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Review

Targeting post-mitochondrial effectors of apoptosis for neuroprotection

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ABSTRACT

Mitochondrial membrane permeabilization (MMP) is commonly regarded as the "point-of-no-return" in the cascade of events that delineate the intrinsic pathway of apoptosis. MMP leads to the functional impairment of mitochondria and to the release into the cytosol of toxic proteins that are normally confined within the mitochondrial intermembrane space. These include direct activators of caspases and caspase-independent effectors of the cell death program. MMP has been implicated in a plethora of pathophysiological settings. In particular, MMP contributes to both the immediate and delayed phases of cell loss that follow acute neuronal injury by ischemia/reperfusion or trauma. Although preventing MMP *a priori* would be the most desirable therapeutic choice, prophylactic interventions are rarely (if ever) achievable in the treatment of stroke and trauma patients. Conversely, interventions that block the post-mitochondrial phase of apoptosis (if administered within the first few hours after the accident) hold great promises for the development of novel neuroprotective strategies. In animal models of acute neuronal injury, the inhibition of caspases, apoptosis-inducing factor (AIF) and other apoptotic effectors can confer significant neuroprotection. Our review recapitulates the results of these studies and proposes novel strategies of inhibiting post-mitochondrial apoptosis in neurons.

1. Introduction

Apoptosis constitutes a highly regulated mechanism for the removal of supernumerary, ectopic, old, damaged or mutated cells, that can be executed by two distinct (yet partially overlapping) molecular mechanisms [1]. On one hand, the extrinsic apoptotic pathway mediates cell death in response to extracellular stimuli. This can occur either through the ligand-induced activation of death receptors at the plasma membrane [2,3], or as a result of signaling cascades that emanate from dependency receptors in the absence of their ligands [4]. On the other hand, intrinsic apoptosis is regulated by mitochondria, which integrate lethal and pro-survival signals, to eventually reach a decision on the cell's fate [5]. If the death sentence is pronounced, mitochondrial membrane permeabilization (MMP) takes place and cells trespass the frontier between life and death, the "point-of-no-return" in the cascade of events that delineates this lethal routine (which is also known as "mitochondrial apoptosis") [6,7]. Irrespective of the initiating signals, the extrinsic and the intrinsic pathway converge on the massive activation of catabolic enzymes (including a class of cysteine proteases known as caspases, non-caspase proteases, lipases and endonucleases), which account for the execution of apoptosis and (at least partially) for its morphological appearance [1,8,9].

MMP has a number of consequences that contribute to cell death, which include (but are not limited to): (1) loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$), in turn leading to the arrest of

Abbreviations: ABD, ATP-binding domain; AIF, apoptosis-inducting factor; ANT, adenine nucleotide translocase; Antp HD, antennapedia homeodomain; Apaf-1, apoptotic peptidase activating factor 1; BH, Bcl-2 homology; BIR, baculoviral IAP repeat; boc-D-fmk, boc-Asp(OMe)-fmk; CCI, controlled cortical impact; CK, creatine kinase; CNS, central nervous system; CsA, cyclosporine A; CypA, cyclophilin A; CypD, cyclophilin D, Cyt *c*, cytochrome *c*; $\Delta\Psi_m$, mitochondrial transmembrane potential; DIABLO, direct IAP-binding protein with a low pI; EndoG, endonuclease G; *fog*, forebrain overgrowth; HK, hexokinase; H/I, hypoxia/ischemia; Hsp, heat-shock protein; *Hq*, Harlequin; HtrA2, high temperature requirement protein A 2; IAP, inhibitor of apoptosis protein; IM, mitochondrial inner membrane; IMS, mitochondrial intermembrane space; I/R, ischemia/reperfusion; JNK, c-Jun N-terminal kinase; MCAO, middle cerebral artery occlusion; MEFs, mouse embryonic fibroblasts; MMP, mitochondrial membrane permeabilization; MPT, mitochondrial permeability transition; NAIP, neuronal apoptosis inhibitory protein; NOS, nitric oxide synthase; OM, mitochondrial outer membrane; Omi, Omi stress-regulated endoprotease; PBR, peripheral-type benzodiazepine receptor; PTD, protein transduction domain; PTPC, permeability transition pore complex; RGCs, retinal ganglion cells; RING, real interesting new gene; ROS, reactive oxygen species; SCI, spinal cord injury; Smac, second direct activator of caspases; SOD1, copper/zinc-superoxide dismutase; tFCI, transient focal cerebral ischemia; TUDCA, tauroursodeoxycholic acid; VDAC, voltage-dependent anion channel; WT, wild type; XIAP, X-linked IAP; Z-DEVD-fmk, Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; Z-VAD-fmk, Z-Val-Ala-Asp(OMe)-fluoromethylketone

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mitochondrial bioenergetic and biosynthetic functions (and in particular of ATP synthesis through the F_0 F_1-ATP synthase); (2) uncoupling of the respiratory chain, which results in the overproduction of reactive oxygen species (ROS) and further amplifies the functional impairment of mitochondria; and (3) release into the cytosol of proteins that in healthy cells are secured within the mitochondrial intermembrane space (IMS), where they serve vital functions [10–12]. These comprehend enzymes with an intrinsic catabolic activity (*e.g.*, apoptosis-inducing factor, *i.e.*, AIF, endonuclease G, *i.e.*, EndoG, several pro-caspases) [13,14], direct activators of caspases (*e.g.*, cytochrome *c*, *i.e.*, Cyt *c*) [15,16], as well as proteins that block and/or degrade endogenous inhibitors of caspases, thereby indirectly favoring the execution of cell death (*e.g.*, Smac/DIABLO, Omi/HtrA2) [17,18] (Table 1). Thus, on one hand MMP leads to a bioenergetic, biosynthetic and redox crisis that coincides with the complete collapse of cellular metabolism, and, on the other hand, it triggers multiple mechanisms that actively degrade the cellular content. Ultimately, MMP results in cell death, which may manifest with the classical morphological hallmarks of apoptosis (*e.g.*, chromatin condensation, karyorrhexis, pyknosis, shedding of apoptotic bodies) or by exhibiting a necrotic and/or an autophagic phenotype. In this regard, it should be noted that the inhibition of caspases often shifts the appearance of cell death from full-blown pictures of apoptosis to a non-apoptotic morphology, and concomitantly delays (but rarely, if ever, prevents) its ultimate occurrence [1,8,9].

At present, two major models have been put forward to describe the mechanisms through which mitochondrial membranes become permeabilized (Fig. 1). In some settings, MMP results from protein-permeable pores formed across the outer mitochondrial membrane (OM) by proapoptotic members of the Bcl-2 protein family (*e.g.*, Bak, Bax) [19,20]. Such pores are responsible for the early release of IMS proteins, followed by blockade of the respiratory chain (due to the loss of IMS-soluble components like Cyt *c*), ROS overgeneration, $\Delta\Psi_m$ dissipation, and functional breakdown of mitochondria [21,22] (Fig. 1A). In response to a plethora of apoptotic stimuli, Bax and Bak, which in healthy cells are in a latent state, undergo conformational changes allowing for homo- and/or hetero-oligomerization. This structural reorganization often involves a direct physical interaction with another class of Bcl-2 family members that function as intracellular sensors of stress, namely BH3-only proteins (*e.g.*, Bid, Bad, Bim) [23–25]. In this context, a third group of proteins from the Bcl-2 family (*e.g.*, Bcl-2, Bcl-X_L) exerts antiapoptotic functions by sequestering their proapoptotic counterparts into inactive complexes [26], as well as via extramitochondrial mechanisms (for instance by modulating Ca^{2+} fluxes at the endoplasmic reticulum) [27].

In other instances, MMP is triggered by an abrupt increase in the permeability of the mitochondrial inner membrane (IM) to low-molecular weight solutes, which is known as mitochondrial permeability transition (MPT). MPT derives from the opening of the so-called permeability transition pore complex (PTPC), a supramolecular entity assembled at the junctions between IM and OM. MPT provokes an osmotic imbalance that leads to a net influx of water into the mitochondrial matrix, swelling of the matrix and eventually physical rupture of the OM (Fig. 1B) [28,29]. Although the precise molecular composition of the PTPC still remains a matter of debate, some consensus has emerged on its minimal scaffold structure, which includes the voltage-dependent anion channel (VDAC) in the OM, the adenine nucleotide translocase (ANT) in the IM, and cyclophilin D (CypD) in the mitochondrial matrix [30,31]. In addition, the activity of the PTPC (which responds to proapoptotic signals such as Ca^{2+} overload and oxidative stress) is regulated by the association with several interacting partners, including the peripheral-type benzodiazepine receptor (PBR) [32], the mitochondrial creatine kinase (CK) [33], multiple isoforms of hexokinase (HK) [34,35], as well as pro- and antiapoptotic Bcl-2-like proteins [36].

Beside its role in numerous physiological settings (*e.g.*, embryonic and post-embryonic development, homeostasis of highly proliferating

Table 1
Consequences of mitochondrial membrane permeabilization (MMP) [12]

Primary event	Outcomes — observations	Ref.
Bioenergetic failure		
Ca^{2+} release	Favors PTPC opening in yet unaffected mitochondria.	[167]
	Favors the activation of Ca^{2+}-dependent catabolic enzymes (*e.g.*, calpains).	[167]
$\Delta\Psi_m$ dissipation	Blockade of ATP synthesis and of other mitochondrial metabolisms.	[28, 29]
ROS overproduction	Uncoupling of the respiratory chain, in turn favoring MPT.	[168]
	ROS favor MPT also in yet unaffected mitochondria.	[28,29]
Morphological, structural and ultrastructural modifications		
Fragmentation of the mitochondrial network	Underscores the implication of the mitochondrial fusion/fission machinery in cell death pathways.	[169,170]
Mitophagy	Permeabilized mitochondria are selectively sent to autophagic removal.	[171,172]
Swelling	Results from the unregulated entry of solutes and water in the mitochondrial matrix that follows MPT.	[12]
Release into the cytosol of IMS proteins (examples)		
AIF	Together with CypA, translocates to the nucleus where it mediates chromatin condensation and DNA degradation.	[14,126]
Cyt *c*	Interacts in a dATP-dependent fashion with Apaf-1 to assemble the apoptosome, a molecular platform for the activation of pro-caspase-9.	[15,16]
	De-inhibits IP_3Rs, thereby favoring the increase of cytosolic Ca^{2+}.	[173]
	Its loss results in the uncoupling of the respiratory chain, in turn favoring ROS overgeneration and the bioenergetic impairment of mitochondria.	[174]
EndoG	Translocates to the nucleus and mediates caspase-independent internucleosomal DNA fragmentation.	[13,143]
Heat-shock proteins (*e.g.*, Hsp10, Hsp60)	Their depletion from the IMS favors the disruption of the structural integrity of mitochondria and the impairment of ATP generation.	[175]
	Consistent with their role of chaperones, they accelerate caspase activation.	[77]
Omi/HtrA2	Binds to and hence sequestrates IAPs, in turn favoring caspase activation.	[150]
	Cleaves IAPs and other substrates, thereby promoting caspase-dependent and -independent executioner mechanisms of cell death.	[17,152]
Pro-caspases (*e.g.*, -3, -9)	Kept in an inactive state by S-nitrosylation in the IMS, upon release they add to the cytosolic pool of caspases ready for proteolytic processing. Pro-caspase-9 can be directly activated by redox stress within mitochondria.	[73,74,176]
Smac/DIABLO	Binds to and hence sequestrates IAPs, in turn favoring the activation of caspases in an indirect fashion.	[18,159,162]

Abbreviations: AIF, apoptosis-inducting factor; Apaf-1, apoptotic peptidase activating factor 1; Ca^{2+}, calcium; CypA, cyclophilin A; Cyt *c*, cytochrome *c*; $\Delta\Psi_m$, mitochondrial transmembrane potential; EndoG, endonuclease G; Hsp, heat-shock protein; IAP, inhibitor of apoptosis protein; IMS, mitochondrial intermembrane space; IP_3R, inositol (1,4,5) trisphosphate receptor; MPT, mitochondrial permeability transition; Omi/HtrA2, Omi stress-regulated endoprotease/high temperature requirement protein A 2; PTPC, permeability transition pore complex; ROS, reactive oxygen species; Smac/DIABLO, second direct activator of caspases/direct IAP-binding protein with a low pI.

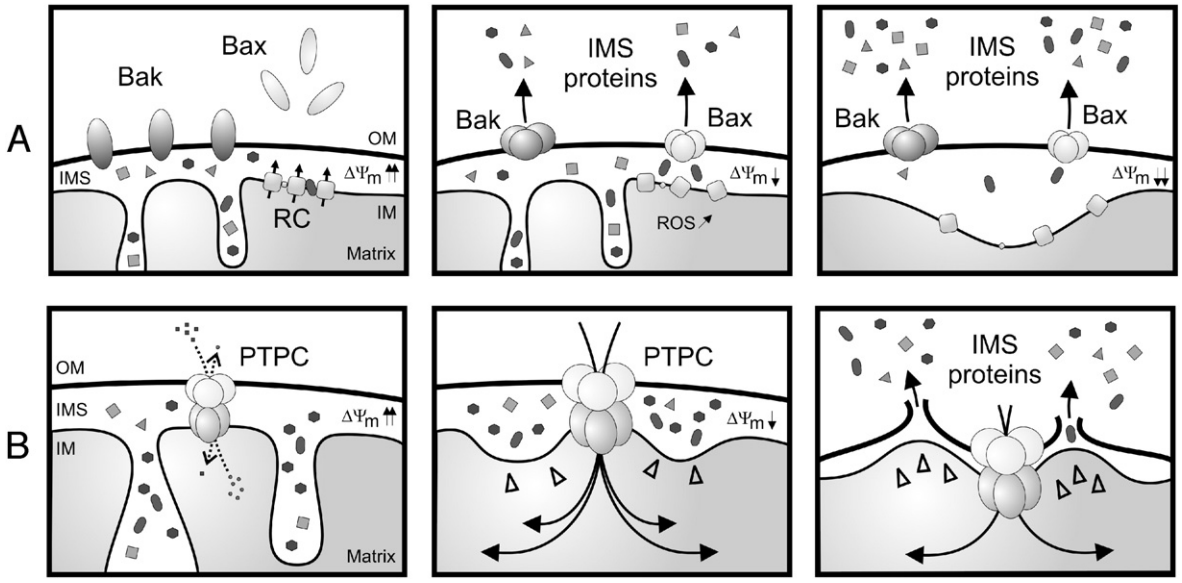

Fig. 1. Models of mitochondrial membrane permeabilization (MMP). (A) In some instances, MMP originates at the mitochondrial outer membrane (OM), due to the pore-forming activity of proapoptotic proteins from the Bcl-2 family (*e.g.*, Bak, Bax). In physiological conditions, Bak and Bax exist in an inactive conformation, loosely associated with the OM and in the cytosol, respectively. Under proapoptotic stimulation, they undergo a conformational change that allows them to fully insert into the OM and to form homo-/heteromeric pores though which mitochondrial intermembrane space (IMS) proteins are released. These include soluble components of the respiratory chain (RC) such as cytochrome *c*. The release of IMS proteins favors mitochondrial uncoupling, overgeneration of reactive-oxygen species (ROS) and dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$), which altogether account for the functional impairment of mitochondria. (B) In other cell death scenarios, MMP is started at the inner mitochondrial membrane (IM), following the activation of the permeability transition pore complex (PTPC). In healthy cells, the PTPC ensures the exchange of metabolites between the mitochondrial matrix and the cytosol. In response to some proapoptotic triggers (*e.g.*, Ca^{2+} overload, oxidative stress), the PTPC opens, thereby allowing for the unregulated entry of solutes and water into the mitochondrial matrix. This abrupt increase in the permeability of the IM – also known as mitochondrial permeability transition (MPT) – results in the osmotic swelling of the mitochondrial matrix, and eventually in the osmotic pressure-driven rupture of the OM.

tissues), mitochondrial apoptosis has been implicated in a number of human diseases. Thus, while disabled apoptosis is associated with both the development and the limited therapeutic response of cancer, disproportionate cell loss contributes to acute and chronic degenerative pathologies in a variety of organs, including liver, heart, kidney and the central nervous system (CNS) [12]. In all these pathological settings, cell death does not occur exclusively with an apoptotic morphology but may also manifest with a necrotic and/or autophagic appearance [12,37,38].

As compared to other cells, neurons have a very high energetic demand. This is a direct consequence of the molecular processes underlying their excitability (all of which require large amounts of ATP), which include the maintenance of a precise ionic homeostasis, axonal and dendritic transports, and the release/reuptake of neurotransmitters at the synaptic cleft. Moreover, since neurons cannot switch to anaerobic glycolysis when oxidative phosphorylation is limited, they are even more dependent than other cell types on mitochondrial metabolism. For these reasons, neurons are especially sensitive to glucose and oxygen deprivation as they occur after ischemic and traumatic insult of the CNS [39,40].

During acute neuronal damage, necrosis is the most prominent cell death phenotype in the core of lesions, whereas apoptosis predominates in adjacent zones, at the boundary between the lesion and healthy tissue (also known as the "penumbra"). Since the necrotic core is presumably damaged beyond any possibility of recovery, therapeutic interventions most frequently aim at inhibiting apoptosis, and therefore at reducing neuronal loss in the penumbra [41]. In this context, the inhibition of MMP confers neuroprotective effects *in vivo*, in numerous animal models of CNS ischemia and trauma (Table 2) [42].

In the best case scenario, patients affected by stroke or trauma are treated within dozens of minutes, but usually this occurs only within hours, that is after MMP has been initiated. Since ischemic and traumatic injuries of the CNS are responsible for the protracted disability and death of millions of individuals throughout the world, and since current treatments are mainly intended to reduce cellular infiltrate, limit edema and favor the resolution of inflammation (and hence in a way represent symptomatic therapies), novel neuroprotective strategies that actively prevent the acute loss of neurons are urgently awaited. Such interventions should target the post-mitochondrial effectors of apoptosis, allowing to block the cascade of events that lead to neuronal cell death when MMP has already occurred. Nonetheless, even the possibility to target post-MMP events would not abolish the need to treat patients affected by stroke or CNS trauma with maximal urgency. First, the early removal of the cause of injury (for instance the dissolution of an arterial clot) is known to limit the area of neuronal damage, by reducing the duration of the insult. In addition, the efficacy of post-mitochondrial apoptosis inhibitors would be greatly improved if they were administered during the first phases of cell death, before catabolic reactions have been ignited. Finally, in case of an early intervention (and in particular before all affected neurons have undergone MMP), combination therapies that associate MMP blockers with inhibitors of post-mitochondrial apoptosis might confer enhanced neuroprotection, by preventing unwarranted cell death both upstream and downstream of mitochondria.

2. Keeping Pandora's box closed: inhibition of mitochondrial membrane permeabilization (MMP)

The inhibition of MMP by pharmacological and/or genetic means has been associated with significant neuroprotective effects *in vivo*, in multiple animal models of stroke or traumatic injury of the spinal cord (Table 2). In this context, the cascade of events that leads to cell death is interrupted well before the functional impairment of mitochondria and the massive activation of catabolic reactions, which represents the most efficient strategy to prevent neuronal loss. In spite of the fact that this approach is not realistic in the clinical setting, several studies have corroborated its (at least) theoretical validity [40, 42].

Pharmacological inhibitors of the pore-forming activity of Bax (but not of its conformational activation nor of its translocation to

Table 2
Examples of neuroprotection conferred *in vivo* by mitochondrial membrane permeabilization (MMP) inhibitors

Target	Strategy/Model	Observations	Ref.
Pre-mitochondrial signaling (Initiation phase)			
JNK	*jnk3* gene knockout	Following H/I, *jnk3*$^{-/-}$ mice exhibited less Casp-3 activation and reduced tissue loss in the cerebral cortex, hippocampus, striatum and thalamus as compared to WT animals.	[177]
	Systemic administration of a JNK-specific peptide inhibitor (Tat-JBD)	Upon I/R, Tat-JBD-treated rats exhibited lower Bax mitochondrial translocation, Cyt *c* release, Casp-3 activation, PARP-1 cleavage and TUNEL reactivity than control animals.	[70]
	Systemic administration of a small JNK-specific inhibitor (SP600125)	Upon focal I/R, treated mice displayed reduced mitochondrial translocation of Bax and Bim, IMS proteins release, Casp-3 and -9 activation, and infarct volume as compared to control animals.	[69]
p53	*p53* gene knockout	Following kainate-induced excitotoxicity, neuronal damage was limited to the dorsal hippocampus in *p53*$^{-/-}$ mice, while it also involved the amygdala, piriform and cerebral cortex, caudate-putamen and thalamus in WT animals.	[67]
	Systemic administration of a small p53-specific inhibitor (pifithrin-α)	Pifithrin-treated mice exhibited increased resistance of cortical and striatal neurons to tFCI and of hippocampal neurons to excitotoxic damage as compared to control animals.	[66]
SOD1	Whole-body overexpression	Upon tFCI, SOD1-overexpressing mice had lower oxidative injury, reduced release of IMS proteins from mitochondria, and enhanced XIAP/Casp-9 interaction than WT animals.	[158,166]
Mitochondrial events (Decision/integration phase)			
ANT	Oral administration of HIV-1 PIs (nelfinavir and ritonavir)	PIs prevented AIF and Cyt *c* release from mitochondria, Casp-9 activation and photoreceptor cell loss in a murine model of RD. Upon MCAO, PI-treated mice showed lower TUNEL reactivity and infarct volumes than control animals.	[108,134]
Bad	*bad* gene knockout	Following H/I, *bad*$^{-/-}$ mice exhibited reduced Casp-3 activation and GFAP positivity in their hippocampi as compared to WT animals, which correlated with reduced tissue loss.	[54]
Bax	*bax* gene knockout	*bax*$^{-/-}$ mice subjected to CCI exhibited fewer TUNEL^{+} cells in the hippocampus than their WT littermates (but performed worse in behavioral tests, independently of the traumatic injury).	[50,51]
	Intraperitoneal injection of small BCIs	Upon tGCI, hippocampi from BCI-treated gerbils exhibited lower Cyt *c* release and more limited tissue damage than hippocampi from saline-treated animals.	[43]
	Intravenous administration of TUDCA before or after the injury	Following tFCI and ICH, TUDCA-treated rats displayed reduced TUNEL positivity, mitochondrial swelling, Casp activation and lesion volume than control animals, which correlated with an improvement in neurobehavioral deficits.	[44,45]
Bcl-2	Neuron-specific overexpression	In mice overexpressing Bcl-2 under the control of a neuron-specific promoter, MCAO-induced brain infarction volume was reduced by approximately 50% as compared to WT animals.	[47]
Bcl-X_L	Neuron-specific overexpression	Mice overexpressing Bcl-X_L under the control of a cortex-specific promoter exhibited a 20% reduction in MCAO-induced brain infarction volume as compared to their WT littermates.	[49]
Bid	*bid* gene knockout	After MCAO or mild focal ischemia, *bid*$^{-/-}$ mice displayed lower Cyt *c* release and infarct volumes than their WT counterparts.	[52,53]
Bim	*bim* gene knockout	Upon H/I, *bim*$^{-/-}$ mice displayed reduced Casp-3 activation and GFAP positivity in their brains as compared to WT animals, which correlated with reduced hippocampal tissue loss.	[54]
CypD	Intraperitoneal injection of a non-immunosuppressive CsA analogue (MeVal-CsA)	Upon MCAO, MeVal-CsA-treated rats exhibited lower cortical and striatal infarct size than vehicle-treated animals.	[56]
	Intravenous administration of CsA after the injury	High CsA concentrations, the presence of an intracerebral needle lesion or the intracarotid infusion of mannitol allowed CsA to cross the BBB and reduced tFCI-induced infarct size in rats.	[57]
	ppif gene knockout	Following MCAO, *ppif*$^{-/-}$ mice displayed a dramatic reduction in brain infarct size as compared to their WT littermates.	[61]
	Systemic administration of CsA before the injury	In response to tFCI, CsA-treated rats showed decreased ischemic brain edema and infarct size as compared to control animals.	[55]

Abbreviations: AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; BBB, blood–brain barrier; BCIs, Bax channel activity inhibitors; BH, Bcl-2 homology; Casp, caspase; CCI, controlled cortical impact; CsA, cyclosporine A; CypD, cyclophilin D, GFAP, glial fibrillary acidic protein; H/I, hypoxia/ischemia; HIV-1, human immunodeficiency virus 1; IAP, inhibitor of apoptosis protein; ICH, intracerebral hemorrhage; IMS, mitochondrial intermembrane space; I/R, ischemia/reperfusion; JBD, JNK binding domain of JIP-1; JIP-1, JNK-interacting protein 1; JNK, c-Jun N-terminal kinase; MCAO, middle cerebral artery occlusion; PARP-1, poly(ADP-ribose) polymerase 1; *ppif*, peptidyl–prolyl cis–trans isomerase f; PI, protease inhibitors; RD, retinal detachment; SOD1, copper/zinc-superoxide dismutase; tFCI, transient focal cerebral ischemia; tGCI, transient global cerebral ischemia; Tat, Tat peptide from HIV-1; TUDCA, tauroursodeoxycholic acid; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling; WT, wild type; XIAP, X-linked IAP.

mitochondria) have been shown to exert neuroprotective effects in an animal model of global brain ischemia [43]. Also, the endogenous bile acid tauroursodeoxycholic acid (TUDCA) protected rats against neurological injury after transient focal cerebral ischemia (tFCI) [44] and intracerebral hemorrhage [45], presumably by inhibiting the association of Bax with mitochondria [46]. The implication of Bcl-2-like proteins in the pathogenesis of CNS ischemia and trauma has been substantiated by the use of genetically engineered animals to achieve whole-body or tissue-specific changes in the expression of Bcl-2 family members. As an example, mice overexpressing antiapoptotic members of the Bcl-2 family such as Bcl-2 itself [47,48] or Bcl-X_L [49] displayed reduced tissue damage after permanent focal ischemia. Similarly, *bax*$^{-/-}$ mice had significantly less hippocampal tissue loss upon neonatal hypoxia/ischemia (H/I, a model of cerebral palsy) [50] or controlled cortical impact (CCI, a model of traumatic brain injury) [51] than *bax*$^{+/-}$ and *bax*$^{+/+}$ animals, and adult mice devoid of the BH3-only protein Bid exhibited decreased Cyt *c* release from mitochondria and reduced infarct volumes – as compared to their wild type (WT) littermates – after transient focal ischemia [52,53]. However, as opposite to *bim*$^{-/-}$ and *bad*$^{-/-}$ animals (which displayed decreased parenchymal loss as compared to control littermates), *bid*$^{-/-}$ mice were not protected from neonatal H/I [54]. This suggests that BH3-only proteins may be selectively implicated in distinct scenarios of ischemic injury.

More than 15 years ago, the immunosuppressant cyclosporine A (CsA), which binds to CypD and hence inhibits PTPC opening, has been shown to confer neuroprotection against focal ischemia, when administered for several days prior to injury [55]. Similar effects were observed with the CsA derivative MeVal-CsA, which blocks MPT but does not bind to calcineurin and therefore lack immunosuppressant activities [56]. This proved that the neuroprotective effects of CsA are (at least partially) due to MPT inhibition, in spite of the fact that other immunosuppressants that do not bind to CypD (*e.g.*, FK-506) may also protect from focal and permanent ischemia [57–59]. More recently, the crucial role of PTPC-dependent MPT in ischemic injury has been unquestionably corroborated by studies in *ppif*$^{-/-}$ mice (which lack the expression of CypD) [60]. CypD-deficient mice displayed a dramatic reduction in brain infarct size following acute

middle cerebral artery occlusion (MCAO) and reperfusion [61]. Since mouse embryonic fibroblasts (MEFs) and mitochondria isolated from *ppif*$^{-/-}$ mice were relevantly resistant to triggers of necrosis (including Ca^{2+} overload and oxidative stress) but normally responsive to apoptotic stimuli, CypD deficiency was suggested to confer neuroprotection *in vivo* by regulating necrotic, rather than apoptotic, cell death [62]. Interestingly, a role for other components of the PTPC (and in particular for ANT and VDAC) in CNS ischemia and trauma has not (yet) been unquestionably demonstrated [12], in line with the fact that no sign of cell death resistance could be detected *in vitro* for *ant*$^{-/-}$ [63] and *vdac*$^{-/-}$ [64,65] cells.

Although this is beyond the scope of the present review, it should be noted that significant levels of neuroprotection have been achieved *in vivo* by inhibiting MMP further upstream, at the initiation phase that precedes mitochondrial commitment. Just to mention some examples, neuroprotective effects against different types of CNS injury (including ischemia, trauma and excitotoxicity) have been recorded upon pharmacological [66] and genetic [67] inhibition of the tumor suppressor protein p53, in mice lacking the neuronal nitric oxide synthase (NOS) gene [68], as well as upon systemic administration of small molecules [69] or peptides [70] designed to block the c-Jun N-terminal kinase (JNK) signaling pathway.

3. When Pandora's box has opened: inhibition of post-mitochondrial apoptotic effectors

When MMP has already occurred, as in the majority of affected neurons in stroke and/or trauma patients, therapeutic interventions to limit neuronal loss should be directed at inhibiting the post-mitochondrial events that lead to cell death. Hereafter, we will summarize the results of studies performed in animal models of acute neuronal injury (Table 3).

3.1. The caspase cascade

Upon MMP, several IMS proteins that directly promote the activation of the caspase cascade are released into the cytosol (Fig. 2) [71]. These include: Cyt *c*, which together with the cytosolic protein apoptotic peptidase activating factor 1 (Apaf-1) and dATP forms the so-called "apoptosome", a molecular platform that recruits and allosterically activates pro-caspase-9 [15,16]; multiple pro-caspases that act as apoptotic executioners downstream of mitochondria and/or that are implicated in feedforward circuitries to amplify proapoptotic signals [72], such as pro-caspase-2 [73], -3 [74], -8 [75] and -9 [73,76]; as well as chaperones of the heat-shock protein (Hsp) family (*e.g.*, Hsp10, Hsp60), which may modulate the activity of the apoptosome [77].

Caspase-3 is classically considered as the prototype of executioner caspases and is the most abundant caspase in the brain [78–80]. Most *casp-3*$^{-/-}$ mice die perinatally and manifest a hyperplasic, disorganized brain [81], which demonstrates that caspase-3 is crucial during the embryonic development of the nervous system. Acute inhibition of caspases via quite unspecific pharmacological agents (such as the pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-fluoromethylketone, *i.e.*, Z-VAD-fmk) administered after the insult has been reported to confer neuroprotection in adult models of ischemia [82–85] and traumatic CNS injury [86–88]. In other rat models of transient focal or global ischemia, however, the activation of caspases could not be demonstrated, and pan-caspase inhibitors such as Z-VAD-fmk or (relatively) caspase-3-specific blockers like Z-Asp(OMe)-Glu(OMe)-Val-Asp (OMe)-fluoromethylketone (Z-DEVD-fmk) failed to provide any neuroprotective effect [89,90]. This argues against a generalized implication of the caspase cascade in ischemic brain injury, and suggests that (at least in some scenarios), caspase-independent mechanisms may prevail. Moreover, a selective caspase-3/-7 inhibitor (M826) administered immediately after the insult [91] as well as more unspecific agents (such as boc-Asp(OMe)-fmk, *i.e.*, boc-D-fmk) [92,93] have been demonstrated to confer impressive protection during neonatal H/I injury [91,92] and after traumatic brain injury [93]. Thus, the involvement of caspases during ischemic CNS injury may not only vary in different pathological settings, but also during aging (from neonatal to adult life), in line with age-associated changes in the expression and activity levels of multiple apoptotic regulators (and in particular of caspases) [94,95]. Although the vast majority of *casp-3*$^{-/-}$ mice die perinatally due to extensive brain hyperplasia and impaired CNS development [81], some animals exhibit milder defects and reach adulthood, during which they are more resistant to ischemic injuries than their WT littermates [96]. Moreover, strain-specific brain phenotypes resulting from caspase-3 deficiency have been reported [97], presumably due to the "rescue" by other caspases, and in particular by caspase-7 [98]. Interestingly, it has been recently suggested that the long-term inhibition of caspase-3 during development (as it occurs in *casp-3*$^{-/-}$ and *casp-3*$^{+/-}$ mice) leads to the upregulation of caspase-independent cell death routines, thereby increasing the vulnerability of the developing brain to neonatal H/I injury [99].

The neurodevelopmental phenotype of *casp-3*$^{-/-}$ mice [81], which include marked ventricular zone expansion, exencephaly and ectopic neuronal structures, is recapitulated in both *apaf-1*$^{-/-}$ [100] and *casp-9*$^{-/-}$ animals [97,101], suggesting that these proteins ensure the correct development of the CNS by participating in a common cell death pathway. The knockout of the other component of the apoptosome, *i.e.*, Cyt *c*, results in an even more severe phenotype (*cyc1*$^{-/-}$ embryos die *in utero* by midgestation) [102], due to its essential function as an electron shuttle in the respiratory chain [15]. This has greatly limited the possibility to analyze *in vivo*, in adult animals, the role of Cyt *c*, Apaf-1 and caspase-9 during ischemic and/or traumatic brain injuries. Recently, mice that express a mutant Cyt *c* (the *KA* mutation), which retains normal electron transfer functions but fails to activate the apoptosome, have been generated [103]. Most *KA*/*KA* mice exhibit embryonic or perinatal lethality due to extensive CNS disarrangement, which demonstrates that the lethal (but not the vital) functions of Cyt *c* are implicated in CNS development [103]. Moreover, the forebrain overgrowth (*fog*) mutation, which was described as a spontaneous recessive mutation mapping to mouse chromosome 10, coincides with an hypomorphic Apaf-1 defect that leads to reduced levels of normal Apaf-1 mRNA [104]. Although *fog*/*fog* mice are characterized by forebrain abnormalities, facial deformities and spina bifida, they survive into adulthood, thereby representing valuable models to study Apaf-1 deficiency *in vivo* [104].

Nevertheless, for the abovementioned reasons, few studies have evaluated the role of the constituents of the apoptosome during acute neuronal injury, mostly based on (unspecific) pharmacological agents. Selective inhibition of caspase-9 after tFCI (as obtained by intraventricular injection of a specific inhibitor 15 min after the insult) was reported to reduce total infarction volume and to improve the neurological score in a rat model of ischemia/reperfusion (I/R) injury [105]. Similarly, repeated intraocular injection of specific caspase-9 inhibitors was shown to prevent the death of retinal ganglion cells upon optic nerve transection (a model of traumatic injury) in rats [106]. At present, other pharmacological inhibitors of the apoptosome are not yet available for *in vivo* use. Interestingly, small molecules that bind to and inhibit Apaf-1 have been recently identified [107], but their activity in animal models of acute neuronal injuries remains to be established. A recent study indicates that Apaf-1 is required (together with AIF, see below) for the death of photoreceptor cells that occurs in a murine model of retinal detachment [108]. In this context, the *fog*/*fog* genotype resulted in decreased activation of caspases and conferred significant neuroprotection (as compared to WT animals).

Given the destructive consequences of massive caspase activation, it is not surprising that cells have evolved multiple caspase-suppressive mechanisms, including the family of inhibitor of apoptosis proteins (IAPs) [109]. Most IAPs share a baculoviral IAP repeat (BIR)

Table 3
Examples of neuroprotection conferred *in vivo* by inhibitors of post-mitochondrial effectors of apoptosis

Target	Strategy/Model	Observations	Ref.
Caspase-dependent cell death mechanisms			
Apaf-1	*fog*/*fog* mice (homozygous hypomorphic Apaf-1 mutation)	Upon RD, retinae from *fog*/*fog* mice exhibited lower Casp-activation, TUNEL positivity, photoreceptor cell loss and overall histological derangement than their counterparts from WT animals.	[108]
Caspases	Intracerebroventricular injections of boc-D-fmk after the insult	24 h after TBI, boc-D-fmk-treated rats displayed decreased Cyt *c* release, Casp-3 activation, TUNEL reactivity and cortical tissue loss as compared to control animals, but this did not resulted in long-term neuroprotection and improvement of motor function.	[93]
	Intracerebroventricular and systemic injections of boc-D-fmk after the insult	In rats subjected to neonatal H/I, boc-D-fmk strikingly reduced Casp-3 activation, TUNEL positivity and striatal, cortical and hippocampal tissue loss, as compared to a control peptide.	[92]
	Intracerebroventricular injection of Z-VAD-fmk before and after the insult	Z-VAD-fmk-treated mice subjected to MCAO or excitotoxic brain damage exhibited limited increase in tissue IL-1β levels and improved behavioral deficits as compared to untreated animals.	[82]
		Upon MCAO, Z-VAD-fmk-treated mice displayed lower TUNEL staining in the striatum than control animals, which correlated with decreased infarct size and reduced neurologic deficits.	[83,85]
		Infarction volumes following cold injury-brain trauma were significantly reduced in Z-VAD-fmk-treated mice as compared to vehicle-treated animals.	[86]
	Intrastriatal injection of Z-VAD-fmk after the insult	Z-VAD-fmk protected mice against malonate-induced striatal histotoxic hypoxic lesions, as assessed by reduced lesion volume as compared to untreated animals.	[84]
	Local application of Z-VAD-fmk-soaked sponges on contused spinal cords	In mice, post-SCI Casp-3 activation, TUNEL reactivity, lesion size and neurologic deficits were significantly ameliorated by the local application of Z-VAD-fmk, as compared to vehicle control.	[87]
	Systemic (?) administration of Z-VAD-fmk	Upon SCI, Z-VAD-fmk-treated mice exhibited reduced TUNEL positivity, tissue injury and inflammatory reactions than untreated animals, which correlated with improved recovery of limb function.	[88]
Casp-3	*casp-3* gene knockout	After MCAO, *casp-3*$^{-/-}$ mice exhibited decreased TUNEL positivity and cortical infarct volume, as compared to their WT littermates.	[96]
	Intracerebroventricular injection of M826 after the insult	Upon H/I, M826-treated mice displayed reduced Casp-3 activation, cleavage of Casp-3 substrates, DNA fragmentation and brain tissue loss, as compared to untreated control animals.	[91]
	Intracerebroventricular injection of Z-DVED-fmk before and after the insult	Following MCAO, the striatum of Z-DVED-fmk-treated mice exhibited reduced TUNEL positivity as compared to control animals, which was accompanied by decreased infarct size and minor neurologic deficits.	[83,85]
Casp-9	Intracerebroventricular injection of Z-LEHD-fmk after the insult	Z-LEHD-fmk-treated rats (as compared to vehicle-treated animals) subjected to MCAO exhibited a decrease in total infarct volume and improved neurological outcomes.	[105]
	Intraocular injection of Casp-9-specific inhibitors	In rats, casp-9 inhibition prevented the death of axotomized RGCs that follows optical nerve transection (a model of TBI).	[106]
NAIP	Intrahippocampal injection of an adenoviral overexpression vector before the insult	In rats subjected to tFCI, adenovirus-mediated overexpression of NAIP (but not of β-gal) reduced DNA fragmentation and hippocampal neuron loss.	[114]
	Systemic administration of the bacterial alkaloid K252a before the insult	Upon tFCI, K252a-treated rats exhibited reduced DNA fragmentation and hippocampal neuron loss, as compared to vehicle-treated animals.	[114]
p35	Adenoviral delivery of a transient overexpression vector after the insult	In adult rats, local administration of an adenoviral vector for the overexpression of p35 (but not of β-gal) at the nerve stump reduced the death of RGCs following axotomy.	[111]
	Oligodendrocyte-specific overexpression	Upon SCI, transgenic mice demonstrated a lesser extent of demyelination and cell death, as well as an improved recovery of hindlimb motor function, as compared to control animals.	[118]
XIAP	Adenoviral delivery of a transient overexpression vector after the insult	In adult rats, local administration of a XIAP-overexpressing (but not of a β-gal-overexpressing) adenoviral vector at the nerve stump inhibited the death of RGCs following axotomy.	[111]
	Adenoviral delivery of a transient overexpression vector before the insult	Transgenic mice undergoing tFCI displayed reduced Casp-3 activation, TUNEL positivity and hippocampal neurodegeneration as compared to their WT littermates.	[113]
	Intraperitoneal injection of PTD-BIR3-RING peptide before and after the insult	Rats treated with PTD-BIR3-RING and subjected to MCAO were characterized by lower Casp-3 activation and TUNEL reactivity than untreated rats, which correlated with improved NSS.	[116]
	Neuron-specific overexpression	Mice subjected to MCAO exhibited lowered TUNEL labeling, decrease in protein synthesis and Casp-3 activation in neurons as compared to WT animals.	[178]
	Whole-body overexpression	As opposed to WT animals, transgenic mice subjected to H/I almost failed to activate casp-9 and -3, which correlated with reduced tissue loss in multiple brain regions.	[112]
Caspase-independent cell death mechanisms			
AIF	Male *Hq*/*Y* mice (hemizygous hypomorphic AIF^{Hq} mutation)	In a model of kainic acid-induced seizure, *Hq* mice exhibited lower levels of neuronal loss and hippocampal damage than WT animals.	[129]
		After MCAO, *Hq* mice exhibited decreased infarct volumes and dramatically reduced cell death in the ischemic penumbra, as compared to their WT littermates subjected to the same insult.	[130]
		Following RD, retinae from *Hq*/*Y* mice displayed lower Casp-activation, TUNEL positivity, photoreceptor cell loss and overall histological derangement than their counterparts from WT animals.	[108,128]
	Male (*Hq*/*Y*) and female (*Hq*/*Hq*) newborn mice (hemi- and homozygous AIF^{Hq} mutation, respectively)	Upon H/I, *Hq* mice showed infarct volumes reduced by approximately 50% as compared to their WT littermates. These neuroprotective effects were further enhanced by the administration of a broad-spectrum caspase inhibitor or antioxidants.	[131,132]
CypA	*cypA* gene knockout	*cypA*$^{-/-}$ and *cypA*$^{+/-}$ mice subjected to H/I failed to exhibit the nuclear translocation of AIF that was observed in WT mice, which correlated with a reduction in infarct size of approximately 50%.	[123]
Modulators of both caspase-dependent and -independent cell death			
Hsp70	Brain-specific overexpression of WT and mutant Hsp70	Following MCAO, mice overexpressing in the brain WT Hsp70 (as well as ATPase-deficient Hsp70 mutants) showed lower levels of nuclear AIF than control animals, had significantly smaller infarcts and higher neurological scores.	[142]
	Whole-body overexpression	Upon H/I, mice overexpressing rat Hsp70 showed limited release of Cyt *c* and increased AIF/Hsp70 binding, which correlated with significant neuroprotection, as compared to WT animals.	[140]

Abbreviations: AIF, apoptosis-inducting factor; Apaf-1, apoptotic peptidase activating factor 1; β-gal, β-galactoside; boc-D-fmk, boc-Asp(OMe)-fmk; Casp, caspase; CypA, cyclophilin A; Cyt *c*, cytochrome *c*; BIR3, baculoviral IAP repeat 3 domain; *fog*, facial overgrowth; H/I, hypoxia/ischemia; *Hq*, Harlequin; Hsp70, heat-shock 70 kDa protein; IL-1β, interleukin 1; MCAO, middle cerebral artery occlusion; NAIP, neuronal apoptosis inhibitory protein; NSS, neurological severity score; PTD, protein transduction domain; RD; retinal detachment RGCs, retinal ganglion cells; RING, real interesting new gene; SCI, spinal cord injury; TBI, traumatic brain injury; tFCI, transient focal cerebral ischemia; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling; WT, wild type; Z-DEVD-fmk, Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; Z-LEDH-fmk, Z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone; Z-VAD-fmk, Z-Val-Ala-Asp(OMe)-fluoromethylketone.

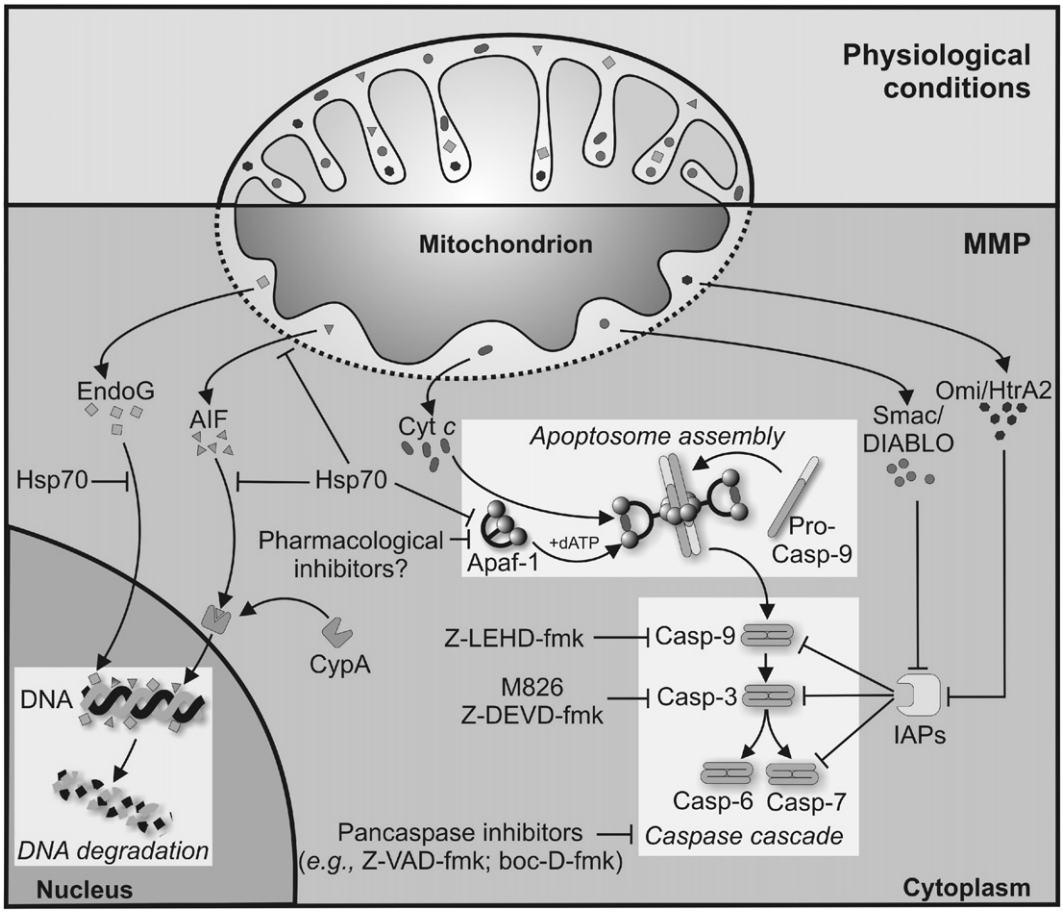

Fig. 2. Inhibition of post-mitochondrial effectors of apoptosis for neuroprotection. Mitochondrial membrane permeabilization (MMP) leads to the release into the cytosol of a plethora of proteins that are normally found within the mitochondrial intermembrane space. Upon MMP, several among these factors contribute to cell death. Pharmacological and genetic approaches that target post-mitochondrial cell death effectors have been shown to confer neuroprotective effects in various animal models of acute neuronal damage, including (but not limited to) models of focal and global brain ischemia, neonatal hypoxia/ischemia and traumatic injury of the spinal cord. Please, refer to the main text for further details. AIF, apoptosis-inducing factor; Apaf-1, apoptotic peptidase activating factor 1; boc-D-fmk, boc-Asp(OMe)-fmk; Casp, caspase; CypA, cyclophilin A; Cyt *c*, cytochrome *c*; EndoG, endonuclease G; Hsp70, heat-shock 70 kDa protein; IAPs, inhibitor of apoptosis proteins; Omi/HtrA2, Omi stress-regulated endoprotease/high temperature requirement protein A 2; Smac/DIABLO, second direct activator of caspases/direct IAP-binding protein with a low pI; Z-DEVD-fmk, Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; Z-LEDH-fmk, Z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone; Z-VAD-fmk, Z-Val-Ala-Asp(OMe)-fluoromethylketone.

domain and a real interesting new gene (RING) finger motif, by which they interact with and therefore prevent the activation of caspases [109,110]. In multiple models of acute neuronal injury, IAPs have been shown to mediate neuroprotective effects. Thus, transgenic over-expression of the X-linked IAP (XIAP) in the brain conferred neuroprotection against axotomy [111], neonatal H/I [112] and tFCI in adult mice [113]. Similarly, enhanced expression of the neuronal apoptosis inhibitory protein (NAIP) reduced tFCI-induced damage in the rat brain [114]. Interestingly, tFCI increases the ubiquitination of XIAP in rat brain, which in turn might modulate its subcellular distribution and activity [115]. More recently, the intraperitoneal injection of a chimeric peptide made by the BIR3-RING domains of XIAP fused to the protein transduction domain (PTD) of antennapedia homeodomain (Antp HD) has been shown to efficiently transduce hippocampal neurons, thereby exerting neuroprotective effects against tFCI in rats [116]. Viral proteins that inhibit caspases have also been used to develop neuroprotective strategies.[117] For instance, the broad spectrum caspase inhibitor p35 has been shown to prevent the death of retinal ganglion cells (RGCs) in rats subjected to optic nerve transection [111], as well as to reduce demyelination and motor function loss in a murine model of spinal cord injury (SCI) [118]. These few examples underscore the notion that the IAP family as well as exogenous caspase inhibitors might represent promising targets to control the caspase cascade for neuroprotection.

3.2. Mitochondrial DNA-degrading enzymes

Apoptosis-inducing factor (AIF) is an IMS NADH oxidase with a local redox activity that is required for the correct assembly and/or function of the respiratory chain [14], as demonstrated by the fact that AIF deficiency results in reduced oxidative phosphorylation and lowered expression of complex I and III subunits, *in vivo* [119]. Mice bearing the Harlequin (*Hq*) mutation express only 20% of the AIF levels of their WT counterparts, due to a retroviral insertion in the first intron of the AIF gene. Although young *Hq* mice do not exhibit obvious phenotypic alterations, adult animals develop ataxia, cerebellar atrophia and blindness [120]. Muscle-specific knockout of AIF leads to severe mitochondrial dysfunction, skeletal muscle atrophy and dilated cardiomiopathy [121]. Moreover, muscle- and liver-specific AIF ablation leads to a pattern of metabolic changes that counteract the development of insulin resistance, diabetes and obesity [122]. Upon MMP, AIF is released into the cytosol and translocates to the nucleus, where it promotes (together with its obligate cofactor cyclophilin A, *i.e.*, CypA) chromatin condensation and DNA degradation[123,124] independently of caspases [125,126]. Although the contribution of AIF to cell death is highly variable according to the specific experimental and/or pathophysiological setting [21], this caspase-independent executioner has been repeatedly described as a major determinant in the demise of neurons after acute injury [127,128].

Adult *Hq* mice exhibited significantly less brain damage upon kainic acid-induced seizures [129] and MCAO [130] than their WT littermates. Similarly, AIF deficiency has been shown to confer neuroprotective effects following neonatal H/I [131,132] that could be further enhanced by the administration of broad spectrum caspase inhibitors or antioxidants [132]. In rodent models of retinal detachment (obtained by the subretinal injection of sodium hyaluronate), AIF is required for the death of photoreceptor cells [128,133]. In this system, the hypomorphic AIF^{Hq} mutation (as well as the *fog*/*fog* genotype, see above) provided significant protection against photoreceptor apoptosis [108]. Even more remarkable effects resulted from the use of an antiapoptotic Bcl-2-derived BH4 peptide coupled to a membrane-permeant sequence, as well as from the oral administration of the HIV protease inhibitors nelfinavir and ritonavir [108], which are known to inhibit MMP at the mitochondrial level by interacting with ANT [134]. In line with *in vitro* data suggesting that AIF must interact with CypA to form a proapoptotic DNA degradation complex [124], neurons from *Hq* mice subjected to H/I failed to manifest the nuclear translocation of CypA that was observed in WT neurons [123]. More importantly, the H/I-induced nuclear relocalization of AIF was suppressed in $cypA^{-/-}$ mice, and this correlated with a reduction in infarct volume by approximately 50% as compared to WT animals [123].

Besides preventing the assembly of the apoptosome by binding to Apaf-1 [135,136], the chaperon Hsp70 has been shown to sequester AIF in the cytosol upon MMP, thereby interfering with its proapoptotic potential [137]. Moreover, Hsp70 has been described to prevent the mitochondrial release of AIF [138], thereby acting at two distinct levels of the molecular pathway that activates AIF-dependent DNA degradation. Accordingly, $hsp70^{-/-}$ mice responded to an ischemic insult by manifesting a greater infarction volume in the cortex than WT animals [139]. Conversely, in a model of neonatal H/I, Hsp70 overexpression limited the mitochondrio-nuclear translocation of AIF and provided significant neuroprotection [140]. While the interaction between Hsp70 and Apaf-1 requires ATP and the ATP-binding domain (ABD) of Hsp70, it is still unclear whether this domain is needed for the cytosolic sequestration of AIF. Early works reported that a Hsp70 variant lacking ABD retains the ability to inhibit apoptosis *in vitro*, in cell death models that are also affected by micro-injection of anti-AIF antibodies as well as by its genetic ablation [141]. In a more recent work also based on cultured cells, it has been shown that both the ATPase and chaperon domains of Hsp70 are critical for impeding the release of AIF from mitochondria, while only the former would be required to sequester AIF in the cytosol and therefore prevent its nuclear translocation [138]. Finally, *in vivo* data obtained in a rat model of ischemic injury (which followed the brain-targeted transfection of plasmids for overexpressing different mutants of Hsp70) suggest that the C-terminal portion of Hsp70 is sufficient for neuroprotection, irrespective of the functionality as well as of the presence of its N-terminal ATPase domain [142]. Taken together, these experimental results provide a solid basis for the development of neuroprotective strategies based on Hsp70-mediated inhibition of AIF.

Endonuclease G (EndoG) is an DNase encoded by the nuclear genome that normally resides in the IMS [13]. Upon MMP, EndoG is released into the cytosol and translocates to the nucleus, where it cleaves chromatin DNA independently of caspases [143]. Although early reports suggested that the $endoG^{-/-}$ genotype is incompatible with life and results in embryonic lethality [144], it has been recently demonstrated that this phenotype was due to the disruption of an adjacent gene, and that $endoG^{-/-}$ mice can develop into adulthood without obvious abnormalities [145]. EndoG nuclear translocation has been detected in the brain of mice subjected to tFCI [146], as well as in sensory cells of the inner ear upon noise trauma [147]. At present, however, no studies have evaluated the response of EndoG-deficient animals to ischemic and traumatic brain injuries. Interestingly, assays based on purified cellular components suggest that the DNase activity of EndoG could be suppressed by Hsp70, in an ATP-dependent manner [148].

3.3. Other mitochondrial apoptotic effectors

The stress-regulated endoprotease Omi (also known as high temperature requirement protein A 2, *i.e.*, HtrA2) is a serine protease that shows extensive homology to the *Escherichia coli HtrA* gene products, which are essential for bacterial survival at high temperatures [149]. Human Omi/HtrA2 has been implicated in both caspase-dependent and caspase-independent cell death mechanisms that originate from MMP, due to its ability to release caspases from IAP-mediated inhibition [150,151] and to promote the cleavage of caspase-unrelated substrates (*e.g.*, cytoskeletal proteins) [152], respectively. In particular, Omi/HtrA2 inhibition of IAPs relies both on their sequestration [150] and on their proteolytic degradation [151]. However, mice in which the *omi* gene has been removed by homologous recombination [153,154], as well as *mnd2* mice (whose phenotype results from a loss-of-function mutation in *omi*) [155], exhibit early onset neurodegeneration with features of Parkinson disease, as well as juvenile lethality. These observations suggest that Omi/HtrA2 has a prominent role in cell survival, rather than in cell death, at least *in vivo* [10,11]. Although the contribution of Omi/HtrA2 to chronic neurodegenerative disorders such as Parkinson disease has been extensively explored [156,157], its implication in acute neuronal injury is poorly characterized. Cytosolic translocation of Omi/HtrA2 and its enhanced interaction with XIAP have been reported to occur *in vivo*, in mice subjected to tFCI [158]. This could not be prevented by Z-VAD-fmk administration, but was significantly reduced in the brain of mice overexpressing the copper/zinc-superoxide dismutase (SOD1), pointing to a contribution of ROS (but not of caspases) to the molecular pathways leading to ischemia-induced Omi/HtrA2 release.

Direct IAP-binding protein with a low pI (DIABLO, whose murine ortholog is known as second mitochondria-derived activator of caspase, *i.e.*, Smac) closely resembles Omi/Htra2 in several aspects: (i) it is a nuclear DNA-encoded factor that normally resides within the IMS and is released into the cytosol following MMP [159,160]; (ii) cytosolic Smac/DIABLO promotes cell death by sequestering IAPs, thereby favoring caspase activation [18]; (iii) $smac^{-/-}$ mice are viable, grow normally into adulthood and do not exhibit any histological abnormalities [161]. At difference with Omi/HtrA2, Smac/DIABLO does not display any proteolytic activity, and hence inhibits IAPs merely by physical engagement [162]. tFCI has been shown to promote the cytosolic translocation of Smac/DIABLO in both rats [163] and mice [164,165], in the latter case though a pathway that could be counteracted by SOD1 overexpression [166].

4. Concluding remarks

The observations discussed in this review underscore the notion that, although blocking cell death at the MMP level would represent the most efficient strategy, the inhibition of post-mitochondrial apoptotic executioners may also result in considerable neuroprotection (Fig. 2). This is of great importance in view of the fact that the majority of stroke and trauma patients are treated hours after the insult. At this stage, most (if not all) neurons in the affected region either have already died or have undergone MMP. Thus, novel therapeutic strategies are urgently awaited to block (or at least inhibit) executioner mechanisms that are already in motion.

Thus far, results from *in vivo* studies based on animal models of acute CNS injury (*e.g.*, focal and global brain ischemia, neonatal H/I, traumatic injury of the spinal cord) have lent strong support to the hypothesis that blocking post-mitochondrial apoptotic mechanisms would exert neuroprotective effects. Multiple lethal pathways are activated upon MMP to execute cell death. Thus, as elegantly demonstrated in murine models of neonatal H/I [132] or retinal

detachment [108], improved efficacy may be attained by combination therapies that inhibit more than a single cell death effector (*e.g.*, the caspase cascade and AIF). Moreover, the possibility to modulate the activity of lethal proteins in an indirect fashion, by pharmacologically activating their endogenous regulators, urgently awaits further investigation. In particular, Hsp70, which has been shown to regulate both caspase-dependent and -independent cell death by interacting with Apaf-1 [135], AIF [137] and EndoG [148], may represent a promising target for neuroprotection.

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