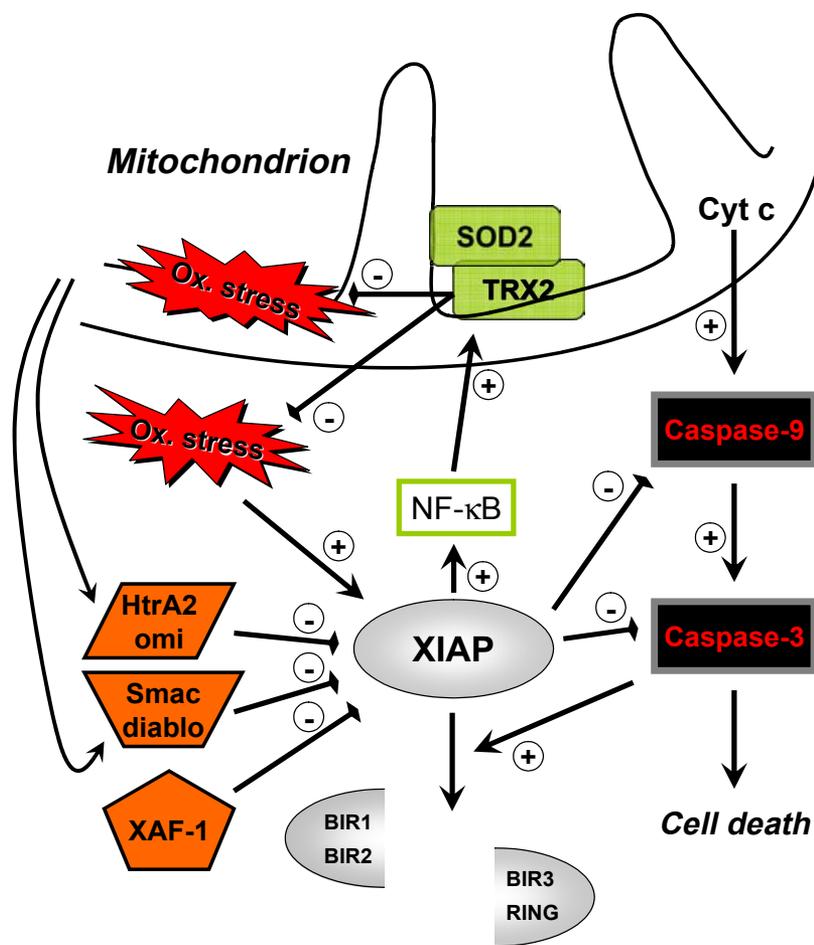
XIAP over-expression did prevent the activation of caspase-9 and caspase-3 observed after HI [172] and provided signifi cant neuroprotection. Activation of caspase-9 and caspase-3 was prevented also in the DG after irradiation [122], but in this paradigm no reduction of cell loss could be observed, so the effects of XIAP were not secondary to tissue protection. Similarly, XIAP was demonstrated to protect against oxidative stress by up-regulating the mitochondrial antioxidant enzymes SOD2 and TRX2 through NF-κB activation after HI and after irradiation, but not under normal condition, in the absence of an insult (unpublished observations) (Fig. 5). This supports the notion that the same effector can be involved in different pathways and that different regulatory mechanisms are utilized during normal and pathological apoptosis.

Calpains and caspases

Cross-talk between caspase-3 and calpains, calcium-activated cysteine proteases with a neutral pH optimum, has been demonstrated. Calpains are cytosolic proteases mainly implicated in excitotoxic cell death and necrosis [173], and

calpains, like caspase-3, are also more highly expressed in the immature brain [174]. In mice, calpain-mediated activation of caspase-3 has been shown to occur by way of caspase-12, such that calpains cleave and release the active form of caspase-12 from the endoplasmic reticulum to the cytosol, where it subsequently can cleave and activate caspase-3 [175] (Fig. 3). After neonatal HI, activation of m-calpain (calpain 2), but not μ-calpain (calpain 1) facilitated the activation of caspase-3 in a synergistic manner by cleavage in the prodomain of caspase-3, yielding a 29 kDa intermediate form, which apparently was more rapidly processed to the fully active forms [132] (Fig. 2). This is supported by another study demonstrating that $CaCl_2$ triggered nuclear apoptosis and increased caspase-3 activity in cell-free systems [176], and one study where caspase-3 activation *in vivo* was decreased in neurons rescued by treatment with the NMDA receptor antagonist MK-801 after HI [177]. This synergistic enhancement of caspase-3 activation after an excitotoxic insult and calpain activation is a prime example of pathological apoptosis, i.e. cross-talk between PCD and mechanisms usually more associated with necrosis. However, there are other reports demonstrating

calpain-mediated inactivation of caspase. Calpains were shown to cleave APAF-1 in neuroblastoma cells challenged with ionophore, thereby compromising caspase-3 activation [178]. In one paradigm, non-canonical cleavage of caspase-3 by calcium-dependent proteolysis yielded weak DEVDase activity, but the truncated caspase-3 thus produced could not be further activated by the cytochrome *c* and dATP pathway [179]. Also, calpain activation in nitric oxide-triggered excitotoxicity was coupled to execution of caspase-independent apoptosis in cerebellar granule neurons [180]. Conversely, caspase-3-mediated cleavage of the endogenous calpain inhibitor calpastatin has been demonstrated in several studies [181–183], thereby promoting calpain activation. Neumar et al. demonstrated a dual role for calpains during staurosporine-induced neuronal apoptosis in neuroblastoma cells. In the early execution phase, calpain down-regulated caspase-3-like activity and slowed progression of apoptotic nuclear morphology. Subsequent calpain activity, facilitated by caspase-mediated degradation of calpastatin, contributed to plasma membrane disruption and secondary necrosis [184]. These and other studies project a complex picture of calpain-caspase interaction in cell death, depending on the cell or tissue type studied and the injury paradigm used.

Differential effects of the caspase-independent AIF

Unlike caspase-3, the caspase-independent Apoptosis-Inducing Factor (AIF) does not appear to be significantl regulated with age. The AIF protein levels in brain were similar from postnatal day zero to adult [143]. In adult rodents extensive translocation of AIF from mitochondria to nuclei was detected after trauma [185], hypoglycemia [186], focal ischemia [187, 188] and neonatal HI [142, 143, 189]. Extensive and rapid (within 30 min) AIF translocation to nuclei after irradiation to the immature brain was also detected in progenitor cells of the dentate gyrus and the subventricular zone, but not in differentiated neurons or glia [122]. In the harlequin (Hq) mouse strain the expression of AIF is reduced to approx. 20% in adults and 40% in P9 mice of the wild type level due to a retroviral insertion into the firs intron of the AIF gene located on chromosome X [190]. Hq mice displayed 43% smaller infarcts after adult focal ischemia [191] and 53% and 43% infarct volumes in male (YX^{Hq}) and female ($X^{Hq}X^{Hq}$) mice, respectively [192]. Interestingly, AIF activation (as judged by nuclear translocation, was much more prominent in P5 and P9 mice (corresponding to premature and term human brains) than in P21 and P60 mice (corresponding to juvenile and adult human brains), despite the fact that the concentration of AIF was the same at all ages [142]. Again, the same effector displays different roles under different conditions, in this case different developmental levels.

Nitric oxide

Activation of NMDA receptors results in the influ of calcium and subsequent calmodulin-mediated activation of neuronal nitric oxide synthase (nNOS), converting l-arginine to citrulline and nitric oxide (NO) [193] (Fig. 3). NOS activity is high in the postnatal brain, with peak levels preceding the period of maximal synaptogenesis [194]. Both nNOS and, surprisingly, inducible (or macrophage) NOS (iNOS) are constitutively expressed at several-fold higher levels during early postnatal development [142]. Neuronal nitric oxide synthase (nNOS) is expressed in a limited number (<4%) of neurons (quisqualate sensitive), but these cells are capable of killing neighboring cells. Elimination of nNOS neurons through injections of quisqualic acid into the cortex of P7 rats rendered these animals resistant to HI [195], and nNOS deficien y through genetic targeting was also neuroprotective [196]. Administration of the combined iNOS/nNOS inhibitor 2-iminobiotin reduced caspase-3 activation [197] and conferred tissue protection [198]. The iNOS inhibitor aminoguanidine [199] (IL-1) also reduced brain injury after HI, further indicating that NO production exerts cytotoxic effects in the developing brain such insults. NO can also mediate neuroprotective actions, through vasodilatation, angiogenesis, inhibition of platelet aggregation and leukocyte activation, explaining why endothelial nitric oxide synthase (eNOS) inhibition resulted in exacerbation of ischemic injury [200]. However, small (or short-lasting) mitochondrial elevations of NO, and subsequently hydrogen peroxide, can trigger protective responses, e.g. stabilization of hypoxia inducible factor-1 alpha, which seems to bring about protective responses also in the immature brain [201]. Also, NO (contrary to peroxynitrite) may exert anti-apoptotic effects through inhibition of cytochrome c release [202] and quenching of excess O_2. See also the paragraph on oxidative stress in the section on intracellular effectors below.

Oxidative stress

There are multiple pathways whereby reactive oxygen species (ROS) are produced in the brain. As mentioned earlier in the preceding paragraph on nitric oxide, NOS activity is a major contributor to generation of free radicals and oxidative stress. Mitochondria are considered to be a major site of ROS production in mammalian cells through electron leakage from the electron transport chain [194, 203], perhaps also after ischemia [204]. Mitochondria also appear to be a major target of ROS attack and the immature brain is particularly susceptible to free radical injury because of its poorly developed scavenging systems and high availability of iron for the catalytic formation of hydroxyl radicals [205]. Formation of ROS in the brain after various insults is respiration-dependent, mitochondria *in vitro* are sensitive to

ROS and peroxynitrite, and most data suggest that oxidative stress contributes to the post-ischemic impairment of mitochondrial respiration [206]. When the ROS levels exceed the capacity of the cell in general and the mitochondria in particular to scavenge and render them harmless, the resulting oxidative stress may initiate mitochondrial permeability transition (mtPT) [207], which then in turn potentiates the oxidative stress. Proapoptotic factors are too large to pass directly through the mtPT pore, but opening of the mtPT can trigger the release of cytochrome-c and other intermembrane proteins. In addition, the release of proapoptotic intermembrane proteins may also occur mtPT independent [208]. Thus, oxidative stress can, directly or indirectly, modulate the release of proapoptotic proteins [209–211] and drugs that block the mtPT pore formation, like cyclosporin A, provide neuroprotection in adult models of ischemia, and prevent release of proapoptotic proteins [212, 213]. In the developing brain, mtPT was demonstrated to occur after HI, as indicated by entrapment of deoxyglucose in mitochondria, but cyclosporin A treatment did not provide neuroprotection after HI using the same, or higher, doses of cyclosporin A as in studies of adult ischemia [214]. It was not shown, however, whether cyclosporin A actually blocked mtPT and the reasons for the lack of protection remain unclear.

Superoxide is dismutated by superoxide dismutase into H_2O_2, which is converted to water and oxygen by catalase or glutathione peroxidase. There are three major superoxide dismutases: SOD1 (Cu,Zn-SOD), SOD2 (Mn-SOD) and SOD3 (EC-SOD). SOD1 is mainly found in the cytosolic and lysosomal fractions, but also in the mitochondrial intermembrane space whereas SOD2 is located in the mitochondrial matrix. The neurological outcome and infarctions are aggravated in SOD2-deficien mice after both transient [215] and permanent [216] focal ischemia in adult mice. Conversely, overexpression of SOD2 prevented apoptosis and reduced tissue damage after focal ischemia [217]. SOD1 overexpression in adult mouse brains also reduced the injury after transient focal ischemia [218], but in the immature brain overexpression of SOD1 unexpectedly aggravated the tissue damage after HI [219]. This was subsequently attributed to a limited capacity of the immature brain to convert the accumulated H_2O_2 into water and oxygen due to lower levels of catalase and glutathione peroxidase [220], emphasizing the importance of glutathione peroxidase for downstream processing of the H_2O_2 produced by SOD.

Autophagy

Autophagy is a process responsible for the bulk degradation of intracellular material in double or multiple-membrane autophagic vesicles and their delivery to and subsequent degradation by the cell's own lysosomal system. Just like the genetically controlled, physiological PCD, autophagy was demonstrated to be more pronounced during embryonic development and tissue remodeling [221]. It has been suggested that autophagy is a caspase-independent, genetically controlled cell death [222]. Emerging evidence points to the importance of autophagy in the protein quality-control process, a process that may be of particular importance in post-mitotic brain neurons. Loss of *Atg5* or *Atg7* (autophagy-related 5/7), two genes essential for autophagy, leads to progressive neurodegeneration, behavioral defects, presence of polyubiquitinated inclusion bodies in neurons and premature death [223, 224]. Visualization of autophagic vesicles by electron microscopy is still the golden standard to demonstrate autophagy. The electrophoretic mobility change of microtubule-associated protein 1, light chain 3 (LC3) from the non-autophagic, cytosolic form (LC3-I; 16 kDa) to the autophagic, membrane-recruited form (LC3-II; 14 kDa) provides a molecular marker-based method for detection of autophagic activity [225–228]. LC3-II was higher in the developing, immature mouse brain, compatible with a role for autophagy in brain growth and tissue remodeling [142]. LC3-II inscreased from 1–3 days after HI, coinciding with the peak of cell death and subsequent tissue degeneration, and the LC3-II increase was more pronounced in the adult than in the immature brain [142]. This was the firs evidence for ischemia-induced autophagy, providing another example of how cell death mechanisms can be activated by different triggers under normal and pathological conditions. It is unclear at present to what extent autophagy is a cellular repair process, a stress response or actually contributing to cell death.

Challenges for the future

Pathological apoptosis involves numerous other pathways that could not be addressed here in detail, as this would be beyond the space limitations and scope and of this concise review. Such key players include MAP kinases, heat shock proteins and mechanisms controlling phagocytosis. The challenge for the future will be to unravel whether the respective pathways and mechanisms are (i) directly involved in neurodegeneration/neuroprotection, (ii) only epiphenomena, or (iii) elicited to actually counteract the main process. An illustrative example of the complexity of the issue is the role of JNK in developmental neuronal death, as this MAP kinase promotes apoptosis on one side of the neural tube and blocks it on the other [229]. Another example is the role of phagocytosis that has until recently been considered a cleanup process after damage, but now is known to also take an active part in killing cells during development [230]. The complexity and heterogeneity of brain tissue obviates the need for new and advanced combinations of histology and molecular biology to solve these important questions.

Summary

In summary, we argue that the intra- and extracellular conditions after an insult like ischemia or irradiation are not compatible with the execution of normal programmed cell death (PCD). Rather, multiple mechanisms of injury, including apoptotic mechanisms, will be activated and interact on many levels, producing biochemical and morphological characteristics distinctly different from those observed during physiological, developmentally regulated PCD. Often a mixed morphology is observed, with characteristics of both necrotic and apoptotic cell death, particularly after injury to the developing brain, where the apoptotic machinery is more prominent. In view of the large differences compared with physiological PCD, we suggest that cell death occurring after insults like ischemia or irradiation not be called apoptosis. We propose *pathological apoptosis* as an alternative term, indicating that classical PCD and other cell death mechanisms interact and contribute to the demise of cells.

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