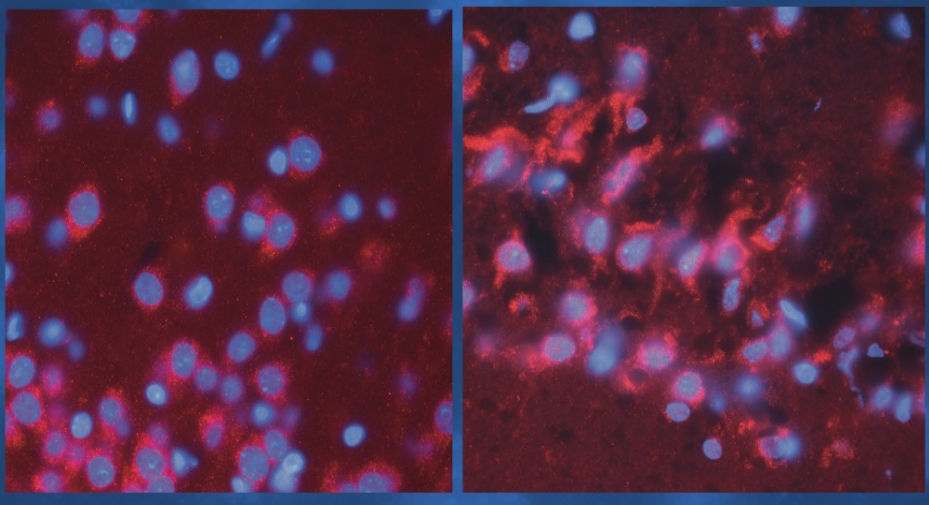
Denson G. Fujikawa
Editor

Acute Neuronal Injury

The Role of Excitotoxic Programmed Cell Death Mechanisms



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The Role of Excitotoxic Programmed Cell Death Mechanisms

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Cover illustration: The cover shows neurons in the piriform cortex of a control rat and a rat 24 hours after generalized seizures, showing seizure-induced translocation of endonuclease G (endoG) from cytoplasmic mitochondria to nuclei. The mitochondrial endoG is red and nuclei are blue in the control brain. Following seizures, nuclei appear purplish-white because of movement of endoG to nuclei.

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This book would not have been possible without the pioneering work of John W. Olney, MD, to whom it is dedicated

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Chapter 1
Introduction

Denson G. Fujikawa

In the early 1980s it was recognized that excessive Ca^{2+} influx, presumably through voltage-gated Ca^{2+} channels, with a resultant increase in intracellular Ca^{2+}, was associated with neuronal death from cerebral ischemia, hypoglycemia, and status epilepticus (Siejö 1981). Calcium activation of phospholipases, with arachidonic acid accumulation and its oxidation, generating free radicals, was thought to be a potential mechanism by which neuronal damage occurs. In cerebral ischemia and hypoglycemia, energy failure was thought to be the reason for excessive Ca^{2+} influx, whereas in status epilepticus it was thought that repetitive depolarizations were responsible (Siejö 1981).

Meanwhile, John Olney found that monosodium glutamate, the food additive, when given to immature rats, was associated with neuronal degeneration in the arcuate nucleus of the hypothalamus, which lacks a blood–brain barrier (Olney 1969). He followed up this observation with a series of observations in the 1970s that administration of kainic acid, which we now know activates the GluR5-7 subtypes of glutamate receptor, and other glutamate analogues, caused not only post-synaptic cytoplasmic swelling, but also dark-cell degeneration of neurons, when viewed by electron microscopy (Olney 1971; Olney et al. 1974).

On the basis of these observations, Olney proposed the excitotoxic hypothesis, namely, that glutamate and aspartate, the principal excitatory neurotransmitters in the central nervous system, are responsible for the excitotoxic death of neurons (Olney 1985). Electron-microscopic studies in the early 1970s and 1980s described dark-cell neuronal degeneration similar to that described by Olney in experimental cerebral ischemia (McGee-Russell et al. 1970), hypoglycemia (Auer et al. 1985a, b; Kalimo et al. 1985) and status epilepticus (Griffiths et al. 1983; Ingvar et al. 1988), but the connection between these pathological states and excitotoxicity was not made.

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In 1983, Rothman showed that synaptic activity was necessary for hypoxic death in hippocampal neuronal cultures (Rothman 1983). Soon thereafter, it was shown that an antagonist that bound to the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor reduced hypoxic-ischemic neuronal death in vivo (Simon et al. 1984). Further studies of cerebral cortical neurons in culture exposed to glutamate or NMDA showed that activation of glutamatergic receptors caused excessive Ca^{2+} influx through NMDA's ionotropic cationic channel (Choi 1987), and that NMDA-receptor activation results in neuronal death (Choi et al. 1987). Antagonist blockade of NMDA receptors reduced Ca^{2+} influx, intracellular Ca^{2+} concentrations, and neuronal death (Choi et al. 1988; Tymianski et al. 1993).

Subsequently, other sources for raising intracellular Ca^{2+} during an excitotoxic insult have been found. For example, Ca^{2+} can enter through the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-receptor subtype of glutamate channel lacking the GluR2 subunit (Pellegrini-Giampietro et al. 1992), Ca^{2+} is extruded from the endoplasmic reticulum (Rao et al. 2004), and the Na^{+}–Ca^{2+} exchanger (NCX) at the plasma membrane is cleaved by calpains during cerebral ischemia or glutamate excitotoxicity, resulting in elevation of intracellular Ca^{2+} (Bano et al. 2007).

Elevated intracellular Ca^{2+} activates two Ca^{2+}-dependent enzymes that in turn activate programmed cell death pathways that result in neuronal death. The first enzyme, neuronal nitric oxide synthase (nNOS), uses L-arginine as a substrate to produce nitric oxide (NO), which reacts with superoxide (O_2^{-}) to produce the toxic free radical, peroxynitrite ($ONOO^{-}$) (Jourd'heuil et al. 2001). Peroxynitrite and other reactive oxygen species damage the plasma membrane, the membranes of intracellular organelles (e.g., lysosomes and mitochondria) and cause double-strand DNA cleavage of nuclear DNA (Adibhatla and Hatcher 2006; Butler and Bahr 2006; Christophe and Nicolas 2006; Wilson and McNeill 2007). Poly(ADP-ribose) polymerase-1 (PARP-1) acts to repair DNA strand breaks by generating poly(ADP-ribose) (PAR) polymers by utilizing NAD^{+}, which in turn depletes ATP (Ha and Snyder 1999). PAR translocates to mitochondria, triggering the release of apoptosis-inducing factor (AIF), which translocates to the nucleus, acting with an unknown endonuclease to produce large-scale (50 kb) DNA cleavage (Yu et al. 2002, 2006; Andrabi et al. 2006).

The second key enzyme activated by elevated intracellular Ca^{2+} is the cysteine protease, calpain I. This protease cleaves structural proteins, e.g., αII-spectrin (fodrin) (Sorimachi et al. 1997), causes permeabilization of lysosomal membranes (Yamashima 2004), cleaves and inactivates the NCX (Bano et al. 2007), and cleaves AIF at its amino end (Polster et al. 2005), releasing it to the cytosol, where it translocates to the nucleus.

In recent years, attention has focused almost exclusively on one of three major morphological forms of developmental cell death, apoptosis (Clarke 1990), and two programmed cell death pathways that are activated in apoptotic cell death, the intrinsic (mitochondrial) caspase pathway, and the extrinsic (death receptor) pathway (Reed 2000; Philchenkov 2004; Riedl and Shi 2004; Kumar 2007). Activation of one or both pathways has been described in the adult rodent brain subjected to cerebral ischemia (Hara et al. 1997; Chen et al. 1998; Benchoua et al. 2001; Cho

et al. 2003), traumatic CNS injury (Qiu et al. 2002; Knoblach et al. 2005) and status epilepticus (SE) (Henshall et al. 2000, 2001a, b). However, the morphological evidence in the adult brain points to another of the three forms of cell death, necrosis (van Lookeren Campagne and Gill 1996; Colbourne et al. 1999; Fujikawa et al. 1999, 2000), and there is also evidence that caspase activation is age-dependent, with little caspase-3 activation in the adult brain (Hu et al. 2000; Liu et al. 2004). Also, there is evidence that the principal effector caspase, caspase-3, is not activated in vulnerable neurons in cerebral ischemia (Gill et al. 2002) and SE (Ananth et al. 2001; Fujikawa et al. 2002; Puig and Ferrer 2002; Narkilahti et al. 2003), and neither the intrinsic nor the extrinsic caspase pathway is activated in SE (Fujikawa et al. 2002; 2007; Narkilahti et al. 2003).

Whereas previously necrotic cell death was thought to be a purely passive occurrence, with cell swelling and lysis, there is accumulating evidence that it is also programmed (Kitanaka and Kuchino 1999; Leist and Jäättelä 2001; Proskuryakov et al. 2003; Syntichaki and Tavernarakis 2003; Festjens et al. 2006; Golstein and Kroemer 2006), and that cell shrinkage and nuclear pyknosis with irregular, dispersed chromatin clumps, are the end result (Fujikawa et al. 1999, 2000, 2002; Fujikawa 2000). Excitotoxic neuronal necrosis, triggered by Ca^{2+}-dependent nNOS and calpain I activation, is an example of such programmed cell death. Excitotoxic mechanisms underlie all of the major examples of acute neuronal injury – cerebral ischemia, traumatic brain injury, hypoglycemia and SE, and are the subject of this book.

References

Adibhatla RM, Hatcher JF (2006) Phospholipase A_2, reactive oxygen species, and lipid peroxidation in cerebral ischemia. Free Rad Biol Med 40:376–387

Ananth C, Tharmeem Dheen S, Gopalakrishnakone P, Kaur C (2001) Domoic acid-induced neuronal damage in the rat hippocampus: changes in apoptosis related genes (bcl-2, bax, caspase-3) and microglial response. J Neurosci Res 66:177–190

Andrabi SA, Kim S-W, Wang H et al (2006) Poly(ADP-ribose) (PAR) polymer is a death signal. Proc Natl Acad Sci USA 103:18308–18313

Auer RN, Kalimo H, Olsson Y, Siesjo BK (1985a) The temporal evolution of hypoglycemic brain damage. I. Light- and electron-microscopic findings in the rat cerebral cortex. Acta Neuropathol (Berl) 67:13–24

Auer RN, Kalimo H, Olsson Y, Siesjo BK (1985b) The temporal evolution of hypoglycemic brain damage. II. Light- and electron-microscopic findings in the hippocampal gyrus and subiculum of the rat. Acta Neuropathol (Berl) 67:25–36

Bano D, Munarriz E, Chen HL, Ziviani E, Lippi G, Young KW, Nicotera P (2007) The plasma membrane Na^+/Ca^{2+} exchanger is cleaved by distinct protease families in neuronal cell death. Ann N Y Acad Sci 1099:451–455

Benchoua A, Guegan C, Couriaud C, Hosseini H, Sampaio N, Morin D, Onteniente B (2001) Specific caspase pathways are activated in the two stages of cerebral infarction. J Neurosci 21:7127–7134

Butler D, Bahr BA (2006) Oxidative stress and lysosomes: CNS-related consequences and implications for lysosomal enhancement strategies and induction of autophagy. Antioxid Redox Signal 8:185–196

Chen J, Nagayama T, Jin K, Stetler RA, Zhu RL, Graham SH, Simon RP (1998) Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J Neurosci 18:4914–4928

Cho S, Liu D, Gonzales C, Zaleska MM, Wood A (2003) Temporal assessment of caspase activation in experimental models of focal and global ischemia. Brain Res 982:146–155

Choi DW (1987) Ionic dependence of glutamate neurotoxicity. J Neurosci 7:369–379

Choi DW, Maulucci-Gedde M, Kriegstein AR (1987) Glutamate neurotoxicity in cortical cell culture. J Neurosci 7:357–368

Choi DW, Koh J-Y, Peters S (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. J Neurosci 8, 185–196

Christophe M, Nicolas S (2006) Mitochondria: A target for neuroprotective interventions in cerebral ischemia-reperfusion. Curr Pharm Des 12:739–757

Clarke PGH (1990) Developmental cell death: Morphological diversity and multiple mechanisms. Anat Embryol 181:195–213

Colbourne F, Sutherland GR, Auer RN (1999) Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J Neurosci 19:4200–4210

Festjens N, Berghe TV, Vandenabeele P (2006) Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response. Biochim Biophys Acta 1757:1371–1387

Fujikawa DG (2000) Confusion between neuronal apoptosis and activation of programmed cell death mechanisms in acute necrotic insults. Trends Neurosci 23:410–411

Fujikawa DG, Shinmei S, Cai B (1999) Lithium-pilocarpine-induced status epilepticus produces necrotic neurons with internucleosomal DNA fragmentation in adult rats. Eur J Neurosci 11:1605–1614

Fujikawa DG, Shinmei SS, Cai B (2000) Kainic acid-induced seizures produce necrotic, not apoptotic, neurons with internucleosomal DNA cleavage: Implications for programmed cell death mechanisms. Neuroscience 98:41–53

Fujikawa DG, Ke X, Trinidad RB, Shinmei SS, Wu A (2002) Caspase-3 is not activated in seizure-induced neuronal necrosis with internucleosomal DNA cleavage. J Neurochem 83:229–240

Fujikawa DG, Shinmei SS, Zhao S, Aviles ER Jr (2007) Caspase-dependent programmed cell death pathways are not activated in generalized seizure-induced neuronal death. Brain Res 1135:206–218

Gill R, Soriano M, Blomgren K et al (2002) Role of caspase-3 activation in cerebral ischemia-induced neurodegeneration in adult and neonatal brain. J Cereb Blood Flow Metab 22:420–430

Golstein P, Kroemer G (2006) Cell death by necrosis: Towards a molecular definition. Trends Biochem Sci 32:37–43

Griffiths T, Evans M, Meldrum BS (1983) Intracellular calcium accumulation in rat hippocampus during seizures induced by bicuculline or L-allylglycine. Neuroscience 10:385–395

Ha HC, Snyder SH (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci USA 96:13978–13982

Hara H, Friedlander RM, Gagliardini V, Ayata C, Fink K, Huang Z, Shimizu-Sasamata M, Yuan J, Moskowitz MA (1997) Inhibition of interleukin-1-beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. Proc Natl Acad Sci USA 94:2007–2012

Henshall DC, Chen J, Simon RP (2000) Involvement of caspase-3-like protease in the mechanism of cell death following focally evoked limbic seizures. J Neurochem 74:1215–1223

Henshall DC, Bonislawski DP, Skradski SL, Lan J-Q, Meller R, Simon RP (2001a) Cleavage of Bid may amplify caspase-8-induced neuronal death following focally evoked limbic seizures. Neurobiol Dis 8:568–580

Henshall DC, Bonislawski DP, Skradski SL, Araki T, Lan J-Q, Schindler CK, Meller R, Simon RP (2001b) Formation of the Apaf-1/cytochrome c complex precedes activation of caspase-9 during seizure-induced neuronal death. Cell Death Diff 8:1169–1181

Hu BR, Liu CL, Ouyang Y, Blomgren K, Siejö BK (2000) Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. J Cereb Blood Flow Metab 20:1294–1300

Ingvar M, Morgan PF, Auer RN (1988) The nature and timing of excitotoxic neuronal necrosis in the cerebral cortex, hippocampus and thalamus due to flurothyl-induced status epilepticus. Acta Neuropathol 75:362–369

Jourd'heuil D, Jourd'heuil FL, Kutchukian PS, Musrah RA, Wink DA, Grisham MB (2001) Reaction of superoxide and nitric oxice with peroxynitrite. Implications for peroxynitrite-mediated oxidation reactions in vivo. J Biol Chem 276:28799–28805

Kalimo H, Auer RN, Siesjo BK (1985) The temporal evolution of hypoglycemic brain damage. III. Light and electron microscopic findings in the rat caudoputamen. Acta Neuropathol (Berl) 67:37–50

Kitanaka C, Kuchino Y (1999) Caspase-independent programmed cell death with necrotic morphology. Cell Death Diff 6:508–515

Knoblach SM, Huang X, VanGelderen J, Calva-Cerqueira D, Faden AI (2005) Selective caspase activation may contribute to neurological dysfunction after experimental spinal cord trauma. J Neurosci Res 80:369–380

Kumar S (2007) Caspase function in programmed cell death. Cell Death Differ 14:32–43

Leist M, Jäättelä M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nature Rev Mol Cell Biol 2:589–598

Liu CL, Siesjö BK, Hu BR (2004) Pathogenesis of hippocampal neuronal death after hypoxia-ischemia changes during brain development. Neuroscience 127:113–123

McGee-Russell SM, Brown AW, Brierley JB (1970) A combined light and electron microscope study of early anoxic-ischaemic cell change in rat brain. Brain Res 20:193–200

Narkilahti S, Pirtillä TJ, Lukasiuk K, Tuunanen J, Pitkänen A (2003) Expression and activation of caspase 3 following status epilepticus. Eur J Neurosci 18:1486–1496

Olney JW (1969) Brain lesions, obesity and other disturbances in mice treated with monosodium glutamate. Science 164:719–721

Olney JW (1971) Glutamate-induced neuronal necrosis in the infant mouse hypothalamus. An electron microscopic study. J Neuropathol Exp Neurol 30:75–90

Olney JW (1985) Excitatory transmitters and epilepsy-related brain damage. In: Smythies JR, Bradley RJ (eds) International review of neurobiology. Academic, Orlando, pp 337–362

Olney JW, Rhee V, Ho OL (1974) Kainic acid: A powerful neurotoxic analogue of glutamate. Brain Res 77:507–512

Pellegrini-Giampietro DE, Zukin RS, Bennett MV, Cho S, Pulsinelli WA (1992) Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. Proc Natl Acad Sci USA 89:10499–10503

Philchenkov A (2004) Caspases: Potential targets for regulating cell death. J Cell Mol Med 8:432–444

Polster BM, Basanez G, Etxebarria A, Hardwick JM, Nicholls DG (2005) Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. J Biol Chem 280: 6447–6454

Proskuryakov SY, Knoplyannikov AG, Gabai VL (2003) Necrosis: A specific form of programmed cell death? Exp Cell Res 283:1–16

Puig B, Ferrer I (2002) Caspase-3-associated apoptotic cell death in excitotoxic necrosis of the entorhinal cortex following intraperitoneal injection of kainic acid in the rat. Neurosci Lett 321:182–186

Qiu J, Whalen MJ, Lowenstein P, Fiskum G, Fahy B, Darwish R, Aarabi B, Yuan J, Moskowitz MA (2002) Upregulation of the Fas receptor death-inducing signaling complex after traumatic brain injury in mice and humans. J Neurosci 22:3504–3511

Rao RV, Ellerby HM, Bredesen DE (2004) Coupling endoplasmic reticulum stress to the cell death program. Cell Death Differ 11:372–380

Reed JC (2000) Mechanisms of apoptosis. Am J Pathol 157:1415–1430

Riedl SJ, Shi Y (2004) Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 5:897–907

Rothman SM (1983) Synaptic activity mediates death of hypoxic neurons. Science 220:536–537

Siejö BK (1981) Cell damage in the brain: A speculative synthesis. J Cereb Blood Flow Metab 1:155–185

Simon RP, Swan JH, Griffiths T, Meldrum BS (1984) Blockade of *N*-methyl-D-aspartate receptors may protect against ischemic damage in the brain. Science 226:850–852

Sorimachi H, Ishiura S, Suzuki K (1997) Structure and physiological function of calpains. Biochem J 328(Pt 3):721–732

Syntichaki P, Tavernarakis N (2003) The biochemistry of neuronal necrosis: Rogue biology? Nat Rev Neurosci 4:672–684

Tymianski M, Charlton MP, Carlen PL, Tator CH (1993) Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. J Neurosci 13:2085–2104

van Lookeren Campagne M, Gill R (1996) Ultrastructural morphological changes are not characteristic of apoptotic death following focal cerebral ischemia in the rat. Neurosci Lett 213:111–114

Wilson DM 3rd, McNeill DR (2007) Base excision repair and the central nervous system. Neuroscience 145:1187–1200

Yamashima T (2004) Ca2+-dependent proteases in ischemic neuronal death: a conserved 'calpain-cathepsin cascade' from nematodes to primates. Cell Calcium 36:285–293

Yu S-W, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Yu S-W, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Proc Natl Acad Sci USA 103:18314–18319

Part I
Caspase-Independent Programmed Cell Death: General Considerations

Chapter 2
Caspase-Independent Cell Death Mechanisms in Simple Animal Models

Matthias Rieckher and Nektarios Tavernarakis

2.1 Introduction

Cells undergo cell death in many forms and due to different insults. Programmed cell death (PCD) is crucial for correct development of the organism and the clearance of harmful cells like tumor cells or autoreactive immune cells. PCD is initiated by the activation of cell death receptors and in most cases it is associated with the activation of the cysteine proteases caspases, which lead to apoptotic cell death; cells shrink, chromatin clumps and forms a large, sharply demacrated, crescent-shaped or round masses, the nucleus condenses, apoptotic bodies are formed and eventually dead cells are engulfed by a neighboring cell or cleared by phagocytosis (Kerr et al. 1972). Other insults can trigger this ordered disposal of a cell, such as radiation leading to DNA damage, via p53 which in turn activates the apoptotic pathway (Xiang et al. 1996). The classical caspase-dependent cell death pathway has been studied in great detail not only in mammalian cells, but also in model organisms *C. elegans* and *Drosophila* (Hengartner 2000; Danial and Korsmeyer 2004; Hay and Guo 2006; Lettre and Hengartner 2006).

The view of apoptosis as the only form of PCD, entirely dependent on caspases, is now challenged by several findings in both *C. elegans* and *Drosophila*. Several paradigms of cell death have been shown to be executed independently of caspases: autophagy, necrosis, and even apoptosis (Broker et al. 2005; Kroemer and Martin 2005; Stefanis 2005). Different cell organelles have been implicated in contributing to cell death in a caspase-independent manner with the mitochondrion playing the central role by releasing death executors from the intermembrane space to the cytosol, triggering the breakdown of the cell (Lorenzo and Susin 2004; Kim et al. 2006). Here, we review caspase-independent cell death mechanisms and relevant genes in the nematode and the fruit fly (Table 2.1). We discuss the roles of autophagy and necrosis and possible interplay between caspase-dependent and -independent

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Table 2.1 Proteins implicated in caspase-independent cell death mechanisms in mammals and their homologs in *C. elegans* and *Drosophila* (Proteins involved in autophagic cell death are discussed in Baehrecke 2003; Samara and Tavernarakis 2008)

Mitotic catastrophy				
Mammals	*C. elegans*	*D. melanogaster*	Protein function	Refs
AIF	WAH-1	fAIF	(FAD)-binding oxidoreductase	Wang et al. (2002)
endoG	CPS-6	CG8862	Sequence-unspecific DNase	Parrish et al. (2001)
Omi/HtrA2	–	dOmi	Serine protease	Challa et al. (2007)
HSpin1	C39E9.10, C13C4.5, CEF09A5	spin		Nakano et al. (2001)
WWOX	–	DmWWOX	Oxido-reductase	O'Keefe et al. (2005)
AMID	–	–	(FAD)-binding oxidoreductase	Wu et al. (2002)
PRG3	–	–	(FAD)-binding oxidoreductase	Ohiro et al. (2002)
Autophagy				
Yeast	*C. elegans*	*D. melanogaster*	Protein function	Refs
Induction of autophagy				
Atg1	UNC-51	Atg1	Ser/Thr protein kinase	Kamada et al. (2000)
TOR1/TOR2	LET-363	Tor	Rapamycin-sensitive Ser/Thr protein kinase	Scott et al. (2004)
Autophagosome nucleation				
Atg6	BEC-1	Atg6	Component of class III PI3-kinase complex	Furuya et al. (2005)
VPS34	VPS-34	Vps34	Class III PI3-kinase	Furuya et al. (2005)
Autophagosome maturation				
Atg3	Y55F3AM.4	Aut1	E2-like enzyme	
Atg4	Y87G2A.3	Atg4	Cys protease	
Atg5	ATGR-5	Atg5		Scott et al. (2004)
Atg7	ATGR-7	Atg7	E1-like enzyme	Juhasz et al. (2007a)
Atg8	LGG-1	–	Ubiquitin-like protein conjugated to PE	Juhasz et al. (2007b)
Atg10	D2085.2	–	E2-like enzyme	
Atg12	LGG-3	Atg12	Ubiquitin-like protein	
Atg16	K06A1.5	–		

AUT2/APG4	–	CG1694	Cysteine-type endopeptidase	Thumm and Kadowaki (2001)
AUT7/APG8/CVT5	–	Atg8a	Microtubule binding; cytoskeleton biogenesis	Simonsen et al. (2008)
–	–	Atg8b	Microtubule binding; cytoskeleton biogenesis	
Autophagic protein retrival				
Atg2	–	Atg2		Samara and Tavernarakis (2008)
Atg9	ATGR-9	Atg9	Integral membrane protein	Samara and Tavernarakis (2008)
Atg18	ATGR-18	Atg18		Samara and Tavernarakis (2008)
Necrosis				
Mammals	*C. elegans*		Protein function	Refs
Adenylyl cyclase	ACY-1		Adenylyl cyclase	Berger et al. (1998)
	ASP-3		Aspartyl protease	Syntichaki et al. (2002)
	ASP-4		Aspartyl protease	Syntichaki et al. (2002)
	CAD-1			Artal-Sanz et al. (2006)
	CLP-1		Calcium-activated cysteine protease	Syntichaki et al. (2002)
Calnexin	CNX-1		ER Ca^{2+} binding chaperone	Xu et al. (2001)
Calreticulin	CRT-1		ER Ca^{2+} binding-storing protein	Xu et al. (2001)
Mucolipin-1	CUP-5			Artal-Sanz et al. (2006)
	DAF-2		Receptor of insulin-like ligands	Scott et al. (2002)
	DAT-1		Dopamine transporter	Nass et al. (2002)
	ITR-1		Inositol triphosphate receptor ion channel	Xu et al. (2001)
	PQE-1		Q/P-rich protein	Faber et al. (2002)
	SGS-1		Adenylyl cyclase	Korswagen et al. (1998)
	SPE-5		Vacuolar H^+-ATPase B subunit	Syntichaki et al. (2005)
	TRA-3		Calcium-activated cysteine protease	Syntichaki et al. (2002)
Ryanodine receptor	UNC-68		ER Ca^{2+} release channel	Xu et al. (2001)
	UNC-32		Vacuolar H^+-ATPase a subunit	Syntichaki et al. (2005)
	VHA-2		Vacuolar H^+-ATPase c subunit	Syntichaki et al. (2005)
	VHA-10		Vacuolar H^+-ATPase G subunit	Syntichaki et al. (2005)
	VHA-12		Vacuolar H^+-ATPase B subunit	Syntichaki et al. (2005)

pathways leading to cell death, as well as their implication in disorders like neurodegenerative diseases. Recent research has also provided evidence for additional novel forms of cell death in *C. elegans* and *Drosophila*, indicating that current cell death classification may need to be revisited in the future.

2.2 Advantages of Invertebrate Model Organisms

Simple model organisms are becoming increasingly important for investigating principal biochemical and molecular mechanisms. *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster* (*Drosophila*) have been instrumental in deciphering the molecular underpinnings of cell death. Both animals are ideal for genetic and molecular studies and additionally they have proved to be highly relevant models for studying human disorders, such as neurodegenerative diseases (Driscoll and Gerstbrein 2003; Celotto and Palladino 2005).

Both are multi cellular organisms with a relatively simple anatomy. In the case of *C. elegans*, the total number of cells of the animal is 959, including 302 neurons that form a simple nervous system. The cell lineage in the development of the nematode is fully deciphered and a complete lineage tree is available (see http://www.wormatlas.org/; Sulston et al. 1983). During development, 131 cells undergo programmed cell death (Ellis and Horvitz 1986). This makes *C. elegans* a powerful tool for investigating developmental biology (Bargmann and Avery 1995).

An important advantage of the nematode is its transparency, which makes microscopy far easier, permitting every cell division throughout development to be tracked. The simple nervous system is well documented, all neurons are mapped, and an almost complete wiring diagram has been created (see http://www.wormatlas.org/; White et al. 1983; Hall and Russell 1991). Specific behaviors, such as locomotion, chemo- or thermotaxis, as well as learning and memory, can be experimentally associated with the relevant neuron(s) (Thomas and Lockery 2005). The detailed characterization of its nervous system renders *C. elegans* particularly suited for the study of neurodegeneration and aging (Murakami 2007). The nervous system of *Drosophila* is far more complex and includes an intricate brain structure. The fly has been utilized with resounding success to study programmed cell death, neurodevelopment, as well as neurodegenerative diseases (Tabata and Takei 2004; Carthew 2007; Leyssen and Hassan 2007; Li and Baker 2007).

Both organisms go through a short life cycle and likewise, have a short mean life span. *C. elegans* develops from the fertilized egg to a self-fertilizing adult hermaphrodite within 3.5 days by undergoing four larval stages (L1 to L4). Due to food starvation or harsh environmental conditions the developing larva can enter the so-called dauer stage before completing the L1 stage, which increases the mean life span for more than 5 months. Favorable food conditions allow the animal to reenter the normal life cycle as an L4 larva. After entering the adult stage an approximately 3 day reproductive period follows, during which the animal lays about 300 eggs. *C. elegans* lives around 20 days, of which the last 2 weeks are characterized by a

decline in locomotion, food pumping, and recognizable tissue degeneration, revealing typical symptoms of aging. A low percentage of male animals (about 0.1 % of the progeny) is generated by hermaphrodites during self fertilization. These males enable genetic crosses that allow easy construction of double or multiple mutants (Riddle et al. 1997).

Drosophila needs about 8.5 days to develop from the zygote to the adult stage. After hatching, the animal undergoes three instar larval stages (first to third), followed by a prepupa and pupa stage, finally giving rise to the reproductive animal, which is either male or female. Females store the sperm of the male after mating and thereafter lay about 400 eggs (Lawrence 1992). Due to their short life span, both the nematode and the fruit fly are particularly popular for studying the mechanisms of aging and senescent decline (Lim et al. 2006).

Another important advantage of both animals is the easy maintenance in the laboratory. *C. elegans* feeds on bacteria (usually *Escherichia coli* strain OP50), which are grown either on solid agar plates or in liquid culture medium, and grows best at a temperature of 20°C. Drosophila is simply cultured at room temperature (25°C) and can be fed on different media containing a sugar source, like malt medium (Lakovaara 1969; Brenner 1974). The culturing temperature affects development timing of both animals. For example, *C. elegans* grows about 30% slower at 16°C compared to 20°C, while *Drosophila* needs about twice the time to complete a life cycle when grown at 18°C instead of 25°C, making it convenient to time experimental procedures. Both organisms can be cultured on a large scale.

Both the *C. elegans* and *Drosophila* genomes have been fully sequenced and annotated (Waterston and Sulston 1995; Kornberg and Krasnow 2000). Physical maps of the genome for both organisms based on the use of cosmids and yeast artificial chromosomes (YACs) have been created (Coulson et al. 1988; Hartl et al. 1992). The *C. elegans* genome is organized in five autosomes plus the sex chromosome X (sequence database: http://www.wormbase.org/). *Drosophila* only carries three autosomes plus the sex chromosome (sequence database: http://flybase.bio.indiana.edu/). Approximately 20,000 open reading frames (ORFs) for the nematode and about 14,000 ORFs for the fruit fly have been predicted (Blumenthal et al. 2002; Halligan and Keightley 2006). Additionally detailed protein interaction networks have been modeled for both organisms (Walhout et al. 2000; Lin et al. 2006).

The availability of fully-charted genomes allows the implementation of large-scale, genome-wide genetic and molecular methodologies such as double-stranded RNA-mediated interference (dsRNAi; Mello and Conte 2004). In *C. elegans* high-throughput RNAi screens against all 20,000 ORFs have been published (Simmer et al. 2003). The use of RNAi in the nervous system of the nematode has been less successful so far, but can be offset by the use of special hypersensitive mutants or the introduction of double-stranded hairpin RNAs (dshRNAs) through microinjection (Tavernarakis et al. 2000; Schmitz et al. 2007).

Both organisms are genetically malleable (Lee et al. 2004; Venken and Bellen 2005). The most straightforward method of creating mutants in both cases is random mutagenesis through the use of the chemical ethyl methanesulfonate (EMS).

Mutants for almost every gene are available or can be ordered. Animals carrying multiple mutations can be constructed and efficient genetic mapping is possible, by utilizing precise single nucleotide polymorphism (SNP) maps available for both model organisms (Jakubowski and Kornfeld 1999; Berger et al. 2001).

In the case of Drosophila loss of function mutants can also be generated by the use of *P* transposable elements or introducing dshRNAs through the GAL4/upstream activating sequence (GAL4/UAS) expression system, which is broadly used for gene overexpression (Brand and Perrimon 1993; Spradling et al. 1995; Cauchi and van den Heuvel 2006). In the fruit fly, the flippase (Flp)/flippase recombinase target (FRT) genetic mosaic system is also used (Golic 1991; Cauchi and van den Heuvel 2006). Other genetic manipulation methods are additionally available in *Drosophila* (Greenspan 1997).

In *C. elegans*, transgenic animals can be obtained by microinjection of engineered DNA samples into the gonad, where they generate inherited extrachromosomal arrays. This extrachromosomal array can further be integrated and stabilized in the genome through mutagenesis-induced integration (Mello and Fire 1995; Jin 2005; Rieckher et al. 2009).

In conclusion, both *C. elegans* and *Drosophila* are exceptionally powerful and convenient model organisms for investigating diverse biological phenomena, including cell death.

2.3 Cell Death by Mitotic Catastrophe

Compromised mitochondrial function irreversibly leads to cell death in both a caspase-dependent and -independent manner (Fig. 2.1). Mitochondrial breakdown is mainly the consequence of either extrinsic or intrinsic signals. Caspase-independent processes induce mitochondrial outer membrane permeabilization (MOMP). Although the precise mechanisms remain controversial, the involvement of Bcl-2 family proteins, among them the BH3-only proteins, is crucial in triggering MOMP (Green and Kroemer 2004). BH3-only proteins either activate or inhibit (Bcl-2 and Bcl-x_L) the proapoptotic Bcl-2 family members Bax and Bak. Oligomerization of these proteins generates pores in the outer membrane of mitochondria, releasing cell death factors from the intermembrane space to the cytoplasm (Kim et al. 2006). The process of MOMP initiated through Bax and Bak has been investigated intensively (Antignani and Youle 2006).

The *C. elegans* gene *ced-9* has been identified as an ortholog of the antiapoptotic members of the Bcl-2 family. CED-9 is anchored to the membrane of mitochondria and acts upstream of CED-3 and CED-4, negatively regulating the caspase-dependent cell death machinery (Igaki and Miura 2004). On the other hand, *egl-1* encodes a protein that belongs to the BH3-only protein subfamily also functioning upstream of CED-3 and CED-4, inducing cell death. CED-4 is released from the CED-4/CED-9 complex, which is localized to mitochondria by EGL-1, inducing caspase-dependent cell death (del Peso et al. 2000; Lettre and Hengartner 2006).

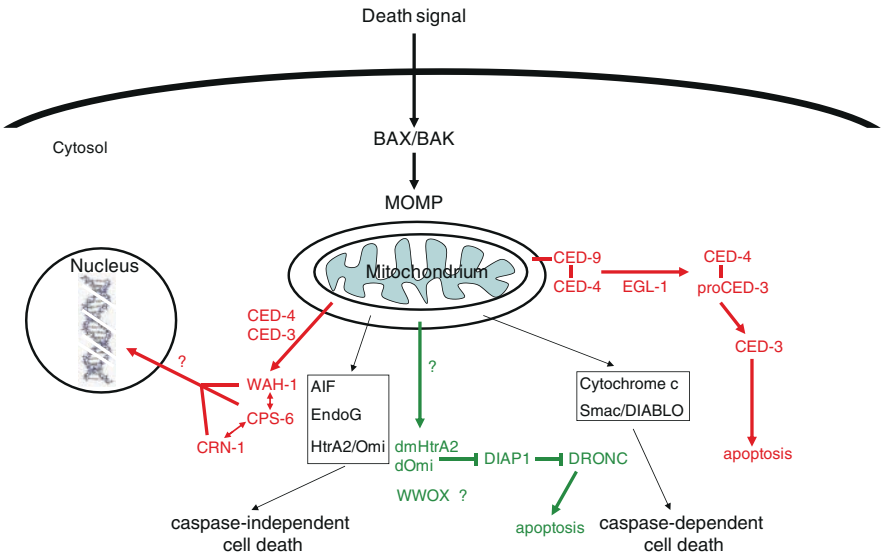

Fig. 2.1 Cell death by mitotic catastrophe. The induction of catastrophic breakdown of the mitochondrion derived from experimental results of mammalian cells (*black*) is shown. Various insults lead to the activation of BAX and BAK, which eventually trigger MOMP, resulting in the release of various enzymes. Depending on different factors, e.g., concentration, these enzymes cause cell death either in a caspase-dependent or -independent manner. *C. elegans* cell death pathways are shown in red and *Drosophila* mechanisms in green. Pathways for both animal models have also been shown to be dependent on caspases: The classical apoptotic pathway in *C. elegans* is based on the release of CED-4 from CED-9 leading to caspase-dependent cell death. The caspases CED-3 and CED-4 are also involved in the release of WAH-1. In *Drosophila*, the process of MOMP and the release of the HtrA2/Omi homologues, result in the downregulation of the caspase inhibitor DIAP1 and eventually the elevated activity of the caspase DRONC. It is not clear yet in which cell death pathway WWOX is involved. *DRONC* Drosophila Nedd2-like caspase; *MOMP* mitochondrial outer membrane permeabilization; *CED* cell death abnormality; *EGL* egg-laying deficiency; *WAH* worm AIF homolog; *CPS* CED-3 protease suppressor; *CRN* cell-death-related nuclease; *AIF* apoptosis inducing factor; *EndoG* endonuclease G; *DIABLO* direct inhibitor of apoptosis-binding protein with low p*I*; *WWOX* WW domain-containing oxidoreductase

In summary, although Bcl-2 family members have been shown to be involved in apoptosis and CED-9 is localized to the mitochondrial membrane, it is not known whether it is associated with MOMP (Estaquier and Arnoult 2006). In the fruit fly *Drosophila,* the involvement of mitochondria in cell death is less clear and they probably do not undergo MOMP (Varkey et al. 1999), although recent research findings may overturn this notion (see below; Challa et al. 2007; Igaki et al. 2007).

In mammalian cells the consequence of MOMP is the release of several factors (cytochrome c, Smac/Diablo, Omi/HtrA2, AIF and EndoG in mammals) from the intermembrane space into the cytosol, where they function as either caspase-dependent or -independent death executors (Kim et al. 2006). Cytochrome c release causes activation of Apaf-1 and leads to the classical apoptotic pathway, in which Smac/Diablo also plays a role as counteractor of IAPs (inhibitor of apoptosis

proteins; Hengartner 2000). The release of apoptosis inducing factor (AIF) HtrA2/Omi and EndoG have been shown to initiate caspase-independent mechanisms of cell death (Lorenzo and Susin 2004).

In mouse cells the endonuclease EndoG was identified as an apoptotic DNase that is released from mitochondria, subsequently localizing to the nucleus and fragmenting DNA independently of the activity of caspases. UV irradiation-induced DNA fragmentation mediated by EndoG still occurs in the presence of caspase inhibitors (Li et al. 2001). The function of the protein in mitochondria is the generation of RNA primers initiating DNA synthesis, a process important during mitochondrial replication (Cote and Ruiz-Carrillo 1993). The *C. elegans* homolog of mammalian EndoG, CPS-6 represents the first mitochondrial protein that has been identified to be involved in developmental programmed cell death in the nematode, indicating that an evolutionarily conserved family of nucleases plays an important role in apoptotic DNA degradation (Parrish et al. 2001). The activity of CPS-6 appears to be caspase-dependent, since down regulated CPS-6 function enhances cell survival in developing nematodes baring mutations in the caspases CED-3 and CED-4 (Parrish et al. 2001). Some interactors of CPS-6 have been identified: WAH-1, the *C. elegans* homolog of AIF (Wang et al. 2002), which is discussed below, and CRN-1, the homolog of human flap endonuclease-1 (FEN-1) (Parrish et al. 2003). CRN-1 possesses a 5′–3′ exonuclease and a structure-specific endonuclease activity. It acts as a co-factor of CPS-6, which is an endonuclease generating single-stranded nicks in DNA. Together they mediate stepwise DNA degradation (Parrish et al. 2003). Several more CRN nucleases might be involved in this process (Parrish and Xue 2003).

AIF was first identified in mammals as an effector of apoptotic cell death causing chromatin condensation and large-scale DNA fragmentation after localizing to the nucleus (Susin et al. 1999). Although it has been connected to the release of caspase-9 and therefore acting in the caspase-dependent pathway of cell death (Susin et al. 1999), AIF is also thought to be involved in a caspase-independent mechanism called “apoptosis-like” cell death (Leist and Jaattela 2001). The mechanism of releasing AIF to the cytosol is still under debate: The protein is embedded in the inner membrane of mitochondria and needs to be cleaved by proteases in order to be released. Cleavage occurs after the permeabilization of the outer membrane of mitochondria and is processed by the cysteine proteases cathepsins and calpains (Yuste et al. 2005). Such a scenario is supported by the fact that AIF is released to the cytosol through the same pore but much slower than cytochrome c, Smac/Diablo and Omi/HtrA2 (Munoz-Pinedo et al. 2006). However this notion contradicts earlier findings, where blocking caspase activity through zVAD-fmk prevents the release of AIF from mitochondria (Arnoult et al. 2003). Given that zVAD-fmk also blocks the activity of cysteine proteases these data need to be re-evaluated (Modjtahedi et al. 2006; Krantic et al. 2007).

The precise mechanism by which AIF promotes apoptosis-like cell death is not fully understood. Human AIF likely interacts with DNA since it shows a strong positive electrostatic potential (Ye et al. 2002) and most likely recruits potential partners such as nucleases to degrade DNA, triggering cell death (Lorenzo and Susin 2004). Indirectly, AIF may activate cell death via generation of free radicals after being

released to the cytosol. AIF exhibits NADH oxidase activity, reducing O_2 (Miramar et al. 2001). However, AIF also plays the role of a free radical scavenger, as shown in the Harlequin mouse (Klein et al. 2002). Thus, AIF might fulfill a dual role depending on its actual localization either to the cytosol (oxidase and cell death executor) or to the inner membrane of mitochondria (free radical scavenger involved in the mitochondrial respiratory chain; Porter and Urbano 2006).

Insight into the mode of AIF action has been obtained by studies of the *C. elegans* AIF homolog *wah-1* in developmental cell death. Wang and colleagues demonstrated that WAH-1 and the *C. elegans* EndoG (CPS-6) can be released from mitochondria by EGL-1 in a way similar to the release of cytochrome c and EndoG from mammalian mitochondria. Both proteins cooperate and act in the same pathway to promote apoptotic DNA degradation (Wang et al. 2002). Surprisingly, speed of WAH-1 release observed in a time-course study is at least partially dependent on caspase CED-3 activity, suggesting that *C. elegans* AIF and EndoG define a single, mitochondria-initiated apoptotic DNA degradation pathway that is conserved between *C. elegans* and mammals (Wang et al. 2002; Wang unpublished results). This assumption was recently confirmed by the discovery that WAH-1 promotes plasma membrane phosphatidylserine externalization and initiates cell engulfment typical for classical apoptosis in the nematode through activation of phospholipid scramblase 1 (SCRM-1; Wang et al. 2007).

The death effector Omi/HtrA2 was first identified in mammals as inhibitor of the X-chromosome linked inhibitor of apoptosis (XIAP) similar to Smac/Diablo. The same investigation showed the induction of a second mechanism of mediating cell death independent of caspases, probably due to its serine protease function (Suzuki et al. 2001). In mammals the protein is processed after import to the mitochondria and 133 of 458 residues are removed, leaving an active form of 36 kDa. The amino-terminus shares high homology with *Drosophila* pro-death proteins Grim, Hid, Reaper and mammalian Smac/Diablo proteins (Lorenzo and Susin 2004). Some evidence about the mechanism of Omi/HtrA2 action comes from studies in *Drosophila*: The mitochondrial proteins dOmi and dmHtrA2 were independently identified as highly homologous to the human HtrA2/Omi, particularly within the serine protease domain. During UV-irradiation-induced cell death, labeled dmHtrA2 or dOmi proteins and also cytochrome c, were observed outside mitochondria (Challa et al. 2007; Igaki et al. 2007). Release is both caspase-dependent and -independent (Challa et al. 2007). In the cytosol dOmi induces cell death in S2 cells and in the developing fly eye by proteolytically degrading DIAP1 (an IAP family caspase inhibitor), which finally displaces DRONC and acts in the classical apoptosis pathway (Challa et al. 2007; Igaki et al. 2007).

Another recently investigated gene involved in caspase-independent cell death is *hspin1*, a homolog of the *Drosophila spin* gene (Yanagisawa et al. 2003). Mutations in *spin* interfere with programmed cell death during the development of *Drosophila* nurse cells and neurons. Persistence of surviving cells leads to neurodegeneration and death of oocytes in the ovary (Nakano et al. 2001). In human cells HSpin1, which contains membrane spanning domains, causes necrotic cell death when overexpressed. HSpin1 binds to the antiapoptotic proteins Bcl-2 and Bcl-x_L and its

activity can be blocked by the necrosis inhibitor pyrrolidine dithiocarbamate (PDTC) but not by the caspase-inhibitors zVAD-fmk and p35. This indicates that HSpin1 titrates Bcl-2 and/or Bcl-x_L by localizing to the mitochondria and thereby promoting cell death in a caspase-independent way (Yanagisawa et al. 2003). Three homologs of the *spin* gene are encoded in the *C. elegans* genome and have not been characterized in detail (Nakano et al. 2001).

Additional proteins that are involved in mitochondrial caspase-independent cell death have been identified in mammalian cells: WWOX or FOR, the AIF homologue mitochondrion-associated inducer of death (AMID) and the p53 regulated gene 3 (PRG3). All these show sequence similarity to AIF (Lorenzo and Susin 2004). WWOX has a homolog in *Drosophila,* which has been shown to protect from ionizing radiation when overexpressed (O'Keefe et al. 2005).

2.4 Autophagic Cell Death

Although identified more than 50 years ago, the process of autophagy remained relatively mysterious until relatively recently. Interest in autophagy markedly increased within the last decade after it was shown to play a role in human pathophysiology (Klionsky 2007). Dual roles of autophagy in cell survival and death have been reported (Baehrecke 2005). Autophagy differs from apoptosis (type I programmed cell death) by the presence of autophagic vacuoles and autophagolysosomes which are involved in degradation of the dying cell.

Three different types of autophagy have been defined: microautophagy, chaperone-mediated autophagy and macroautophagy (Majeski and Dice 2004; Baehrecke 2005). Hereafter, we will refer to macroautophagy using the term autophagy for simplicity.

During autophagy, cytoplasmic double membrane vesicles, called autophagosomes or autophagic vacuoles are initially formed, primed from a yet unknown membrane source (Wang and Klionsky 2003). As autophagosomes form they engulf parts of the cytoplasm and/or organelles. Ultimately, their outer membrane fuses with lysosomes. The inner-single membrane vesicle (autophagic body) is released into the lumen, where it is digested, together with its content, by various enzymes (Yorimitsu and Klionsky 2007).

Most proteins involved in autophagy have been identified in the yeast *Saccharomyces cerevisiae* and are encoded by autophagy related genes (Atg; Klionsky et al. 2003; Yorimitsu and Klionsky 2007). These genes regulate every step of autophagy from induction, through cargo selection and packaging up to fusion with the lysosomes and degradation. Nevertheless, many unanswered questions about each phase of the process still remain (Klionsky 2005).

Cells use autophagy as a "regulated self-cannibalism" process. Cells degrade and recycle their contents in order to maintain viability in the absence of food. By sensing the presence of nutrients through the class I and class III phosphatidylinositol 3-kinase (PI3K) signaling pathways, the downstream acting target of rapamycin

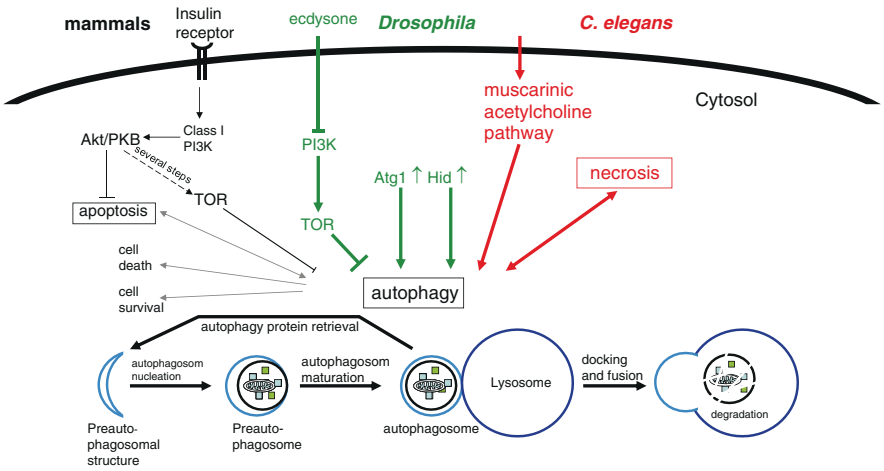

Fig. 2.2 Autophagy and cell death. Pathways leading to or interacting with autophagy that result in cell survival or cell death are shown. *C. elegans* mechanisms are shown in *red* and *Drosophila* in *green*. The main autophagic mechanism is also presented. Initially, a pre-autophagosomal structure arises from an unknown membrane source and develops into the preautophagosome. While developing the autophagosome engulfs parts of the cytosol, containing proteins and whole organelles. After completing maturation, the autophagosome docks to the lysosome and the outer membrane fuses with the lysosomal membrane. The inner autophagic body and its contents are degraded by lysosomal proteases. The genes involved in each step are presented in Table 1. *Atg* Autophagy-related gene; *PI3K* phosphoinositol 3 kinase; *TOR* target of rapamycin; *Akt/PKB* serine/threonine kinase/protein kinase B; *Hid* head involution defective

(TOR) kinase suppresses autophagy. In the absence of food, low insulin levels abrogate suppression (Klionsky 2004). Under harsh nutrient deprivation conditions excessive autophagy may lead to cell death (Fig. 2.2). Investigation of physiological as well as aberrant autophagy in invertebrate model organisms has provided new insight into the role of autophagy in cell survival and cell death. These studies in *Drosophila* and *C. elegans* point to the involvement of autophagy in caspase-independent cell death.

During the last larval stage of *Drosophila*, fat body cells, which are part of the fat body, a nutrient storage organ similar to the human liver, undergo programmed cell death and show induced autophagic vesicle formation in response to starvation. Cells appear to die in response to the hormone ecdysone, which down regulates PI3K signaling, resulting in pronounced induction of autophagy (Rusten et al. 2004). TOR was shown to be an important downstream effector in the pathway leading to the suppression of autophagy in the fruit fly (Scott et al. 2004). Additionally, the *Drosophila* homologue of the yeast protein Vps18, Deep Orange (Dor) has been shown to control programmed autophagy in fat body cells. Dor is required for ecdysone signaling and also mediates the fusion between autophagosome and lysosome (Lindmo et al. 2006; Lindmo and Stenmark 2006). Very recently, the importance of Atg1 in this process has been confirmed by overexpression studies in the fly, where it leads to suppression

of TOR and triggers autophagy, leading to caspase-dependent cell death in fat body cells (Neufeld 2007; Scott et al. 2007). Earlier overexpression studies suggest that the intracellular executor of programmed autophagy is the protein *head involution defective* (*hid* – counterpart of Smac/DIABLO), which kills cells in a caspase-independent way. However, this effect might be the result of overexpression (Juhasz and Sass 2005).

Dying salivary glands have been intensively studied in Drosophila by serial analysis of gene expression (SAGE) which revealed the involvement of both autophagy, apoptosis and other genes (total: 1,244 transcripts) in cell death (Gorski et al. 2003). In another genome-wide analysis, the activation of genes involved in cell death of salivary glands caused by radiation (usually triggering apoptosis) and steroids (ecdysone) has been investigated by the use of microarrays. The response to the two different death triggers is radically different: ecdysone significantly increases the RNA levels of 932 gene transcripts, while in response to radiation only 34 genes were activated. Five genes were commonly activated in both cases, indicating a cross-talk between different cell death programs (Lee et al. 2003). Supporting this point, a recent study by Martin and Baehrecke suggests that salivary gland cells die via autophagy in a caspase-dependent manner (Martin and Baehrecke 2004). A recent shotgun proteome analysis of purified, dying (steroid-triggered) larval salivary glands, combined with whole-genome microarrays, revealed upregulation of proteins usually involved in apoptosis and autophagy. Known players, such as the ecdysone-response proteins, caspases and caspase-regulators were identified as well as proteins from caspase-independent acting mechanisms. Besides establishing a powerful screening method in *Drosophila* cells, this study confirmed earlier studies and strengthened the point that both caspase-independent (such as autophagy) and caspase-dependent mechanisms contribute to cell death in specific tissues (Martin et al. 2007).

Both *Drosophila* and *C. elegans* are particularly suited for investigating the role of autophagy in cell death associated with pathological conditions such as neurodegenerative diseases. For example, a *Drosophila* model of Huntington`s disease has been established (Ravikumar et al. 2004). The disease is associated with expanded polyglutamine repeats (polyQ) in the protein huntingtin, which causes aggregation of the protein and cytotoxicity (Lee and Kim 2006). Huntingtin aggregates are mostly cleared by the ubiquitin proteasome system (UPS). Autophagy also contributes to the clearance of aggregates when the UPS system becomes impaired. The *Drosophila* histone deacetylase HDAC6 appears to be involved in coordinating both mechanisms (Pandey et al. 2007). Autophagy is engaged in the turnover of polyQ and other aggregates by downregulation of TOR signaling. This has been shown in mammalian cells and also in flies expressing mutant huntingtin in the photoreceptor cells of the eye (Ravikumar et al. 2004). Rapamycin and its analog CCI-779 protect cells by inhibiting TOR and inducing autophagy, which clears aggregated huntingtin (Rubinsztein et al. 2007). In addition, small molecule enhancers (SMER) and inhibitors (SMIR) of rapamycin cytostatic effects have been studied in yeast. These molecules induce autophagy independent from rapamycin and enhance the clearance of huntingtin aggregates and also mutant α-synuclein aggregates associated with Parkinson's disease (Sarkar et al. 2007).

Autophagic cell death has also been studied in *C. elegans*. The physiological function of autophagy in the nematode is associated with dauer larva formation. L2 animals enter the arrested dauer developmental state due to unfavorable environmental conditions such as high temperature, absence of food or the presence of a pheromone, which indicates a highly dense population (Riddle 1988). Entering this state is negatively regulated by the insulin-like signaling pathway. *Bec-1*, the *C. elegans* ortholog of the yeast and mammalian autophagy gene *Atg6/Vps60/beclin1* plays an important role in dauer morphogenesis. Additionally, the orthologs of the yeast autophagy genes *Atg1*, *Atg7*, *Atg8,* and *Atg10* (which now are defined as *unc-51*, *atgr-7*, *lgg-1* and *atgr-18*; see Table 1) are involved in the process; their downregulation results in defect in dauer formation (Melendez et al. 2003; Riddle and Gorski 2003). *bec-1* forms a complex with CED-9/Bcl-2 and has also been found to play a role in apoptosis in *C. elegans*. Deletion of *bec-1* triggers CED-3/caspase-dependent cell death. BEC-1 is necessary for the function of the class III PI3 kinase LET-512/Vps34, which is involved in autophagy, membrane trafficking, and endocytosis (Takacs-Vellai et al. 2005). The muscarinic acetylcholine pathway signals induction of autophagy in pharyngeal muscles of *C. elegans* during starvation. Pumping rates are enhanced by activation of the muscarinic acetylcholine pathway and the energy needed for this process is likely provided by autophagy. Indeed, *bec-1* RNAi knockdown decreases autophagy and pumping rates during starvation (Kang et al. 2007). Autophagy is also associated with endocytosis in *C. elegans*. *Ce*VPS-27 is the ortholog of the yeast endosomal Vps27p, which regulates the formation of endosomal sorting complexes. *Ce*VPS-27 is important for larval development and inactivation of the corresponding gene leads to defects in endosome formation, as well as accumulation of autophagosomes, suggesting a role in autophagy (Roudier et al. 2005).

Recent findings in *C. elegans* indicate that autophagy also contributes to necrotic cell death. In an *unc-51* deficient background, necrotic cell death, triggered either by *deg-3(d)* or *mec-4(d)* or hypoxia (see the following section on necrosis for details) is significantly suppressed. This effect is also observed after downregulation of other autophagy-related genes such as *bec-1*, *lgg-1* and *atgr-18* by RNAi. In addition, increase of autophagosomes formation is observed under conditions of neurodegeneration. Calpain proteases and autophagy appear to act in the same pathway (Samara et al. 2008). *Ce*TOR signaling eventually prevents and starvation promotes neuronal cell death in a *mec-4(d)* background (Toth et al. 2007). Thus, autophagy appears to play a dual role in *C. elegans*, either by promoting survival (physiological autophagy) or causing death (insufficient or excessive autophagy; Samara et al. 2008; Samara and Tavernarakis 2008)

2.5 Necrotic Cell Death

Necrosis is considered to be one of the main caspase-independent cell death types and morphologically distinct from apoptosis. Among the major features of necrosis are the extensive swelling of the cell and various cellular organelles, the random

degradation and clumping of nuclear DNA, the formation of small, tightly wrapped membrane whorls, the rupture of the plasma membrane and the appearance of autophagosomes (Edinger and Thompson 2004). The word necrosis is derived from the Greek expression "necros," standing for "dead" and was traditionally considered as the chaotic breakdown of the cell. In humans, necrotic cell death accompanies prolonged hypoxia, ischemia, hypoglycemia, toxin exposure, exposure to reactive oxygen metabolites, extreme changes in temperature, and nutrient deprivation (Nicotera et al. 1999). Necrosis is also involved in neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis and epilepsy (Stefanis 2005). Necrotic cell death, unlike apoptosis, was thought to be a passive process, not requiring energy, synthesis of new proteins and specific regulatory mechanisms. However, recent findings in *Drosophila* and *C. elegans* have forced a shift of this simplistic view (Syntichaki and Tavernarakis 2002; Kourtis and Tavernarakis 2007).

In the nematode necrotic neuronal death can be triggered by a great variety of extrinsic and intrinsic signals, mainly by the expression of ion channels bearing a hyperactive mutation (Fig. 2.3; Syntichaki and Tavernarakis 2003). The most thoroughly studied case of necrotic cell death is the one induced by hyperactive

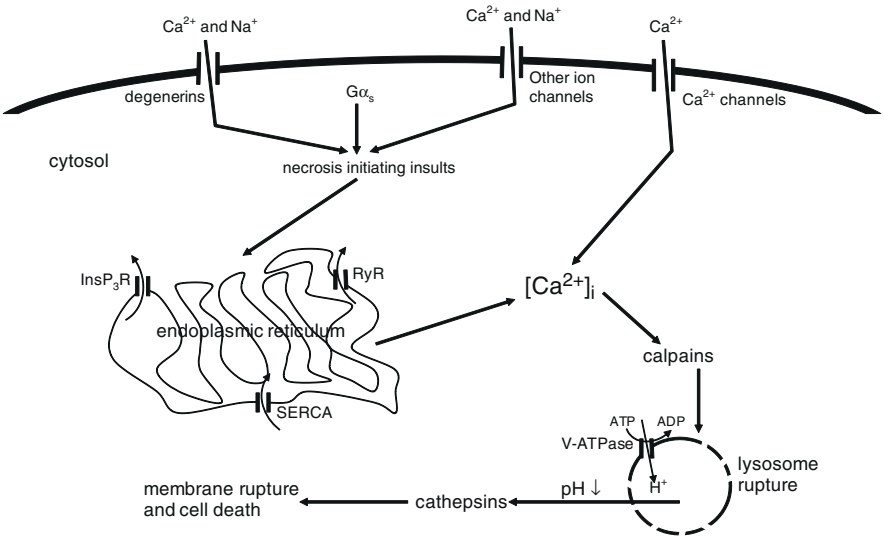

Fig. 2.3 Main necrotic pathways in *C. elegans*. Necrosis is triggered by mutant ion channels, as well as mutant $G\alpha_s$ (for more details see text). These insults stimulate Ca^{2+} release from the endoplasmic reticulum (ER) through the transporters RyR and $InsP_3R$, reuptake of calcium is facilitated through SERCA. Elevation of Ca^{2+} levels is also mediated directly by plasma membrane Ca^{2+} channels. Subsequently, calpain proteases become activated. Lysosomal rupture is the consequence of calpain activity, which leads to the release of lysosomal cathepsin proteases and a decrease in local pH, facilitated through the action of V-ATPase. *$G\alpha_s$* G-protein subunit; *$InsP_3R$* inositol triphosphate receptor; *RyR* ryanodin receptor; *SERCA* sarco-endoplasmic reticulum Ca^{2+}-ATPase; *V-ATPase* vacuolar H^+-ATPase)

deg-1(d) (*deg*enerin) and *mec-4*(d) (*mec*hanosensory) both carrying dominant mutations and causing necrosis in special neurons of *C. elegans*: Gain of function mutations in *deg-1* induce necrosis in a group of interneurons of the posterior touch sensory circuit (Chalfie and Wolinsky 1990). *mec-4* gain of function mutations cause similar effects in the six touch receptor neurons, which are required for the sensation of gentle touch of the body (Syntichaki and Tavernarakis 2004). Both genes belong to the family of degenerins, which induce cell degeneration when mutated to a hyperactive form. Dying cells exhibit the typical morphological characteristics of necrotic cell death. Degenerins are similar in sequence to the subunits of the amiloride-sensitive epithelial Na^+ channel (ENaC) in mammals (Tavernarakis and Driscoll 2001). Large side chain substitutions of amino acids close to the pore forming region of degenerins enhance sodium and calcium conductivity leading to necrotic cell death (Syntichaki and Tavernarakis 2004). Ultimately, extensive ion influx disrupts cellular Ca^{2+} homeostasis (Syntichaki and Tavernarakis 2003). Calcium imbalance caused by mutated ion channels triggers further release of Ca^{2+} from the endoplasmic reticulum (ER)via the ryanodine (RyR) and inositol-1,4,5-triphosphate receptors ($Ins(1,4,5)P_3PR$).

The ionic imbalance and subsequent cell death induced by mutant degenerins resembles excitotoxicity in vertebrates, where the collapse of presynaptic neuron membrane potential due to energy depletion results in the release of high amounts of the excitatory neurotransmitter glutamate into the synaptic cleft (Olney 1994). Accumulation of glutamate at the synapse causes hyper-excitation and necrotic cell death of postsynaptic neurons. Excitotoxicity is the prominent mechanism of neuronal loss during stroke, when nutrient and energy supply to neuronal cells is disrupted by blockage of the blood flow. Degenerin-induced neuronal death in *C. elegans* is an attractive model of excitotoxicity that renders the nematode a suitable and powerful tool for dissecting the molecular mechanisms of neurodegeneration.

In addition to mutant degenerins, several other triggers of nonprogrammed cell death in *C. elegans* have been described. Constitutive activation of the GTP-binding protein $G\alpha_s$, chemical inhibitors of the respiratory chain (e.g., NaN_3), hypoxic treatment, toxins, polyglutamine repeat proteins and macromolecular damage caused by radiation are potent inducers of cell death (Kourtis and Tavernarakis 2007). These inducers have been exploited in genetic and molecular studies that have elucidated key facets of necrotic cell death mechanisms (Artal-Sanz and Tavernarakis 2005).

Null mutations in calreticulin and knock-down of calnexin, which are calcium-binding chaperones, suppress necrotic cell death in *C. elegans* neurons triggered by *mec-4*(d). Also the blockage of Ca^{2+} release from the ER, either by mutations in the calcium release channels encoded by *unc-68* (RyR) and *itr-1* ($Ins(1,4,5)P_3PR$) or by pharmacological treatment results in similar suppression. These findings indicate that Ca^{2+} release from the ER plays an essential role in necrotic cell death (Xu et al. 2001).

The cytoplasmic protease calpain, which is activated by calcium and functions in several signaling and metabolic pathways, also plays a role in necrosis. High levels of calcium activate calpains which then localize to lysosomes and cause

disintegration of the lysosomal membrane. Subsequent release of lysosomal aspartyl proteases and cathepsins into the cytoplasm causes the breakdown of the cell and rupturing of the plasma membrane. Detailed studies of cell death following brain ischemia in monkeys have led to the formulation of the "calpain–cathepsin" hypothesis for the execution of necrosis (Yamashima 2000, 2004). Genetic studies in *C. elegans* support the involvement of a calpain–cathepsin axis during neurodegeneration. Downregulation of the calpains CLP-1 and TRA-3 and cathepsins ASP-3 and ASP-4 by RNAi ameliorates neurodegeneration in the nematode (Syntichaki et al. 2002). The proteolytic action of cathepsins in the cytoplasm is further enhanced by the drop of pH in the cell, mediated by the vacuolar H^+-ATPase, which acidifies lysosomes and other cell organelles. Alkalization of those organelles prevents necrosis in *C. elegans*, supporting the involvement of cytoplasmic acidification in the process (Syntichaki et al. 2005).

The active involvement of lysosomes in necrotic, caspase-independent cell death mechanisms is corroborated by observations in mutant nematodes, defective in lysosomal function (Artal-Sanz et al. 2006). *cup-5(lf)* mutants, which show increased number of enlarged lysosomes (Hersh et al. 2002) are significantly more sensitive to necrotic cell death inducing insults. Visualization of lysosomal morphology during necrosis reveals aggregation of lysosomes around a swollen nucleus and ultimately lysosomal rupture, consistent with the calpain–cathepsin hypothesis (Artal-Sanz et al. 2006).

In *Drosophila*, a similar model of excitotoxicity has been utilized to gain insight into the mechanisms of neurodegeneration. The excitatory amino acid transporters (EAATs) are high-affinity transporters for L-glutamate (Glu) involved in clearing Glu from the synaptic cleft and preventing over-excitation of the postsynaptic neuron (Beart and O'Shea 2007). Downregulation of *Drosophila* dEAAT1, which is expressed in glia, reduces Glu uptake and clearing, which leads to degeneration of neuropil. Similarly to excitotoxicity, degeneration is accompanied by the formation of vacuoles, electron-dense material, and swollen mitochondria (Rival et al. 2004), which are typical features of necrotic cell death.

2.6 Novel Programs of Caspase-Independent Cell Death

During development and morphogenesis of multicellular organisms, programmed cell death controls cell number and also shapes organs (Vaux and Korsmeyer 1999). The most common type of cell death in this context is caspase-dependent apoptosis (Edinger and Thompson 2004). Recent research in *C. elegans* and *Drosophila* has revealed that specific cells also die in a caspase-independent manner (Kumar and Rothman 2007). Cell corpses are subsequently removed through engulfment by neighboring or specialized phagocytic cells in both *C. elegans* and *Drosophila*. The process of engulfment in the nematode assists apoptotic cell killing itself and is also involved in the clearance of necrotic cells (Zhou et al. 2004). In *Drosophila,* cells dying in a caspase-independent manner are removed by a similar mechanism

(Mergliano and Minden 2003). Interestingly, observations in cell-death deficient *H99 Drosophila* embryos revealed removal of cells in the epidermis via caspase-independent cell death that may involve engulfment of living cells (Mergliano and Minden 2003).

A nonapoptotic, caspase-independent cell death mechanism is involved in the removal of the linker cell of *C. elegans*, which is born during the second larval stage (L2) and is essential for male gonadal development in the nematode (Sulston et al. 1983). As the linker cell migrates it directs the extension of the male gonad and mediates the fusion of the vas deferens and cloaca. The linker cell finally dies at L4/adult stage. Death was thought to be dependent on the neighboring engulfing cells (Sulston et al. 1980). However, after laser ablation of the grandparental precursor engulfing cells the linker cell still dies, which hints at a linker cell intrinsic death program. Cell death is independent of genes typically involved in developmental timing, engulfment, and all types of cell death characterized in *C. elegans*, such as necrosis, apoptosis, and autophagy (Abraham et al. 2007). Some morphological features of the linker cell death such as nuclear crenellation, the absence of chromatin compaction and cytoplasmic changes such as dilation of cell organelles are reminiscent of caspase-independent cell death in other organisms (Clarke 1990). Thus, the linker cell death program in *C. elegans* may represent a conserved caspase-independent mode of cell death in diverse species (Abraham et al. 2007).

In *Drosophila,* 15 nurse cells assure the development of one growing oocyte each, by supplying it with essential macromolecules, such as proteins, mRNA, and organelles. Finally they die after extruding all their remaining cytoplasmic contents into the oocyte (McCall 2004). This type of cell death was thought to be classical, caspase-dependent apoptosis but recent research illuminated that nurse cells die in a caspase-independent manner (Mazzalupo and Cooley 2006). Visualization and inhibition of caspase activity demonstrates that caspases do not play a role during the death of the nurse cells. While the possibility of necrosis cannot be excluded, no signs of autophagic or apoptotic cell death have been detected (Mazzalupo and Cooley 2006). Similar to the linker cell death in *C. elegans*, a yet unidentified cell death mechanism likely underlies the demise of nurse cells.

2.7 Concluding Remarks

Several paradigms of caspase-independent cell death have been characterized in diverse species. Most can be grouped into three main types, mitotic catastrophe, autophagy, and necrosis. Proteins that normally serve physiological functions can be released from mitochondria after MOMP and once in the cytosol they act as death executors. The functions of some such proteins have been investigated in mammalian cells as well as in *C. elegan*s and *Drosophila*. These studies point towards conserved mechanisms of caspase-independent cell death. Interestingly, while some of these effectors trigger caspase-independent cell death in mammals, they preferentially engage caspase-dependent apoptotic cell death in invertebrates.

This indicates that caspase-independent cell death mechanisms may represent more recent additions to the cell death program.

Studies in yeast and in mammalian cells indicate that autophagy is a mediator of both cell survival and cell death. Starvation causes formation of autophagosomes, partial degradation of cell contents and recycling of the degraded components, which provides the cell with the energy required to overcome the shortage of nutrients. Nevertheless, abnormally high levels of autophagy may promote cellular destruction instead. Furthermore, the process of autophagy is intimately linked with both apoptosis and necrotic cell death. Necrosis was traditionally considered as merely the chaotic breakdown of cells. However, several recent studies in *C. elegans* indicate that specific molecular mechanisms are involved in the necrotic destruction of the cell. Because necrosis is implicated in many devastating human disorders, such as neurodegenerative diseases and stroke, elucidation of the biochemical events that transpire during necrosis has the potential to provide targets for effective pharmacological interventions.

In addition to the three major categories of caspase-independent cell death, novel cell death paradigms that do not involve caspase function are emerging. Genetic and molecular dissection of these examples of cell death in invertebrate models may reveal new mediators of cell death with relevance to human pathological conditions.

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References

Abraham MC, Lu Y, Shaham S (2007) A morphologically conserved nonapoptotic program promotes linker cell death in Caenorhabditis elegans. Dev Cell 12:73–86

Antignani A, Youle RJ (2006) How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane? Curr Opin Cell Biol 18:685–689

Arnoult D, Gaume B, Karbowski M, Sharpe JC, Cecconi F, Youle RJ (2003) Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J 22:4385–4399

Artal-Sanz M, Tavernarakis N (2005) Proteolytic mechanisms in necrotic cell death and neurodegeneration. FEBS Lett 579:3287–3296

Artal-Sanz M, Samara C, Syntichaki P, Tavernarakis N (2006) Lysosomal biogenesis and function is critical for necrotic cell death in Caenorhabditis elegans. J Cell Biol 173:231–239

Baehrecke EH (2003) Autophagic programmed cell death in Drosophila. Cell Death Differ 10:940–945

Baehrecke EH (2005) Autophagy: dual roles in life and death? Nat Rev Mol Cell Biol 6:505–510

Bargmann CI, Avery L (1995) Laser killing of cells in Caenorhabditis elegans. Methods Cell Biol 48:225–250

Beart PM, O'Shea RD (2007) Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. Br J Pharmacol 150:5–17

Berger AJ, Hart AC, Kaplan JM (1998) G alphas-induced neurodegeneration in Caenorhabditis elegans. J Neurosci 18:2871–2880

Berger J, Suzuki T, Senti KA, Stubbs J, Schaffner G, Dickson BJ (2001) Genetic mapping with SNP markers in Drosophila. Nat Genet 29:475–481

Blumenthal T, Evans D, Link CD, Guffanti A, Lawson D, Thierry-Mieg J, Thierry-Mieg D, Chiu WL, Duke K, Kiraly M, Kim SK (2002) A global analysis of Caenorhabditis elegans operons. Nature 417:851–854

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415

Brenner S (1974) The genetics of Caenorhabditis elegans. Genetics 77:71–94

Broker LE, Kruyt FA, Giaccone G (2005) Cell death independent of caspases: a review. Clin Cancer Res 11:3155–3162

Carthew RW (2007) Pattern formation in the Drosophila eye. Curr Opin Genet Dev 17:309–313

Cauchi RJ, van den Heuvel M (2006) The fly as a model for neurodegenerative diseases: is it worth the jump? Neurodegener Dis 3:338–356

Celotto AM, Palladino MJ (2005) Drosophila: a "model" model system to study neurodegeneration. Mol Interv 5:292–303

Chalfie M, Wolinsky E (1990) The identification and suppression of inherited neurodegeneration in Caenorhabditis elegans. Nature 345:410–416

Challa M, Malladi S, Pellock BJ, Dresnek D, Varadarajan S, Yin YW, White K, Bratton SB (2007) Drosophila Omi, a mitochondrial-localized IAP antagonist and proapoptotic serine protease. EMBO J 26:3144–3156

Clarke PG (1990) Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol (Berl) 181:195–213

Cote J, Ruiz-Carrillo A (1993) Primers for mitochondrial DNA replication generated by endonuclease G. Science 261:765–769

Coulson A, Waterston R, Kiff J, Sulston J, Kohara Y (1988) Genome linking with yeast artificial chromosomes. Nature 335:184–186

Danial NN, Korsmeyer SJ (2004) Cell death: critical control points. Cell 116:205–219

del Peso L, Gonzalez VM, Inohara N, Ellis RE, Nunez G (2000) Disruption of the CED-9.CED-4 complex by EGL-1 is a critical step for programmed cell death in Caenorhabditis elegans. J Biol Chem 275:27205–27211

Driscoll M, Gerstbrein B (2003) Dying for a cause: invertebrate genetics takes on human neurodegeneration. Nat Rev Genet 4:181–194

Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16:663–669

Ellis HM, Horvitz HR (1986) Genetic control of programmed cell death in the nematode C. elegans. Cell 44:817–829

Estaquier J, Arnoult D (2006) CED-9 and EGL-1: a duo also regulating mitochondrial network morphology. Mol Cell 21:730–732

Faber PW, Voisine C, King DC, Bates EA, Hart AC (2002) Glutamine/proline-rich PQE-1 proteins protect Caenorhabditis elegans neurons from huntingtin polyglutamine neurotoxicity. Proc Natl Acad Sci USA 99:17131–17136

Furuya N, Yu J, Byfield M, Pattingre S, Levine B (2005) The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. Autophagy 1:46–52

Golic KG (1991) Site-specific recombination between homologous chromosomes in Drosophila. Science 252:958–961

Gorski SM, Chittaranjan S, Pleasance ED, Freeman JD, Anderson CL, Varhol RJ, Coughlin SM, Zuyderduyn SD, Jones SJ, Marra MA (2003) A SAGE approach to discovery of genes involved in autophagic cell death. Curr Biol 13:358–363

Green DR, Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science 305:626–629

Greenspan RJ (1997) Fly pushing. Cold Spring Harbor Laboratory Press, New York

Hall DH, Russell RL (1991) The posterior nervous system of the nematode Caenorhabditis elegans: serial reconstruction of identified neurons and complete pattern of synaptic interactions. J Neurosci 11:1–22

Halligan DL, Keightley PD (2006) Ubiquitous selective constraints in the Drosophila genome revealed by a genome-wide interspecies comparison. Genome Res 16:875–884

Hartl DL, Ajioka JW, Cai H, Lohe AR, Lozovskaya ER, Smoller DA, Duncan IW (1992) Towards a Drosophila genome map. Trends Genet 8:70–75

Hay BA, Guo M (2006) Caspase-dependent cell death in Drosophila. Annu Rev Cell Dev Biol 22:623–650

Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770–776

Hersh BM, Hartwieg E, Horvitz HR (2002) The Caenorhabditis elegans mucolipin-like gene cup-5 is essential for viability and regulates lysosomes in multiple cell types. Proc Natl Acad Sci USA 99:4355–4360

Igaki T, Miura M (2004) Role of Bcl-2 family members in invertebrates. Biochim Biophys Acta 1644:73–81

Igaki T, Suzuki Y, Tokushige N, Aonuma H, Takahashi R, Miura M (2007) Evolution of mitochondrial cell death pathway: Proapoptotic role of HtrA2/Omi in Drosophila. Biochem Biophys Res Commun 356:993–997

Jakubowski J, Kornfeld K (1999) A local, high-density, single-nucleotide polymorphism map used to clone Caenorhabditis elegans cdf-1. Genetics 153:743–752

Jin Y (2005) C. elegans – a practical approach. Oxford University press, Oxford

Juhasz G, Sass M (2005) Hid can induce, but is not required for autophagy in polyploid larval Drosophila tissues. Eur J Cell Biol 84:491–502

Juhasz G, Erdi B, Sass M, Neufeld TP (2007a) Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. Genes Dev 21:3061–3066

Juhasz G, Puskas LG, Komonyi O, Erdi B, Maroy P, Neufeld TP, Sass M (2007b) Gene expression profiling identifies FKBP39 as an inhibitor of autophagy in larval Drosophila fat body. Cell Death Differ 14:1181–1190

Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol 150:1507–1513

Kang C, You YJ, Avery L (2007) Dual roles of autophagy in the survival of Caenorhabditis elegans during starvation. Genes Dev 21:2161–2171

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257

Kim R, Emi M, Tanabe K (2006) Role of mitochondria as the gardens of cell death. Cancer Chemother Pharmacol 57:545–553

Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, Bronson RT, Ackerman SL (2002) The harlequin mouse mutation downregulates apoptosis-inducing factor. Nature 419:367–374

Klionsky DJ (2004) Cell biology: regulated self-cannibalism. Nature 431:31–32

Klionsky DJ (2005) The molecular machinery of autophagy: unanswered questions. J Cell Sci 118:7–18

Klionsky DJ (2007) Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol 8:931–937

Klionsky DJ, Cregg JM, Dunn WA Jr, Emr SD, Sakai Y, Sandoval IV, Sibirny A, Subramani S, Thumm M, Veenhuis M, Ohsumi Y (2003) A unified nomenclature for yeast autophagy-related genes. Dev Cell 5:539–545

Kornberg TB, Krasnow MA (2000) The Drosophila genome sequence: implications for biology and medicine. Science 287:2218–2220

Korswagen HC, van der Linden AM, Plasterk RH (1998) G protein hyperactivation of the Caenorhabditis elegans adenylyl cyclase SGS-1 induces neuronal degeneration. EMBO J 17:5059–5065

Kourtis N, Tavernarakis N (2007) Non-developmentally programmed cell death in Caenorhabditis elegans. Semin Cancer Biol 17:122–133
Krantic S, Mechawar N, Reix S, Quirion R (2007) Apoptosis-inducing factor: a matter of neuron life and death. Prog Neurobiol 81:179–196
Kroemer G, Martin SJ (2005) Caspase-independent cell death. Nat Med 11:725–730
Kumar A, Rothman JH (2007) Cell death: hook, line and linker. Curr Biol 17:R286–R289
Lakovaara S (1969) Malt as a culture medium for Drosophila species. Drosoph Inf Serv 44:128
Lawrence PA (1992) The making of a fly. The genetics of animal design, 1st edn. Blackwell, Oxford
Lee ST, Kim M (2006) Aging and neurodegeneration. Molecular mechanisms of neuronal loss in Huntington's disease. Mech Ageing Dev 127:432–435
Lee CY, Clough EA, Yellon P, Teslovich TM, Stephan DA, Baehrecke EH (2003) Genome-wide analyses of steroid- and radiation-triggered programmed cell death in Drosophila. Curr Biol 13:350–357
Lee J, Nam S, Hwang SB, Hong M, Kwon JY, Joeng KS, Im SH, Shim J, Park MC (2004) Functional genomic approaches using the nematode Caenorhabditis elegans as a model system. J Biochem Mol Biol 37:107–113
Leist M, Jaattela M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2:589–598
Lettre G, Hengartner MO (2006) Developmental apoptosis in C. elegans: a complex CEDnario. Nat Rev Mol Cell Biol 7:97–108
Leyssen M, Hassan BA (2007) A fruitfly's guide to keeping the brain wired. EMBO Rep 8:46–50
Li W, Baker NE (2007) Engulfment is required for cell competition. Cell 129:1215–1225
Li LY, Luo X, Wang X (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412:95–99
Lim HY, Bodmer R, Perrin L (2006) Drosophila aging 2005/06. Exp Gerontol 41:1213–1216
Lin CY, Chen SH, Cho CS, Chen CL, Lin FK, Lin CH, Chen PY, Lo CZ, Hsiung CA (2006) FlyDPI: database of protein interactomes for D. melanogaster in the approach of systems biology. BMC Bioinformatics 7(Suppl 5):S18
Lindmo K, Stenmark H (2006) How a RING finger protein and a steroid hormone control autophagy. Autophagy 2:321–322
Lindmo K, Simonsen A, Brech A, Finley K, Rusten TE, Stenmark H (2006) A dual function for Deep orange in programmed autophagy in the Drosophila melanogaster fat body. Exp Cell Res 312:2018–2027
Lorenzo HK, Susin SA (2004) Mitochondrial effectors in caspase-independent cell death. FEBS Lett 557:14–20
Majeski AE, Dice JF (2004) Mechanisms of chaperone-mediated autophagy. Int J Biochem Cell Biol 36:2435–2444
Martin DN, Baehrecke EH (2004) Caspases function in autophagic programmed cell death in Drosophila. Development 131:275–284
Martin DN, Balgley B, Dutta S, Chen J, Rudnick P, Cranford J, Kantartzis S, DeVoe DL, Lee C, Baehrecke EH (2007) Proteomic analysis of steroid-triggered autophagic programmed cell death during Drosophila development. Cell Death Differ 14:916–923
Mazzalupo S, Cooley L (2006) Illuminating the role of caspases during Drosophila oogenesis. Cell Death Differ 13:1950–1959
McCall K (2004) Eggs over easy: cell death in the Drosophila ovary. Dev Biol 274:3–14
Melendez A, Talloczy Z, Seaman M, Eskelinen EL, Hall DH, Levine B (2003) Autophagy genes are essential for dauer development and life-span extension in C. elegans. Science 301:1387–1391
Mello CC, Conte D Jr (2004) Revealing the world of RNA interference. Nature 431:338–342
Mello C, Fire A (1995) Methods in cell biology: Caenorhabditis elegans: modern biological analysis of an organism. Academic, San Diego, p 451
Mergliano J, Minden JS (2003) Caspase-independent cell engulfment mirrors cell death pattern in Drosophila embryos. Development 130:5779–5789

Miramar MD, Costantini P, Ravagnan L, Saraiva LM, Haouzi D, Brothers G, Penninger JM, Peleato ML, Kroemer G, Susin SA (2001) NADH oxidase activity of mitochondrial apoptosis-inducing factor. J Biol Chem 276:16391–16398

Modjtahedi N, Giordanetto F, Madeo F, Kroemer G (2006) Apoptosis-inducing factor: vital and lethal. Trends Cell Biol 16:264–272

Munoz-Pinedo C, Guio-Carrion A, Goldstein JC, Fitzgerald P, Newmeyer DD, Green DR (2006) Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. Proc Natl Acad Sci USA 103:11573–11578

Murakami S (2007) Caenorhabditis elegans as a model system to study aging of learning and memory. Mol Neurobiol 35:85–94

Nakano Y, Fujitani K, Kurihara J, Ragan J, Usui-Aoki K, Shimoda L, Lukacsovich T, Suzuki K, Sezaki M, Sano Y, Ueda R, Awano W, Kaneda M, Umeda M, Yamamoto D (2001) Mutations in the novel membrane protein spinster interfere with programmed cell death and cause neural degeneration in Drosophila melanogaster. Mol Cell Biol 21:3775–3788

Nass R, Hall DH, Miller DM 3rd, Blakely RD (2002) Neurotoxin-induced degeneration of dopamine neurons in Caenorhabditis elegans. Proc Natl Acad Sci USA 99:3264–3269

Neufeld TP (2007) Contribution of Atg1-dependent autophagy to TOR-mediated cell growth and survival. Autophagy 3:477–479

Nicotera P, Leist M, Manzo L (1999) Neuronal cell death: a demise with different shapes. Trends Pharmacol Sci 20:46–51

Ohiro Y, Garkavtsev I, Kobayashi S, Sreekumar KR, Nantz R, Higashikubo BT, Duffy SL, Higashikubo R, Usheva A, Gius D, Kley N, Horikoshi N (2002) A novel p53-inducible apoptogenic gene, PRG3, encodes a homologue of the apoptosis-inducing factor (AIF). FEBS Lett 524:163–171

O'Keefe LV, Liu Y, Perkins A, Dayan S, Saint R, Richards RI (2005) FRA16D common chromosomal fragile site oxido-reductase (FOR/WWOX) protects against the effects of ionizing radiation in Drosophila. Oncogene 24:6590–6596

Olney JW (1994) Excitatory transmitter neurotoxicity. Neurobiol Aging 15:259–260

Pandey UB, Nie Z, Batlevi Y, McCray BA, Ritson GP, Nedelsky NB, Schwartz SL, DiProspero NA, Knight MA, Schuldiner O, Padmanabhan R, Hild M, Berry DL, Garza D, Hubbert CC, Yao TP, Baehrecke EH, Taylor JP (2007) HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature 447:859–863

Parrish JZ, Xue D (2003) Functional genomic analysis of apoptotic DNA degradation in C. elegans. Mol Cell 11:987–996

Parrish J, Li L, Klotz K, Ledwich D, Wang X, Xue D (2001) Mitochondrial endonuclease G is important for apoptosis in C. elegans. Nature 412:90–94

Parrish JZ, Yang C, Shen B, Xue D (2003) CRN-1, a Caenorhabditis elegans FEN-1 homologue, cooperates with CPS-6/EndoG to promote apoptotic DNA degradation. EMBO J 22:3451–3460

Porter AG, Urbano AG (2006) Does apoptosis-inducing factor (AIF) have both life and death functions in cells? Bioessays 28:834–843

Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ, Rubinsztein DC (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet 36:585–595

Riddle D (1988) The nematode C. elegans. Cold Spring Harbor Laboratory Press, New York, pp 393–412

Riddle DL, Gorski SM (2003) Shaping and stretching life by autophagy. Dev Cell 5:364–365

Riddle DL, Blumenthal T, Meyer BJ, Priess JR (1997) C. elegans II. Cold Spring Harbor Laboratory Press, New York

Rieckher M, Kourtis N, Pasparaki A, Tavernarakis N (2009) Transgenesis in Caenorhabditis elegans. Method Mol Biol 561:21–39

Rival T, Soustelle L, Strambi C, Besson MT, Iche M, Birman S (2004) Decreasing glutamate buffering capacity triggers oxidative stress and neuropil degeneration in the Drosophila brain. Curr Biol 14:599–605

Roudier N, Lefebvre C, Legouis R (2005) CeVPS-27 is an endosomal protein required for the molting and the endocytic trafficking of the low-density lipoprotein receptor-related protein 1 in Caenorhabditis elegans. Traffic 6:695–705

Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ (2007) Potential therapeutic applications of autophagy. Nat Rev Drug Discov 6:304–312

Rusten TE, Lindmo K, Juhasz G, Sass M, Seglen PO, Brech A, Stenmark H (2004) Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. Dev Cell 7:179–192

Samara C, Tavernarakis N (2008) Autophagy and cell death in Caenorhabditis elegans. Curr Pharm Des 14:1–19

Samara C, Syntichaki P, Tavernarakis N (2008) Autophagy is required for necrotic cell death in Caenorhabditis elegans. Cell Death Differ 15(1):105–112

Sarkar S, Perlstein EO, Imarisio S, Pineau S, Cordenier A, Maglathlin RL, Webster JA, Lewis TA, O'Kane CJ, Schreiber SL, Rubinsztein DC (2007) Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. Nat Chem Biol 3:331–338

Schmitz C, Kinge P, Hutter H (2007) Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive Caenorhabditis elegans strain nre-1(hd20) lin-15b(hd126). Proc Natl Acad Sci USA 104:834–839

Scott BA, Avidan MS, Crowder CM (2002) Regulation of hypoxic death in C. elegans by the insulin/IGF receptor homolog DAF-2. Science 296:2388–2391

Scott RC, Schuldiner O, Neufeld TP (2004) Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev Cell 7:167–178

Scott RC, Juhasz G, Neufeld TP (2007) Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. Curr Biol 17:1–11

Simmer F, Moorman C, van der Linden AM, Kuijk E, van den Berghe PV, Kamath RS, Fraser AG, Ahringer J, Plasterk RH (2003) Genome-wide RNAi of C. elegans using the hypersensitive rrf-3 strain reveals novel gene functions. PLoS Biol 1:E12

Simonsen A, Cumming RC, Brech A, Isakson P, Schubert DR, Finley KD (2008) Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. Autophagy 4(2):176–184

Spradling AC, Stern DM, Kiss I, Roote J, Laverty T, Rubin GM (1995) Gene disruptions using P transposable elements: an integral component of the Drosophila genome project. Proc Natl Acad Sci USA 92:10824–10830

Stefanis L (2005) Caspase-dependent and -independent neuronal death: two distinct pathways to neuronal injury. Neuroscientist 11:50–62

Sulston JE, Albertson DG, Thomson JN (1980) The Caenorhabditis elegans male: postembryonic development of nongonadal structures. Dev Biol 78:542–576

Sulston JE, Schierenberg E, White JG, Thomson JN (1983) The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Biol 100:64–119

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell 8:613–621

Syntichaki P, Tavernarakis N (2002) Death by necrosis. Uncontrollable catastrophe, or is there order behind the chaos? EMBO Rep 3:604–609

Syntichaki P, Tavernarakis N (2003) The biochemistry of neuronal necrosis: rogue biology? Nat Rev Neurosci 4:672–684

Syntichaki P, Tavernarakis N (2004) Genetic models of mechanotransduction: the nematode Caenorhabditis elegans. Physiol Rev 84:1097–1153
Syntichaki P, Xu K, Driscoll M, Tavernarakis N (2002) Specific aspartyl and calpain proteases are required for neurodegeneration in C. elegans. Nature 419:939–944
Syntichaki P, Samara C, Tavernarakis N (2005) The vacuolar H+ -ATPase mediates intracellular acidification required for neurodegeneration in C. elegans. Curr Biol 15:1249–1254
Tabata T, Takei Y (2004) Morphogens, their identification and regulation. Development 131:703–712
Takacs-Vellai K, Vellai T, Puoti A, Passannante M, Wicky C, Streit A, Kovacs AL, Muller F (2005) Inactivation of the autophagy gene bec-1 triggers apoptotic cell death in C. elegans. Curr Biol 15:1513–1517
Tavernarakis N, Driscoll M (2001) Degenerins. At the core of the metazoan mechanotransducer? Ann N Y Acad Sci 940:28–41
Tavernarakis N, Wang SL, Dorovkov M, Ryazanov A, Driscoll M (2000) Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. Nat Genet 24:180–183
Thomas JH, Lockery S (2005) C. elegans – a practical approach: neurobiology. Oxford University press, Oxford
Thumm M, Kadowaki T (2001) The loss of Drosophila APG4/AUT2 function modifies the phenotypes of cut and Notch signaling pathway mutants. Mol Genet Genomics 266:657–663
Toth ML, Simon P, Kovacs AL, Vellai T (2007) Influence of autophagy genes on ion-channel-dependent neuronal degeneration in Caenorhabditis elegans. J Cell Sci 120:1134–1141
Varkey J, Chen P, Jemmerson R, Abrams JM (1999) Altered cytochrome c display precedes apoptotic cell death in Drosophila. J Cell Biol 144:701–710
Vaux DL, Korsmeyer SJ (1999) Cell death in development. Cell 96:245–254
Venken KJ, Bellen HJ (2005) Emerging technologies for gene manipulation in Drosophila melanogaster. Nat Rev Genet 6:167–178
Walhout AJ, Sordella R, Lu X, Hartley JL, Temple GF, Brasch MA, Thierry-Mieg N, Vidal M (2000) Protein interaction mapping in C. elegans using proteins involved in vulval development. Science 287:116–122
Wang CW, Klionsky DJ (2003) The molecular mechanism of autophagy. Mol Med 9:65–76
Wang X, Yang C, Chai J, Shi Y, Xue D (2002) Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans. Science 298:1587–1592
Wang X, Wang J, Gengyo-Ando K, Gu L, Sun CL, Yang C, Shi Y, Kobayashi T, Shi Y, Mitani S, Xie XS, Xue D (2007) C. elegans mitochondrial factor WAH-1 promotes phosphatidylserine externalization in apoptotic cells through phospholipid scramblase SCRM-1. Nat Cell Biol 9:541–549
Wang X, Yang C, Xue D (unpublished results)
Waterston R, Sulston J (1995) The genome of Caenorhabditis elegans. Proc Natl Acad Sci USA 92:10836–10840
White JG, Southgate E, Thomson JN, Brenner S (1983) Factors that determine connectivity in the nervous system of Caenorhabditis elegans. Cold Spring Harb Symp Quant Biol 48(Pt 2):633–640
Wu M, Xu LG, Li X, Zhai Z, Shu HB (2002) AMID, an apoptosis-inducing factor-homologous mitochondrion-associated protein, induces caspase-independent apoptosis. J Biol Chem 277:25617–25623
Xiang H, Hochman DW, Saya H, Fujiwara T, Schwartzkroin PA, Morrison RS (1996) Evidence for p53-mediated modulation of neuronal viability. J Neurosci 16:6753–6765
Xu K, Tavernarakis N, Driscoll M (2001) Necrotic cell death in C. elegans requires the function of calreticulin and regulators of Ca(2+) release from the endoplasmic reticulum. Neuron 31:957–971
Yamashima T (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. Prog Neurobiol 62:273–295

Yamashima T (2004) Ca2+-dependent proteases in ischemic neuronal death: a conserved 'calpain-cathepsin cascade' from nematodes to primates. Cell Calcium 36:285–293

Yanagisawa H, Miyashita T, Nakano Y, Yamamoto D (2003) HSpin1, a transmembrane protein interacting with Bcl-2/Bcl-xL, induces a caspase-independent autophagic cell death. Cell Death Differ 10:798–807

Ye H, Cande C, Stephanou NC, Jiang S, Gurbuxani S, Larochette N, Daugas E, Garrido C, Kroemer G, Wu H (2002) DNA binding is required for the apoptogenic action of apoptosis inducing factor. Nat Struct Biol 9:680–684

Yorimitsu T, Klionsky DJ (2007) Eating the endoplasmic reticulum: quality control by autophagy. Trends Cell Biol 17:279–285

Yuste VJ, Moubarak RS, Delettre C, Bras M, Sancho P, Robert N, D'Alayer J, Susin SA (2005) Cysteine protease inhibition prevents mitochondrial apoptosis-inducing factor (AIF) release. Cell Death Differ 12:1445–1448

Zhou Z, Mangahas PM, Yu X (2004) The genetics of hiding the corpse: engulfment and degradation of apoptotic cells in C. elegans and D. melanogaster. Curr Top Dev Biol 63:91–143

Chapter 3
Programmed Necrosis: A "New" Cell Death Outcome for Injured Adult Neurons?

Slavica Krantic and Santos A. Susin

3.1 Cell Death Outcomes: General Considerations

Historically, cell death has been divided into two generic categories: apoptosis, which requires energy and in which the cell plays an active role, and necrosis, which occurs accidentally, does not require energy consumption and is considered as a passive, uncontrolled cell death program. Among the conceptually opposite cell death forms, apoptosis is the best understood. This death program has been defined as developmentally programmed and ordered cellular response. Apoptosis is initiated by cell rounding and subsequent detachment from the surrounding cells. Chromatin condenses into "crescent-like" forms abutting the inner nuclear membrane. Plasma membrane convolutes and gives rise to characteristic vesicles containing cellular organelles and cytoplasm, known as the "apoptotic bodies." Apoptosis is generally not accompanied by inflammation since macrophages or neighbouring cells engulf the formed apoptotic bodies before the loss of plasma membrane integrity (Kerr et al. 1972). In contrast to apoptosis, necrosis is characterized by disruption of the plasma membrane with a subsequent water influx and leakage of cell content to the surroundings. Cell death by necrosis can elicit an inflammatory response (Edinger and Thompson 2004).

It now seems clear that the above-described apoptosis versus necrosis dichotomy is an artificial division, and that "programmed" cell death is a more complex physiological process than initially thought. The cell can use different mechanisms/pathways with underlying apoptotic or necrotic features to accomplish its proper

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demise in a controlled manner (Jaattela 2002; Broker et al. 2005; Jaattela and Tschopp 2003; Okada and Mak 2004; Golstein and Kroemer 2007; Golstein et al. 2003; Gozuacik and Kimchi 2007; Krantic et al. 2005, 2007; Susin et al. 1996, 1999, 2000; Lorenzo and Susin 2004; Barkla and Gibson 1999; Chautan et al. 1999; Holler et al. 2000; Colbourne et al. 1999; Nicotera et al. 1999a, b; Saelens et al. 2005; Vanden Berghe et al. 2004; Aarts et al. 2003; Moubarak et al. 2007; Festjens et al. 2006; Srivastava et al. 2007; Borst and Rottenberg 2004; Bras et al. 2007; Boujrad et al. 2007; Niquet et al. 2006; Li et al. 2007; Han et al. 2007; Fujikawa et al. 2000a).

To better understand the complexity of neuronal cell death linked to acute injury, we will first briefly discuss two of the most broadly used PCD classifications (Bredesen 2007). One of them bears the overall morphology of the dying cell and divides PCDs into three types: type 1 or "classical" apoptosis, type 2 or "autophagic" death, and type 3 or vesicular, nonlysosomal degradation (Schweichel and Merker 1973; Clarke 1990). The second classification is based on the type of chromatin condensation and comprises classical apoptosis, apoptosis-like PCD, and necrosis-like PCD (Jaattela and Tschopp 2003). According to this classification, apoptosis is characterized by compact (stage 2) chromatin condensation, whereas apoptosis-like PCD displays partial/peripheral (stage 1) chromatin condensation. In necrosis-like PCD chromatin is either noncondensed at all, or only slightly granulated (Jaattela and Tschopp 2003; Leist and Jaattela 2001).

Type 1 PCD or classical apoptosis, is the best-characterized cell death outcome at both genetic and biochemical levels (Danial and Korsmeyer 2004; Hengartner 2000). This mode of PCD involves the activation of a family of cysteine proteases named caspases (Thornberry and Lazebnik 1998) and it can be triggered via "death receptors" along the "extrinsic pathway" or via mitochondrial "intrinsic pathway" (Green and Reed 1998). The extrinsic pathway is initiated by interaction of death receptors (Fas, TNF-R) with their cognate ligands (FasL, TNF). Death receptors contain death domains (DD) and death effector domains (DED), which, upon ligand binding, engage into homeotypic protein–protein interactions. Receptor assembly into oligomers results in the formation of a Death-Inducing Signaling Complex (DISC) through conformational alterations. Adaptor proteins (e.g. TRADD, RaiDD, FADD) then associate with the relevant receptors via their DD and DED domains. Procaspase-8/-10 are further recruited to the complex and proteolytically cross-activated by the local accumulation of their pro-enzymes in DISC vicinity (Jaattela and Tschopp 2003; Danial and Korsmeyer 2004). The intrinsic pathway consists of two branches. One is triggered by endoplasmic reticulum stress resulting from the accumulation of unfolded or misfolded proteins and is associated with caspase-12 activation (Rao et al. 2002). The second is initiated by mitochondrial outer membrane permeabilization (MOMP), and is regulated by the Bcl-2 (B-cell lymphoma-2) family of proteins (Tsujimoto 2002). By regulating MOMP, these proteins control the release of key death regulatory proteins from mitochondria (Danial and Korsmeyer 2004). Although many aspects of this regulation remain poorly understood, it is clear that multidomain Bcl-2 proapoptotic proteins such as Bax (Bcl-2-associated X protein) and Bak (Bcl-2 homologous antagonist killer) are required for this cell death induction because their double genetic ablation confers resistance to type 1 PCD (Letai et al. 2002;

Scorrano and Korsmeyer 2003). Indeed, Bax or Bak create pores in the mitochondrial outer membrane. These pores allow the release of cytochrome *c* and other proteins, such as Smac/DIABLO or Omi/HtrA2, into the cytoplasm. The process progresses further by antagonistic interaction of Smac/DIABLO or Omi/HtrA2 with caspase inhibitors, which results in their inhibition and indirectly allows for caspase activation. The latter can also be achieved directly by cytochrome *c* binding to Apaf-1 into complexes that aggregate to form apoptosomes. This aggregation requires energy from ATP. The apoptosomes activate caspase-9, which then cleaves and activates caspase-3 and caspase-7. These executioner caspases engage a cascade of proteolytic activity that leads to the digestion of structural proteins and DNA degradation. This "caspase-dependent" DNA degradation, which relates to Caspase activated DNase (CAD), leads to a typical "apoptotic" internucleosomal DNA fragmentation (180–200 bp) and chromatin compaction into spherical or crescent masses abutting on the nuclear envelope (Hengartner 2000).

Autophagic type 2 PCD is characterized morphologically by the appearance of autophagic, double membraned vacuoles. These cytoplasmic vesicles contain cellular organelles, such as mitochondria or endoplasmic reticulum (Gozuacik and Kimchi 2007). It is important to distinguish "autophagic death" from autophagy. Indeed, autophagy is an adaptive process involved in survival response to low-nutrient states (in hypoglycemia, after axonal injury, etc), allowing for the catabolism of cellular constituents to produce energy or to remove damaged organelles. However, autophagy is also associated with cell death with either necrotic or apoptotic phenotype [for review, see Nixon (2006)]. The criteria for detection of this type of PCD were until recently exclusively morphological and even the existence of this PCD outcome is not yet generally accepted. It is in particular not clear whether autophagy represents a consequence (cell dies following autophagy) or a cause (cell death requires autophagy) of the cell death process. However, identification of the autophagy-related genes such as Atg5 or Atg6/Becn1 and the fact that their inactivation precludes "autophagic" PCD (Shimizu et al. 2004) strengthened the experimental evidence supporting the existence of this PCD as a specific and distinct cell death modality.

Type 3 PCD has been initially characterized by the presence of swelling organelles followed by the appearance of "empty" spaces in the cytoplasm which merge and make connections with the extra-cellular space (Schweichel and Merker 1973). The plasma membrane is fragmented, but nuclear disintegration is retarded. This type of PCD has subsequently been subdivided into sub-types 3A (i.e. "non-lysosomal disintegration") and 3B (i.e. "cytoplasmic degeneration") (Clarke 1990). Sub-type 3A is characterized by nuclear disintegration whereas sub-type 3B displays karyolysis (Clarke 1990; Beaulaton and Lockshin 1982).

Above-described types 2 and 3 PCD occur without pronounced nuclear chromatin condensation. This nuclear feature is used as the main criterion to classify a given type of cell death as necrosis-like PCD (Jaattela and Tschopp 2003; Leist and Jaattela 2001). Apoptosis-like PCD can be considered as an intermediate morphological phenotype. Indeed, chromatin condensation, which accompanies apoptosis-like PCD, is not as pronounced as in apoptosis but it is more prominent than in necrosis-like PCD.

Apoptosis-like PCD occurs through a caspase-independent mitochondrial route. Apoptosis-inducing factor (AIF) is currently considered the major apoptosis-like PCD effector (Krantic et al. 2007; Susin et al. 1996, 1997, 1999, 2000; Boujrad et al. 2007; Lorenzo and Susin 2007; Dawson and Dawson 2004; Yu et al. 2002; Hong et al. 2004). Upon MOMP, AIF is released from the intermembrane mitochondrial space. The kinetics of AIF release is, however, slower than that of cytochrome *c* release (Munoz-Pinedo et al. 2006) and caspase-dependent apoptosis appears consistently as more rapid cellular response to death-inducing insult. It is not clear yet whether apoptosis-like PCD represents a secondary cell death outcome, expressed only when apoptosis is inhibited. Indeed, it is still unknown whether AIF release can occur without previous cytochrome *c* release or under conditions in which released cytochrome *c* concentration is kept low by mitochondrial recovery. Such a mechanism has been suggested to occur in terminally differentiated rabbit aortic smooth muscle cells at least in vitro (Seye et al. 2004). In this light, the reversibility of cytochrome *c* release is of particular importance in neurons since these long-lived postmitotic cells can survive cytochrome *c* release step, at least during a limited time period (Martinou et al. 1999).

After release from the intermembrane mitochondrial space, AIF translocates to the nucleus, leading to large-scale DNA degradation into 50–200 kb fragments (Cregan et al. 2004). Given the absence of an intrinsic endonuclease activity (Mate et al. 2002; Ye et al. 2002), the DNA-degrading capacity of AIF relies on the recruitment of downstream nucleases, such as cyclophilin A (cyp A) (Cande et al. 2004) or endonuclease G (EndoG) (Wang et al. 2002; Bajt et al. 2006; Whiteman et al. 2007).

Necrosis-like PCD occurs by yet poorly understood molecular mechanisms, but it is usually independent of caspase activation, with some exceptions [see Edinger and Thompson (2004), Sperandio et al. (2000), Boise and Collins (2001), Meurette et al. (2007), and Zong and Thompson (2006)]. Note that, although necrosis-like PCD (Edinger and Thompson 2004; Zong and Thompson 2006) as well as programmed necrosis (Moubarak et al. 2007; Boujrad et al. 2007) are morphologically indistinguishable from necrosis, they both differ from that nonregulated, accidental process described by Kerr, Wyllie, and Curie in 1972 (Kerr et al. 1972). These morphological resemblances include principally the fact that in accidental necrosis (Edinger and Thompson 2004), as in necrosis-like PCD (Jaattela and Tschopp 2003) and programmed necrosis [as described recently by us (Moubarak et al. 2007; Boujrad et al. 2007) and others (Vande Velde et al. 2000; Hirt et al. 2000; Gharibyan et al. 2007)], the integrity of the plasma membrane is lost early. Nevertheless, it should be stressed that this loss of plasma membrane integrity occurs in an orderly and similar way in all cases, although its kinetics is certainly different between the programmed and accidental modalities of necrosis. Thus, plasma membrane becomes permeable (as measured by propidium iodide, PI, diffusion) relatively early after initiation of the death process (Chen et al. 2001a; Liu and Schnellmann 2003) whereas leakage of cytoplasmic proteins, such as lactate dehydrogenase (LDH), indicates the terminal phase of permeabilization (Nishimura and Lemasters 2001) in all known death modalities with a necrotic phenotype.

The difference between the molecular mass of PI (0.67 kDa) and LDH (140 kDa) suggests that the size of the membrane pores increases as the death process progresses. If proceeding slowly, such process of membrane permeabilization appears compatible with the programmed (or controlled) character of certain necrotic death outcomes. For the sake of clarity, in the forthcoming text we will use exclusively the term of programmed necrosis to point out the PCD outcome occurring with a necrosis-like morphological phenotype through activation of identified effectors/proteins (Moubarak et al. 2007).

3.2 Programmed Necrosis

3.2.1 General Considerations

As already stated, the clear morphological and underlying mechanistic distinctions between accidental and programmed necrosis are still lacking (Bredesen 2007). Intuitively, it can be assumed that if the process of cell death is programmed, in the sense of controlled over the time, it can be temporally distinguished from accidental necrosis, which occurs instantly and in a "violent" manner. These theoretical considerations are primordial since they point to the importance of the kinetic analysis of necrotic death phenotypes, which remains the most accurate approach allowing to distinguish between the following outcomes of necrosis (1) primary accidental; (2) primary programmed (controlled) and (3) secondary (which theoretically might occur in both a controlled and uncontrolled manner). However, this does not mean that in extremis [provided that the competent death stimulus is strong enough (Orrenius and Zhivotovsky 2006)], all cell death outcomes should be considered as accidental necrosis and that the study of different death modalities is irrelevant. Indeed, in vivo, the cells are exposed to different death signals of which each can occur in an infinite panel of intensities yielding a great diversity of resulting cell death outcomes. Programmed necrosis might represent one, among a multitude, of end-stage phenotypic expressions.

3.2.2 Definition

Knowledge of the mechanisms underlying programmed necrosis is yet too incomplete and does not allow for a universal (in terms of considering a panoply of different cell types) definition. The definition thus remains descriptive (based principally on the morphological features) and negative (based on the elimination of other cell death mechanisms). There are currently only two discriminatory criteria coming from the genetic (for the first) and biochemical (for the second) studies. First criterion concerns the apparent dependency of the programmed necrosis on cyclophilin D (cypD): genetic ablation of this component of the mitochondrial

permeability transition (PT) pore confers resistance to (programmed) necrosis without preventing other forms of cell death (Baines et al. 2005; Basso et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005). The second criterion comes from the biochemical studies and deals with the structural alterations of the plasma membrane at the early stages of programmed necrosis. Cells undergoing programmed necrosis display Annexin V-detectable phosphatidylserine (PS) exposure, but in contrast to what is seen in apoptosis, Annexin V-labelling never appears before plasma membrane permeabilization (assessed by PI labelling and LDH release). The programmed necrosis-related Annexin V-labelling does not appear as an artefact since the cells dying by programmed necrosis can be efficiently phagocyted (Boujrad et al. 2007). Altogether, this strongly suggests that PS exposed to the extra-cellular space serves as a functional "eat me" signal. The capacity of monocytes/macrophages to eliminate MNNG-treated cells before they undergo lysis due to plasma membrane permeabilization (Boujrad et al. 2007), argues in favour of a very short delay between PS exposure and plasma membrane permeabilization. Although such delay is probably undetectable by current methods (meaning that both Annexin V-labelling and LDH leakiness are detected concomitantly), it might be a specific biochemical hallmark of programmed necrosis. Consistent with this is the fact that the delay between Annexin V-exposure and LDH release is much longer in apoptosis, allowing for a detection of Annexin V-positive/PI-negative cells before detection of double-positive cells. However, it should be kept in mind that, alternatively, cells dying by programmed necrosis might display specific (i.e. different from those used to clear apoptotic cells) and yet unknown "eat me" signals that can be used by macrophages to clear necrotic cells (Krysko et al. 2006).

3.2.3 Effectors of Programmed Necrosis

Although necrotic PCD involves the loss of mitochondrial membrane potential ($\Delta\Psi_m$), this cell death program can be initiated by the organelles other than mitochondria (e.g. lysosomes, endoplasmatic reticulum, or nucleus) and proteases other than caspases (e.g., calpains or cathepsins). Indeed, it seems that initial, organelle-specific death responses finally lead to mitochondrial alteration. Mitochondria might function as a central integrator of the programmed necrotic pathway, thereby streamlining lysosome-, endoplasmatic reticulum-, or nucleus-elicited responses into a common pathway. A good example of such integration is programmed necrosis induced by extensive DNA-damage (Moubarak et al. 2007). This form of cell death is regulated by mitochondrial (AIF), cytoplasmic (Bax and calpains), and nuclear (PARP-1) effectors. Indeed, this recent work from our laboratory provides novel insights into the molecular mechanisms of programmed necrosis, since we have demonstrated the sequential activation of PARP-1, calpains, Bax, and AIF in a linear cell death pathway. Single ablation of PARP-1, the proapoptotic Bcl-2 member Bax, but not Bak, as well as inactivation of calpains or AIF prevented programmed necrosis.

In the following sections we will describe these key programmed necrosis effectors in further detail.

3.2.3.1 AIF

AIF was the first identified protein involved in caspase-independent cell death (Susin et al. 1999). AIF is expressed as a precursor of 67 kDa, which is addressed and compartmentalized into mitochondria by two mitochondrial localization sequences (MLS) located within the N-terminal prodomain of the protein. Once in mitochondria, this prodomain is removed, giving rise to a mature form of ~62 kDa (Susin et al. 1999; Otera et al. 2005). This form comprises three structural domains: FAD-binding domain, NADH-binding domain, and C-terminal domain (Lorenzo and Susin 2004, 2007). Under physiological conditions, AIF remains confined to the internal mitochondrial membrane, with its N-terminal region oriented towards the matrix (Otera et al. 2005). AIF is here a mitochondrial FAD-dependent oxidoreductase that plays a role in oxidative phosphorylation (Mate et al. 2002; Vahsen et al. 2004; Miramar et al. 2001). However, after a cellular insult, AIF is cleaved by calpains and/or cathepsins to yield tAIF (truncated AIF) a 57 kDa form of AIF (Otera et al. 2005; Polster et al. 2005; Yuste et al. 2005a). tAIF then translocates from mitochondria to cytosol and further to the nucleus, where it interacts with DNA and causes caspase-independent chromatin condensation and participates in large-scale (~50 kb) DNA fragmentation (Susin et al. 1999; Ye et al. 2002). The mitochondrial release, nuclear translocation, and DNA fragmentation associated with AIF have now been extensively demonstrated in several cell death systems and cell types (~900 references in Medline to date).

Three additional AIF isoforms have been characterized (1) AIF-exB, generated by an alternative use of exon 2b (rather than exon 2) (Loeffler et al. 2001); (2) AIFsh (AIF short) derived from an alternative transcriptional start site located at intron 9 of *AIF* (Delettre et al. 2006a); and (3) AIFsh2 (AIF short 2), produced by the alternative use of the newly discovered exon 9b (Delettre et al. 2006b). While AIF-exB is similar to AIF in terms of regulation and subcellular distribution (Loeffler et al. 2001), AIFsh and AIFsh2 are respectively restricted to cytoplasm and mitochondria, at least under physiological conditions (Delettre et al. 2006a, b). Upon the induction of cell death, like AIF, AIFsh translocates to the nucleus leading to large-scale (~50 kb) DNA-fragmentation (Delettre et al. 2006a). In contrast, AIFsh2, which presents oxidoreductase but no proapoptotic activity, translocates from mitochondria to cytosol after an apoptotic insult but cannot be further translocated to the nucleus (Delettre et al. 2006b).

As indicated above, AIF release from mitochondria occurs in a number of cell types, independently of the nature of the death-triggering signal (Kim et al. 2003a; Murahashi et al. 2003; Zhu et al. 2003). The requirement of nuclear translocation for AIF-mediated induction of caspase-independent, apoptosis-like PCD has been explicitly demonstrated by microinjection experiments of AIF-directed antibodies, which potently inhibited AIF targeting to the nucleus as well as cell

death (Susin et al. 1999; Yu et al. 2002; Cregan et al. 2004). Following nuclear translocation, the integrity of AIF's C-terminal domain, but not of the oxidoreductase-related (NADH) and FAD-binding N-terminal domains, is required to trigger PCD (Miramar et al. 2001; Loeffler et al. 2001). This was confirmed by a study showing that AIFsh can trigger cell death despite not having NADH and FAD-binding domains (Delettre et al. 2006a).

Interestingly, it is currently unknown whether the mechanisms of mitochondrial AIF release might be different and conditioned by the organelle at which the cell death program has been initiated (i.e. nucleus in the case of DNA damage-induced death vs. mitochondria in the case of Ca^{2+} overload). This is of a particular interest because both of these pathological alterations have been involved in triggering programmed necrosis (see sections "Increase in intracellular Ca^{2+} concentration" and "DNA damage"). It is important to keep in mind that different organelles (e.g. nucleus and mitochondria) might be engaged in an orderly manner to mediate induction of the cell death program. For example, the nucleus might be the site of cell death process initiation in the paradigm of genotoxic DNA damage, whereas it can be involved in a secondary fashion when oxidative DNA damage is triggered by reactive oxygen species (ROS) generated after mitochondrial impairment (Fig. 3.1).

3.2.3.2 Bax

The Bcl-2 family includes proteins with antagonistic functions that either cause programmed cell death or inhibit it. Their structures in solution are similar to those of bacterial channel-forming toxins. Indeed, just like these toxins, the Bcl-2 proteins cycle between soluble and membrane-associated forms, and form channels in mitochondrial membranes. Bax is a member of the Bcl-2 family that, together with Bak, belongs to the multidomain BH1-BH3 (Bcl-2 homology domains 1-3) pro-apoptotic Bcl-2 proteins (Kuwana et al. 2002, 2005). Both Bax and Bak act by sequestering antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xL (proteins containing BH1-BH4 domains). Other proapoptotic

Fig. 3.1 (continued) compatible with the reported Bax-dependency (Moubarak et al. 2007). It has to be noted that the endonucleases recruited by tAIF (*circle* 9 and *square* 12) are probably distinct. In addition, there might exist a cross-talk between these two distinct pathways leading to different AIF-mediated PCD outcomes. Indeed, intra-cellular Ca^{2+} over-load might lead to increased ROS production subsequent to mitochondrial metabolism impairment. Among other deleterious effects, ROS might mediate oxidative damage of DNA, which can in turn trigger PARP-1 activation. This will result in the switch from the Ca^{2+}-induced pathway depicted in the *lower part* of the figure to the DNA-damage-induced pathway depicted in the *upper part* of the figure. An interesting possibility is that these two pathways might operate in parallel in neurons exposed to some acute injuries, and thus explain the existence of "hybrid" death phenotypes seen, for example, in neurons exposed to ischemia that display both necrotic features and TUNEL-positivity (Unal-Cevik et al. 2004)

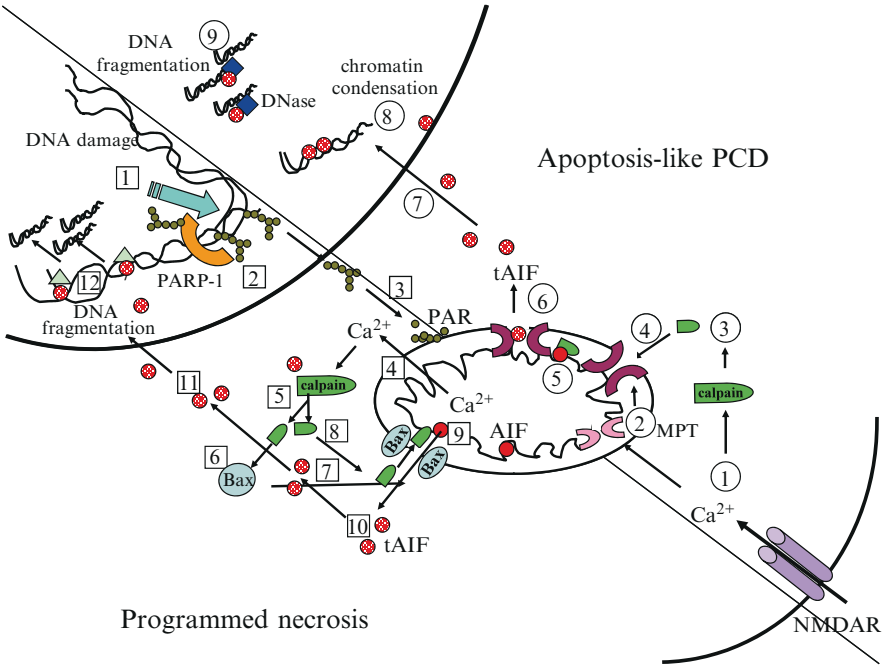

Fig. 3.1 Unifying model for AIF-mediated PCDs. The proposed model is based on the initial trigger of PCD: the increase in Ca^{2+} intracellular level for apoptosis-like PCD (*lower part* of the scheme) and DNA damage for programmed necrosis (*upper part* of the scheme). Initial rise in Ca^{2+} level (*circle* 1) in neurons is mainly due to the activation of the NMDA type of glutamate receptors (NMDAR) (Cheung et al. 2005). The increase in Ca^{2+} level may lead to the opening of the high conductance permeability transition (PT) pores in the mitochondrial inner membrane (IMM), as well as to subsequent mitochondrial membrane depolarization associated with mitochondrial permeability transition (MPT), oxidative phosphorylation uncoupling, and mitochondrial swelling (*circle* 2) (Kim et al. 2003b). In parallel, the increase in intracellular Ca^{2+} level leads to calpain activation (*circle* 3); activated calpain might enter the mitochondrial inter-membrane space via putative PT pore (*circle* 4) and subsequently cleave AIF from the inner mitochondrial membrane yielding truncated AIF (tAIF) (*circle* 5). The latter is then released from mitochondria (*circle* 6) and is translocated to the nucleus (*circle* 7), where it participates in chromatin condensation (*circle* 8) and DNA fragmentation (*circle* 9) (Susin et al. 1999; Susin et al. 2000; Susin et al. 1996; Moubarak et al. 2007; Susin et al. 1997; Yuste et al. 2005b). This hypothetical model is compatible with reported Bax-independence of NMDA-induced apoptosis-like PCD (Cheung et al. 2005). When the initial lesion concerns DNA damage (*square* 1), PARP-1 activation and increased synthesis of PAR polymers occur (*square* 2). PARs can be released from the nucleus (Andrabi et al. 2006; Yu et al. 2006) and can trigger the loss of mitochondrial membrane potential by a yet unknown mechanism. The latter might be associated with the release of Ca^{2+} from mitochondrial Ca^{2+} stores (*square* 4) and subsequent calpain activation (*square* 5) (Heeres and Hergenrother 2007). Activated calpain might first activate (*square* 6) and then trigger Bax translocation to the mitochondria (*square* 7). Bax-formed pores on the outer mitochondrial membrane allow activated calpain to enter the inter-membrane space (*square* 8) and reach AIF, which is imbedded at the inner mitochondrial membrane. AIF is then cleaved by calpain (*square* 9) and tAIF is released from mitochondria (*square* 10). After translocation of tAIF to the nucleus (*square* 11), tAIF might associate with an unknown endonuclease (*square* 12) to cleave DNA in TUNEL-positive fragments (Moubarak et al. 2007). This hypothetical model for programmed necrosis induction is

members are characterized by the presence of only one BH domain (BH3-only). Among them, two groups can be distinguished based on the mechanism of action. BH3-only proteins Bim and tBid (truncated Bid) cooperate with Bax and Bak to induce PCD (Youle and Strasser 2008), whereas BH3-only proteins such as Bad, Puma, and Noxa act as apoptosis derepressors. These derepressors act through a competitive binding of Bcl-2 and Bcl-xL which, by rendering Bax and Bak free, favors the mitochondrial-dependent PCD program (Kuwana et al. 2005; Certo et al. 2006).

Bax and Bak are key players in triggering apoptosis. However, genetic deletion of either Bak (Lindsten et al. 2000) or Bax (Knudson et al. 1995) displays relatively low phenotypic impact, although Bax deficiency results (among other alterations) in a mild neuronal overgrowth (Knudson et al. 1995). By contrast, double Bax/Bak deletion leads to severe developmental defects pointing to a functional redundancy of these two Bcl-2 multidomain proteins (Lindsten et al. 2000). Importantly, the cells lacking both Bax and Bak are resistant to death-inducing signals that might trigger programmed necrosis (Wei et al. 2001).

Bax activation is causally related to the initiation of MOMP. In the absence of apoptogenic stimuli, Bax is an inactive cytoplasmic protein that is constitutively expressed at a relatively constant level. Its activation results mainly from its interaction with other members of Bcl-2 family. It has been shown (at least in cell-free membrane permeabilization assays) that Bax acts in synergy with the BH3-only proteins tBid or Bim to trigger apoptosis, probably through a direct (physical) interaction (Kuwana et al. 2005; Certo et al. 2006; Willis and Adams 2005). However, neither of these two BH3-only proteins appears to be required for Bax activation, as suggested by the data from double Bim/Bid knockout mice (Willis et al. 2007). Alternatively, Bax could be activated by a large panel of apoptotic regulators, such as JNK, through Bad phosphorylation (Donovan et al. 2002), or calpains, by calpain-dependent proteolytic cleavage (Wood et al. 1998; Gao and Dou 2000).

Since Bax is mostly a cytoplasmic, monomeric protein (in contrast to Bcl-2 which is mainly embedded in the outer mitochondrial membrane, OMM), the translocation from cytoplasm to mitochondria is an essential step for its death-inducing activity. Translocation is followed by Bax insertion into OMM via its C terminus, resulting in a conformational change revealing a hidden epitope in the N terminus. This translocation-related conformational alteration coincides with the increased oligomerization capacity of Bax, which is in turn directly linked to its membrane pore-forming activity. It has been shown that in liposomes, Bax dimers form a pore of approximately 11 Å whereas tetramers give rise to 22-Å pores (Korsmeyer et al. 2000). Thus, Bax alone is capable of forming pores of increasing size in mitochondrial membranes. Consequently, translocation of more Bax from cytoplasm to mitochondria increases the probability of multimeric Bax complex formation, giving rise to pores of increasing size. It is then reasonable to assume that the increase in the size of Bax-formed pores is time-dependent, which is compatible with the release of proteins with increasing mass from the intermembrane mitochondrial space (Munoz-Pinedo et al. 2006).

3.2.3.3 Calpains

Calpains encompass a family of calcium-dependent, nonlysosomal proteases characterized by a cysteine-protease domain that includes a conserved catalytic sequence Cys-His-Arg combined with a calmodulin-like Ca^{2+}-binding site (Sorimachi and Suzuki 2001). The configuration of the cysteine-protease domain determines the formation of an active catalytic pocket, which only occurs when calcium is present. Calpains are involved in a large variety of calcium-regulated processes, such as signal transduction, cell proliferation, and PCD (Goll et al. 2003).

The calpain family comprises a heterogeneous group of cysteine proteases with a large expression pattern. The proteases belonging to this family have been subdivided into three groups depending on their primary structure and the presence or absence of regulatory subunits. These three groups are defined as: typical (also called ubiquitous or conventional), atypical, and other EF-calpains (Goll et al. 2003; Saez et al. 2006). The most important and most studied calpains are the typical μ-calpain (calpain I) and m-calpain (calpain II). The terms μ- and m-calpain were initially used to refer to the micromolar or millimolar Ca^{2+} concentration needed to activate μ- and m-calpain, respectively (Goll et al. 2003). Both μ- and m-calpain are heterodimeric enzymes (80 kDa) sharing a common small regulatory subunit protein of 28 kDa encoded by the *CAPN4* gene. This regulatory subunit protein is critical for calpain function, since its genetic ablation leads to a complete blockage of calpain activity (Tan et al. 2006). Calpains are activated by binding of Ca^{2+}, followed by an autolytic cleavage at the N-terminal moiety of the protein. These proteases do not recognize any specific amino acid sequence. The only amino acid specificity that has been reported involves small hydrophobic amino acids (e.g. leucine) at the P2 position, and large hydrophobic amino acids (e.g. phenylalanine) at the P1 position (Cuerrier et al. 2005).

Calpains are involved in the regulation of both apoptosis (type 1 PCD) (Toyota et al. 2003; Cartron et al. 2004; Cao et al. 2003a) and AIF-mediated programmed necrosis (Moubarak et al. 2007). The mechanism of calpain involvement in apoptosis is relatively well understood. It has been initially associated with the cleavage of the cytoskeleton protein fodrin (Nath et al. 1996) and to proteolytic activation of caspases-3 and -12. In addition to such positive regulation, calpain-mediated degradation of p53 and caspases-7 and -9 inhibits apoptosis (Goll et al. 2003). By contrast, the mechanisms by which calpains control programmed necrosis are less known. It has, however, been demonstrated that calpains control mitochondrial AIF-release subsequent to the extensive DNA damage provoked by high doses of alkylating agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). Chemical inhibitors of the enzyme activity, calpain knock-down, or *CAPN4* genetic ablation, preclude mitochondrial AIF-release and DNA damage-induced programmed necrosis (Moubarak et al. 2007).

The relationship between calpains, AIF, and the Bcl-2 family of proteins deserves particular attention. Indeed, proapoptotic members from the Bcl-2 family, such as Bax (Cao et al. 2003a) and Bid (Mandic et al. 2002), are processed and activated by calpains. Using a model of sympathetic neurons, it has been shown that Bax translocation from cytosol to mitochondria is a critical event for neuronal type

1 PCD (apoptosis). In neurons, calpains cleave inactive Bax (21 kDa) into an 18-kDa proapoptotic protein that redistributes from cytoplasm to mitochondria and promotes PCD (Cao et al. 2003a). In a similar way, cleavage of Bid by calpains has also been implicated in mitochondrial permeabilization, AIF release, and PCD (Chen et al. 2001b). Indeed, it has been recently proposed that μ-calpain cleaves Bid into tBid (Polster et al. 2005). Next, tBid appears involved in the permeabilization of OMM and favors calpain access to the mitochondrial intermembrane space (Polster et al. 2005). Once in mitochondria, μ-calpain cleaves AIF yielding its proapoptotic form, tAIF. This interaction has been further confirmed in focal cerebral ischemia (Culmsee et al. 2005).

3.2.3.4 PARP-1

Poly(ADP-ribose) polymerase (PARP) is a generic term for a family of enzymes catalyzing the production of poly(ADP-ribose) or PAR polymers from NAD^+. The family consists of five members (PARP-1 to 5) with distinct cellular and subcellular localizations (Moroni 2008). PARP enzymes participate in the control of chromosomal segregation, DNA replication, and gene transcription along the relevant phases of the cell cycle. They are also involved in the regulation of chromatin structure and DNA repair (Schreiber et al. 2006).

After DNA-damage, activation of PARP results in poly(ADP-ribosyl)ation of key DNA-repair proteins at the expense of NAD^+ that is cleaved into ADP-ribose and nicotinamide. When DNA-damage is limited, this physiological machinery could repair the injury (Haince et al. 2005; Shall and de Murcia 2000). If DNA breaks are repaired, the damaged cell survives and the cellular NAD^+ levels are restored by recycling nicotinamide with two ATP molecules. If DNA repair is not completely achieved, cells undergo apoptosis by a caspase-dependent mechanism. When DNA-damage is extensive, the cell cannot repair the injury. In this case, disproportionate activation of PARP (mainly PARP-1) depletes the cellular pools of NAD^+ and ATP, driving the cell to a programmed necrosis PCD pathway (Haince et al. 2005; Shall and de Murcia 2000).

Traditionally, this kind of cell death was attributable to the fact that PARP-1 activation correlated with a rapid cellular depletion of NAD^+ and ATP. For example, IL-3-dependent hematopoietic cells represent one of the relatively well-known paradigms of this cell death, where PARP-1 activation results in a rapid loss of cellular ATP and programmed necrosis (Zong et al. 2004). In these cells, which are highly glycolytic, IL-3 deprivation, glucose withdrawal, or glycolytic blockade renders MNNG inefficient (Zong et al. 2004). Moreover, IL-3 deprivation, which blocks the cellular glycolysis, preserves ATP levels after MNNG treatment, even if PARP-1 remains activated. This suggests that the glycolytic control of the metabolic state of the cell regulates DNA-damage mediated death (Zong and Thompson 2006; Zong et al. 2004; Nelson 2004). Albeit necessary, PARP-1 activity is not sufficient to induce programmed necrosis in IL-3-dependent hematopoietic cells. In contrast, our recent results demonstrated that in MEFs, glucose deprivation,

glycolytic blockade by methyl-pyruvate, or oxidative phosphorylation inhibition did not preclude either ATP loss or necrosis associated with MNNG (Moubarak et al. 2007). Moreover, PARP-1 genetic ablation blocks both NAD^+/ATP loss and alkylating DNA-damage induced PCD. Hence, in MEFs, PARP-1 activity is mandatory. Therefore, it seems that depending on the cell type, there are different mechanisms governing DNA-damage mediated programmed necrosis.

New exciting studies confirm the hypothesis that energy collapse is not the sole mechanism by which PARP-1 contributes to cell death. A new "death link" between PARP-1, mitochondria, and AIF has now been established in an in vitro model of glutamate excitotoxicity via *N*-methyl-D-aspartate (NMDA)-type receptors (Andrabi et al. 2006; Yu et al. 2006). These recent studies from the Dawson laboratory elegantly demonstrated that the PAR polymers, a major product of PARP-1 activation (Haince et al. 2005), are the death signals that provoke AIF release from mitochondria to cytosol (Andrabi et al. 2006; Yu et al. 2006). The precise mechanism by which PAR polymer induces AIF mitochondrial release is not totally elucidated. The authors propose two possibilities (1) induction of $\Delta\Psi_m$ dissipation due to the charged nature of PAR polymers and (2) binding of PAR polymers to a yet unknown mitochondrial partner that, in turn, induces AIF release (Yu et al. 2006). Further analysis is necessary to clarify if there exists a relationship between these polymers and other AIF activators such as calpains and/or cathepsins. In this regard, high PAR levels associated with the opening of TRPM2 Ca^{2+} channels and subsequent lethal rise in intra-cellular Ca^{2+} (Fonfria et al. 2004) could provoke the calpain activation needed for AIF release. In any case, it becomes clear that a better understanding of the connection between PAR and AIF can guide the development of new therapeutic strategies regulating AIF-mediated PCD pathways in NMDA-mediated excitotoxicity.

The involvement of PARP in glutamate-mediated neuronal death with necrotic morphology has also been reported in animal models of ischemia and stroke [for review, see Moroni (2008)]. In this case, the mechanisms underlying glutamate-triggered neuronal cell death have been related to excitotoxicity via both ionotropic NMDA- (Moroni et al. 2001; Meli et al. 2004) or metabotropic mGluR1 receptors (Meli et al. 2005).

3.2.4 Mechanism of Programmed Necrosis Induction

In the following section, we will consider the three main mechanisms implicated in the initiation of the programmed necrosis pathway (1) energy failure; (2) increase in intracellular Ca^{2+} concentration; and (3) oxidative DNA damage.

3.2.4.1 Decreased ATP Production/Energy Failure

Decreased energy production may originate from excessive ATP consumption or defective ATP production. Excessive ATP consumption due to the over-activation

of PARP has been discussed in the section "PARP-1." Here, we will consider only the main reasons of loss in ATP production.

Defective ATP production occurs, for example, when the mitochondrial respiratory chain is inhibited or impaired (Ohgoh et al. 2000). This happens upon peroxidation/nitration of protein and lipid constituents of mitochondrial membranes by reactive oxygen species (ROS, see section "Increase in intracellular Ca^{2+} concentration") and results in the loss of $\Delta\Psi_m$, subsequent collapse of electron transport along the respiratory chain and inhibition of ATP synthesis. Importantly, it has been shown that in brain mitochondria, loss of $\Delta\Psi_m$ also occurs upon the local increase of Ca^{2+} in the peri-mitochondrial cytoplasmic compartment (Andreyev et al. 1998).

A second mechanism leading to decreased ATP production is related to the prolonged opening of the mitochondrial permeability transition (PT) pore (Pastorino et al. 1998). In this case, CypD, which is a part of a PT multiprotein complex, appears as a key mediator of programmed necrosis. Indeed, pharmacological inhibition or genetic ablation of CypD precludes this type of PCD (Baines et al. 2005; Basso et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005).

Decreased ATP production can further result from the incapacity of the cell to increase ATP production by switching from glycolysis coupled to oxidative phosphorylation (Holt 1983; Baggetto 1992) to ATP-generating oxidative phosphorylation using nonglucose substrates (such as amino- or fatty acids). This happens in pathological conditions, such as in ischemic neurons where glucose supply is limited (Baggetto 1992). Physiologically, it occurs in highly proliferating cells that shunt amino- and fatty acids away from ATP-generating oxidative phosphorylation into anabolic pathways (Bauer et al. 2004).

Finally, ATP production can be disturbed as a consequence of the inhibition of glycolysis by depletion of the cytosolic NAD^+ pool (Berger et al. 1983) subsequent to PARP-1 overactivation, as described in IL-3-dependent hematopoietic cells (see section "PARP-1") (Zong et al. 2004). ATP production via glycolysis (occurring in the cytoplasm) and oxidative phosphorylation (occurring in mitochondria) requires two independent and nonexchangeable NAD^+ pools (i.e. cytoplasmic and mitochondrial, respectively). By consequence, the fact that PARP-1 overactivation selectively depletes the cytoplasmic NAD^+ pool suggests that cells maintaining their ATP production by preferential catabolism of glucose (e.g. neurons, but also tumor or highly proliferative hematopoïetic cells) are extremely sensitive to death by controlled necrosis due to rapid ATP drop and energy failure. In contrast, quiescent cells, capable of ATP production from nonglucose substrates (i.e. amino- and fatty acids) are relatively resistant to programmed necrosis via PARP activation (Zong et al. 2004).

3.2.4.2 Increase in Intracellular Ca^{2+} Concentration

Deregulated Ca^{2+} influx via plasma membrane Ca^{2+} channels or uncompensated Ca^{2+} liberation from the intracellular stores (endoplasmic reticulum ER, mitochondria) can trigger programmed necrosis. Thus, plasma membrane and ER redox-active Ca^{2+} channels have been involved in triggering programmed necrosis by increasing Ca^{2+} influx (Waring 2005).

In neurons, Ca^{2+} influx can be increased pathologically by overstimulation of ionotropic glutamatergic receptors leading to excitotoxic cell death (Sattler and Tymianski 2001), which displays some characteristics of programmed necrosis (Lindsten et al. 2003). Other routes of increasing intracellular Ca^{2+} level are activation of (1) voltage-sensitive L-type Ca^{2+} channels in excitable cells, including neurons (Fryer et al. 1999); (2) transient receptor potential (TRP) cation channels (Aarts et al. 2003); and (3) acid-sensing ion channels (ASICs) resulting in increased Ca^{2+} influx. ASICs are particularly relevant for the Ca^{2+} influx during increased anaerobic glycolysis in low oxygen conditions (Xiong et al. 2004). In all these circumstances, intracellular Ca^{2+} loading results in mitochondrial depolarization with subsequent PT (Dubinsky and Levi 1998) and calpain activation (see section "Calpains"), which in turn may trigger programmed necrosis. Because the Na^+/Ca^{2+} exchanger involved in Ca^{2+} extrusion is cleaved by calpain, its inactivation leads to a positive feed-back amplification of the Ca^{2+} influx (Bano et al. 2005). Furthermore, the Ca^{2+} buffering ability of mitochondria (allowing for the clearance of excessive cytoplasmic Ca^{2+}) is prevented by the inhibition of Ca^{2+} uptake following $\Delta\Psi_m$ loss and mitochondrial PT. This general scheme has been confirmed in brain mitochondria (Andreyev et al. 1998).

Relevantly, the loss of $\Delta\Psi_m$ is associated with increased production of ROS. ROS are defined as redox-active molecules which can structurally alter the cellular constituents. They are generated as by-products of incomplete oxygen reduction to water in the course of oxidative phosphorylation, mostly due to the activity of the mitochondrial respiratory chain complex I and III. Physiologically, cells cope with ROS production by few lines of defence mechanisms, including their enzymatic inactivation by catalase and super-oxide dismutase (SOD) [reviewed in (Balaban et al. 2005)].

ROS family comprises different types of free radicals (i.e. species with highly reactive unpaired electrons), such as superoxide anion ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$), and hydroxyl ($OH^{\cdot}$) radicals, as well as other molecular species (e.g. hydrogen peroxide, H_2O_2, and peroxynitrite $ONOO^-$). These reactive species generally affect unsaturated bonds in lipids yielding peroxides, hydroperoxides, and aldehydes (Kehrer 1993) and their deleterious effects are particularly important in neurons because of the high brain lipid content (Ikeda and Long 1990). ROS-mediated alterations of proteins affect their side-chains, which are oxidized to carbonyl groups (Stadtman and Oliver 1991). This structural modification often leads to the functional inactivation of proteins. For example, ROS-mediated oxidation of plasma membrane proteins like Na^+-K^+-ATPase, renders these targets more susceptible to degradation by calpain, thus, further contributing to the intracellular ion imbalance. In the particular case of Na^+-K^+-ATPase in excitable cells, including neurons, this is associated with plasma membrane depolarization (Zolotarjova et al. 1994).

3.2.4.3 DNA Damage

The mechanism of DNA damaging agent actions involves structural DNA modifications such as alkylation and methylation, which, if exceeding DNA repair activity of the cell, trigger a death response. These agents are generally recognized as potent

inducers of both apoptosis and necrosis. Apoptosis involves transcription factor p53, which is involved in genome integrity sensing (Bose and Ghosh 2007). Necrosis [considered as p53 independent cell death outcome based on the capacity of p53-deficient cells to die a nonapoptotic death (Moubarak et al. 2007; Zong and Thompson 2006)] is commonly induced by DNA damaging agents, particularly in neurons, since these terminally differentiated cells display low DNA repair capacity (Fonnum and Lock 2004). In addition to the chemical DNA damaging agents, ROS can induce necrotic cell deaths by mediating DNA damage and subsequent PARP activation (see section "PARP-1").

Programmed necrosis triggered in non-neuronal cells by DNA-damaging agents (e.g. MNNG or MNU) is associated with stage 1 chromatin condensation. Such chromatin condensation is also accompanied by TUNEL-detectable 3′OH DNA breaks (Moubarak et al. 2007). Interestingly, studies on different in vivo models of epilepsy by Fujikawa and coworkers have reported almost ten years before that dying neurons in these models display TUNEL-positivity and typical ultra-structural morphology of necrotic cells (Fujikawa et al. 1999, 2000a, b). In these pioneer works, the authors have suggested that these neurons die by programmed necrosis.

3.3 Neuronal Death Outcomes

3.3.1 General Considerations

It is widely accepted that apoptosis may represent the predominant neuronal death phenotype occurring during synaptogenesis (Oppenheim et al. 2001). By contrast, the importance of classical apoptosis in the adult nervous system is still under debate. According to some studies, apoptosis is down regulated (at least under physiological conditions) due to a differentiation-associated reduction in Apaf-1 expression and increased efficacy of IAPs (inhibitors of apoptosis proteins) (Wright et al. 2004). Moreover, the over-expression of IAPs confers neuroprotection in vivo (Perrelet et al. 2000) whereas neuronal IAP (NAIP)-deleted mice display increased vulnerability to kainic acid injury (Holcik et al. 2000). Besides these genetic factors, inhibition of caspases can result from cross-inhibition by other proteases activated simultaneously (Chua et al. 2000), silencing by viral proteins in the course of infection (Strasser et al. 2000), nitrative/oxidative stress (Leist et al. 1999), and energy depletion (Leist et al. 1997). Neurons are highly sensitive to the latter, since they depend entirely on the aerobic metabolism of glucose, which generates ROS as by-products (see section "Increase in intracellular Ca^{2+} concentration").

At this stage, there exists reasonable evidence arguing that, in physiological conditions, apoptosis is inhibited in adult neurons, whereas in pathological conditions it can be re-activated. Predominance of alternative (i.e. caspase-independent) PCD outcomes such as autophagic and apoptosis-like cell death programmes is currently emerging as a consensus [for review, see Krantic et al. (2007) and Krantic et al. (2005)]. Concerning these alternative PCD phenotypes, there is so

far no explicit evidence for the involvement of AIF-mediated programmed necrosis in neurons, which would be similar to that described in MEFs (Moubarak et al. 2007). However, given that AIF has been involved in neuronal death along the apoptosis-like pathway, it is important to re-examine the relevant neuronal PCDs. Thus, some of neuronal PCD phenotypes, originally considered as apoptosis-like, might turn to be programmed necrosis. This can be the case, for example, of peroxide-treated cerebella granule neurons (CGN) dying by AIF-mediated apoptosis-like PCD, where inhibition of mPT by cyclosporin A provides a partial protection against cell death (Hans et al. 2005), thus revealing a putative programmed necrosis component of the cell death process triggered by peroxide. Indeed, as already discussed in the section "Decreased ATP production/energetic failure," inhibition of PT by targeting CypD pharmacologically with cyclosporin A prevents (programmed?) necrosis. Distinguishing apoptosis-like PCD from programmed necrosis is crucial not only from a theoretical (taxonomic) point of view but also from a practical standpoint, since this knowledge might orient future neuroprotective strategies more accurately.

Clear distinction between accidental and AIF-mediated programmed necrosis requires a careful re-examination of the underlying pathways. It can be done, for example, by testing the sensitivity of the death process to cyclosporin A, to help distinguish those processes that are programmatic in nature, hence allowing for therapeutic intervention.

3.4 AIF-Dependent Alternative PCD Phenotypes in Neurons

The goal of the following two sections is to discuss the arguments pointing to the necessity of re-examining death phenotypes occurring in adult-injured neurons in the light of recent data on AIF-mediated PCD programs.

3.4.1 Apoptosis-Like PCD

Discovery of Harlequin (Hq) mice bearing a mutation in AIF gene, which results in 80% reduction of the normal AIF expression (Klein et al. 2002), brought a valuable model to study the involvement of AIF in neuronal cell death. Thus, the fact that decreased expression of AIF in Hq mice confers neuroprotection to cortical neurons exposed to glutamate in vitro provided a direct evidence for the causal involvement of apoptosis-like, AIF-dependent PCD in neuronal death, with obvious implications for the acute neuronal injuries (Cheung et al. 2005). Currently, it is, however, clear that AIF-mediated neuronal death by apoptosis-like PCD can be triggered concomitantly with classical apoptosis (see discussion in section "Excitotoxic Neuronal Death and Programmed Necrosis in Acute Injuries"). Indeed, ceramide, which accumulates in neurons in a variety of acute (and chronic) neurodegenerative processes, has the

capacity to induce the mitochondrial release of AIF in conjunction with other, classical apoptosis-associated mitochondrial proteins (cytochrome *c*, Omi/HtrA2, or Smac/DIABLO) (Stoica et al. 2005). This complexity makes it difficult to clearly distinguish between caspase-dependent and -independent PCD outcomes in this particular paradigm, and also more broadly highlights the difficulties in accurately establishing the PCD phenotype(s) involved in response to a majority of cell death triggers.

Glutamate-induced PCD in cortical and CGN neurons has been defined as apoptosis-like PCD based on its insensitivity to caspase inhibitors and AIF translocation to the nucleus (Cheung et al. 2005; Wang et al. 2004). Such apoptosis-like PCD involves mainly the NMDA type of glutamate receptors since NMDA antagonist (MK-801) has the capacity to inhibit it, at last in vitro (Cheung et al. 2005; Wang et al. 2004). Similarly, trophic factors like hepatocyte growth factor (HCG), capable of preventing NMDA- (Ishihara et al. 2005) or glutamate-induced AIF nuclear translocation (Niimura et al. 2006), also prevent neuronal death. Most importantly, both glutamate- and NMDA-triggered apoptosis-like PCD appears Bax-independent (in contrast to caspase-dependent apoptosis), since genetic ablation of Bax does not confer neuroprotection (Cheung et al. 2005; Miller et al. 1997; Dargusch et al. 2001). Such Bax-independence is an important finding as it provides a clear-cut criterion to distinguish between apoptosis and apoptosis-like PCD, at least in this particular model of neuronal death.

In addition to these in vitro data, excitotoxic neuronal death of an apoptosis-like type via NMDA receptor activation has been convincingly demonstrated in vivo in the following acute neuronal injuries: traumatic brain injury in rat (Zhang et al. 2002), ischemia in rat and mice (Zhu et al. 2003, 2006, 2007; Cao et al. 2003b) and stroke (Plesnila et al. 2004).

3.4.2 Necrotic PCDs

As noted above, there is currently no direct evidence for the involvement of programmed necrosis in neuronal death. However, indirect evidence suggests that at least some of the necrotic phenotypes observed in dying neurons might turn to be programmatic rather than accidental. Before discussing this evidence, it has to be stressed that clear distinction of programmed necrosis among other types of programmed cell death outcomes is further complicated by the fact that even apoptosis, triggered by pathological insults in adult neurons, can display some clearly necrotic features. The related morphologies seen in acutely injured neurons have been designated as atypical, hybrid cell death phenotypes because they present the characteristics of both apoptosis and necrosis in the single neuron (Unal-Cevik et al. 2004).

Among these atypical morphologies, absent or mild chromatin aggregation in irregular or reticular forms has been reported (Fukuda and Yamamoto 1999; Oo et al. 1996; Stadelmann et al. 1998; Liu et al. 2004; Strosznajder and Gajkowska 2006). In many instances, these morphologies are reminiscent of necrosis seen in DNA damage-triggered cell death in neuroblastoma cell lines (Daugas et al. 2000).

Moreover, some biochemical findings reveal the involvement of calpains (proteases implicated in programmed necrosis) in acute neuronal injury. Thus, it has been shown that there is calpain activation in a rat model of acute neurodegeneration such as status epilepticus (Araujo et al. 2005). In addition, the aberrant expression (or impaired activity) of calpains has been related with neuronal injuries seen in ischemia and strokes (Goll et al. 2003; Saez et al. 2006; Cao et al. 2007).

Finally, recent evidence points to the existence of a Bax-sensitive component of DNA damage-induced AIF translocation in campothecin treated neurons (Cheung et al. 2005). Furthermore, AIF-mediated DNA fragmentation in campothecin-induced cell death is TUNEL positive (Cheung et al. 2005), as is programmed necrosis in MEFs (Moubarak et al. 2007). The existence of a Bax-positive component of DNA-damage-induced AIF translocation combined with its TUNEL-positivity (Cheung et al. 2005), further suggests that programmed necrosis, similar to that characterized in MEFs (Moubarak et al. 2007), can be induced in neurons.

3.5 Excitotoxic Neuronal Death and Programmed Necrosis in Acute Injuries

Neuronal over-stimulation by glutamate accumulation in the synaptic cleft has been repeatedly involved in acute neurodegenerative processes. The resulting excitotoxicity is considered as an initial trigger of neuronal PCDs in stroke (and associated experimental hypoxia-ischemia models), epileptic seizures, and traumatic brain injuries (Friberg and Wieloch 2002). However, it is important to keep in mind that the mechanisms underlying these acute neuronal injuries occurring in vivo are not identical to those underlying excitotoxicity. Indeed, death-inducing signals are much more complex in acute neuronal injuries occurring in vivo than is the simple application of glutamate in vitro (experimentally used to model excitotoxicity). For example, in vivo, leukocyte invasion and local production of cytokines (which are absent in vitro) are known to considerably contribute to excitotoxicity resulting from the excessive accumulation of glutamate during cerebral ischemia [for review, see Lipton (1999)].

According to a generally accepted view, glutamate excitotoxicity is mainly mediated by overactivation of NMDA receptors. Kainate receptors are also activated, but to a lesser extent (Cheung et al. 2005). The activation of the relevant receptor-associated ion channels leads to excessive Ca^{2+} influx. The intracellular Ca^{2+} level is further increased by NMDA-related Ca^{2+}-induced Ca^{2+} release (Alford et al. 1993) from intracellular stores (ER and mitochondria) and allows for the subsequent calpain activation (Amadoro et al. 2006). It has also been shown that NMDA-driven increase in intracellular Ca^{2+} may be generated by activation of second messenger-producing enzymes, like neuronal nitric oxide synthase (Zhu et al. 2004). All these mechanisms converge on the uncompensated increase of intracellular Ca^{2+} level, which then triggers depolarization of mitochondrial membrane, loss of $\Delta\Psi_m$, and mPT (Dubinsky and Levi 1998). These mitochondrial alterations are in turn associated with increased ROS generation [Dykens (1994) and Dugan et al. (1995);

reviewed in van Wijk and Hageman (2005)]. DNA damage resulting from ROS production triggers PARP-1 overactivation and a subsequent drop in ATP/ADP level down to 30% of the physiological level (Budd and Nicholls 1996). AIF then translocates from mitochondria to the nucleus in a PARP-1-dependent manner [i.e. it is abolished in PARP-1 knockout mice (Yu et al. 2002; Wang et al. 2003)]. Consistently, AIF-deficient neurons are resistant to PARP-1 dependent cell death mediated by PARs (Yu et al. 2006), thereby demonstrating that both effectors function along the same biochemical pathway.

AIF involvement in excitotoxicity has been consistently reported in more detail by a number of studies. In the original study by Klein and collaborators, CGN neurons derived from AIF hypomorphic Hq mice and treated with high glutamate concentrations (mM range) were more sensitive to cell death induction than neurons derived from their wild-type counterparts (Klein et al. 2002). In contrast, Cheung and colleagues found that, compared to wild-type neurons, cultured cerebellar neurons from Hq mice were protected from glutamate (100 μM) cell death induction (Cheung et al. 2005). These authors further reported that cortical neurons derived from Hq mice and treated with selective agonists of different types of glutamate receptors were less vulnerable to NMDA- and kainate-, but not AMPA, -induced cell death than wild-type neurons (Cheung et al. 2005). Furthermore, Hq mice were more resistant to excitotoxicity in vivo since they displayed reduced neuronal injury in the ischemic penumbra after middle cerebral artery occlusion (Culmsee et al. 2005), as well as decreased hippocampal damage resulting from kainic acid-induced seizures (Cheung et al. 2005). These findings, combined with in vitro data demonstrating a significant reduction of cell death by siRNA-mediated AIF down regulation in glutamate- and oxygen-glucose deprivation neuronal injury models (Culmsee et al. 2005), pointed to AIF as a major mediator of glutamate excitotoxicity.

What remains to be established is whether AIF mediates all excitotoxic cell deaths exclusively by an apoptosis-like PCD pathway (see section “Apoptosis-Like PCD”). Indeed, the possibility exists that AIF contributes to excitotoxic neuronal death also by (a yet undiscovered) programmed necrosis. The main argument supporting the latter hypothesis concerns the Bax-insensitive component of excitotoxic neuronal death. Thus, although excitotoxic neuronal death appears to be mostly independent of Bax, genetic ablation of Bax does confer a partial protection to neurons injured with glutamate or its agonist NMDA (Cheung et al. 2005; Dargusch et al. 2001). This raises the possibility of AIF involvement in programmed necrosis that might occur either as a primary or secondary response, after the initiation of the death process by apoptosis-like PCD. In this latter case, ROS generated along the excitotoxicity-induced apoptosis-like PCD may trigger DNA damage and shift to secondary induction of programmed necrosis (Fig. 3.2). Clarifying these points is critical, since it is increasingly clear that excitotoxic cell death actually involves a mixture of distinct cell death programmes, and that apoptosis-like PCD is certainly not the only PCD involved. The additional cell death outcomes that have been involved in excitotoxicity are AMPA receptor-mediated apoptosis (Cheung et al. 2005; Wang et al. 2004), and also kainate receptor-mediated autophagic PCD (Wang et al. 2008) and necrosis (Chihab et al. 1998).

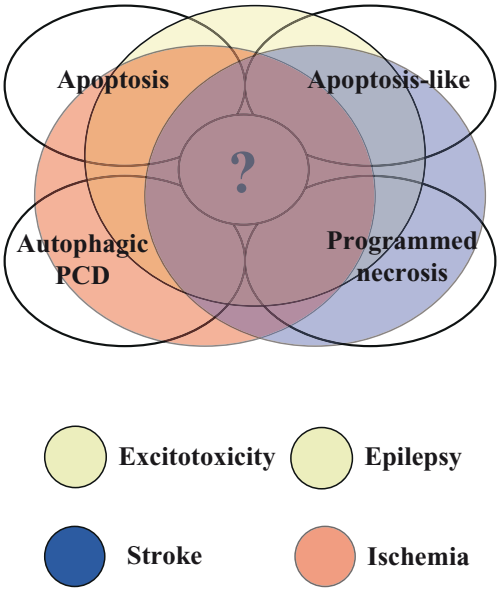

Fig. 3.2 A model for a putative contribution of different PCD outcomes to acute neuronal injuries. All known acute neuronal injuries might trigger concomitantly different types of PCD, but their proportion might vary according to the initial lesion (epileptic seizure: *yellow circle*; stroke: *blue circle*; ischemia: *pink circle*). Excitotoxicity is represented by the *yellow circle* as is epilepsy because, among the selected injuiries, excitotoxicity probably overlaps the best with epileptic seizures. The latter are associated with excessive neuronal network activity related to non-compensated liberation of glutamate and glutamate receptor over-stimulation (Ben-Ari 2001) leading to cell death (Fujikawa et al. 2000a). Ischemia and stroke (where cell death is triggered by excess synaptic liberation of glutamate) also involve an excitotoxic component but they comprise additional triggers like instantaneous unavailability of glucose (more specifically related to ischemia) or fast ROS over-load (more specifically related to reperfusion in stroke). Indeed, because glucose is physiologically the only metabolite substrate that is rapidly transported across the blood–brain-barrier, in ischemia where blood supply is insufficient there is a rapid energy failure that is related to glutamate accumulation in the synaptic cleft (Camacho and Massieu 2006). Decline in ATP production due to the insufficient supply of glucose might activate defence mechanisms such as autophagy, to compensate for glucose absence by providing metabolic sources from degraded cell constituents. However, excessive autophagy might in turn trigger autophagic PCD (Adhami et al. 2007), meaning that autophagic PCD might be more involved in ischemia than, for example, in epilepsy. Similarly, during the reperfusion phase of stroke, there is a massive and rapid ROS production (Saito et al. 2005) which is, among other factors, related to DNA damage and PARP-1 activation (Skaper 2003). The latter has been involved in apoptosis-like PCD and in programmed necrosis (see section "PARP-1"), thus both of them might contribute more to stroke-induced neuronal death than to the type of death seen in other acute injuries

The reported involvement of (accidental) necrosis in excitotoxicity (and adjacent necrotic morphology of dying neurons) should motivate additional studies aimed at the assessment of the putative programmed character of this cell death outcome. Additional argument for the relevance of programmed necrosis in excitotoxicity is the involvement of effectors such as calpain and PARP-1, which in addition to AIF,

has been demonstrated to contribute to neuronal apoptosis-like PCD in excitotoxicity, and are also shared by recently characterized programmed necrosis (Moubarak et al. 2007). The experimental evidence which supports this view comes from the data obtained after combined disabling of apoptosis (by Apaf-1 ablation) and apoptosis-like PCD (by siRNA-mediated AIF downregulation), which still leaves about 30% of neurons unprotected against the excitotoxic death (Cheung et al. 2005). The reported Bax independency of excitotoxic neuronal death observed in that study (Cheung et al. 2005) appears at first sight incompatible with programmed necrosis, since this PCD displays Bax-dependency in MEFs (Moubarak et al. 2007). However, in the previous study, Bax dependency was assessed in the paradigm that the excitotoxic insult was applied for a relatively short time period (1 h), then removed and cell death assessed only 24 h later. It is thus possible that initial, transient Bax activation, which would argue for the involvement of programmed necrosis, remained unnoticed. The involvement of Bax in excitotoxicity should be definitely re-examined during the early steps of cell death triggering in order to answer the question of a possible involvement of programmed necrosis in this type of neuronal death. In this light, very recently it was reported that μ-calpain plays a crucial role in the release of AIF in neuronal ischemic injury, thus confirming that the same scenario as the one we proposed for programmed necrosis in MEFs is also functional in neurons (Cao et al. 2007).

3.5 Conclusion

It is currently reasonably certain that not one, but a myriad of different cell death outcomes contribute to neuronal demise in the course of acute neuronal injuries. It is moreover generally accepted that these different death outcomes include both accidental (uncontrolled) and programmed (thus obviously controlled) modalities. It is also reasonably certain that excitotoxic cell death, induced by excessive release and accumulation of the excitatory neurotransmitter glutamate, represents a common determinant of various acute (and chronic) neurodegenerations. However, it is also clear that excitotoxicity cannot be considered as a synonym for acute neuronal injury, and that other factors (local accumulation of immune cells, release of the relevant signalling mediators by these cells, etc.) do contribute to brain damage in vivo.

This being said, it remains true that excitotoxicity is probably the main pathological component of the acute neuronal injury. A great amount of experimental evidence supports the view that excitotoxic neuronal death actually corresponds to a mixture of different cell death phenotypes, although the existence of excitotoxic cell death, stricto sensu, as a single entity corresponding to a peculiar form of cell death has also been proposed (Kroemer et al. 2005). However, to date there is no convincing experimental data to support the latter hypothesis. By contrast, as discussed here, different PCD modalities have been demonstrated to take part in neuronal demise in the course of acute injuries. These include caspase-independent

phenotypes such as apoptosis-like and autophagic PCD and, probably to a lesser extent, caspase-dependent apoptosis.

In addition, the involvement of nonprogrammed cell death such as necrosis has been repeatedly and convincingly demonstrated by a number of morphological studies. In recent years, a novel concept has begun to emerge concerning necrosis in general. According to this new concept, at least part of the cell death outcomes that appear necrotic at the ultrastructural level might in fact be the end-point of a death programme designated as programmed necrosis. Although not yet tested, the assumption is that programmed necrosis also occurs in neurons. Morphological evidence (e.g. the absence of pronounced chromatin condensation) and biochemical (e.g. involvement of the effectors demonstrated to mediate programmed necrosis in cell lines such as Bax, calpain, AIF, and PARP-1) is in agreement with this assumption. Future studies are now required to assess them experimentally in different models of acute neuronal injuries. The task is very difficult to accomplish because the biochemical pathways underlying programmed necrosis remain poorly understood. This means that we have to work backwards from the described necrotic morphologies and attempt to discover and ascribe effectors responsible for the phenotypic expression to each of these morphologies seen in the acute neuronal injuries. It is very probable that the programmed necrosis that we have recently described in non-neuronal cells (Moubarak et al. 2007) will be unravelled in neurons dying in the course of acute injuries. It is moreover reasonable to postulate that this particular phenotype of programmed necrosis represents only the tip of the iceberg, as it was for caspase-dependent apoptosis, considered upon its discovery as the sole existing PCD. The efforts similar to those made in understanding molecular mechanisms of apoptosis at the time of its re-discovery by Kerr, Wylie, and Curie (Kerr et al. 1972) should now be oriented towards understanding the molecular mechanisms of programmed necrosis in general, and those occurring in neurons in particular. The goal is ambitious, but our hope is that the invested efforts will bring results as exciting as those obtained in the field of apoptosis. The understanding of the mechanisms of programmed necrosis would have a great impact not only in the domain of neurodegeneration, but probably in the fields of cancer and developmental diseases as well.

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References

Aarts M, Iihara K, Wei WL, Xiong ZG, Arundine M, Cerwinski W, MacDonald JF, Tymianski M (2003) A key role for TRPM7 channels in anoxic neuronal death. Cell 115:863–877

Adhami F, Schloemer A, Kuan CY (2007) The roles of autophagy in cerebral ischemia. Autophagy 3:42–44

Alford S, Frenguelli BG, Schofield JG, Collingridge GL (1993) Characterization of Ca2+ signals induced in hippocampal CA1 neurones by the synaptic activation of NMDA receptors. J Physiol 469:693–716

Amadoro G, Ciotti MT, Costanzi M, Cestari V, Calissano P, Canu N (2006) NMDA receptor mediates tau-induced neurotoxicity by calpain and ERK/MAPK activation. Proc Natl Acad Sci USA 103:2892–2897

Andrabi SA et al (2006) Poly(ADP-ribose) (PAR) polymer is a death signal. Proc Natl Acad Sci USA 103:18308–18313

Andreyev AY, Fahy B, Fiskum G (1998) Cytochrome *c* release from brain mitochondria is independent of the mitochondrial permeability transition. FEBS Lett 439:373–376

Araujo IM et al (2005) Proteolysis of NR2B by calpain in the hippocampus of epileptic rats. Neuroreport 16:393–396

Baggetto LG (1992) Deviant energetic metabolism of glycolytic cancer cells. Biochimie 74:959–974

Baines CP et al (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434:658–662

Bajt ML, Cover C, Lemasters JJ, Jaeschke H (2006) Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. Toxicol Sci 94:217–225

Balaban RS, Nemoto S, Finkel T (2005) Mitochondria, oxidants, and aging. Cell 120:483–495

Bano D et al (2005) Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. Cell 120:275–285

Barkla DH, Gibson PR (1999) The fate of epithelial cells in the human large intestine. Pathology 31:230–238

Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, Bernardi P (2005) Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. J Biol Chem 280:18558–18561

Bauer DE, Harris MH, Plas DR, Lum JJ, Hammerman PS, Rathmell JC, Riley JL, Thompson CB (2004) Cytokine stimulation of aerobic glycolysis in hematopoietic cells exceeds proliferative demand. FASEB J 18:1303–1305

Beaulaton J, Lockshin RA (1982) The relation of programmed cell death to development and reproduction: Comparative studies and an attempt at classification. Int Rev Cytol 79:215–235

Ben-Ari Y (2001) Cell death and synaptic reorganizations produced by seizures. Epilepsia 42(Suppl 3):5–7

Berger NA, Sims JL, Catino DM, Berger SJ (1983) Poly(ADP-ribose) polymerase mediates the suicide response to massive DNA damage: studies in normal and DNA-repair defective cells. Princess Takamatsu Symp 13:219–226

Boise LH, Collins CM (2001) Salmonella-induced cell death: Apoptosis, necrosis or programmed cell death? Trends Microbiol 9:64–67

Borst P, Rottenberg S (2004) Cancer cell death by programmed necrosis? Drug Resist Update 7:321–324

Bose I, Ghosh B (2007) The p53-MDM2 network: From oscillations to apoptosis. J Biosci 32:991–997

Boujrad H, Gubkina O, Robert N, Krantic S, Susin SA (2007) AIF-mediated programmed necrosis: A highly regulated way to die. Cell Cycle 6:2612–2619

Bras M et al (2007) Drp1 mediates caspase-independent type III cell death in normal and leukemic cells. Mol Cell Biol 27:7073–7088

Bredesen DE (2007) Key note lecture: Toward a mechanistic taxonomy for cell death programs. Stroke 38:652–660

Broker LE, Kruyt FA, Giaccone G (2005) Cell death independent of caspases: A review. Clin Cancer Res 11:3155–3162

Budd SL, Nicholls DG (1996) Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells. J Neurochem 67:2282–2291

Camacho A, Massieu L (2006) Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death. Arch Med Res 37:11–18

Cande C et al (2004) AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. Oncogene 23:1514–1521

Cao X, Deng X, May WS (2003a) Cleavage of Bax to p18 Bax accelerates stress-induced apoptosis, and a cathepsin-like protease may rapidly degrade p18 Bax. Blood 102:2605–2614

Cao G, Clark RS, Pei W, Yin W, Zhang F, Sun FY, Graham SH, Chen J (2003b) Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation. J Cereb Blood Flow Metab 23:1137–1150

Cao G et al (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. J Neurosci 27:9278–9293

Cartron PF, Oliver L, Juin P, Meflah K, Vallette FM (2004) The p18 truncated form of Bax behaves like a Bcl-2 homology domain 3-only protein. J Biol Chem 279:11503–11512

Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA, Letai A (2006) Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell 9:351–365

Chautan M, Chazal G, Cecconi F, Gruss P, Golstein P (1999) Interdigital cell death can occur through a necrotic and caspase-independent pathway. Curr Biol 9:967–970

Chen J, Liu X, Mandel LJ, Schnellmann RG (2001a) Progressive disruption of the plasma membrane during renal proximal tubule cellular injury. Toxicol Appl Pharmacol 171:1–11

Chen M, He H, Zhan S, Krajewski S, Reed JC, Gottlieb RA (2001b) Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion. J Biol Chem 276:30724–30728

Cheung EC et al (2005) Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms. J Neurosci 25:1324–1334

Chihab R, Oillet J, Bossenmeyer C, Daval JL (1998) Glutamate triggers cell death specifically in mature central neurons through a necrotic process. Mol Genet Metab 63:142–147

Chua BT, Guo K, Li P (2000) Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. J Biol Chem 275:5131–5135

Clarke PG (1990) Developmental cell death: Morphological diversity and multiple mechanisms. Anat Embryol (Berl) 181:195–213

Colbourne F, Sutherland GR, Auer RN (1999) Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J Neurosci 19:4200–4210

Cregan SP, Dawson VL, Slack RS (2004) Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 23:2785–2796

Cuerrier D, Moldoveanu T, Davies PL (2005) Determination of peptide substrate specificity for mu-calpain by a peptide library-based approach: The importance of primed side interactions. J Biol Chem 280:40632–40641

Culmsee C, Zhu C, Landshamer S, Becattini B, Wagner E, Pellecchia M, Blomgren K, Plesnila N (2005) Apoptosis-inducing factor triggered by poly(ADP-ribose) polymerase and Bid mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia. J Neurosci 25:10262–10272

Danial NN, Korsmeyer SJ (2004) Cell death: Critical control points. Cell 116:205–219

Dargusch R, Piasecki D, Tan S, Liu Y, Schubert D (2001) The role of Bax in glutamate-induced nerve cell death. J Neurochem 76:295–301

Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N, Kroemer G (2000) Apoptosis-inducing factor (AIF): A ubiquitous mitochondrial oxidoreductase involved in apoptosis. FEBS Lett 476:118–123

Dawson VL, Dawson TM (2004) Deadly conversations: Nuclear-mitochondrial cross-talk. J Bioenerg Biomembr 36:287–294

Delettre C, Yuste VJ, Moubarak RS, Bras M, Lesbordes-Brion JC, Petres S, Bellalou J, Susin SA (2006a) AIFsh, a novel apoptosis-inducing factor (AIF) pro-apoptotic isoform with potential pathological relevance in human cancer. J Biol Chem 281:6413–6427

Delettre C, Yuste VJ, Moubarak RS, Bras M, Robert N, Susin SA (2006b) Identification and characterization of AIFsh2, a mitochondrial apoptosis-inducing factor (AIF) isoform with nadh oxidase activity. J Biol Chem 281:18507–18518

Donovan N, Becker EB, Konishi Y, Bonni A (2002) JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery. J Biol Chem 277:40944–40949

Dubinsky JM, Levi Y (1998) Calcium-induced activation of the mitochondrial permeability transition in hippocampal neurons. J Neurosci Res 53:728–741

Dugan LL, Sensi SL, Canzoniero LM, Handran SD, Rothman SM, Lin TS, Goldberg MP, Choi DW (1995) Mitochondrial production of reactive oxygen species in cortical neurons following exposure to *N*-methyl-D-aspartate. J Neurosci 15:6377–6388

Dykens JA (1994) Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated CA^{2+} and Na^{+}: Implications for neurodegeneration. J Neurochem 63:584–591

Edinger AL, Thompson CB (2004) Death by design: Apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16:663–669

Festjens N, Vanden Berghe T, Vandenabeele P (2006) Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response. Biochim Biophys Acta 1757:1371–1387

Fonfria E et al (2004) TRPM2 channel opening in response to oxidative stress is dependent on activation of poly(ADP-ribose) polymerase. Br J Pharmacol 143:186–192

Fonnum F, Lock EA (2004) The contributions of excitotoxicity, glutathione depletion and DNA repair in chemically induced injury to neurones: Exemplified with toxic effects on cerebellar granule cells. J Neurochem 88:513–531

Friberg H, Wieloch T (2002) Mitochondrial permeability transition in acute neurodegeneration. Biochimie 84:241–250

Fryer HJ, Knox RJ, Strittmatter SM, Kalb RG (1999) Excitotoxic death of a subset of embryonic rat motor neurons in vitro. J Neurochem 72:500–513

Fujikawa DG, Shinmei SS, Cai B (1999) Lithium-pilocarpine-induced status epilepticus produces necrotic neurons with internucleosomal DNA fragmentation in adult rats. Eur J Neurosci 11:1605–1614

Fujikawa DG, Shinmei SS, Cai B (2000a) Seizure-induced neuronal necrosis: Implications for programmed cell death mechanisms. Epilepsia 41(Suppl 6):S9–S13

Fujikawa DG, Shinmei SS, Cai B (2000b) Kainic acid-induced seizures produce necrotic, not apoptotic, neurons with internucleosomal DNA cleavage: Implications for programmed cell death mechanisms. Neuroscience 98:41–53

Fukuda K, Yamamoto M (1999) Acquisition of resistance to apoptosis and necrosis by Bcl-xL over-expression in rat hepatoma McA-RH8994 cells. J Gastroenterol Hepatol 14:682–690

Gao G, Dou QP (2000) N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome *c* release and apoptotic cell death. J Cell Biochem 80:53–72

Gharibyan AL, Zamotin V, Yanamandra K, Moskaleva OS, Margulis BA, Kostanyan IA, Morozova-Roche LA (2007) Lysozyme amyloid oligomers and fibrils induce cellular death via different apoptotic/necrotic pathways. J Mol Biol 365:1337–1349

Goll DE, Thompson VF, Li H, Wei W, Cong J (2003) The calpain system. Physiol Rev 83:731–801

Golstein P, Kroemer G (2007) Cell death by necrosis: Towards a molecular definition. Trends Biochem Sci 32:37–43

Golstein P, Aubry L, Levraud JP (2003) Cell-death alternative model organisms: Why and which? Nat Rev Mol Cell Biol 4:798–807

Gozuacik D, Kimchi A (2007) Autophagy and cell death. Curr Top Dev Biol 78:217–245

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281:1309–1312

Haince JF, Rouleau M, Hendzel MJ, Masson JY, Poirier GG (2005) Targeting poly(ADP-ribosyl) ation: A promising approach in cancer therapy. Trends Mol Med 11:456–463
Han W, Li L, Qiu S, Lu Q, Pan Q, Gu Y, Luo J, Hu X (2007) Shikonin circumvents cancer drug resistance by induction of a necroptotic death. Mol Cancer Ther 6:1641–1649
Hans G et al (2005) Beta-carbolines induce apoptosis in cultured cerebellar granule neurons via the mitochondrial pathway. Neuropharmacology 48:105–117
Heeres JT, Hergenrother PJ (2007) Poly(ADP-ribose) makes a date with death. Curr Opin Chem Biol 11:644–653
Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770–776
Hirt UA, Gantner F, Leist M (2000) Phagocytosis of nonapoptotic cells dying by caspase-independent mechanisms. J Immunol 164:6520–6529
Holcik M, Thompson CS, Yaraghi Z, Lefebvre CA, MacKenzie AE, Korneluk RG (2000) The hippocampal neurons of neuronal apoptosis inhibitory protein 1 (NAIP1)-deleted mice display increased vulnerability to kainic acid-induced injury. Proc Natl Acad Sci USA 97:2286–2290
Holler N et al (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495
Holt JA (1983) Cancer, a disease of defective glucose metabolism. Med Hypotheses 10:133–150
Hong SJ, Dawson TM, Dawson VL (2004) Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. Trends Pharmacol Sci 25:259–264
Ikeda Y, Long DM (1990) The molecular basis of brain injury and brain edema: The role of oxygen free radicals. Neurosurgery 27:1–11
Ishihara N et al (2005) Inhibition of apoptosis-inducing factor translocation is involved in protective effects of hepatocyte growth factor against excitotoxic cell death in cultured hippocampal neurons. J Neurochem 95:1277–1286
Jaattela M (2002) Programmed cell death: Many ways for cells to die decently. Ann Med 34:480–488
Jaattela M, Tschopp J (2003) Caspase-independent cell death in T lymphocytes. Nat Immunol 4:416–423
Kehrer JP (1993) Free radicals as mediators of tissue injury and disease. Crit Rev Toxicol 23:21–48
Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257
Kim GT, Chun YS, Park JW, Kim MS (2003a) Role of apoptosis-inducing factor in myocardial cell death by ischemia-reperfusion. Biochem Biophys Res Commun 309:619–624
Kim JS, He L, Lemasters JJ (2003b) Mitochondrial permeability transition: A common pathway to necrosis and apoptosis. Biochem Biophys Res Commun 304:463–470
Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, Bronson RT, Ackerman SL (2002) The harlequin mouse mutation downregulates apoptosis-inducing factor. Nature 419:367–374
Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270:96–99
Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH (2000) Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. Cell Death Differ 7:1166–1173
Krantic S, Mechawar N, Reix S, Quirion R (2005) Molecular basis of programmed cell death involved in neurodegeneration. Trends Neurosci 28:670–676
Krantic S, Mechawar N, Reix S, Quirion R (2007) Apoptosis-inducing factor: A matter of neuron life and death. Prog Neurobiol 81:179–196
Kroemer G et al (2005) Classification of cell death: Recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ 12(Suppl 2):1463–1467
Krysko DV, Denecker G, Festjens N, Gabriels S, Parthoens E, D'Herde K, Vandenabeele P (2006) Macrophages use different internalization mechanisms to clear apoptotic and necrotic cells. Cell Death Differ 13:2011–2022
Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 111:331–342

Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD (2005) BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol Cell 17:525–535

Leist M, Jaattela M (2001) Four deaths and a funeral: From caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2:589–598

Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P (1997) Intracellular adenosine triphosphate (ATP) concentration: A switch in the decision between apoptosis and necrosis. J Exp Med 185:1481–1486

Leist M, Single B, Naumann H, Fava E, Simon B, Kuhnle S, Nicotera P (1999) Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis to necrosis. Exp Cell Res 249:396–403

Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2:183–192

Li L, Han W, Gu Y, Qiu S, Lu Q, Jin J, Luo J, Hu X (2007) Honokiol induces a necrotic cell death through the mitochondrial permeability transition pore. Cancer Res 67:4894–4903

Lindsten T et al (2000) The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell 6:1389–1399

Lindsten T, Golden JA, Zong WX, Minarcik J, Harris MH, Thompson CB (2003) The proapoptotic activities of Bax and Bak limit the size of the neural stem cell pool. J Neurosci 23:11112–11119

Lipton P (1999) Ischemic cell death in brain neurons. Physiol Rev 79:1431–1568

Liu X, Schnellmann RG (2003) Calpain mediates progressive plasma membrane permeability and proteolysis of cytoskeleton-associated paxillin, talin, and vinculin during renal cell death. J Pharmacol Exp Ther 304:63–70

Liu T, Brouha B, Grossman D (2004) Rapid induction of mitochondrial events and caspase-independent apoptosis in Survivin-targeted melanoma cells. Oncogene 23:39–48

Loeffler M et al (2001) Dominant cell death induction by extramitochondrially targeted apoptosis-inducing factor. FASEB J 15:758–767

Lorenzo HK, Susin SA (2004) Mitochondrial effectors in caspase-independent cell death. FEBS Lett 557:14–20

Lorenzo HK, Susin SA (2007) Therapeutic potential of AIF-mediated caspase-independent programmed cell death. Drug Resist Update 10:235–255

Mandic A, Viktorsson K, Strandberg L, Heiden T, Hansson J, Linder S, Shoshan MC (2002) Calpain-mediated Bid cleavage and calpain-independent Bak modulation: Two separate pathways in cisplatin-induced apoptosis. Mol Cell Biol 22:3003–3013

Martinou I, Desagher S, Eskes R, Antonsson B, Andre E, Fakan S, Martinou JC (1999) The release of cytochrome *c* from mitochondria during apoptosis of NGF-deprived sympathetic neurons is a reversible event. J Cell Biol 144:883–889

Mate MJ et al (2002) The crystal structure of the mouse apoptosis-inducing factor AIF. Nat Struct Biol 9:442–446

Meli E, Pangallo M, Picca R, Baronti R, Moroni F, Pellegrini-Giampietro DE (2004) Differential role of poly(ADP-ribose) polymerase-1in apoptotic and necrotic neuronal death induced by mild or intense NMDA exposure in vitro. Mol Cell Neurosci 25:172–180

Meli E, Baronti R, Pangallo M, Picca R, Moroni F, Pellegrini-Giampietro DE (2005) Group I metabotropic glutamate receptors stimulate the activity of poly(ADP-ribose) polymerase in mammalian mGlu1-transfected cells and in cortical cell cultures. Neuropharmacology 49(Suppl 1):80–88

Meurette O, Rebillard A, Huc L, Le Moigne G, Merino D, Micheau O, Lagadic-Gossmann D, Dimanche-Boitrel MT (2007) TRAIL induces receptor-interacting protein 1-dependent and caspase-dependent necrosis-like cell death under acidic extracellular conditions. Cancer Res 67:218–226

Miller TM, Moulder KL, Knudson CM, Creedon DJ, Deshmukh M, Korsmeyer SJ, Johnson EM Jr (1997) Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. J Cell Biol 139:205–217

Miramar MD et al (2001) NADH oxidase activity of mitochondrial apoptosis-inducing factor. J Biol Chem 276:16391–16398
Moroni F (2008) Poly(ADP-ribose)polymerase 1 (PARP-1) and postischemic brain damage. Curr Opin Pharmacol 8:96–103
Moroni F et al (2001) Poly(ADP-ribose) polymerase inhibitors attenuate necrotic but not apoptotic neuronal death in experimental models of cerebral ischemia. Cell Death Differ 8:921–932
Moubarak RS, Yuste VJ, Artus C, Bouharrour A, Greer PA, Menissier-de Murcia J, Susin SA (2007) Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. Mol Cell Biol 27:4844–4862
Munoz-Pinedo C, Guio-Carrion A, Goldstein JC, Fitzgerald P, Newmeyer DD, Green DR (2006) Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. Proc Natl Acad Sci USA 103:11573–11578
Murahashi H et al (2003) Possible contribution of apoptosis-inducing factor (AIF) and reactive oxygen species (ROS) to UVB-induced caspase-independent cell death in the T cell line Jurkat. J Leukoc Biol 73:399–406
Nakagawa T et al (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 434:652–658
Nath R et al (1996) Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. Biochem J 319(Pt 3):683–690
Nelson WG (2004) Prostate cancer prevention. J Nutr 134:3211S–3212S
Nicotera P, Leist M, Manzo L (1999a) Neuronal cell death: A demise with different shapes. Trends Pharmacol Sci 20:46–51
Nicotera P, Leist M, Ferrando-May E (1999b) Apoptosis and necrosis: Different execution of the same death. Biochem Soc Symp 66:69–73
Niimura M et al (2006) Prevention of apoptosis-inducing factor translocation is a possible mechanism for protective effects of hepatocyte growth factor against neuronal cell death in the hippocampus after transient forebrain ischemia. J Cereb Blood Flow Metab 26:1354–1365
Niquet J, Seo DW, Wasterlain CG (2006) Mitochondrial pathways of neuronal necrosis. Biochem Soc Trans 34:1347–1351
Nishimura Y, Lemasters JJ (2001) Glycine blocks opening of a death channel in cultured hepatic sinusoidal endothelial cells during chemical hypoxia. Cell Death Differ 8:850–858
Nixon RA (2006) Autophagy in neurodegenerative disease: Friend, foe or turncoat? Trends Neurosci 29:528–535
Ohgoh M, Shimizu H, Ogura H, Nishizawa Y (2000) Astroglial trophic support and neuronal cell death: Influence of cellular energy level on type of cell death induced by mitochondrial toxin in cultured rat cortical neurons. J Neurochem 75:925–933
Okada H, Mak TW (2004) Pathways of apoptotic and non-apoptotic death in tumour cells. Nat Rev Cancer 4:592–603
Oo TF, Blazeski R, Harrison SM, Henchcliffe C, Mason CA, Roffler-Tarlov SK, Burke RE (1996) Neuron death in the substantia nigra of weaver mouse occurs late in development and is not apoptotic. J Neurosci 16:6134–6145
Oppenheim RW, Flavell RA, Vinsant S, Prevette D, Kuan CY, Rakic P (2001) Programmed cell death of developing mammalian neurons after genetic deletion of caspases. J Neurosci 21:4752–4760
Orrenius S, Zhivotovsky B (2006) The future of toxicology – does it matter how cells die? Chem Res Toxicol 19:729–733
Otera H, Ohsakaya S, Nagaura Z, Ishihara N, Mihara K (2005) Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. EMBO J 24:1375–1386
Pastorino JG, Chen ST, Tafani M, Snyder JW, Farber JL (1998) The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition. J Biol Chem 273:7770–7775

Perrelet D et al (2000) IAP family proteins delay motoneuron cell death in vivo. Eur J Neurosci 12:2059–2067

Plesnila N, Zhu C, Culmsee C, Groger M, Moskowitz MA, Blomgren K (2004) Nuclear translocation of apoptosis-inducing factor after focal cerebral ischemia. J Cereb Blood Flow Metab 24:458–466

Polster BM, Basanez G, Etxebarria A, Hardwick JM, Nicholls DG (2005) Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. J Biol Chem 280:6447–6454

Rao RV, Castro-Obregon S, Frankowski H, Schuler M, Stoka V, del Rio G, Bredesen DE, Ellerby HM (2002) Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. J Biol Chem 277:21836–21842

Saelens X, Festjens N, Parthoens E, Vanoverberghe I, Kalai M, van Kuppeveld F, Vandenabeele P (2005) Protein synthesis persists during necrotic cell death. J Cell Biol 168:545–551

Saez ME, Ramirez-Lorca R, Moron FJ, Ruiz A (2006) The therapeutic potential of the calpain family: New aspects. Drug Discov Today 11:917–923

Saito A et al (2005) Oxidative stress and neuronal death/survival signaling in cerebral ischemia. Mol Neurobiol 31:105–116

Sattler R, Tymianski M (2001) Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. Mol Neurobiol 24:107–129

Schinzel AC et al (2005) Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. Proc Natl Acad Sci USA 102:12005–12010

Schreiber V, Dantzer F, Ame JC, de Murcia G (2006) Poly(ADP-ribose): Novel functions for an old molecule. Nat Rev Mol Cell Biol 7:517–528

Schweichel JU, Merker HJ (1973) The morphology of various types of cell death in prenatal tissues. Teratology 7:253–266

Scorrano L, Korsmeyer SJ (2003) Mechanisms of cytochrome *c* release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun 304:437–444

Seye CI, Knaapen MW, Daret D, Desgranges C, Herman AG, Kockx MM, Bult H (2004) 7-Ketocholesterol induces reversible cytochrome *c* release in smooth muscle cells in absence of mitochondrial swelling. Cardiovasc Res 64:144–153

Shall S, de Murcia G (2000) Poly(ADP-ribose) polymerase-1: What have we learned from the deficient mouse model? Mutat Res 460:1–15

Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y (2004) Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol 6:1221–1228

Skaper SD (2003) Poly(ADP-Ribose) polymerase-1 in acute neuronal death and inflammation: A strategy for neuroprotection. Ann N Y Acad Sci 993:217–228; discussion 287–288

Sorimachi H, Suzuki K (2001) The structure of calpain. J Biochem (Tokyo) 129:653–664

Sperandio S, de Belle I, Bredesen DE (2000) An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci USA 97:14376–14381

Srivastava S et al (2007) Apoptosis-inducing factor regulates death in peripheral T cells. J Immunol 179:797–803

Stadelmann C, Bruck W, Bancher C, Jellinger K, Lassmann H (1998) Alzheimer disease: DNA fragmentation indicates increased neuronal vulnerability, but not apoptosis. J Neuropathol Exp Neurol 57:456–464

Stadtman ER, Oliver CN (1991) Metal-catalyzed oxidation of proteins. Physiological consequences. J Biol Chem 266:2005–2008

Stoica BA, Movsesyan VA, Knoblach SM, Faden AI (2005) Ceramide induces neuronal apoptosis through mitogen-activated protein kinases and causes release of multiple mitochondrial proteins. Mol Cell Neurosci 29:355–371

Strasser A et al (2000) The role of bim, a proapoptotic BH3-only member of the Bcl-2 family in cell-death control. Ann N Y Acad Sci 917:541–548

Strosznajder R, Gajkowska B (2006) Effect of 3-aminobenzamide on Bcl-2, Bax and AIF localization in hippocampal neurons altered by ischemia-reperfusion injury. The immunocytochemical study. Acta Neurobiol Exp (Wars) 66:15–22

Susin SA et al (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J Exp Med 184:1331–1341

Susin SA et al (1997) The central executioner of apoptosis: Multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. J Exp Med 186:25–37

Susin SA et al (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446

Susin SA et al (2000) Two distinct pathways leading to nuclear apoptosis. J Exp Med 192:571–580

Tan Y, Dourdin N, Wu C, De Veyra T, Elce JS, Greer PA (2006) Conditional disruption of ubiquitous calpains in the mouse. Genesis 44:297–303

Thornberry NA, Lazebnik Y (1998) Caspases: Enemies within. Science 281:1312–1316

Toyota H, Yanase N, Yoshimoto T, Moriyama M, Sudo T, Mizuguchi J (2003) Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax. Cancer Lett 189:221–230

Tsujimoto Y (2002) Bcl-2 family of proteins: life-or-death switch in mitochondria. Biosci Rep 22:47–58

Unal-Cevik I, Kilinc M, Can A, Gursoy-Ozdemir Y, Dalkara T (2004) Apoptotic and necrotic death mechanisms are concomitantly activated in the same cell after cerebral ischemia. Stroke 35:2189–2194

Vahsen N et al (2004) AIF deficiency compromises oxidative phosphorylation. EMBO J 23:4679–4689

van Wijk SJ, Hageman GJ (2005) Poly(ADP-ribose) polymerase-1 mediated caspase-independent cell death after ischemia/reperfusion. Free Radic Biol Med 39:81–90

Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, Hakem R, Greenberg AH (2000) BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. Mol Cell Biol 20:5454–5468

Vanden Berghe T, van Loo G, Saelens X, Van Gurp M, Brouckaert G, Kalai M, Declercq W, Vandenabeele P (2004) Differential signaling to apoptotic and necrotic cell death by Fas-associated death domain protein FADD. J Biol Chem 279:7925–7933

Wang X, Yang C, Chai J, Shi Y, Xue D (2002) Mechanisms of AIF-mediated apoptotic DNA degradation in *Caenorhabditis elegans*. Science 298:1587–1592

Wang HM, Shimoji M, Yu SW, Dawson TM, Dawson VL (2003) Apoptosis inducing factor and PARP-mediated injury in the MPTP mouse model of Parkinson's disease. In: Federoff H (eds) Parkinson's disease: The life cycle of the dopamine neuron. New York Acad Sciences, New York, pp 132–139

Wang H et al (2004) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. J Neurosci 24:10963–10973

Wang Y, Han R, Liang ZQ, Wu JC, Zhang XD, Gu ZL, Qin ZH (2008) An autophagic mechanism is involved in apoptotic death of rat striatal neurons induced by the non-*N*-methyl-D-aspartate receptor agonist kainic acid. Autophagy 4:214–226

Waring P (2005) Redox active calcium ion channels and cell death. Arch Biochem Biophys 434:33–42

Wei MC et al (2001) Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. Science 292:727–730

Whiteman M et al (2007) The pro-inflammatory oxidant hypochlorous acid induces Bax-dependent mitochondrial permeabilisation and cell death through AIF-/EndoG-dependent pathways. Cell Signal 19:705–714

Willis SN, Adams JM (2005) Life in the balance: How BH3-only proteins induce apoptosis. Curr Opin Cell Biol 17:617–625

Willis SN et al (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 315:856–859

Wood DE, Thomas A, Devi LA, Berman Y, Beavis RC, Reed JC, Newcomb EW (1998) Bax cleavage is mediated by calpain during drug-induced apoptosis. Oncogene 17:1069–1078

Wright KM, Linhoff MW, Potts PR, Deshmukh M (2004) Decreased apoptosome activity with neuronal differentiation sets the threshold for strict IAP regulation of apoptosis. J Cell Biol 167:303–313

Xiong ZG et al (2004) Neuroprotection in ischemia: Blocking calcium-permeable acid-sensing ion channels. Cell 118:687–698

Ye H et al (2002) DNA binding is required for the apoptogenic action of apoptosis inducing factor. Nat Struct Biol 9:680–684

Youle RJ, Strasser A (2008) The BCL-2 protein family: Opposing activities that mediate cell death. Nat Rev Mol Cell Biol 9:47–59

Yu SW et al (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Yu SW, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Proc Natl Acad Sci USA 103:18314–18319

Yuste VJ et al (2005a) The contribution of apoptosis-inducing factor, caspase-activated DNase, and inhibitor of caspase-activated DNase to the nuclear phenotype and DNA. J Biol Chem 280:35670–35683

Yuste VJ, Moubarak RS, Delettre C, Bras M, Sancho P, Robert N, d'Alayer J, Susin SA (2005b) Cysteine protease inhibition prevents mitochondrial apoptosis-inducing factor (AIF) release. Cell Death Differ 12:1445–1448

Zhang X et al (2002) Intranuclear localization of apoptosis-inducing factor (AIF) and large scale DNA fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. J Neurochem 82:181–191

Zhu C, Qiu L, Wang X, Hallin U, Cande C, Kroemer G, Hagberg H, Blomgren K (2003) Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. J Neurochem 86:306–317

Zhu C, Wang X, Qiu L, Peeters-Scholte C, Hagberg H, Blomgren K (2004) Nitrosylation precedes caspase-3 activation and translocation of apoptosis-inducing factor in neonatal rat cerebral hypoxia-ischaemia. J Neurochem 90:462–471

Zhu C, Xu F, Wang X, Shibata M, Uchiyama Y, Blomgren K, Hagberg H (2006) Different apoptotic mechanisms are activated in male and female brains after neonatal hypoxia-ischaemia. J Neurochem 96:1016–1027

Zhu C et al (2007) Apoptosis-inducing factor is a major contributor to neuronal loss induced by neonatal cerebral hypoxia-ischemia. Cell Death Differ 14:775–784

Zolotarjova N, Ho C, Mellgren RL, Askari A, Huang WH (1994) Different sensitivities of native and oxidized forms of Na+/K(+)-ATPase to intracellular proteinases. Biochim Biophys Acta 1192:125–131

Zong WX, Thompson CB (2006) Necrotic death as a cell fate. Genes Dev 20:1–15

Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB (2004) Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 18:1272–1282

Chapter 4
Age-Dependence of Neuronal Apoptosis and of Caspase Activation

Denson G. Fujikawa

4.1 Morphological Classification of Cell Death

Prior to the morphological description of cellular apoptosis in 1972, cell death was described as "coagulative necrosis," which had the ultrastructural appearance of *in vitro* autolysis (Kerr et al. 1972). Apoptotic cells were described as having condensed cytoplasm with normal-appearing cytoplasmic organelles and the compaction of nuclear chromatin into one or more large masses, with subsequent breaking up of cells into membrane-bound, well-preserved fragments, or "apoptotic bodies" (Kerr et al. 1972) (Fig. 4.1). It was subsequently discovered that glucocorticoid-induced thymocytic apoptosis was associated with internucleosomal DNA cleavage, or DNA "laddering," due to endonuclease activation (Wyllie 1980). Shortly afterward, Wyllie classified cell death into two categories: necrosis and apoptosis (Wyllie 1981). Necrosis differs from apoptosis in that there is cytoplasmic edema, with dilation of endoplasmic reticulum, mitochondrial swelling, polysome disaggregation and multiple small clumps of condensed chromatin within nuclei (Wyllie 1981) (Fig. 4.2). These changes are irreversible when there is high-amplitude mitochondrial swelling with rupture of cristae and flocculent or granular matrix densities and plasma membrane rupture (Fig. 4.2).

Several years later, Peter Clarke divided developmental cell death into three categories: apoptosis (type I), autophagy (type II) and necrosis (type IIIb) (Clarke 1990). This classification has been widely adopted by cell death researchers. Autophagic cell death differs from apoptosis and necrosis morphologically by the presence of double membrane-bound autophagosomes within the cytoplasm, which fuse with lysosomes to form autophagolysosomes, where their contents are degraded by acidic lysosomal hydrolases (Rubinsztein et al. 2005; Uchiyama et al. 2008)

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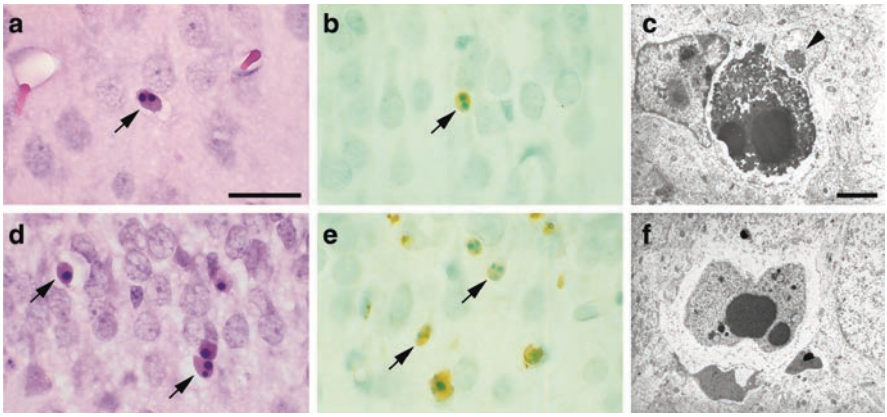

Fig. 4.1 Apoptosis and necrosis are morphologically distinct events (compare with Fig. 4.2). (**a**) and (**d**) are hematoxylin and eosin (H&E)-stained sections of the left retrosplenial cortex in a postnatal day 8 (P8) rat, (**b**) and (**e**) are terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-stained adjacent sections, with methyl green counterstain, and (**c**) and (**f**) are electron-micrographic ultrathin sections from the right hemisphere of the same rat. (**a**–**c**) show naturally occurring apoptotic neurons, and D-F apoptotic neurons induced by administration of 1 mg/kg MK-801 i.p. 24 h earlier. The large, round, basophilic chromatin clumps and acidophilic cytoplasm in A and D are clearly visible (*arrows point to some*). In (**b**) and (**e**) the lightly positive TUNEL staining of the entire cell bodies except for the large, round chromatin clumps, which are stained with methyl green, are also clearly seen (*arrows point to some*). The DNA fragments labeled with TUNEL disperse throughout apoptotic neurons because of disruption of nuclear membranes. In (**c**) a naturally occurring apoptotic neuron in an advanced stage of degeneration is being phagocytosed by an adjacent astrocyte; the large chromatin clumps are still present. The *arrowhead* points to an apoptotic body that has separated from the cell body. (**f**) shows a more newly formed apoptotic neuron, with large, round chromatin clumps and a less electron-dense cytoplasm. Scale bars are 20 μm for (**a**), (**b**), (**d**) and (**e**) and 2 μm for (**c**) and (**f**) (from Fujikawa et al. 2000, with permission from Elsevier)

(Fig. 4.3). There is also a lack of the nuclear and cytoplasmic changes that accompany apoptotic and necrotic cell death.

4.2 Programmed Cell Death

Research into the biochemical basis of cell death began with cellular apoptosis, and in recent years, the programmed cell death (PCD) mechanisms contributing to cell death and apoptosis became synonymous, which has created confusion, because "apoptosis" refers to a particular morphology and not to a biochemical pathway (Fujikawa 2000, 2002). Cell death investigators with a simplistic view of morphology, for example, that cells with shrunken, condensed nuclei are apoptotic, created confusion because cells with shrunken, condensed but intact nuclei are necrotic, not apoptotic (Fujikawa et al. 1999, 2000, 2002, 2007; Fujikawa 2000, 2002).

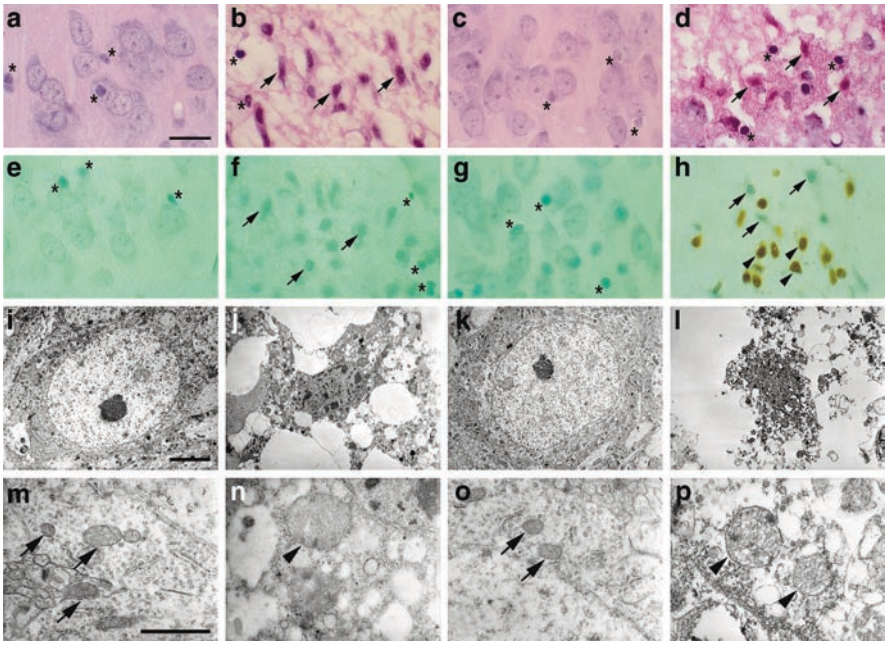

Fig. 4.2 Apoptosis and necrosis are morphologically distinct events (compare with Fig. 4.1). (**a**–**d**) are H&E-stained sections, (**e**–**h**) are TUNEL-stained sections with methyl green counterstain, (**i**–**l**) are lower magnification and (**m**–**p**) higher magnification electron photomicrographs. (**a**) and (**e**) show the left, and (**i**) and (**m**) the right piriform cortex of a control rat given normal saline, and (**b**) and (**f**) show the left, and (**j**) and (**n**) the right piriform cortex of a rat with 3-h kainic acid-induced SE (KASE) and a 24-h recovery period. (**c**) and (**g**) show the left, and (**k**) and (**o**) the right piriform cortex of a control rat given normal saline, and (**d**) and (**h**) show the *left*, and (**l**) and (**p**) the right piriform cortex of a rat with 3-h KASE and a 72-h recovery period. Control light-microscopic sections show round nuclei with nucleoli and reticular chromatin stained with H&E (**a** and **c**) and methyl green (**e** and **g**). SE light-microscopic sections show necrotic acidophilic neurons with pyknotic (shrunken), condensed nuclei and eosinophilic cytoplasm stained with H&E (**b** and **d**) and with TUNEL/methyl green (**f** and **h**). TUNEL positivity in the condensed nuclei of necrotic neurons appeared 72 h but not 24 h after SE. *Arrows* point to necrotic neurons and arrowheads in (**h**) point to some neurons with TUNEL-positive nuclei. (**i**) and (**k**) are electron photomicrographs showing normal nuclei and (**m**) and (**o**) normal mitochondria (*arrows point to some*) in control rats. (**j**) and (**l**) show the shrunken, electron-dense necrotic neurons with pyknotic nuclei containing scattered chromatin clumps (much smaller at 72 h recovery) and degenerating cytoplasm with many vacuoles and dilated mitochondria. At higher magnification (**n** and **p**) the swollen mitochondria are apparent, containing dark particles (flocculent densities) indicating irreversible damage (*arrowheads*). Scale bars are 20 μm (**a**–**h**), 4 μm (**i**–**l**) and 1 μm (**m**–**p**) (from Fujikawa et al. 2000, with permission from Elsevier)

4.2.1 Caspase-Dependent Programmed Cell Death

Research within the past decade has shown the importance of cysteine proteases that cleave after aspartate residues (caspases) in PCD (Cohen 1997; Earnshaw et al. 1999). Two caspase-dependent PCD pathways, the intrinsic (mitochondrial) pathway

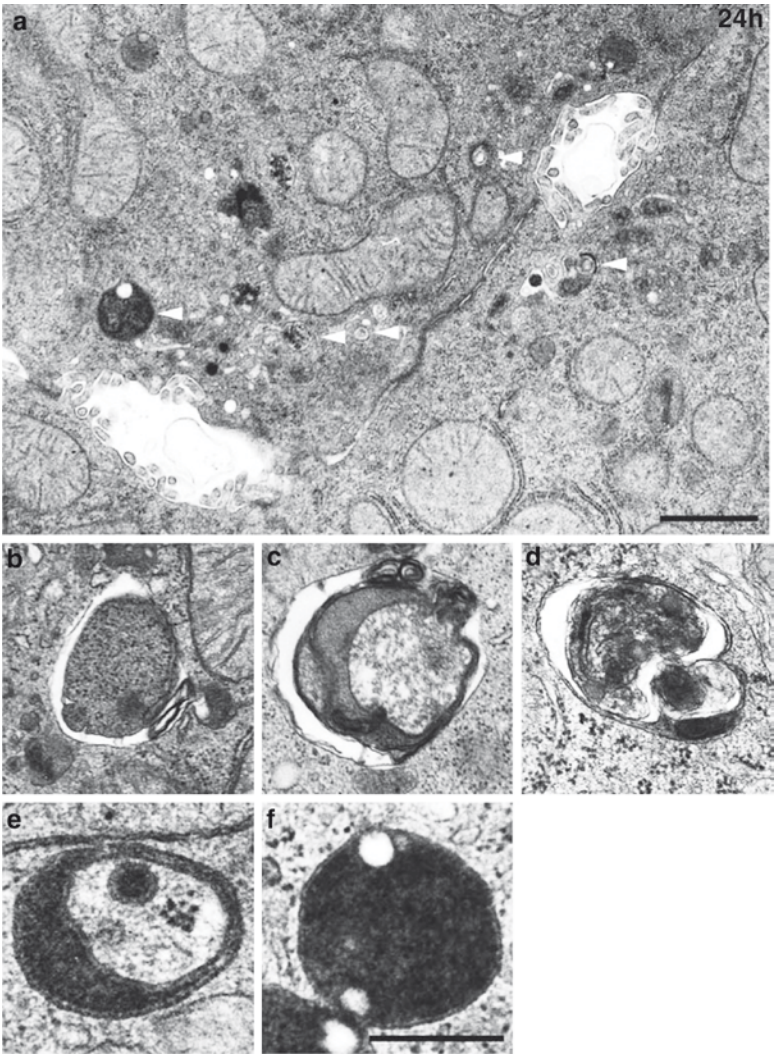

Fig. 4.3 Autophagy involves the formation of cytoplasmic autophagosomes. These photomicrographs show mouse hepatocytes in a mouse not given food for 24 h. (**a**) shows the cytoplasm of a hepatocyte with normal mitochondria but with vacuolar structures in the cytoplasm (*arrowheads*). (**b**) through (**e**) show autophagosomes containing recognizable cytoplasmic structures, whereas (**f**) shows a lysosome with unidentifiable electron-dense material (from Uchiyama et al. 2008, with permission from Springer)

involving caspase-9 (casp-9) activation, and the extrinsic (death-receptor) pathway involving caspase-8 (casp-8) activation, both result in activation of caspase-3 (casp-3), the principal executioner caspase. Following an apoptotic stimulus with mitochondrial membrane permeabilization, cytochrome *c* (cyt *c*) translocates to the cytosol, where it forms the "apoptosome," together with apoptotic protease activating factor-1

(Apaf-1) and dATP, which cleaves casp-9, activating it (Liu et al. 1996, 1997; Zou et al. 1997). Active casp-9 cleaves casp-3 (Li et al. 1997), and the cleaved (active) casp-3 translocates to nuclei, activating caspase-activated DNase (CAD)/DNA fragmentation factor 40 (DFF40) by cleaving inhibitor of CAD (ICAD)/DNA fragmentation factor 45 (DFF45) (Liu et al. 1997, 1998; Enari et al. 1998; Sakahira et al. 1998). CAD/DFF40 then cleaves double-stranded DNA at internucleosomal sites, producing 180 base pair fragments that have a ladder-like appearance on electrophoresed DNA agarose gels (DNA laddering) (Liu et al. 1997, 1998; Enari et al. 1998; Sakahira et al. 1998).

In the CNS morphological apoptosis, both naturally occurring and that induced by an acute insult, occurs during the neonatal period, becoming undetectable in older rats (Ikonomidou et al. 1999; Liu et al. 2004) (Fig. 4.4). Exceptions in the adult rat have been observed (Sloviter et al. 1993, 1996). It has also been shown that casp-3 activation is age-dependent in cerebral ischemia, occurring during the neonatal period and becoming negligible in the adult rat (Hu et al. 2000; Liu et al. 2004) (Fig. 4.5). Thus, casp-3 activation occurs in morphologically apoptotic neurons in the neonatal brain. Earlier studies have shown casp-3 activation in adult rodents following cerebral ischemia (Hara et al. 1997; Chen et al. 1998) and focal status epilepticus (SE) (Henshall et al. 2000), or activation of upstream casp-8 and casp-9 in focal SE (Henshall et al. 2001a, b), but none of the studies showed morphological neuronal apoptosis, and the preponderance of subsequent studies, to be discussed in the next subsection, have pointed to caspase-independent PCD pathways in excitotoxic neuronal death, of which cerebral ischemia and SE are examples.

4.2.2 Caspase-Independent Programmed Cell Death

As mentioned previously, apoptosis and PCD have been used interchangeably, and until recently, necrosis was assumed to be a passive process of cell swelling and lysis, based on *in vitro* evidence. However, there has been for many years *in vivo* evidence in cerebral ischemia, status epilepticus and hypoglycemia that what was then called "ischemic cell change" and what we now describe as neuronal necrosis involves cell shrinkage and condensation, with nuclear pyknosis (shrinkage), scattered irregular chromatin clumps, and mitochondrial swelling, with disrupted cristae and flocculent densities (Fig. 4.6) (Brown and Brierley 1972, 1973; Griffiths et al. 1984; Auer et al. 1985a, b; Ingvar et al. 1988; Colbourne et al. 1999; Fujikawa et al. 1999, 2000, 2002). As discussed in the section "Morphological Classification of Cell Death," these changes were described independently by Wyllie (1981), and were recognized as being necrotic.

Recently, evidence has accumulated that necrotic cell death can also be programmed, but that it involves caspase-independent mechanisms (Kitanaka and Kuchino 1999; Proskuryakov et al. 2003; Syntichaki and Tavernarakis 2003; Vanlangenakker et al. 2008). For example, in cerebral ischemia and SE activation of both the calcium-dependent cysteine protease calpain I and the DNA repair

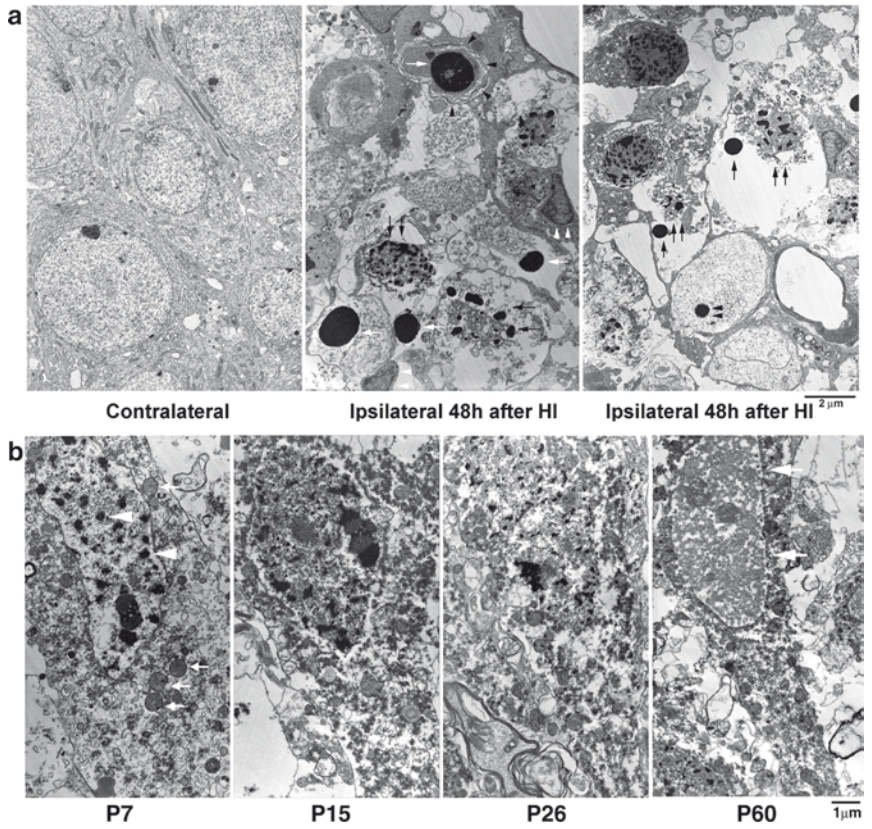

Fig. 4.4 (**a**) shows electron photomicrographs of neurons in the upper blade of the dentate granule cell layer in contralateral (control) hemisphere and ipsilateral hemisphere in rats with left common carotid artery ligation and hypoxia (8% O_2) for 60 min in P7 rats 48 h after hypoxia-ischemia. The contralateral hemisphere showed normal neuronal nuclei and cytoplasm. Ipsilaterally, there were two types of degenerating neurons: those with pyknotic nuclei and scattered, irregular chromatin clumps (*double black arrows*) and those with large, round, chromatin clumps (white arrows in the middle panel and single black arrow in the right panel). These corresponded to necrotic and apoptotic neurons, respectively. (**b**) shows necrotic neurons in the hippocampal CA1 pyramidal cell layer ipsilaterally 48 h after hypoxia-ischemia in P7, P15, P26 and P60 rats (the P7 and P15 rats had 60 min of hypoxia, the P26 and P60 rats, 30 min of hypoxia). The shrunken neurons are electron-dense, with pyknotic nuclei containing scattered, irregular chromatin clumps, which decreased in size from P7 to P26 and were not visible in P60 rats. The cytoplasm at all ages showed extensive degeneration, with swollen, round mitochondria and disrupted endoplasmic reticulum, with ribosomal disaggregation. The white arrows in the P7 rat point to round, swollen mitochondria without obvious disruption of cristae, and the arrowheads point to the scattered chromatin clumps. The white arrows in the P60 rat point to the nucleus, with no visible chromatin clumps and with an intact nuclear membrane (from Liu et al. 2004, with permission from Elsevier)

Fig. 4.5 (continued) granule cell layer, showing progressively less active caspase-3 IR with age, and absent caspase-3 IR at P60. Large arrows point to nuclei with active caspase-3 IR, small arrows to large, round chromatin masses without active caspase-3 IR and *arrowheads* to condensed nuclei without active caspase-3 IR (from Liu et al. 2004, with permission from Elsevier)

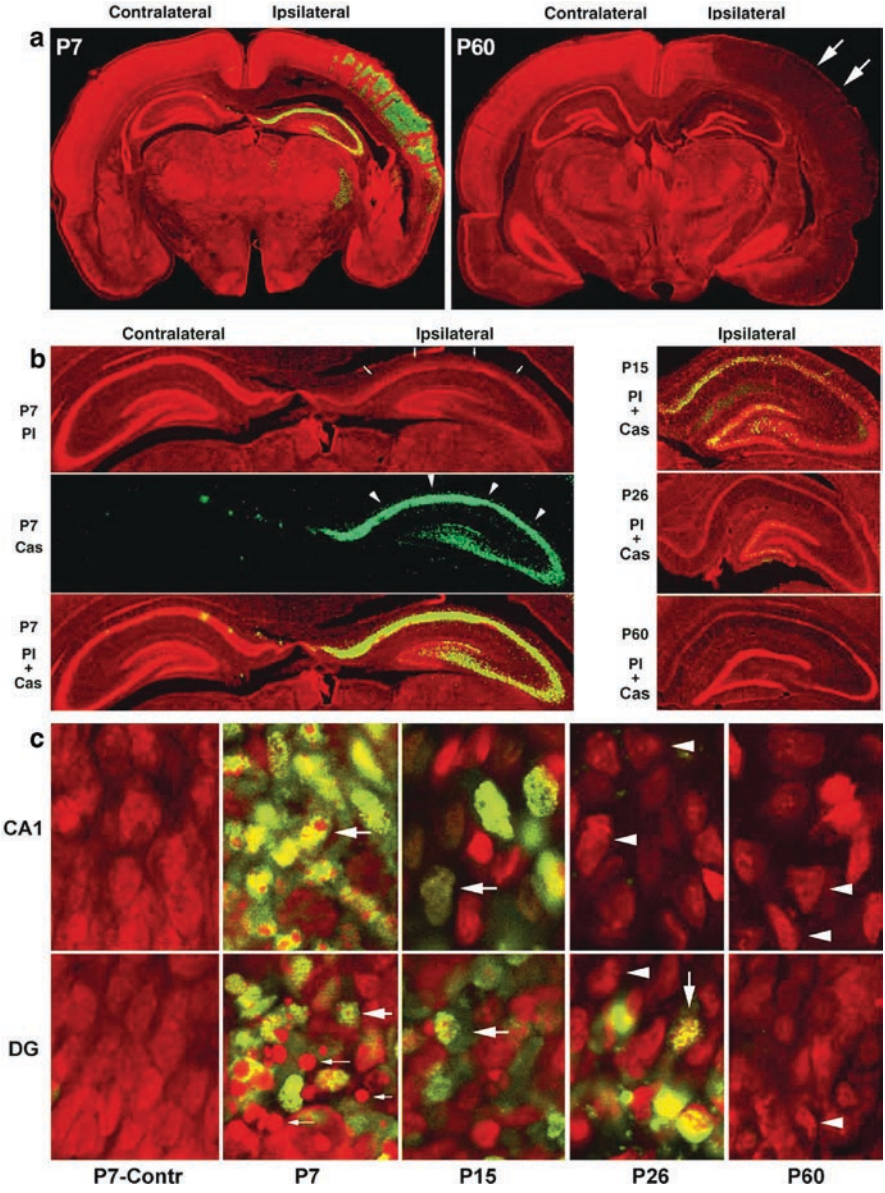

Fig. 4.5 These are confocal images from brain sections of P7 and P60 rats (**a**), double-labeled with an antibody to active caspase-3 (*green*) and propidium iodide (PI), which, since brains were perfused with 4% phosphate-buffered paraformaldehyde, penetrates cells and stains nucleic acids of normal and degenerating neurons, essentially serving as a nuclear stain. Rats were subjected to left common carotid ligation and hypoxia, as in Fig. 4.4. Active caspase-3 expression is present in the ipsilateral hippocampal pyramidal cell layer and neocortex of the P7 rat, but there is no expression in the P60 rat. Arrows point to weaker PI staining in the neocortex of the P60 rat. (**b**) shows left hippocampal PI staining, active caspase-3 immunoreactivity (IR) and a merged image in a P7 rat, with merged images in P15, P26 and P60 rats. There is substantially less active caspase-3 IR in P15 hippocampus, which is only faintly seen in dentate granule cells at P26, and which is absent throughout the hippocampus at P60. (**c**) shows higher magnification images in CA1 and the upper blade of the dentate

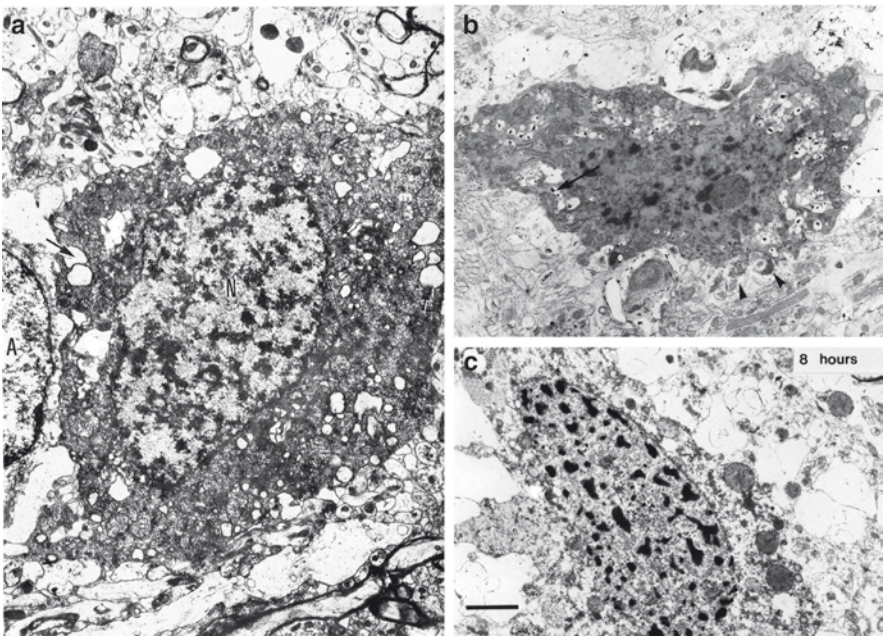

Fig. 4.6 Early evidence that necrotic neurons are produced by cerebral ischemia (**a**), status epilepticus (**b**) and hypoglycemia (**c**). (**a**) shows an ischemic necrotic hippocampal pyramidal neuron in a rat subjected to right common carotid artery ligation and 40 min of hypoxia (Levine preparation) with a short (less than 2 h) recovery period. It is shrunken, electron-dense, with a pyknotic (shrunken) nucleus (N) containing diffuse, irregular chromatin clumps and cytoplasm with numerous vacuoles, some of which are dilated endoplasmic reticulum (arrows point to two) and dilated mitochondria with disrupted cristae. The neuron is surrounded by dilated astrocytic processes and an adjacent astrocytic nucleus (**A**) (from Brown and Brierley 1972, with permission from Elsevier). (**b**) shows a seizure-induced necrotic neuron in the hippocampal "stratum polymorph" 60 min after 2-h L-allylglycine-induced status epilepticus. It is also shrunken, electron-dense, with a pyknotic nucleus with scattered, irregular chromatin clumps and cytoplasmic vacuoles, most of which contain Ca^{2+} pyroantimonate deposits (the *arrow* points to one such vacuole; arrowheads point to synaptic terminals) (from Griffiths et al. 1984, with permission from Elsevier). (**c**) shows a hypoglycemia-induced necrotic neuron in the cerebral cortex of a rat subjected to 60 min of an isoelectric EEG, induced by hypoglycemia, and an 8-h recovery period. It is also shrunken, electron-dense, with prominent irregular chromatin clumps in the pyknotic nucleus, large, swollen mitochondria containing flocculent densities and surrounding swollen astrocytic processes. The scale bar is 1.5 μm (from Auer et al. 1985a, with permission from Springer)

enzyme poly(ADP-ribose) polymerase-1 (PARP-1) occur and contribute to neuronal death (Eliasson et al. 1997; Cao et al. 2007). Calpain I translocates to both mitochondrial and lysosomal membranes, causing release from mitochondria of cyt *c* and apoptosis-inducing factor (AIF) (Gao and Dou 2000; Lankiewicz et al. 2000; Ding et al. 2002; Volbracht et al. 2005) and of lysosomal cathepsins and DNase II from lysosomes (Yamashima et al. 1998, 2003; Tsukada et al. 2001). PARP-1 forms poly(ADP-ribose) (PAR) polymers, which translocate from nuclei to mitochondria, with resultant AIF release (Yu et al. 2002, 2006; Andrabi et al. 2006). AIF translocates to neuronal nuclei, and together with an as yet unknown endonuclease, is responsible for large-scale, 50-kb DNA cleavage (Susin et al. 1999).

The actions of cytosolic cyt *c* in caspase-independent programmed cell death are largely unknown. Aside from apoptosome formation, cytosolic cyt *c* translocates to the endoplasmic reticulum, where it binds to inositol (1,4,5) trisphosphate receptors, inducing Ca^{2+} release that amplifies mitochondrial cyt *c* release (Boehning et al. 2003). In addition, cyt *c* translocates to HeLa cell or cerebellar granule cell nuclei following DNA damage, where it induces cytoplasmic translocation of acetylated histone H2A and chromatin condensation (Nur-E-Kamal et al. 2004). We also have evidence that cyt *c* translocates to neuronal nuclei within 60 min after the onset of generalized SE, together with mitochondrial AIF and endoG and lysosomal cathepsin B (cath-B) and cathepsin D (cath-D) and DNase II (Zhao et al. Submitted for publication). However, the functional consequences of cyt *c*, cath-B and cath-D nuclear translocation are not known, and potential large-scale DNA cleavage by AIF and DNA laddering by endoG and DNase II have yet to be shown following SE.

References

Andrabi SA, Kim S-W, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD, Poirier GG, Dawson VL, Dawson TM (2006) Poly(ADP-ribose) (PAR) polymer is a death signal. Proc Natl Acad Sci USA 103:18308–18313

Auer RN, Kalimo H, Olsson Y, Siesjo BK (1985a) The temporal evolution of hypoglycemic brain damage. I. Light- and electron-microscopic findings in the rat cerebral cortex. Acta Neuropathol (Berl) 67:13–24

Auer RN, Kalimo H, Olsson Y, Siesjo BK (1985b) The temporal evolution of hypoglycemic brain damage. II. Light- and electron-microscopic findings in the hippocampal gyrus and subiculum of the rat. Acta Neuropathol (Berl) 67:25–36

Boehning D, Patterson RL, Sedaghat L, Glebova NO, Kurosaki T, Snyder SH (2003) Cytochrome c binds to inositol (1, 4, 5) trisphosphate receptors, amplifying calcium-dependent apoptosis. Nat Cell Biol 5:1051–1061

Brown AW, Brierley JB (1972) Anoxic-ischaemic cell change in rat brain light microscopic and fine-structural observations. J Neurol Sci 16:59–84

Brown AW, Brierley JB (1973) The earliest alterations in rat neurones and astrocytes after anoxia-ischaemia. Acta Neuropathol 23:9–22

Cao G, Xing J, Xiao X, Liou AK, Gao Y, Yin XM, Clark RS, Graham SH, Chen J (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. J Neurosci 27:9278–9293

Chen J, Nagayama T, Jin K, Stetler RA, Zhu RL, Graham SH, Simon RP (1998) Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J Neurosci 18:4914–4928

Clarke PGH (1990) Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol 181:195–213

Cohen GM (1997) Caspases: the executioners of apoptosis. Biochem J 326:1–16

Colbourne F, Sutherland GR, Auer RN (1999) Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J Neurosci 19:4200–4210

Ding WX, Shen HM, Ong CN (2002) Calpain activation after mitochondrial permeability transition in microcystin-induced cell death in rat hepatocytes. Biochem Biophys Res Commun 291:321–331

Earnshaw WC, Martins LC, Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68:383–424

Eliasson MJL, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang Z-Q, Dawson TM, Snyder SH, Dawson VL (1997) Poly (ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. Nature Med 3:1089–1095

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391:43–50

Fujikawa DG (2000) Confusion between neuronal apoptosis and activation of programmed cell death mechanisms in acute necrotic insults. Trends Neurosci 23:410–411

Fujikawa DG (2002) Apoptosis: ignoring morphology and focusing on biochemical mechanisms will not eliminate confusion. Trends Pharmacol Sci 23:309–310

Fujikawa DG, Shinmei SS, Cai B (1999) Lithium-pilocarpine-induced status epilepticus produces necrotic neurons with internucleosomal DNA fragmentation in adult rats. Eur J Neurosci 11:1605–1614

Fujikawa DG, Shinmei SS, Cai B (2000) Kainic acid-induced seizures produce necrotic, not apoptotic, neurons with internucleosomal DNA cleavage: implications for programmed cell death mechanisms. Neuroscience 98:41–53

Fujikawa DG, Ke X, Trinidad RB, Shinmei SS, Wu A (2002) Caspase-3 is not activated in seizure-induced neuronal necrosis with internucleosomal DNA cleavage. J Neurochem 83:229–240

Fujikawa DG, Shinmei SS, Zhao S, Aviles ER Jr (2007) Caspase-dependent programmed cell death pathways are not activated in generalized seizure-induced neuronal death. Brain Res 1135:206–218

Gao G, Dou QP (2000) N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. J Cell Biochem 80:53–72

Griffiths T, Evans MC, Meldrum BS (1984) Status epilepticus: the reversibility of calcium loading and acute neuronal pathological changes in the rat hippocampus. Neuroscience 12:557–567

Hara H, Friedlander RM, Gagliardini V, Ayata C, Fink K, Huang Z, Shimizu-Sasamata M, Yuan J, Moskowitz MA (1997) Inhibition of interleukin-1-beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. Proc Natl Acad Sci USA 94:2007–2012

Henshall DC, Chen J, Simon RP (2000) Involvement of caspase-3-like protease in the mechanism of cell death following focally evoked limbic seizures. J Neurochem 74:1215–1223

Henshall DC, Bonislawski DP, Skradski SL, Araki T, Lan J-Q, Schindler CK, Meller R, Simon RP (2001a) Formation of the Apaf-1/cytochrome c complex precedes activation of caspase-9 during seizure-induced neuronal death. Cell Death Diff 8:1169–1181

Henshall DC, Bonislawski DP, Skradski SL, Lan J-Q, Meller R, Simon RP (2001b) Cleavage of Bid may amplify caspase-8-induced neuronal death following focally evoked limbic seizures. Neurobiol Dis 8:568–580

Hu BR, Liu CL, Ouyang Y, Blomgren K, Siejö BK (2000) Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. J Cereb Blood Flow Metab 20:1294–1300

Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler V, Dikranian K, Tenkova TI, Stefovska V, Turksi L, Olney JW (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283:70–74

Ingvar M, Morgan PF, Auer RN (1988) The nature and timing of excitotoxic neuronal necrosis in the cerebral cortex, hippocampus and thalamus due to flurothyl-induced status epilepticus. Acta Neuropathol 75:362–369

Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257

Kitanaka C, Kuchino Y (1999) Caspase-independent programmed cell death with necrotic morphology. Cell Death Diff 6:508–515

Lankiewicz S, Luetjens CM, Bui NT, Krohn AJ, Poppe M, Cole GM, Saido TC, Prehn JHM (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J Biol Chem 275:17064–17071

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–489

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86:147–157

Liu X, Zou H, Slaughter C, Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89:175–184

Liu X, Peng L, Widlak P, Zou H, Luo X, Garrard WT, Wang X (1998) The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc Natl Acad Sci USA 95:8461–8466

Liu CL, Siesjö BK, Hu BR (2004) Pathogenesis of hippocampal neuronal death after hypoxia-ischemia changes during brain development. Neuroscience 127:113–123

Nur-E-Kamal A, Gross SR, Pan Z, Balklava Z, Ma J, Liu LF (2004) Nuclear translocation of cytochrome c during apoptosis. J Biol Chem 279:24911–24914

Proskuryakov SY, Knoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? Exp Cell Res 283:1–16

Rubinsztein DC, DiFiglia M, Heintz N, Nixon RA, Qin ZH, Ravikumar B, Stefanis L, Tolkovsky A (2005) Autophagy and its possible roles in nervous system diseases, damage and repair. Autophagy 1:11–22

Sakahira H, Enari M, Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96–99

Sloviter RS, Dean E, Neubort S (1993) Electron microscopic analysis of adrenalectomy-induced hippocampal granule cell degeneration in the rat: apoptosis in the adult central nervous system. J Comp Neurol 330:337–351

Sloviter RS, Dean E, Sollas AL, Goodman JH (1996) Apoptosis and necrosis induced in different hippocampal neuron populations by repetitive perforant path stimulation in the rat. J Comp Neurol 366:516–533

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446

Syntichaki P, Tavernarakis N (2003) The biochemistry of neuronal necrosis: rogue biology? Nat Rev Neurosci 4:672–684

Tsukada T, Watanabe M, Yamashima T (2001) Implications of CAD and DNase II in ischemic neuronal necrosis specific for the primate hippocampus. J Neurochem 79:1196–1206

Uchiyama Y, Shibata M, Koike M, Yoshimura K, Sasaki M (2008) Autophagy-physiology and pathophysiology. Histochem Cell Biol 129:407–420

Vanlangenakker N, Vanden Berghe T, Krysko D, Festjens N, Vandenabeele P (2008) Molecular mechanisms and pathophysiology of necrotic cell death. Curr Mol Med 8:207–220

Volbracht C, Chua BT, Ng CP, Bahr BA, Hong W, Li P (2005) The critical role of calpain versus caspase activation in excitotoxic injury induced by nitric oxide. J Neurochem 93:1280–1292

Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284:555–556

Wyllie A (1981) Cell death: a new classification separating apoptosis from necrosis. In: Bowen I, Lockshin R (eds) Cell death in biology and pathology. Chapman and Hall, London, pp 9–34

Yamashima T, Kohda Y, Tsuchiya K, Ueno T, Yamashita J, Yoshioka T, Kominami E (1998) Inhibition of ischaemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074: a novel strategy for neuroprotection based on 'calpain-cathepsin hypothesis'. Eur J Neurosci 10:1723–1733

Yamashima T, Tonchev AB, Tsukada T, Saido TC, Imajoh-Ohmi S, Momoi T, Kominami E (2003) Sustained calpain activation associated with lysosomal rupture executes necrosis of the postischemic CA1 neurons in primates. Hippocampus 13:791–800

Yu S-W, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Yu S-W, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Proc Natl Acad Sci USA 103:18314–18319

Zhao S, Aviles ER Jr, Fujikawa DG. Nuclear translocation of mitochondrial cytochrome *c* and lysosomal cathepsins B and D within the first 60 minutes of generalized seizures (submitted for publication).

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405–413

Chapter 5
Excitotoxic Programmed Cell Death Involves Caspase-Independent Mechanisms

Ho Chul Kang, Ted M. Dawson, and Valina L. Dawson

5.1 Introduction

Cell death is a strictly programmed physiological process during the development of living organisms. In conditions like brain trauma, ischemia/reperfusion, brain injury, and cellular stress, cell death becomes unregulated, leading to neurodegenerative processes and stroke. Cell death, in general, is a complex process involving multiple pathways. Apoptosis, necrosis and autophagy are the three traditionally described pathways of cell death. These pathways share many common biochemical events in cell death execution. Apoptosis, in general, is executed by the activation of caspases; and involves the formation of apoptotic bodies without eliciting inflammatory responses. Necrosis, on the other hand, involves massive cell swelling, inflammation and rupture of cellular structures. Autophagy, the third pathway of cell death, was originally identified in the recycling of organelles and proteins. Recent data suggest that autophagy plays a critical role in neurodegenerative processes. Excitotoxicity is a common pathological process in many neurodegenerative disorders, and this process involves over-stimulation of glutamate receptors and an excessive influx of calcium into cells. Cell death in excitotoxicity is unique in that, for the most part, it does not involve caspase-dependent pathways. Overactivation of poly (ADP-ribose) polymerase-1 (PARP-1) is an early pathological event in excitotoxicity that leads to a unique form of cell death called parthanatos. Biochemical events in parthanatos include early accumulation of poly (ADP-ribose) (PAR) and nuclear translocation of apoptosis inducing factor (AIF) from the mitochondria.

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5.2 Excitotoxicity

Excitotoxicity is a process of neuronal injury mediated by excitatory amino acids (Olney and Sharpe 1969). Glutamate is the most abundant amino acid in the brain and is an essential neurotransmitter in the central nervous system. It plays a primary role in excitotoxicity (Arundine and Tymianski 2003). Overstimulation of glutamate receptors results in an excessive influx of calcium to mediate excitotoxic responses in nerve cells. Glutamate can act on many receptor types in the nervous system, including ionotropic and metabotropic receptors. Ionotropic NMDA-receptors are activated by the glutamate analogue N-methyl-D-aspartate (NMDA) and play a major role in excitotoxicity. α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA) and kainate receptors are activated by AMPA and by kainate, respectively. These non-NMDA-type receptors are also involved in excessive intracellular calcium influx and excitotoxicity (Arundine and Tymianski 2003; Danbolt 2001). Ionotropic receptors are ion channel-linked receptors and cause ion-influx when stimulated. Metabotropic glutamate receptors (mGluR) are G-protein coupled receptors. Glutamate receptors may take part in excitotoxicity by modulating the function of other receptors either directly or indirectly. The Ca^{2+} influx following glutamate receptor activation in excitotoxicity can induce cell death by activating Ca^{2+}-dependent enzyme systems, such as nitric oxide (NO) synthase (nNOS), calpains, and phospholipases. nNOS activation leads to the overproduction of NO through the conversion of L-arginine of L-citrulline. NO can exert many roles as a signaling molecule in neurons. Generation of excess NO can be neurotoxic (Dawson et al. 1993; Evans et al. 2001; Lankiewicz et al. 2000). NO can combine with O_2^- in the mitochondria to generate more toxic peroxynitrite (ONOO-), which can cause oxidative or nitrosative injury to cellular proteins, lipids and DNA (Stamler et al. 2001). Injury to DNA causes a massive activation of poly (ADP-ribose) polymerase-1, which ultimately triggers cell death via the process of parthanatos (Mandir et al. 2000; Wang et al. 2004) (Fig. 5.1).

5.3 Role of PARP-1 and PAR Polymer in Excitotoxicity

Poly (ADP-ribose) polymerases (PARPs), are known to play key roles in DNA repair (Jeggo 1998; Poirier et al. 1982). PARP-1 is the founding member of PARP family, which includes 18 different isoforms based on protein sequence homology to the PARP-1 catalytic domain (Ame et al. 2004; D'Amours et al. 1999; Hong et al. 2004; Smith 2001; Virag and Szabo 2002). PARP-1 accounts for more than 90% of PARP activity in living cells. In response to DNA damage, PARP-1 uses NAD^+ as a substrate and attaches polymers of PAR on different acceptor proteins (hetero-modification) or on PARP-1 itself (auto-modification)

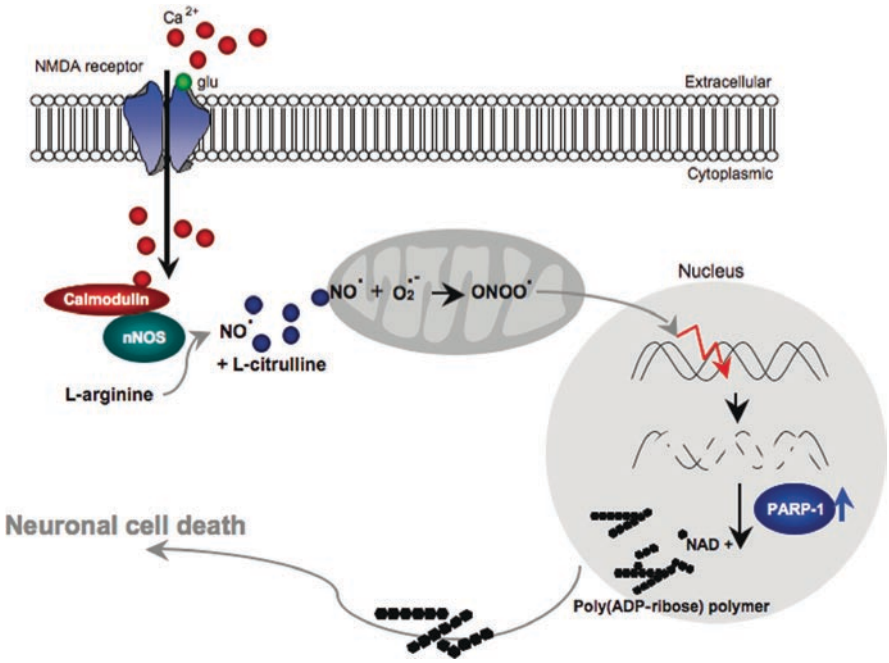

Fig. 5.1 PARP-1 and PAR mediated cell death in excitotoxicity. Over-stimulation of NMDA receptors by glutamate results in the influx of Ca^{2+}, which binds calmodulin and activates nNOS, to convert L-arginine to NO and L-citrulline. Even though NO is an essential molecule in neuronal signal transduction, excess NO can be neurotoxic. Neuronal toxicity by excess NO is mediated by peroxynitrite, a reaction product from NO and superoxide anion (O_2^-). Peroxynitrite causes severe damage to DNA, which results in over activation of PARP (PARP↑), depletion of NAD^+, and generation of PAR polymer, leading to neuronal death

(D'Amours et al. 1999; Virag and Szabo 2002). PARP-1 is considered a "genome guardian," because it takes part in DNA repair under physiological conditions (Jeggo 1998; Poirier et al. 1982). Under mild genomic stress, PARP-1 is activated to induce DNA repair, whereas severe cell stress induces massive PARP-1 activation that ultimately leads to cell death (Virag and Szabo 2002). Both gene deletion and pharmacological inhibition studies have shown that PARP-1 activation plays a key role in cytotoxicity following ischemia/reperfusion, neurodegeneration, spinal cord injury, ischemic injury in heart, liver, and lungs, and in retinal degeneration, arthritis and diabetes (Virag and Szabo 2002). In the nervous system, massive PARP-1 activation is triggered by excitotoxic stimuli. It was originally presumed that cell death in PARP-1 toxicity was induced by the intracellular energy depletion from PARP-1's use of NAD^+ (Virag and Szabo 2002). NAD^+ is an important cellular molecule for many physiological processes. Energy-generating processes, like glycolysis, the Krebs cycle and the

pentose phosphate pathway, utilize NAD^+ as a cofactor (Belenky et al. 2007). While PARP-1 activation leads to decreased cellular NAD^+ and energy levels (Ha and Snyder 1999), it is difficult to obtain evidence that proves that PARP-1 activation depletes enough cellular energy to kill the cell (Fossati et al. 2007; Moubarak et al. 2007). Numerous studies show that cellular ATP and NAD^+ levels drop significantly following PARP-1 activation (Eliasson et al. 1997; Yu et al. 2002). The drop in cellular energy levels following PARP-1 activation may primarily be due to alterations in mitochondrial function and defective oxidative phosphorylation as opposed to PARP-1 mediated catabolism of NAD^+ (Virag and Szabo 2002). Along these lines, it was shown by many studies that mitochondrial depolarization, loss of mitochondrial function and increased mitochondrial membrane permeability are required factors for PARP-1-dependent cell death (Alano et al. 2004). Conclusions that NAD^+ utilization by PARP-1 is a death inducer were drawn from studies that used direct exogenous delivery of NAD^+ or energy substrates as cytoprotective agents. It is important to note that the off-target effects of these substrates may contribute to the observed effects. For example, consumption of NAD^+ by PARP-1 generates nicotinamide (NAM) as a by-product. NAM is a potent PARP-1 inhibitor, so the protective mechanism mediated by exogenous NAD^+ should be interpreted with caution. Recent studies indicate that energy depletion following PARP-1 activation is not a critical factor for cell death. Following PARP-1 activation, we recently demonstrated that cells die as a result of toxic accumulation of PAR. PAR, generated by PARP-1 in the nucleus, travels to the cytosol to induce cell death. Neutralization of cytosolic PAR by PAR-specific antibodies protects against NMDA-induced cell death in mouse primary neurons (Andrabi et al. 2006). Conversely, exogenous delivery of purified PAR kills cells (Andrabi et al. 2006). The toxic potential of PAR increases with dose and polymer complexity. Highly complex and long chain polymers are more toxic than shorter and less complex polymers (Andrabi et al. 2006). Among the PARP family members, there are at least six different PARPs that are confirmed to synthesize PAR. The heterogeneity in the complexity and structure of PAR may vary depending upon the PARP involved. This may contribute to the possible different contributions of individual PARP isoforms to cell survival or cell death. PARP-1-dependent cell death, known as parthanatos, is distinct from classic necrosis or apoptosis in its biochemical and morphological features, although many of the morphologic features are similar to those previously described in excitotoxic/necrotic neurons. The biochemical features of parthanatos are distinct from classically defined pathways of cell death, and include rapid PARP-1 activation, early PAR accumulation, mitochondrial depolarization, early nuclear AIF translocation, loss of cellular NAD and ATP, and late caspase activation. Caspase activation, which is a hallmark of apoptotic cell death, does not play a primary role in parthanatos, as broad-spectrum caspase inhibitors are unable to protect cells. Morphological features of parthanatos include shrunken and condensed nuclei, disintegrating membranes and cells becoming propidium iodide-positive within a few hours after the onset of parthanatos (Figs. 5.2 and 5.3).

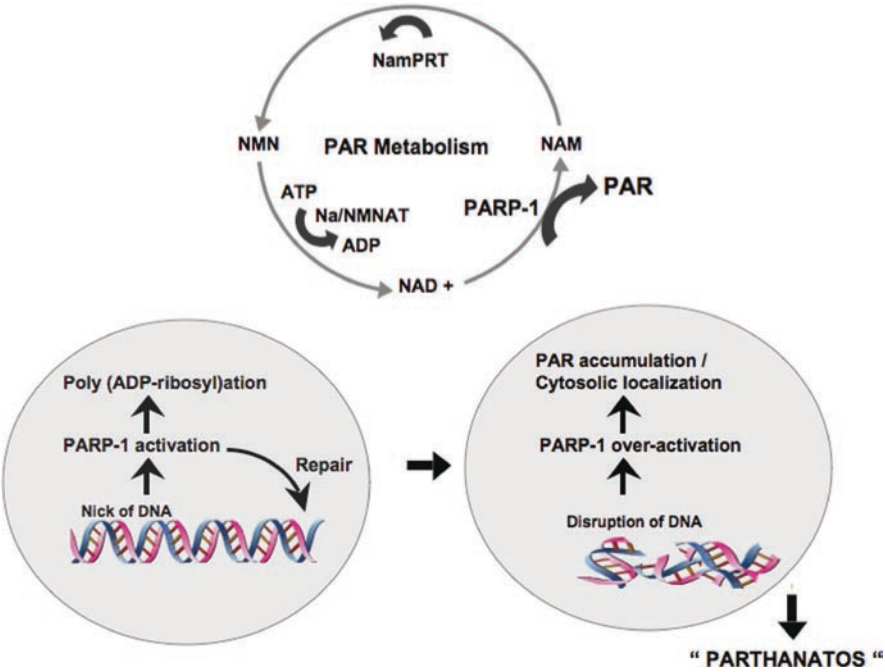

Fig. 5.2 Parthanatos. PARP-1 utilizes NAD^+ as a substrate for synthesis of PAR polymers. In the process of PAR formation, nicotinamide (NAM), a product of NAD^+ hydrolysis, is first converted into nicotinamide mononucleotide (NMN) and then into NAD^+ by nicotinamide phosphoribosyl transferase (NamPRT) and nicotinamide mononucleotide adenylyl transferases (Na/NMNAT-1, -2, and -3), respectively. Mild DNA damage or breaks activate the PARP proteins, where they play a role in the DNA repair process. Under conditions of severe DNA damage, parthanatos is initiated through excessive PAR polymer formation

5.4 Role of PARG in Excitotoxicity and PAR-Mediated Cell Death

Poly(ADP-ribose) glycohydrolase (PARG) is an important cellular enzyme that together with PARPs plays an important role in balancing PAR levels in cells. Many genes encoding different PARPs have been identified, whereas only a single gene encoding for PARG has been identified so far. The full length nuclear PARG in humans is 111 kDa with two cytosolic splice variants, 102 and 99 kDa (Meyer-Ficca et al. 2004). PARG catalyzes the hydrolysis of PAR to ADP-ribose units through its glycosidic activity (Davidovic et al. 2001). Evidence from recent data shows that PARG is critical for cell survival. Genetic deletion of PARG results in accumulation of PAR, which leads to early embryonic lethality in drosophila and mice (Hanai et al. 2004; Koh et al. 2004). Conversely, overexpression of PARG leads to protection against excitotoxicity and PARP-1 dependent cell death (Andrabi et al. 2006; Cozzi et al. 2006).

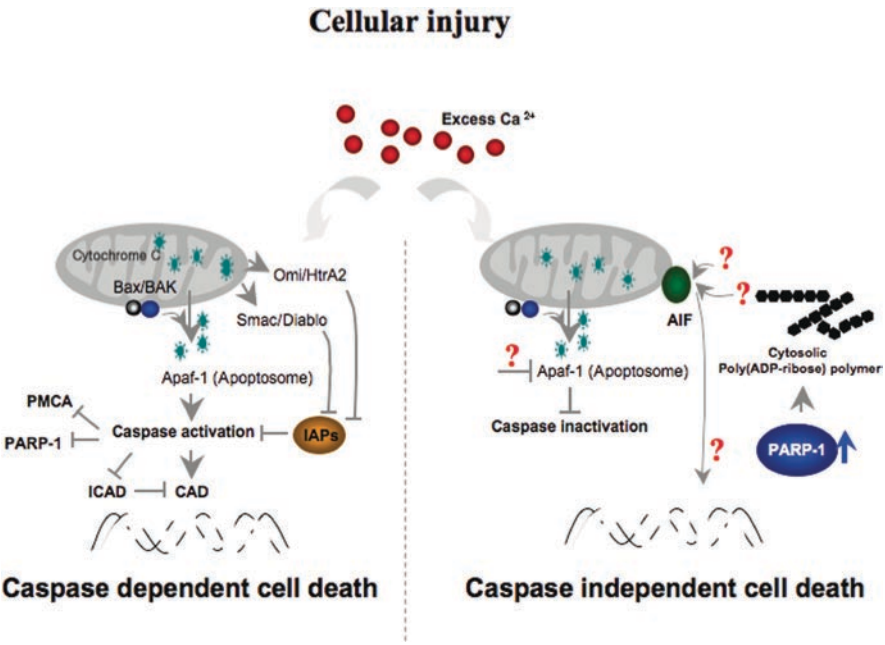

Fig. 5.3 Caspase-dependent vs. caspase-independent neuronal cell death. Caspase-dependent and caspase-independent cell death is signaled by factors released from the mitochondria. In traditional caspase-dependent apoptotic cell death, Bcl family proteins and caspase activation are required for execution of cell death. In caspase-independent cell death, AIF acts as a factor to mediate cell death. Release of AIF from mitochondria can be triggered by different cell signaling mechanisms. In parthanatos, it is PAR polymer generated by over-activation of PARP-1 that induces AIF release. Ongoing questions for investigation are: how PAR travels from the nucleus to the mitochondria, how PAR induces the release of AIF and how nuclear AIF results in DNA fragmentation and nuclear shrinkage

Mouse trophoblasts from E3.5 PARG null mice survive only in the presence of the PARP inhibitor benzamide. Withdrawal of the PARP inhibitor results in cell death in the PARG trophoblasts via toxic accumulation of PAR (Koh et al. 2004). Delivery of purified PAR results in parthanatos in cultured cells. PAR-mediated cell death is inhibited by PARG overexpression. Consistent with these findings, pre-digestion of PAR with recombinant PARG results in the inability of PAR to induce cell death (Andrabi et al. 2006). The inactivation of PAR by PARG predigestion, shows that PARG is important for cell survival and that PAR is a death signaling molecule. Although only one gene for PARG has been discovered, recent data show that a 39-kDa ADP-ribose-(arginine) protein hydrolase (ARH3) has PARG-like activity with unknown biological significance (Oka et al. 2006). The role of ARH3 in PARP-1 dependent cell death and excitotoxicity is still to be characterized. The cell survival role of ARH3 seems to be less important based on the lack of compensation in PARG null mice and drosophila.

5.5 Mitochondria in PAR-Induced Cell Death: Role of AIF

Mitochondria have important roles in cellular energy generating processes. However, in cellular stress, mitochondria participate in cell death signaling by releasing pro-death proteins such as cytochrome c, AIF, Smac/Diablo, and Omi/HtrA2 (Green and Kroemer 2004; Newmeyer and Ferguson-Miller 2003; Suzuki et al. 2001; Verhagen et al. 2000). Among these, Smac/Diablo and Omi/HtrA2 proteins act as inhibitors of cytosolic inhibitor apoptosis proteins (IAPs), which act by inhibiting caspase 9, 3, and 7 (Richter and Duckett 2000). Thus, the release of Smac/Diablo and Omi/HtrA2 into cytoplasm ensures that the brake that IAPs provide on caspase activation is removed. Release of cytochrome c in the cytosol leads to apaf-1 binding and initiates cell death through assembly of the apoptosome complex. Besides cytochrome c and Apaf-1, the apoptosome requires pro-caspase 9 (initiator caspase) and dATP. In this complex, caspase 9 gets cleaved and activated, which in turn activates downstream caspases that include the effector caspase, caspase 3. Active caspase 3 has many cellular substrates, including alpha foldrin, PARP-1, PMCA (plasma membrane Ca2+ pump) and ICAD (Inhibitor of caspase-activated DNase). CAD (caspase-activated DNase) is normally sequestered to an inactive form in a complex with ICAD (CAD-ICAD complex). On ICAD degradation by caspases, CAD is activated to induce large scale DNA-fragmentation and cell death (Liu et al. 1997; Sakahira et al. 1998).

AIF, on the other hand, does not seem to activate a proteolytic cascade but it directly translocates to the nucleus to initiate large scale chromatin condensation and caspase-independent cell death (Cregan et al. 2004; Krantic et al. 2007; Modjtahedi et al. 2006; Wang et al. 2004; Yu et al. 2002). In certain models of cell death, AIF release from mitochondria can be mediated through caspase activation. AIF is a mitochondrial flavoprotein with important functions in oxidative phosphorylation (Pospisilik et al. 2007). Originally, AIF was discovered as a death inducing factor (Susin et al. 1999). Numerous studies have clearly demonstrated that AIF induces cell death upon its translocation to the nucleus (Krantic et al. 2007; Modjtahedi et al. 2006). AIF as a cell death effector in PARP-1 toxicity became evident through studies using AIF-neutralizing antibodies or genetic knock down of AIF (Culmsee et al. 2005; Yu et al. 2006; Yu et al. 2002). In the mitochondria, AIF is involved in oxidative phosphorylation and energy production. Although mitochondrial localization of AIF is required for cell survival, recent studies indicate that nuclear translocation is a required factor to induce cell death and chromatin condensation. Along these lines, it was shown that recombinant AIF induced nuclear shrinkage in isolated nuclear preparations, which strengthens the concept that AIF is a factor that induces chromatin condensation. It still remains unclear how AIF induces chromatin condensation given that AIF has no intrinsic nuclease activity and nuclear AIF translocation is required for chromatin condensation and PARP-1 dependent cell death. It is likely that AIF in the nucleus activates certain proteins that either directly act as nucleases or indirectly activate nuclear condensation systems. PARP-1 activation induces mitochondrial AIF release through PAR polymer acting as a death signal. PAR is

generated in the nucleus and signals the mitochondria to release AIF. It is not yet clear how PAR induces AIF release. Recent data suggests that PARP-1 activation induces mitochondrial permeability transition (mPT). Inhibition of mPT protects against PARP-1 dependent cell death (Alano et al. 2004; Cregan et al. 2004). It remains to be determined how PARP-1 activation induces mPT and whether mPT is critical for AIF release. The data that PAR in the cytosol localizes with mitochondria and induces AIF release hints at the role of PAR as a mediator of mPT. Recent discoveries also suggest that BAX/BAK activation is a required factor for AIF release (Arnoult et al. 2003; Arnoult et al. 2002). However, it remains to be determined whether BAX/BAK and mPT are activated as a consequence of PARP-1 toxicity or whether they act as by-standers in PARP-1 dependent cell death. The intriguing aspect emerging from these studies is how PAR is related to BAX/BAK and mPT activation in PARP-1 toxicity and mitochondrial AIF release (Fig. 5.3).

5.6 Conclusion

Glutamate excitotoxicity is largely a caspase-independent process. Depending on the length and strength of the insult, PARP-1 plays a primary role in the death process. Parthanatos is a unique form of cell death mediated by cytotoxic PAR polymer in cytosol due to overactivation of PARP-1. PAR polymer is synthesized primarily in the nucleus and translocates into the cytosol to induce cell death by regulating mitochondria function. Mitochondria act as the core organelle to release proapoptotic factors. In the case of parthanatos, cell death is initiated by nuclear translocation and mitochondrial release of AIF (Fig. 5.3). PAR polymer induces the structural change of a number of cellular proteins by either the process of poly(ADP-ribosyl)ation by PARP or through non-covalent interactions. Identification of PAR-binding proteins and their characterization may provide a novel opportunity to understand the PAR-signaling mechanisms and to identify novel therapeutics that interfere with PAR dependent cell death.

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References

Alano CC, Ying W, Swanson RA (2004) Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD+ depletion and mitochondrial permeability transition. J Biol Chem 279:18895–18902

Ame JC, Spenlehauer C, de Murcia G (2004) The PARP superfamily. Bioessays 26:882–893

Andrabi SA, Kim NS, Yu SW, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD, Poirier GG, Dawson VL, Dawson TM (2006) Poly(ADP-ribose) (PAR) polymer is a death signal. Proc Natl Acad Sci USA 103:18308–18313

Arnoult D, Gaume B, Karbowski M, Sharpe JC, Cecconi F, Youle RJ (2003) Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. Embo J 22:4385–4399

Arnoult D, Parone P, Martinou JC, Antonsson B, Estaquier J, Ameisen JC (2002) Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome c release in response to several proapoptotic stimuli. J Cell Biol 159:923–929

Arundine M, Tymianski M (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium 34:325–337

Belenky P, Bogan KL, Brenner C (2007) NAD+ metabolism in health and disease. Trends Biochem Sci 32:12–19

Cozzi A, Cipriani G, Fossati S, Faraco G, Formentini L, Min W, Cortes U, Wang ZQ, Moroni F, Chiarugi A (2006) Poly(ADP-ribose) accumulation and enhancement of postischemic brain damage in 110-kDa poly(ADP-ribose) glycohydrolase null mice. J Cereb Blood Flow Metab 26:684–695

Cregan SP, Dawson VL, Slack RS (2004) Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 23:2785–2796

Culmsee C, Zhu C, Landshamer S, Becattini B, Wagner E, Pellecchia M, Blomgren K, Plesnila N (2005) Apoptosis-inducing factor triggered by poly(ADP-ribose) polymerase and Bid mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia. J Neurosci 25:10262–10272

D'Amours D, Desnoyers S, D'Silva I, Poirier GG (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem J 342(Pt 2):249–268

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105

Davidovic L, Vodenicharov M, Affar EB, Poirier GG (2001) Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism. Exp Cell Res 268:7–13

Dawson VL, Dawson TM, Bartley DA, Uhl GR, Snyder SH (1993) Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. J Neurosci 13:2651–2661

Eliasson MJ, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang ZQ, Dawson TM, Snyder SH, Dawson VL (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. Nat Med 3:1089–1095

Evans JH, Spencer DM, Zweifach A, Leslie CC (2001) Intracellular calcium signals regulating cytosolic phospholipase A2 translocation to internal membranes. J Biol Chem 276:30150–30160

Fossati S, Cipriani G, Moroni F, Chiarugi A (2007) Neither energy collapse nor transcription underlie in vitro neurotoxicity of poly(ADP-ribose) polymerase hyper-activation. Neurochem Int 50:203–210

Green DR, Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science 305:626–629

Ha HC, Snyder SH (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci USA 96:13978–13982

Hanai S, Kanai M, Ohashi S, Okamoto K, Yamada M, Takahashi H, Miwa M (2004) Loss of poly(ADP-ribose) glycohydrolase causes progressive neurodegeneration in Drosophila melanogaster. Proc Natl Acad Sci USA 101:82–86

Hong SJ, Dawson TM, Dawson VL (2004) Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. Trends Pharmacol Sci 25:259–264

Jeggo PA (1998) DNA repair: PARP - another guardian angel? Curr Biol 8:R49–R51

Koh DW, Lawler AM, Poitras MF, Sasaki M, Wattler S, Nehls MC, Stoger T, Poirier GG, Dawson VL, Dawson TM (2004) Failure to degrade poly(ADP-ribose) causes increased sensitivity to cytotoxicity and early embryonic lethality. Proc Natl Acad Sci USA 101:17699–17704

Krantic S, Mechawar N, Reix S, Quirion R (2007) Apoptosis-inducing factor: a matter of neuron life and death. Prog Neurobiol 81:179–196

Lankiewicz S, Marc Luetjens C, Truc Bui N, Krohn AJ, Poppe M, Cole GM, Saido TC, Prehn JH (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J Biol Chem 275:17064–17071

Liu X, Zou H, Slaughter C, Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89:175–184

Mandir AS, Poitras MF, Berliner AR, Herring WJ, Guastella DB, Feldman A, Poirier GG, Wang ZQ, Dawson TM, Dawson VL (2000) NMDA but not non-NMDA excitotoxicity is mediated by Poly(ADP-ribose) polymerase. J Neurosci 20:8005–8011

Meyer-Ficca ML, Meyer RG, Coyle DL, Jacobson EL, Jacobson MK (2004) Human poly(ADP-ribose) glycohydrolase is expressed in alternative splice variants yielding isoforms that localize to different cell compartments. Exp Cell Res 297:521–532

Modjtahedi N, Giordanetto F, Madeo F, Kroemer G (2006) Apoptosis-inducing factor: vital and lethal. Trends Cell Biol 16:264–272

Moubarak RS, Yuste VJ, Artus C, Bouharrour A, Greer PA, Menissier-de Murcia J, Susin SA (2007) Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. Mol Cell Biol 27:4844–4862

Newmeyer DD, Ferguson-Miller S (2003) Mitochondria: releasing power for life and unleashing the machineries of death. Cell 112:481–490

Oka S, Kato J, Moss J (2006) Identification and characterization of a mammalian 39-kDa poly(ADP-ribose) glycohydrolase. J Biol Chem 281:705–713

Olney JW, Sharpe LG (1969) Brain lesions in an infant rhesus monkey treated with monsodium glutamate. Science 166:386–388

Poirier GG, de Murcia G, Jongstra-Bilen J, Niedergang C, Mandel P (1982) Poly(ADP-ribosyl) ation of polynucleosomes causes relaxation of chromatin structure. Proc Natl Acad Sci USA 79:3423–3427

Pospisilik JA, Knauf C, Joza N, Benit P, Orthofer M, Cani PD, Ebersberger I, Nakashima T, Sarao R, Neely G, Esterbauer H, Kozlov A, Kahn CR, Kroemer G, Rustin P, Burcelin R, Penninger JM (2007) Targeted Deletion of AIF Decreases Mitochondrial Oxidative Phosphorylation and Protects from Obesity and Diabetes. Cell 131:476–491

Richter BW, Duckett CS (2000) The IAP proteins: caspase inhibitors and beyond. Sci STKE 2000:PE1

Sakahira H, Enari M, Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96–99

Smith S (2001) The world according to PARP. Trends Biochem Sci 26:174–179

Stamler JS, Lamas S, Fang FC (2001) Nitrosylation. the prototypic redox-based signaling mechanism. Cell 106:675–683

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell 8:613–621

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102:43–53

Virag L, Szabo C (2002) The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. Pharmacol Rev 54:375–429

Wang H, Yu SW, Koh DW, Lew J, Coombs C, Bowers W, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2004) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. J Neurosci 24:10963–10973

Yu SW, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Proc Natl Acad Sci USA 103:18314–18319

Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Part II
Focal Cerebral Ischemia

Chapter 6
Significant Role of Apoptosis-Inducing Factor (AIF) for Brain Damage Following Focal Cerebral Ischemia

Nikolaus Plesnila and Carsten Culmsee

6.1 Introduction

Every year, stroke is responsible for the death of 5.5 million people and thus accounts for 10% of all deaths in industrialized countries worldwide (Mackay and Mensah 2004). Despite such a high incidence and mortality, therapeutic options for stroke patients are still very limited (Lo et al. 2003). Currently, the only clinical treatment option for stroke is reperfusion therapy by local or systemic administration of recombinant tissue plasminogen activator (rtPA). A major drawback of rtPA, however, is that it may be fatal if given in hemorrhagic stroke, which has clinical symptoms very similar to ischemic stroke. Accordingly, rtPA therapy can only be initiated after cerebral hemorrhage has been ruled out by brain CT or NMR imaging. By the time diagnostic procedures have been completed, the therapeutic window for rtPA, i.e. 3 h after the onset of ischemia, has commonly closed. As a result less than 5% of all stroke patients are eligible for rtPA lysis according to current protocols (Adams et al. 2007). The remaining 95% may only hope for spontaneous reperfusion, which in most cases, however, occurs too late to prevent penumbral cell death and the subsequent loss of neurological function (Molina et al. 2001). Hence, a treatment strategy is required, which prolongs neuronal survival in the ischemic penumbra, i.e. under compromised cerebral blood flow conditions, until reperfusion occurs.

A strategy that keeps neurons alive until reperfusion occurs may, however, not be the only goal for the development of novel stroke therapies. The main reason for this statement is that even after reperfusion, cell death signaling pathways triggered

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by the initial ischemic event remain activated and result in additional neuronal cell death under completely normal blood flow conditions. This was first demonstrated in experimental approaches where very brief ischemic episodes were induced, i.e. 30 min of middle cerebral artery occlusion in mice or rats (MCAo), which may resemble transient ischemic attacks (TIA) in patients. Under this condition neuronal cell death may occur with a delay of up to 24 h following reperfusion (Du et al. 1996; Endres et al. 1998). Subsequently, post-reperfusion cell death was also demonstrated following more severe ischemic episodes, which are associated with acute infarction and, hence, resemble acute stroke in humans. In the ischemic penumbra of mice subjected to 60 min MCAo, neurons die with a delay of only 3–6 h (Fig. 6.1a), i.e. also post-reperfusion, cell death seems to have a clinically relevant therapeutic window. Accordingly, an optimal therapeutic approach towards the treatment of stroke should include not only the protection of neuronal cells during the period of compromised blood flow, but also the prevention of cell death after reperfusion.

6.2 Mechanisms of Delayed Cell Death Following Focal Cerebral Ischemia

The morphological hallmarks of neuronal cell death following focal cerebral ischemia are cell shrinkage and nuclear condensation. These features are found in brain areas affected by immediate and delayed cell death. Accordingly, the mechanisms leading to ischemic cell death seem to be very similar whether or not affected cells are located in the infarct core where blood flow is almost absent or in the ischemic penumbra where collateral blood flow may keep cells alive for several hours (Astrup et al. 1981). For many years it remained unclear how ischemia causes the morphological findings described above. Nuclear condensation is the morphological sequel of DNA damage, which usually occurs in a highly regulated manner during programmed cell death. That nuclear condensation following cerebral ischemia was the result of DNA damage and endonuclease activation was finally demonstrated by Linnik et al. and Charriaut-Merlangue et al, respectively, more than 10 years ago (Linnik et al. 1995; Charriaut-Marlangue et al. 1996). This finding triggered intense search for the upstream signaling responsible for post-ischemic endonuclease activation, which was finally believed to be the activation of caspase-3 (Namura et al. 1998). Namura and colleagues showed constitutive expression of inactive caspase-3 in neurons throughout the brain, most prominently in neuronal perikarya within piriform cortex and, most importantly, caspase-like enzyme activity in ischemic brain 30–60 min after reperfusion following 2 h MCAo. Active caspase-3 was detected in ischemic neurons at the time of reperfusion by immunohistochemistry. DNA laddering and TUNEL-positive cells as indicators of DNA fragmentation were detected 6–24 h after reperfusion (Namura et al. 1998). Further proof for the role of active caspase-3 for ischemic cell death came in the same year from experiments from the same laboratory using pan-caspase and caspase-3 specific

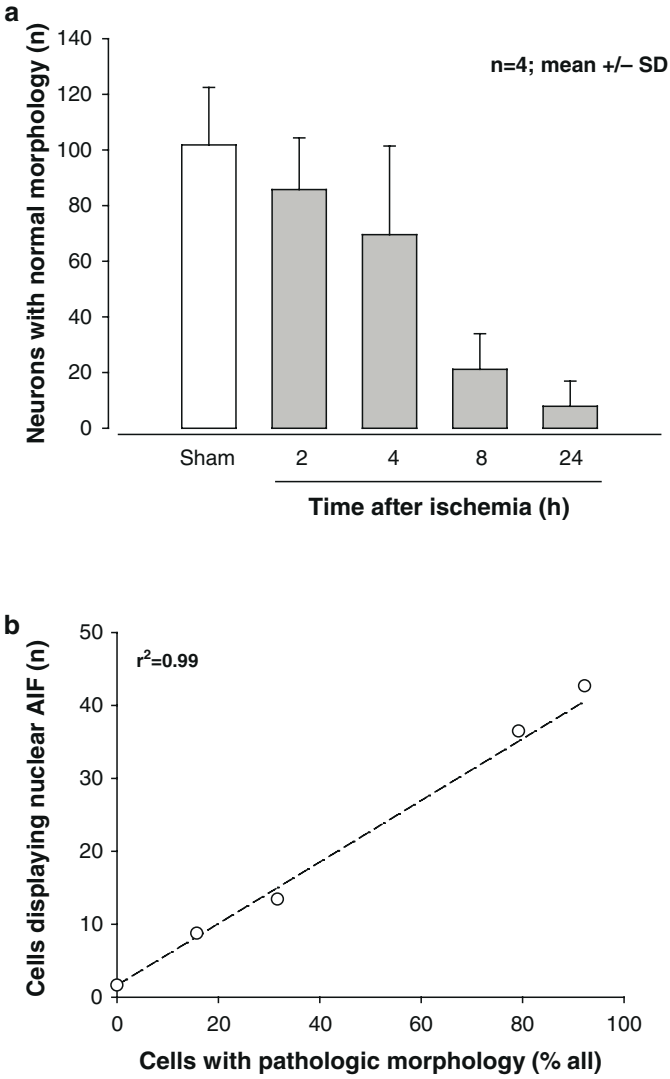

Fig. 6.1 Delayed neuronal cell death in the ischemic penumbra and correlation with nuclear AIF following transient focal cerebral ischemia in mice, (**a**), Following 60 min of middle cerebral artery occlusion (MCAo) the majority of neurons (~70%) in the ischemic penumbra, i.e. the cerebral cortex, stay alive for at least 4 h. Despite sufficient blood flow 24 h after MCAo, over 90% of neurons that were viable 2 h after ischemia display altered membrane and nuclear morphology indicating cell death. (**b**), Correlation of neurons displaying pathological morphology with cells showing nuclear AIF (Culmsee et al. 2005)

peptide inhibitors. Post-ischemic neuronal cell death was prevented and neuronal function was improved when caspase activation was inhibited up to 6 h following reperfusion from 30 min MCAo (Endres et al. 1998). The ultimate mechanistic

link between caspase-3 activation and post-ischemic DNA fragmentation was established by Cao and co-workers by showing that caspase-activated Dnase (CAD), a molecule known to be cleaved and thereby activated by caspase-3, was responsible for post-ischemic DNA-fragmentation (Cao et al. 2001).

In consequence, many research groups concentrated on the upstream mechanisms of caspase-3 activation. Due to very low expression and activation levels of potentially involved molecules it turned out to be technically very challenging to identify respective mechanisms. Caspase-8, a molecule able to cleave caspase-3 in non-neuronal cells, was found to be activated following experimental stroke. However, caspase-8 was described to be activated in a population of neurons (lamina V) distinct from that where active caspase-3 was observed (lamina II/III) (Velier et al. 1999) and a direct link between caspase-8 and caspase-3 activation could never be demonstrated in models of cerebral ischemia. Further, upstream factors in the cascade of caspase activation such as Fas/CD95 receptors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), were found to be upregulated following MCAo, and lpr mice, which express dysfunctional Fas receptors, were protected from focal ischemic brain damage (Martin-Villalba et al. 1999). Despite these interesting findings, it still remained unclear how caspase-3 was activated following cerebral ischemia until 2001, when it was demonstrated that the BH3-only Bcl-2 family member Bid, which has a caspase-8 specific cleavage site, was truncated after experimental stroke (Plesnila et al. 2001). Cleaved/truncated Bid (tBid) translocates from the cytoplasm to the outer mitochondrial membrane where together with Bax it induces the formation of an oligomeric membrane pore (Zha et al. 2000) thereby releasing cytochrome c from mitochondria (Wei et al. 2000). After focal cerebral ischemia, mitochondria of Bid-deficient mice released far less cytochrome c and cortical infarction was significantly reduced compared to wildtype littermates, thereby demonstrating the prominent role of mitochondria in post-ischemic cell death. These data further imply that after focal cerebral ischemia caspase-3 may be activated through the mitochondrial pathway, i.e. by the mitochondrial release of cytochrome c (Fujimura et al. 2000) and apoptosome formation (Plesnila et al. 2001; Yin et al. 2002; Plesnila 2004). Not much later, however, this view was challenged by the fact that caspase-3 knock out mice, which became available at that time, showed much less neuroprotection than expected based on the anticipated prominent role of caspase-3 activation for ischemic neuronal cell death (Le et al. 2002). Together with the pronounced neuroprotective effect achieved by interactions with mitochondrial cell death signaling, (Martinou et al. 1994; Wiessner et al. 1999; Plesnila et al. 2001; Cao et al. 2002; Kilic et al. 2002), i.e. mechanisms upstream of caspase-3 activation, it became clear that alternative cell death pathways distinct from caspase-3 may be present downstream of mitochondria.

The hypothesis that caspase-independent neuronal cell death signaling exists downstream of mitochondria was also suggested by in vitro experiments showing that caspase inhibition provided only transient neuroprotection, which was followed by a more delayed type of DNA-fragmentation-related cell death [see (Rideout and Stefanis 2001) for review]. It was Ruth Slack and her colleagues who identified a mitochondrial protein, apoptosis-inducing factor (AIF), to be one of the most potent

molecular candidates for caspase-independent death in neurons (Cregan et al. 2002). AIF translocation from mitochondria to the nucleus was detected in damaged neurons in vitro in models of neuronal cell death relevant to the pathology of ischemic brain damage, such as glutamate toxicity, DNA damage or oxygen-glucose deprivation, whereas neutralizing AIF antibodies, pharmacological inhibition of AIF release or AIF siRNA prevented neuronal cell death in these in vitro approaches (Cao et al. 2002; Cregan et al. 2002; Culmsee et al. 2005; Becattini et al. 2006).

AIF is a 67 kDa flavoprotein with significant homology to bacterial and plant oxidoreductases located in the mitochondrial intermembranous space (Susin et al. 1999). Upon release from mitochondria, AIF migrates to the nucleus where it induces large-scale (~50 kbp) DNA fragmentation and cell death by a yet not completely understood, but certainly caspase-independent mechanism (Daugas et al. 2000).

In the brain, AIF was shown to be expressed in all so far investigated cell types, i.e. neurons and glial cells (Cao et al. 2003; Zhu et al. 2003). The expression in normal neuronal cells was confined to the mitochondria as shown by co-immunostaining with the mitochondrial marker cytochrome oxidase (Plesnila et al. 2004). Interestingly, in contrast to the expression pattern of many other apoptotic proteins, the expression of AIF protein increases gradually with brain maturation and peaks in adulthood, indicating that in contrast to, e.g. caspase-3, AIF may exert its main function in adult neurons (Cao et al. 2003).

The first pathological condition where AIF was shown to play an important role for neuronal damage was cerebral hypoxia-ischemia, a model for stroke in newborn children. Hypoxia-ischemia in 7-day-old rats induced by ligation of the left carotid artery for 55 min, together with the reduction of ambient oxygen to 7.7% in a hypoxia chamber, resulted in AIF release from mitochondria and translocation to the nucleus in neurons displaying DNA fragmentation and pyknosis (Zhu et al. 2003). Since AIF translocation was not influenced by inhibition of caspases by the pan-caspase inhibitor BAF, these experiments stressed the caspase-independent manner of AIF-induced cell death. Similar findings were also observed following cardiac arrest induced brain damage in rats, i.e. following transient global ischemia. Following 15 min of four-vessel occlusion (4-VO), AIF was found to translocate from mitochondria to the nucleus in hippocampal CA1 neurons. The temporal profile of AIF translocation coincided with the induction of large-scale DNA fragmentation (50 kbp; 24–72 h after 4-VO), a well-characterized hallmark of delayed neuronal cell death (Cao et al. 2003). In line with findings in the rodent models of transient hypoxia-ischemia in immature animals, treatment with an caspase-3 inhibitor had no effect on nuclear AIF accumulation and did not provide any long-lasting neuroprotective effects after global ischemia in adult rats (Cao et al. 2003).

At about the same time, we demonstrated the translocation of AIF from mitochondria to the nucleus following transient focal cerebral ischemia, an experimental model of ischemic stroke followed by reperfusion (Plesnila et al. 2004). Nuclear AIF was detected in single neuronal cells very early, i.e. within one hour after 45 min of middle cerebral artery occlusion (MCAo) and peaked 24 h thereafter. The time course of AIF translocation paralleled mitochondrial cytochrome c release and

apoptosis-like DNA damage as identified by hair-pin probe (HPP) staining, indicating ischemia-induced mitochondrial permeabilization and AIF-induced DNA fragmentation (Plesnila et al. 2004). Further, we showed that in the same experimental paradigm of ischemic stroke that AIF nuclear translocation was mainly found in neurons (Culmsee et al. 2005) and that the number of cells displaying pathological morphology following cerebral ischemia correlated very well ($r^2 = 0.99$) with the number of neurons showing nuclear AIF (Fig. 6.1b).

That nuclear translocation of AIF was indeed responsible for post-ischemic cell death and not only a byproduct of the morphological changes associated with neuronal cell death was first shown in 2005. Small interfering RNA (siRNA)-mediated downregulation of AIF expression (~80%) in HT22 hippocampal neurons and in primary cultured neurons resulted in a significant reduction of glutamate and oxygen-glucose deprivation-induced neuronal cell death, respectively (Fig. 6.2). Reduction of cell death was associated with a lack of nuclear AIF translocation, thereby demonstrating that AIF plays a causal role for excitotoxic and hypoxic-hypoglycemic cell death in vitro (Culmsee et al. 2005). In the same study, we demonstrated that AIF is also relevant for post-ischemic cell death in vivo. Harlequin mutant mice carry a pro-viral insertion in the AIF-gene, thereby expressing only 10–20% of normal AIF protein levels (Klein et al. 2002). These mutant mice show significantly reduced post-ischemic brain damage as compared to their wild-type littermates, which express AIF at normal levels (Culmsee et al. 2005) (Fig. 6.3).

In vivo nuclear AIF translocation was dependent on poly(ADP-ribose) polymerase-1 (PARP-1) activation, as shown by using the specific PARP-1 inhibitor PJ-34 (Culmsee et al. 2005). Accordingly, these results suggest that PARP-1 activation is located upstream of AIF release from mitochondria and that AIF is the major factor mediating PARP1-induced cell death, findings also supported by other laboratories using different strategies to inhibit PARP, i.e. by cilostazol or gallotannin (Wei et al. 2007; Lee et al. 2007). Further, activation of neuronal nitric oxide synthase (nNOS) and formation of free radicals were linked to PARP activation and AIF-mediated neuronal cell death following experimental stroke. Gene deletion of nNOS or application of a metalloporphyrin-based superoxide dismutase mimic reduced post-ischemic cell death together with a reduction of the number of neurons displaying nuclear AIF, thereby suggesting that free radical and peroxynitrite formation may cause direct or indirect mitochondrial damage and subsequent AIF release, nuclear translocation, and large-scale DNA fragmentation (Lee et al. 2005; Li et al. 2007). Results from our and other laboratories on the direct upstream mechanisms responsible for the release of AIF from mitochondria suggest that proteins of the Bcl-2 family of cell death proteins play an important role for this process. Small molecule inhibitors of Bid, a pro-apoptotic BH3-only member of the bcl-2 family, prevented cell death together with translocation of AIF from mitochondria to the nucleus in primary cultured neurons following oxygen-glucose deprivation and completely preserved cell and nuclear morphology following glutamate toxicity in HT22 hippocampal cells (Culmsee et al. 2005; Landshamer et al. 2008).

In conclusion, the current literature suggests that AIF-mediated caspase-independent signaling pathways are of major importance for delayed neuronal cell

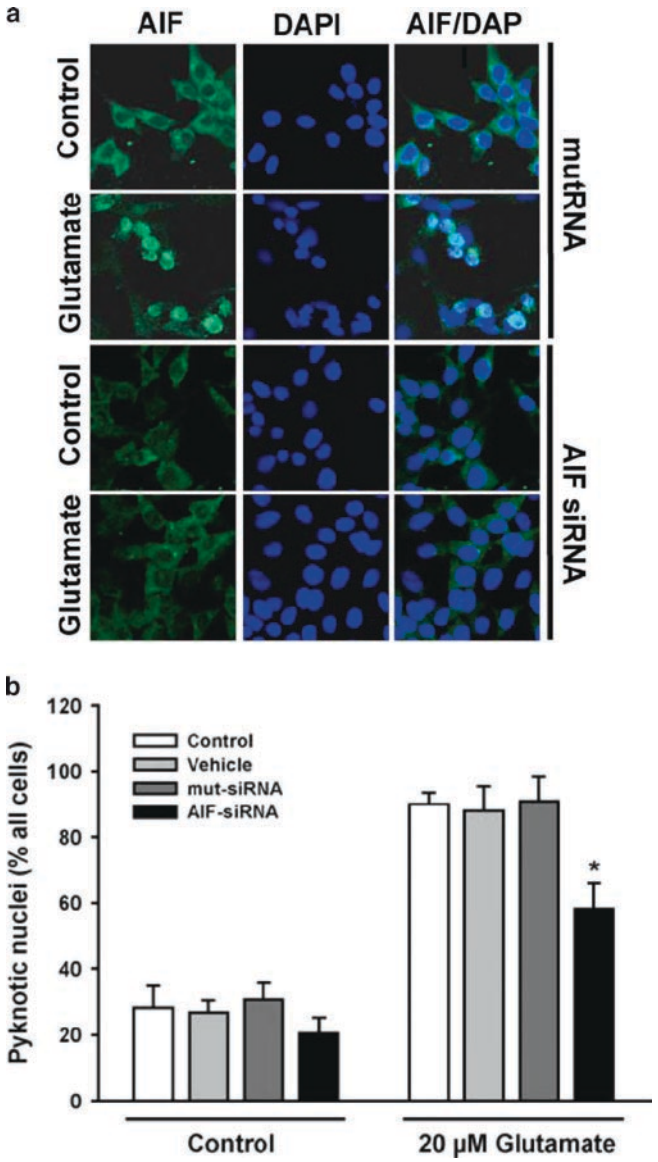

Fig. 6.2 AIF-siRNA knockdown attenuates glutamate-induced neuronal cell death in primary cultured neurons. (**a**), Primary cultured neurons were pre-treated with vehicle (lipofectamine), non-functional mutant RNA (mutRNA), or AIF siRNA for 48 h. Confocal laser scanning microscope images of AIF immunoreactivity (*green*) were obtained after further 18 h of exposure to glutamate (2 mM). Co-staining with DAPI (*dark blue*) allowed the identification of nuclear translocation of AIF (AIF/DAPI, *light blue*) in glutamate-damaged cells. Neurons pre-treated with AIF siRNA did not display nuclear AIF translocation. (**b**), Quantification of data obtained from the experiments described above. Cell death was quantified by counting of cells with pyknotic nuclei. In AIF siRNA-treated neurons, the number of cells displaying pyknotic nuclei was reduced by ~50% ($n=4$; $p<0.01$ vs. control) (Culmsee et al. 2005)

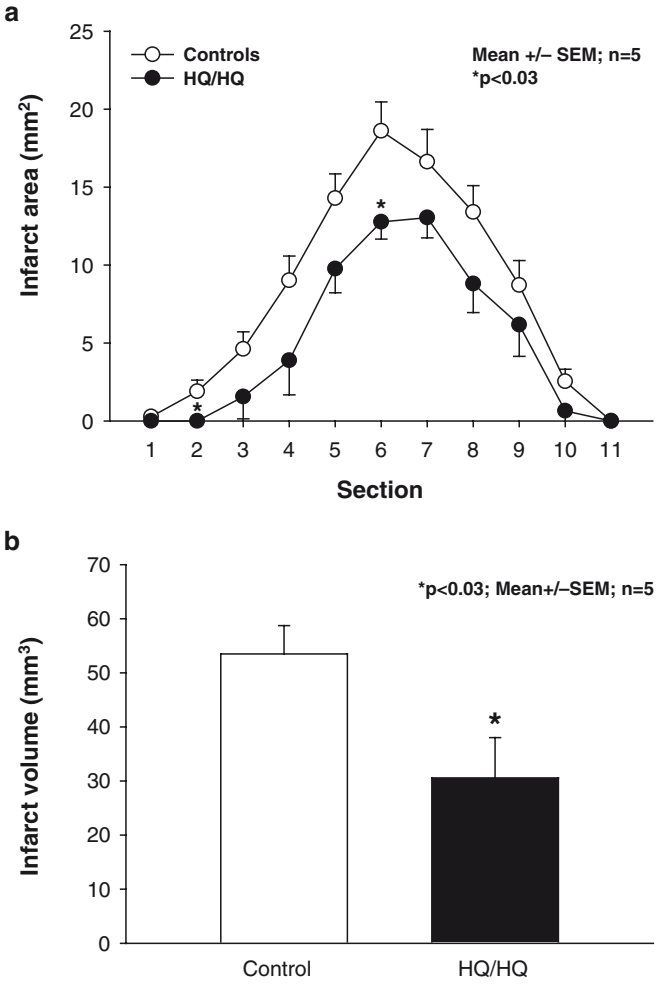

Fig. 6.3 Less ischemic brain damage in mice with reduced AIF expression. (**a**), Infarct volume of Harlequin mutant mice (HQ) which have a reduced expression of AIF protein due to a proviral insertion in the *aif* gene. Infarct areas were evaluated histomorphometrically on 11 consecutive Nissl-stained brain sections (500 μm apart) throughout the infarct of HQ animals and their wild type littermates (Control). The infarcted brain area in HQ mice was reduced on each investigated section as compared to controls. (**b**), In HQ mice, the infarct volume, calculated on the basis of the histomorphometric data from the individual sections, showed a 43% reduction as compared to wild type littermates ($n=5$, $^*p<0.03$) (Culmsee et al. 2005)

death following experimental stroke. Caspase activation occurs during this process, however, inhibition of caspases seems to only delay and not to prevent neuronal death following focal cerebral ischemia. These findings suggest that AIF may be a novel target for drug development aimed to mitigate cell death following stroke.

References

Adams HP Jr, Del ZG, Alberts MJ, Bhatt DL, Brass L, Furlan A, Grubb RL, Higashida RT, Jauch EC, Kidwell C, Lyden PD, Morgenstern LB, Qureshi AI, Rosenwasser RH, Scott PA, Wijdicks EF (2007) Guidelines for the early management of adults with ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council, Clinical Cardiology Council, Cardiovascular Radiology and Intervention Council, and the Atherosclerotic Peripheral Vascular Disease and Quality of Care Outcomes in Research Interdisciplinary Working Groups: The American Academy of Neurology affirms the value of this guideline as an educational tool for neurologists. Circulation 115:e478–e534

Astrup J, Siesjo BK, Symon L (1981) Thresholds in cerebral ischemia - the ischemic penumbra. Stroke 12:723–725

Becattini B, Culmsee C, Leone M, Zhai D, Zhang X, Crowell KJ, Rega MF, Landshamer S, Reed JC, Plesnila N, Pellecchia M (2006) Structure-activity relationships by interligand NOE-based design and synthesis of antiapoptotic compounds targeting Bid. Proc Natl Acad Sci USA 103:12602–12606

Cao G, Pei W, Lan J, Stetler RA, Luo Y, Nagayama T, Graham SH, Yin XM, Simon RP, Chen J (2001) Caspase-activated DNase/DNA fragmentation factor 40 mediates apoptotic DNA fragmentation in transient cerebral ischemia and in neuronal cultures. J Neurosci 21:4678–4690

Cao G, Pei W, Ge H, Liang Q, Luo Y, Sharp FR, Lu A, Ran R, Graham SH, Chen J (2002) In Vivo Delivery of a Bcl-xL Fusion Protein Containing the TAT Protein Transduction Domain Protects against Ischemic Brain Injury and Neuronal Apoptosis. J Neurosci 22:5423–5431

Cao G, Clark RS, Pei W, Yin W, Zhang F, Sun FY, Graham SH, Chen J (2003) Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation. J Cereb Blood Flow Metab 23:1137–1150

Charriaut-Marlangue C, Margaill I, Represa A, Popovici T, Plotkine M, Ben Ari Y (1996) Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis. J Cereb Blood Flow Metab 16:186–194

Cregan SP, Fortin A, MacLaurin JG, Callaghan SM, Cecconi F, Yu SW, Dawson TM, Dawson VL, Park DS, Kroemer G, Slack RS (2002) Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. J Cell Biol 158:507–517

Culmsee C, Zhu C, Landshamer S, Becattini B, Wagner E, Pellecchia M, Blomgren K, Plesnila N (2005) Apoptosis-inducing factor triggered by poly(ADP-ribose) polymerase and Bid mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia. J Neurosci 25:10262–10272

Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N, Kroemer G (2000) Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. FEBS Lett 476:118–123

Du C, Hu R, Csernansky CA, Hsu CY, Choi DW (1996) Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? J Cereb Blood Flow Metab 16:195–201

Endres M, Namura S, Shimizu-Sasamata M, Waeber C, Zhang L, Gomez-Isla T, Hyman BT, Moskowitz MA (1998) Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family. J Cereb Blood Flow Metab 18:238–247

Fujimura M, Morita-Fujimura Y, Noshita N, Sugawara T, Kawase M, Chan PH (2000) The cytosolic antioxidant copper/zinc-superoxide dismutase prevents the early release of mitochondrial cytochrome c in ischemic brain after transient focal cerebral ischemia in mice. J Neurosci 20:2817–2824

Kilic E, Dietz GP, Hermann DM, Bahr M (2002) Intravenous TAT-Bcl-Xl is protective after middle cerebral artery occlusion in mice. Ann Neurol 52:617–622

Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, Bronson RT, Ackerman SL (2002) The harlequin mouse mutation downregulates apoptosis-inducing factor. Nature 419:367–374

Landshamer S, Hoehn M, Barth N, Duvezin-Caubet S, Schwake G, Tobaben S, Kazhdan I, Becattini B, Zahler S, Vollmar A, Pellecchia M, Reichert A, Plesnila N, Wagner E, Culmsee C (2008) Bid-induced release of AIF from mitochondria causes immediate neuronal cell death. Cell Death Differ 15(10):1553–1563

Le DA, Wu Y, Huang Z, Matsushita K, Plesnila N, Augustinack JC, Hyman BT, Yuan J, Kuida K, Flavell RA, Moskowitz MA (2002) Caspase activation and neuroprotection in caspase-3- deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. Proc Natl Acad Sci USA 99:15188–15193

Lee BI, Chan PH, Kim GW (2005) Metalloporphyrin-based superoxide dismutase mimic attenuates the nuclear translocation of apoptosis-inducing factor and the subsequent DNA fragmentation after permanent focal cerebral ischemia in mice. Stroke 36:2712–2717

Lee JH, Park SY, Shin HK, Kim CD, Lee WS, Hong KW (2007) Poly(ADP-ribose) polymerase inhibition by cilostazol is implicated in the neuroprotective effect against focal cerebral ischemic infarct in rat. Brain Res 1152:182–190

Li X, Nemoto M, Xu Z, Yu SW, Shimoji M, Andrabi SA, Haince JF, Poirier GG, Dawson TM, Dawson VL, Koehler RC (2007) Influence of duration of focal cerebral ischemia and neuronal nitric oxide synthase on translocation of apoptosis-inducing factor to the nucleus. Neuroscience 144:56–65

Linnik MD, Miller JA, Sprinkle-Cavallo J, Mason PJ, Thompson FY, Montgomery LR, Schroeder KK (1995) Apoptotic DNA fragmentation in the rat cerebral cortex induced by permanent middle cerebral artery occlusion. Brain Res Mol Brain Res 32:116–124

Lo EH, Dalkara T, Moskowitz MA (2003) Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci 4:399–415

Mackay J, Mensah G (2004) Atlas of heart disease and stroke. World Health Organization.

Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13:1017–1030

Martin-Villalba A, Herr I, Jeremias I, Hahne M, Brandt R, Vogel J, Schenkel J, Herdegen T, Debatin KM (1999) CD95 ligand (Fas-L/APO-1L) and tumor necrosis factor-related apoptosis-inducing ligand mediate ischemia-induced apoptosis in neurons. J Neurosci 19:3809–3817

Molina CA, Montaner J, Abilleira S, Ibarra B, Romero F, Arenillas JF, Alvarez-Sabin J (2001) Timing of spontaneous recanalization and risk of hemorrhagic transformation in acute cardioembolic stroke. Stroke 32:1079–1084

Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J, Moskowitz MA (1998) Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci 18:3659–3668

Plesnila N (2004) Role of mitochondrial proteins for neuronal cell death after focal cerebral ischemia. Acta Neurochir Suppl 89:15–19

Plesnila N, Zinkel S, Le DA, Amin-Hanjani S, Wu Y, Qiu J, Chiarugi A, Thomas SS, Kohane DS, Korsmeyer SJ, Moskowitz MA (2001) BID mediates neuronal cell death after oxygen/ glucose deprivation and focal cerebral ischemia. Proc Natl Acad Sci USA 98:15318–15323

Plesnila N, Zhu C, Culmsee C, Groger M, Moskowitz MA, Blomgren K (2004) Nuclear translocation of apoptosis-inducing factor after focal cerebral ischemia. J Cereb Blood Flow Metab 24:458–466

Rideout HJ, Stefanis L (2001) Caspase inhibition: a potential therapeutic strategy in neurological diseases. Histol Histopathol 16:895–908

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446

Velier JJ, Ellison JA, Kikly KK, Spera PA, Barone FC, Feuerstein GZ (1999) Caspase-8 and caspase-3 are expressed by different populations of cortical neurons undergoing delayed cell death after focal stroke in the rat. J Neurosci 19:5932–5941

Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev 14:2060–2071

Wei G, Wang D, Lu H, Parmentier S, Wang Q, Panter SS, Frey WH, Ying W (2007) Intranasal administration of a PARG inhibitor profoundly decreases ischemic brain injury. Front Biosci 12:4986–4996

Wiessner C, Allegrini PR, Rupalla K, Sauer D, Oltersdorf T, McGregor AL, Bischoff S, Bottiger BW, van der PH (1999) Neuron-specific transgene expression of Bcl-XL but not Bcl-2 genes reduced lesion size after permanent middle cerebral artery occlusion in mice. Neurosci Lett 268:119–122

Yin XM, Luo Y, Cao G, Bai L, Pei W, Kuharsky DK, Chen J (2002) Bid-mediated mitochondrial pathway is critical to ischemic neuronal apoptosis and focal cerebral ischemia. J Biol Chem 277:42074–42081

Zha J, Weiler S, Oh KJ, Wei MC, Korsmeyer SJ (2000) Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science 290:1761–1765

Zhu C, Qiu L, Wang X, Hallin U, Cande C, Kroemer G, Hagberg H, Blomgren K (2003) Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. J Neurochem 86:306–317

Chapter 7
The Role of Poly(ADP-Ribose) Polymerase-1 (PARP-1) Activation in Focal Cerebral Ischemia

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7.1 PARP-1 and Poly(ADP-ribosyl)ation

Poly(ADP-ribosyl)ation is the post-translational modification of proteins operated by poly(ADP-ribose) polymerases (PARPs). PARPs are enzymes that are able to catalyze the transfer of ADP-ribose units from nicotinamide adenine dinucleotide (NAD) to target proteins and are particularly abundant in cell nuclei, where they play a key role in the maintenance of homeostasis. Poly(ADP-ribosyl)ation significantly affects protein functioning because of the high negative charge and steric hindrance conferred by the chains of poly(ADP-ribose) (PAR). PARP-1 is the founding member and the most commonly studied of these enzymes and shows the highest poly(ADP-ribosyl)ating activity. Sequences encoding novel PARPs have been identified and, overall, the PARP superfamily is a growing family of enzymes with numerous members with roles that are yet to be identified (Ame et al. 2004; Smith 2001).

Under physiological conditions, basal DNA strand breaks or cruciform DNA structures (Potaman et al. 2005) activates PARPs and ongoing cycles of PAR formation and hydrolysis onto acceptor proteins finely regulates key cellular functions such as DNA duplication, repair, and transcription (D'Amours et al. 1999; Herceg and Wang 2001; Kraus and Lis 2003) as well as mitosis (Chang et al. 2004) and protein degradation (Ullrich et al. 2001a). However, in the presence of widespread DNA damage, excessive activation of PARPs turns PAR from a homeostatic regulator into a highly cytotoxic molecule (Andrabi et al. 2006; Herceg and Wang 2001) and may cause cell death.

Neurons are particularly sensitive to deregulation of PAR homeostasis and numerous studies demonstrate that hyper-poly(ADP-ribosyl)ation is a key trigger of neurotoxicity (Ha and Snyder 2000). During the past decade, poly(ADP-ribosyl)ation has been identified as a key event in neurodegeneration occurring in a

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large number of experimental models of brain disorders, including excitotoxicity (Mandir et al. 2000), *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism (Mandir et al. 1999), neurotrauma (Whalen et al. 1999), encephalomyelitis (Chiarugi 2002a; Scott et al. 2001), subarachnoid hemorrhage (Satoh et al. 2001), oxidative stress, amyloid neurotoxicity, neuroinflammation (Chiarugi and Moskowitz 2003; Ullrich et al. 2001b), meningitis (Koedel and Pfister 1999) and brain ischemia (Szabo and Dawson 1998).

7.2 PARP-1 in the Pathobiology of Ischemic Stroke

It is now clearly established that cerebral ischemia overactivates PARP-1 in several cell types of the ischemic region and significantly contributes to the extension of ischemic damage. PARP-1 activation occurs not only in injured neurons but also in endothelial and glial cells as well as in pericytes and infiltrating leukocytes. Consistent with PARP-1 involvement in ischemic brain damage, enzymatic activity of PARP-1 in gerbils subjected to transient (5 min) ischemia increases in the injured tissue 4.3- and 1.7-fold at 1 and 24 h of reperfusion, respectively (Nagayama et al. 2000). Prolonging duration of ischemia (10 min) leads to significant increases of PARP-1 activity up to the seventh day of reperfusion (Strosznajder et al. 2003). Accordingly, various studies report PAR accumulation in the ischemic brain tissue. In a focal and transient model of brain ischemia in mice, PAR formation is highly increased in the nuclei of cells of cerebral cortex compared to those present in the contralateral one. Of note, polymer formation is drastically decreased both in the ischemic and contralateral cortex of PARP-1−/− mice subjected to MCAo (Eliasson et al. 1997). A parallel study in mice reports that following 2 h MCAo, increased PAR formation in the ischemic cortex occurs as early as 5 min after reperfusion in cells showing swelling and nuclear disruption (Endres et al. 1997). PAR accumulation, however, is not evident at later times (3–6 h) of reperfusion or after milder ischemic insult (1 h MCAo) (Endres et al. 1997). PAR formation has also been investigated in a permanent model of MCAo in rats. In this study, it is reported that PAR immunoreactivity increases in the ischemic core and penumbra 2–8 h after ischemia, returning to basal levels 16 h post-ischemia. Importantly, PAR immunoreactive cells of the ischemic cortex show the classical morphology of pyramidal neurons (Tokime et al. 1998). Similarly, the ischemic cortex of rats subjected to distal MCAo and transient bilateral common carotid artery occlusion have a three-fold increase of PAR immunoreactive cells 10 min after 1.5 h ischemia. Notably, inhibition of PARP-1 completely abrogates increase of PAR immunoreactivity (Takahashi et al. 1999). Remarkably, PAR formation also occurs in neural cells of the infarcted human brain at 18–24 h post-insult and rapidly declines, thereafter. A second wave of poly(ADP-ribosyl)ation is due to PAR-positive macrophages, which start infiltrating the ischemic human brain 3 days after the ischemic injury (Love et al. 2000). In a subsequent study in humans, it was reported that brain ischemia causes accumulation of PAR in the ischemic core and penumbra mostly during the

first 2 days after cardiac arrest. Importantly, double immunostaining for PAR and the neuronal marker MAP2 indicates that the majority of PAR-positive cells are neurons. It has also been reported that brain ischemia alters PARP-1 expression levels. For example, Love and colleagues show that expression of PARP-1 increases in the infarcted tissue of the human brain 18–24 h post-insult and, similarly to PAR, declines thereafter (Love et al. 2000). Accordingly, PARP-1 protein as well as its mRNA levels increase in the nucleus of cultured cerebellar granular cells upon exposure to neurotoxic concentrations of glutamate (Cosi et al. 1994). Results obtained in a model of transient global ischemia in gerbils are contradictory. Indeed, the group of Sharp reports an increase of PARP-1 mRNA in the dentate gyrus of gerbil brains 4 h after 10 min of global ischemia and return to basal levels 8 h after ischemia (Liu et al. 2000). Conversely, Nagayama and associates (Nagayama et al. 2000) as well as Strosznajder and colleagues (Strosznajder et al. 2003) show that PARP-1 mRNA and protein do not increase in the gerbil hippocampus in the same ischemia model. Nevertheless, these results taken together unambiguously establish a central role of cerebral ischemia in altering the homeostasis of poly(ADP-ribosyl)ation in neurons of different species including humans.

7.3 Molecular Mechanism of PARP-1 Activation-Induced Cell Death

Various hypotheses on the possible molecular mechanisms underpinning PARP-1-dependent cell death during cerebral ischemia have been proposed. Overall, three main mechanisms can be postulated: (1) energy failure induced necrosis; (2) changes in ischemia-induced gene expression profile with reduced expression of prosurvival factors and excessive expression of inflammatory mediators; (3) activation of cell death signaling (Fig. 7.1).

7.3.1 PARP-1 Activation-Induced Energy Failure: The Suicide Hypothesis

Under homeostatic conditions, the main endoergonic processes of neurons are ion-pumping (responsible for about 50% of ATP hydrolysis), biosynthesis of macromolecules (proteins, lipids, carbohydrates and nucleic acids) and neurotransmitters, intracellular molecular transport and phosphorylation. All these ATP-consuming processes are almost totally impaired in neurons of the ischemic core. Conversely, in the ischemic penumbra, physiologic cellular functions are partially maintained. At this level, numerous strategies capable of reducing energy consumption by neurons and glia have profound neuroprotective effects (Beal 2000). In this regard, several lines of evidence demonstrate that inhibition of poly(ADP-ribosyl)ation in

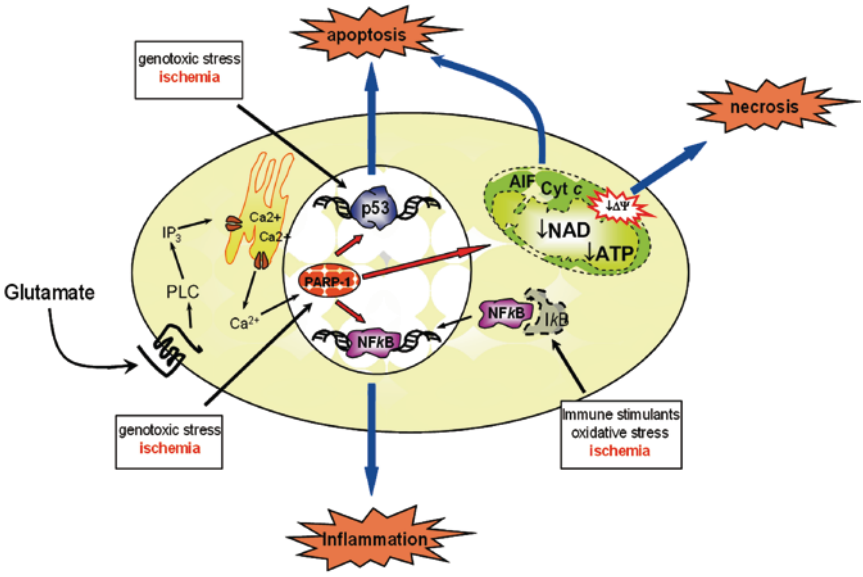

Fig. 7.1 Proposed role of PARP-1 in ischemic neuronal death. In the ischemic brain, overactivation of neuronal glutamate receptors occurs because of glutamate release triggered by energy failure and peri-infarct depolarization. The ensuing excitotoxic cascade prompts intracellular accumulation of Ca^{2+} ($\uparrow[Ca^{2+}]_i$), oxidative and nitrosative stress eventually leading to massive DNA damage. Recruitment of immune cells in the infarcted area also causes genotoxic stress and activation of transcription factors involved in cell death signaling such as NF-kB and p53. Accumulation of intracellular Ca^{2+} can also directly activate nuclear PARP-1 activity (Homburg et al. 2000). Overall, these events cause hyperactivation of PARP-1, which consumes excessive NAD and ATP, leading to glycolytic block and mitochondrial dysfunction, leading to cellular necrosis. Impairment of mitochondrial functioning also prompts AIF release and excitotoxic cell death. Hyperactivation of PARP-1 also alters the enzyme-dependent fine-tuning of transcription factor activation, resulting in transcriptional derangements, and abnormalities in gene expression that eventually contribute to neuronal death

the ischemic brain tissue preserves energy dynamics. It is well appreciated that massive genotoxic stress occurs in tissues subjected to ischemia/reperfusion or mild ischemia because of formation of reactive oxygen and nitrogen radicals such as superoxide ion (O_2^{-}), hydroxyl radical ($OH^{\cdot}$), NO and peroxynitrite ($ONOO^{-}$) (Lee et al. 2000; Lipton 1999). Profuse DNA damage in turn prompts PARP-1 hyperactivation, ATP consumption and NAD depletion, thereby worsening energy dynamics already compromised by the ischemic insult. Under these stressful conditions, the NAD salvage pathway is activated with re-synthesis of NAD, thanks to the concerted actions of nicotinamide phosphoribosyl transferase (NaPRT) and nicotinamide mononucleotide adenylyl transferase (NaMNAT). Notably, because both enzymes utilize ATP, DNA damage-dependent PARP-1 hyperactivation ultimately depletes cellular ATP pools and triggers cell death. This pathogenetic interpretation, the so-called "suicide hypothesis", is the oldest interpretation of PAR-dependent neurotoxicity. It originally stems from the pioneering studies of Berger and colleagues on the role of PARP-1 in radiation-induced cell death (Berger 1985).

In light of the remarkable therapeutic efficacy of PARP-1 inhibitors in models of brain ischemia, several researchers consider energy utilization by hyper-poly(ADP-ribosyl)ation as causative in post-ischemic brain damage (Szabo and Dawson 1998). Several reports are in keeping with this interpretation. For instance, in line with the "suicide hypothesis" the PARP-1 inhibitor 3-aminobenzamide (3-ABA) prevents NAD depletion in the ischemic tissue of mice subjected to 2 h MCAo (Endres et al. 1997). Also, NAD and ATP shortage in rat brains subjected to transient focal ischemia is significantly reduced by inhibiting PARP-1 with nicotinamide (Yang et al. 2002). Similarly, the PARP-1 inhibitor FR247304 prompts NAD rescue in a transient ischemia model in the rat (Iwashita et al. 2004). As further evidence that hyperactivation of PARP-1 triggers death of neural cells because of energy failure, energetic substrates such as tricarboxylic acid cycle intermediates prevent death of cultured neurons undergoing massive poly(ADP-ribosyl)ation (Ying et al. 2002), and NAD repletion rescues astrocytes exposed to alkylating agents (Ying et al. 2002). However, as for its relevance to the pathogenesis of ischemic neuronal death, conflicting results have been reported. Plaschke and colleagues (Plaschke et al. 2000) show that the neuroprotective effect of PARP-1 inhibition in a rat model of global ischemia (15 min) is associated with early rescue of NAD contents without a parallel increase in those of ATP. Similarly, NAD concentrations are neither reduced in the hippocampus of rats subjected to sub-lethal transient global ischemia and reperfusion (Nagayama et al. 2000), nor in the brain of mice exposed to 1 h MCAo and different times of reperfusion (Paschen et al. 2000). Finally, the study by Goto and associates (Goto et al. 2002) shows that the brains of PARP KO mice are more resistant to ischemic stroke despite undergoing an energy depletion similar to those of wild type (WT) animals. This report provides a significant challenge to the relevance of the suicide hypothesis to ischemic brain injury and suggest that mechanisms in addition to energy derangement underlie the detrimental role of PARP-1 in ischemic brain injury. The "suicide hypothesis", therefore, might explain the neurotoxic effects of PAR only in conditions of massive DNA rupture and intense PARP-1 activation within the CNS.

7.3.2 PARP-1 Induced Changes in Gene Expression: The Transcriptional Hypothesis

As mentioned before, the neuroprotective effect obtained by suppression of poly(ADP-ribosyl)ation in the ischemic brain might be related to changes in ischemia-induced gene expression profiles. Numerous findings establish a role of PARP-1 in nuclear functioning under homeostatic conditions, and imply that inhibition of PAR formation significantly affects ongoing transcription, the gene expression profile and resistance to stress. The role of PARP-1 in regulating transcription led to the formulation of the "transcriptional hypothesis" (Chiarugi 2002b). This pathogenetic interpretation states that some of the neurotoxic effects of PAR formation within the brain are transcription-dependent and related to changes in ischemia-induced gene expression profile.

Pioneering work by Poirier and associates demonstrates that PAR unravels chromatin superstructure (de Murcia et al. 1986, 1988; Poirier et al. 1982). Further studies also report a key role of the polymer in regulating histone H1 shuttling on chromatin fibers (Althaus et al. 1990), as well as gene transcription (D'Amours et al. 1999; Kraus and Lis 2003; Ziegler and Oei 2001). Indeed, PARP-1 has been identified as the previously described transcriptional coactivator TFIIC (Slattery et al. 1983), and PARP-1 activity regulates expression of iNOS (Le Page et al. 1998), chemokines (Nirodi et al. 2001) and integrins (Ullrich et al. 2001b). Consistently, recent reports demonstrate that PARP-1 binding to promoter elements (Akiyama et al. 2001; Butler and Ordahl 1999; Nirodi et al. 2001), specific DNA superstructures (Kun et al. 2002) and to RNA-polymerase-II (Carty and Greenleaf 2002) is of relevance to chromatin organization and transcription. Indeed, PARP-1 is a key regulator of numerous transcription factors, including NF-kB (Chiarugi and Moskowitz 2003; Hassa et al. 2001; Hassa and Hottinger 1999; Oliver et al. 1999), AP-1 (Chiarugi 2002a; Ha et al. 2002; Zingarelli et al. 2004) and p53 (Agarwal et al. 1997; Schmid et al. 1999; Wang et al. 1998; Wesierska-Gadek et al. 1996; Wesierska-Gadek and Schmid 2000). Of note, inhibition of these trans-activating factors affords protection from ischemic brain injury (for review see (Chiarugi 2002b; Lo et al. 2003). Also, NF-kB and AP1 are central in activation of microglia and astrocytes and ensuing release of pro-inflammatory, neurotoxic mediators during post-ischemic brain damage (Feuerstain et al. 1998; Mattson et al. 2000; Mattson and Camandola 2001). Accordingly, inhibition of PARP-1 activity by different means impairs glia activation and ensuing neurodegeneration (Chiarugi and Moskowitz 2003; Ha et al. 2002; Nakajima et al. 2004b; Ullrich et al. 2001b).

In agreement with this assumption, inhibition of PARP-1 reduces expression of pro-inflammatory mediators such as CD11b, ICAM-1 and COX2 in the peri-infarcted region in the rat brain (Koh et al. 2004). Consistently, a recent study reports that the potent PARP-1 inhibitor PJ34 reduces ischemia-induced iNOS expression in the brain of mice subjected to 20 min MCAo/72 h reperfusion. Accordingly, treatment with PJ34 (25 mg/Kg) also decreased the raised levels of TNF-α protein and of mRNAs for TNF-α, IL-6, ICAM-1 and E-selectin in brain tissue after focal cerebral ischemia (Haddad et al. 2006). Finally, a recent study by Lenzser et al. (Lenzser et al. 2007) investigates the contribution of PARP activation to blood-brain barrier (BBB) disruption and edema formation after reperfusion in a *in vivo* model of global cerebral ischemia. The permeability of the BBB increases after ischemia-reperfusion compared with the nonischemic animals after 24 and 48 h reperfusion. The administration of the potent PARP inhibitor PJ34 (10 mg/kg), before ischemia, attenuates this increase and decreases brain edema seen at 48 h. PARP inhibition also reduces neutrophil infiltration and decreases ICAM-1 expression, a marker of leukocyte infiltration into the brain, at both 24 and 48 h. Importantly, a recent study reports that the activation of PARP-1 also regulates the translocation of HMGB-1 from the nucleus to the cytosol. HMGB-1 is a nuclear protein, highly expressed in the adult mouse brain, that when released into the extracellular space can elicit a potent inflammatory response (Scaffidi et al. 2002).

Indeed, the extracellular presence of recombinant HMGB1 increases excitotoxic and ischemic neuronal death *in vitro*. In addition, brain microinjection of HMGB1 increases the transcript levels of pro-inflammatory mediators and sensitizes the tissue to the ischemic injury (Faraco et al. 2007). Importantly, down-regulation of HMGB1 brain levels by siRNA correlates with diminished infarct volumes in the rat (Kim et al. 2006). Following N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) treatment, translocation of HMGB-1 is observed in wild-type cells, whereas HMGB-1 remained nuclear in mouse embryonic fibroblasts lacking PARP-1; thus, these results suggests a role for PARP-1 in mediating relocalization of pro-inflammatory molecules in cells that have sustained DNA damage (Ditsworth et al. 2007). However, the PARP inhibitor PJ34 does not affect extracellular release of HMGB-1 from both neurons and astrocytes exposed to necrotic stimuli, indicating that poly (ADP-ribosyl)ation does not regulate HMGB-1 release during necrosis, at least in these cell types (Faraco et al. 2007). These results taken together highlight the significance of transcriptional regulation by PARP-1 in stroke pathogenesis, and suggest that the remarkable stroke neuroprotection afforded by inhibitors of PARP-1 activity is related, at least in part, to changes in gene expression profiles in the ischemic brain tissue (Chiarugi 2002b; Skaper 2003a, 2003b).

7.3.3 PARP-1 Activation of Neuronal Death: The Signaling Hypothesis

Recent findings allow us to hypothesize an additional mechanism through which deregulated PAR formation exerts a detrimental role in the CNS. Several lines of evidence support the so-called "signalling hypothesis" (Chiarugi 2002b). For instance, PARP-1 hyperactivation is a powerful trigger of mitochondrial release of apoptosis inducing factor (AIF) in cultured neurons undergoing excitotoxicity (Hong et al. 2004; Yu et al. 2002) as well as in astrocytes (Alano et al. 2004) and ischemic brain (Komjati et al. 2004). These findings, together with knowledge that AIF may contribute to post-ischemic neurodegeneration (Cao et al. 2003; Culmsee et al. 2005; Plesnila et al. 2004; Zhu et al. 2003), suggest that PAR formation is a pivotal event in neuronal cell death that follows the ischemic insult. Recently, Andrabi et al. reported that PAR polymer is endowed with intrinsic cytotoxicity and that its toxic effect is abolished by pre-treatment with the PAR-degrading enzymes phosphodiesterase (PD1) or poly (ADP-ribose) glycohydrolase (PARG). Moreover, interfering with PAR polymer signalling by means of neutralizing PAR antibodies or PARG overexpression reduces PARP-1-dependent NMDA excitotoxicity and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced cell death (Andrabi et al. 2006; Wang et al. 2003) In keeping with this, transgenic mice overexpressing PARG have markedly reduced infarct volumes after 2 h of MCAo. Consistent with the notion that PAR chains are toxic, experimental evidence indicates that (1) siRNA knockdown of PARG leads to enhanced MNNG toxicity, (2) PARG +/– cortical cultures are more sensitive to NMDA excitotoxicity and (3) PARG +/– mice have

larger infarct volumes 24 h after 1.5 h of MCAo. These results suggest that PAR is a key signal in the nucleus–mitochondria cross-talk during PARP-1-dependent neuronal death, substantiating the relevance of the signaling hypothesis to PAR neurotoxicity (Andrabi et al. 2006a; Wang et al. 2003). To date, molecular mechanisms responsible for PAR-dependent AIF release in neurons are unknown. Of note, recent findings support an important role of PARP-1 in the release of AIF from mitochondria after ischemic brain injury (Culmsee et al. 2005). Inhibition of PARP-1 prevented AIF translocation from mitochondria to the nucleus after oxygen-glucose deprivation *in vitro* and in focal cerebral ischemia *in vivo*. It has been suggested that mitochondrial NAD depletion may represent a link between PARP-1 overactivation and the release of AIF from mitochondria (Yu et al. 2002). Based on these data, these authors suggest that DNA damage and PARP-1 activation subsequent to cerebral ischemia, lead to a decrease of mitochondrial NAD+ contents that induces Bid-mediated mitochondrial membrane pore formation and hence the AIF release from mitochondria to nucleus in which it initiates nuclear condensation (Susin et al. 1999). More recently, a report provides novel insights into the molecular mechanism involved in mitochondrial AIF release upon MNNG-dependent PARP-1 activation (Moubarak et al. 2007). In light of these new findings, calpain appears to link PARP-1 activation to Bax activation and AIF release during MNNG-induced necrosis. Recently, both Bax and AIF have been identified as targets of calpains. Indeed, generation of a Bax fragment is an early event in the induction of apoptosis via calpains (Altznauer et al. 2004; Gao and Dou 2000; Polster et al. 2005) and AIF becomes a soluble and death-promoting protein after its cleavage by calpains (Polster et al. 2005). These results indicate that calpains control mitochondrial AIF release during PARP-1 activation.

Finally, ADP-ribose, the monomeric constituent of PAR and product of PARG, could also be involved in mediating mitochondrial disfunction consequent to PARP-1 hyperactivation (Dumitriu et al. 2004). Following PARP-1 activation, the increasing levels of poly(ADP-ribose) recruit PARG to the nucleus. PARG hydrolyses the protein-bound poly(ADP-ribose), thereby generating free oligo- and monomers of ADP-ribose. ADP-ribose potently inhibits the activity of the ATP-binding cassette (ABC) transporters, involved in the transport of multiple substrates across cellular membranes, among them mitochondrial membranes. Therefore, considering the homology between ADP-ribose, ADP and ATP, it is tempting to speculate that ADP-ribose might compete with these metabolites for its binding site on ATP-dependent membrane transporters (Dumitriu et al. 2004).

7.4 Suppression of Par Neo-Synthesis and Its Effects on Ischemic Brain Injury

Given the relevance of poly(ADP-ribosyl)ation to disease pathogenesis, a range of strategies have been used to suppress PARP-1 activity such as gene deletion, anti-sense oligonucleotides (Simbulan-Rosenthal et al. 1998), expression of

dominant negative forms (Schreiber et al. 1995), RNA interference (Gan et al. 2002) and chemical inhibitors (Costantino et al. 2001; Southan and Szabo 2003). Remarkably, repression of poly(ADP-ribosyl)ation provides impressive protection from ischemic brain injury. Significant reduction of brain infarcts in PARP-1 KO mice has been first reported by two pioneering studies (Eliasson et al. 1997; Endres et al. 1997). In particular, Dawson and her group report that brain infarct is 80% smaller in PARP KO mice compared to wild type (WT) 22 h after 2 h MCAo. Of note, in an identical ischemia/reperfusion model used by Moskowitz and colleagues, infarct volume is significantly smaller in PARP-1 KO mice compared to WT animals with a 45% reduction (Endres et al. 1997). In a similar study, ischemic volume is smaller early (50 min) after occlusion and remains less at 21 h and 3 days of reperfusion in PARP-1 KO versus WT mice (Goto et al. 2002). A recent report has shown that deletion of the gene encoding PARP-2 also leads to neuroprotection in focal cerebral ischemia, reducing infarction by approximately 40% of that observed in the wild-type mice. In the same PARP-2 KO mice, in global cerebral ischemia, where cell death is delayed, neuronal loss is increased, not ameliorated (Kofler et al. 2006). Although PARP-2 has a much lower activity than PARP-1, PARP-2 deficiency is quite effective in reducing ischemic brain injury. It has been suggested that PARP-1 and -2 have to act as heterodimers to initiate single-strand break repair and base excision repair (Schreiber et al. 2002); thus the absence of one of each would have the same consequence on repair efficiency.

Numerous studies showing the neuroprotective effects of chemical inhibitors of PARP-1 in *in vitro* and *in vivo* models of brain ischemia have been reported. For instance, the PARP-1 inhibitor 3-amino-benzamide (3-ABA) affords significant neuroprotection when pre-injected in mice and rats subjected to transient (Endres et al. 1997; Lo et al. 1998) or permanent (Tokime et al. 1998) brain ischemia. Similarly, 3,4-dihydro-5-[4-1(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ) is a potent PARP-1 inhibitor capable of preventing neuronal death in *in vivo* (Takahashi et al. 1997) and *in vitro* models of brain ischemia (Chiarugi et al. 2003; Eliasson et al. 1997). Recently, the phenanthridinone-analog [thieno[2,3-c] isoquinolin-5-one (TIQ-A) has been shown to inhibit PARP-1 with high potency and to provide higher neuroprotection than DPQ in mixed cerebral cultures subjected to oxygen-glucose deprivation (OGD) (Chiarugi et al. 2003). Other compounds with different molecular moieties but potent inhibitory activity on PARP-1, such as aza-5[H]-phenanthridin-6-ones (aza-PHE) (Ferraris et al. 2003a–c), N-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide (PJ-34) (Abdelkarim et al. 2001), indeno-isoquinolinone (INO-1001) (Komjati et al. 2004), 5-chloro-2-[3-(4-phenyl-3,6-dihydro-1(2H)-pyridinyl)propyl]-4(3H)-quinazolinone (FR247304) (Iwashita et al. 2004) and 2-methyl-3,5,7,8-tetrahydrothiopyrano[4,3-d]pyrimidine-4-one (DR2313) (Nakajima et al. 2004a) also protect the brain tissue from the ischemic insult. In contrast, neither nicotinamide nor DPQ reduces hippocampal neuronal death in a transient (5 min) global ischemia model in the gerbil (Moroni et al. 2001). The therapeutic potential of PARP-1 suppression in ischemic stroke is also underscored by studies that use different experimental approaches.

For instance, RNA interference-dependent PARP-1 repression induces resistance to OGD in differentiated neuroblastoma cells (Gan et al. 2002). In addition, transfection of PARP-1 into the brain of PARP-1 KO mice by means of Sindbis viruses increases the animal sensitivity to ischemic stroke relative to lacZ-injected PARP-1 KO mice. Notably, cerebral injection of Sindbis viruses carrying PARP-1 worsens ischemic brain damage in WT animals as well (Goto et al. 2002).

Further corroborating the relevance of poly(ADP-ribosyl)ation to cerebral ischemia, and underscoring the therapeutic potential of PARP-1 inhibitors in stroke treatment, ischemic neurodegeneration can be significantly reduced also when chemical inhibitors of PARP-1 are used in post-treatment paradigms. Indeed, DPQ provides neuroprotection when injected in rats 30 min after a focal brain ischemic insult (Takahashi et al. 1999). Similarly, treatment with DR2313 2 h after ischemia still reduces progression of infarction in rats (Nakajima et al. 2004a), and INO-1001 improves neurological functions with a time window of 6 h in a transient ischemia model in the rat (Komjati et al. 2004). Efficacy of post-treatment is not only related to the length of the time window but also to the inhibitor used. For example, 3-ABA loses its neuroprotective effects when injected in rats as early as 15 min after the ischemic insult (Takahashi and Greenberg 1999). Interestingly, two different studies report that ischemic neuroprotection by DPQ has a bell-shaped curve (Takahashi et al. 1997, 1999). In particular, in a focal cerebral ischemia model in the rat DPQ-mediated neuroprotection becomes less effective when the drug is used in a pre-treatment paradigm at doses higher than 10 mg/kg (Takahashi et al. 1997) and in a post-treatment paradigm when DPQ doses are higher than 40 mg/kg (Takahashi et al. 1999). These findings suggest that inhibition of the homeostatic functions of PARP-1 above a certain threshold and/or for too long periods is detrimental for the ischemic brain tissue.

7.5 Future Perspectives

To date, the considerable amount of information on poly(ADP-ribosyl)ation and stroke undoubtedly indicates a pathogenetic role of PAR formation in ischemic brain injury. Also, a battery of compounds displaying high inhibitory potency toward PARP-1 has been developed and looks promising for ischemic neuroprotection. However, critical issues still wait to be resolved. For instance, current drugs do not exhibit significant selectivity toward PARP-1. This is of particular significance given the role of PARP-2 in DNA repair (Schreiber et al. 2002), and the mutagenic potential of non-selective PARP-1 inhibitors (Tong et al. 2001; Virag and Szabo 2002). Also, acceptable water solubility with satisfactory blood-brain barrier penetrability is an important feature of PARP-1 inhibitors to be optimized. Finally, research aimed at identifying mechanisms underpinning ischemic neuroprotection by inhibition of poly(ADP-ribosyl)ation may also disclose novel players involved in post-ischemic brain damage and provide innovative targets of therapeutic relevance to treatment of cerebral ischemia.

Bibliography

Abdelkarim GE, Gertz K, Harms C, Katchanov J, Dirnagl U, Szabo C, Endres M (2001) Protective effects of PJ34, a novel, potent inhibitor of poly(ADP-ribose) polymerase (PARP) in in vitro and in vivo models of stroke. Int J Mol Med 7:255–260

Agarwal ML, Agarwal A, Taylor WR, Wang ZQ, Wagner EF, Stark GR (1997) Defective induction and normal activation and function of p53 in mouse cells lacking poly-ADP-ribose polymerase. Oncogene 15:1035–1041

Akiyama T, Takasawa S, Nata K, Kobayashi S, Abe M, Shervani NJ, Ikeda T, Nakagawa K, Unno M, Matsuno S, Okamoto H (2001) Activation of Reg gene, a gene for insulin-producing β-cell regeneration: Poly(ADP-ribose) polymerase binds Reg promoter and regulates the transcription by autopoly(ADP-ribosyl)ation. Proc Natl Acad Sci 98:48–53

Alano CC, Ying W, Swanson RA (2004) Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD+ depletion and mitochondrial permeability transition. J Biol Chem 279:18895–18902

Althaus FR, Naegeli H, Realini C, Mathis G, Loetscher P, Mattenberger M (1990) The poly-ADP-ribosylation system of higher eukaryotes: a protein shuttle mechanism in chromatin? Acta Biol Hung 41:9–18

Altznauer F, Conus S, Cavalli A, Folkers G, Simon HU (2004) Calpain-1 regulates Bax and subsequent Smac-dependent caspase-3 activation in neutrophil apoptosis. J Biol Chem 279:5947–5957

Ame JC, Spenlehauer C, de Murcia G (2004) The PARP superfamily. Bioessays 26:882–893

Andrabi SA, Kim NS, Yu SW, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD, Poirier GG, Dawson VL, Dawson TM (2006) Poly(ADP-ribose) (PAR) polymer is a death signal. Proc Natl Acad Sci U S A 103:18308–18313

Beal MF (2000) Energetics in the pathogenesis of neurodegenerative diseases. Trends Neurosci 23:298–304

Berger NA (1985) Poly (ADP-ribose) in the cellular response to DNA damage. Radiat Res 101:4–15

Butler AJ, Ordahl CP (1999) Poly(ADP-ribose)polymerase binds with transcription enhancer factor 1 to MCAT1 elements to regulate muscle-specific transcription. Mol Cell Biol 19:296–306

Cao G, Clark RS, Pei W, Yin W, Zhang F, Sun FY, Graham SH, Chen J (2003) Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation. J Cereb Blood Flow Metab 23:1137–1150

Carty SM, Greenleaf AL (2002) Hyperphosphorylated C-terminal repeat domain-associating proteins in the nuclear proteome link transcription to DNA/chromatin modification and RNA processing. Mol Cell Proteomics 1:598–610

Chang P, Jacobson MK, Mitchinson TJ (2004) Poly(ADP-ribose is required for spindle assembly and structure. Nature 432:645–649

Chiarugi A (2002a) Inhibitors of poly(ADP-ribose) polymerase-1 suppress transcriptional activation in lymphocytes and ameliorate autoimmune encephalomyelitis in rats. Br J Pharmacol 137:761–770

Chiarugi A (2002b) PARP-1: killer or conspirator? The suicide hypothesis revisited. Trends Pharmacol Sci 23:122–129

Chiarugi A, Meli E, Calvani M, Picca R, Baronti R, Camaioni E, Costantino G, Marinozzi M, Pellegrini-Giampietro DE, Pellicciari R, Moroni F (2003) Novel isoquinolinone-derived inhibitors of poly(ADP-ribose) polymerase-1: pharmacological characterization and neuroprotective effects in an in vitro model of cerebral ischemia. J Pharmacol Exp Ther 305:943–949

Chiarugi A, Moskowitz MA (2003) Poly(ADP-ribose) polymerase-1 activity promotes NF-kappaB-driven transcription and microglial activation: implication for neurodegenerative disorders. J Neurochem 85:306–317

Cosi C, Suzuki H, Milani D, Facci L, Menegazzi M, Vantini G, Kanai Y, Skaper SD (1994) Poly(ADP-ribose) polymerase: early involvement in glutamate-induced neurotoxicity in cultured cerebellar granule cells. J Neurosci Res 39:38–46

Costantino G, Macchiarulo A, Camaioni E, Pellicciari R (2001) Modeling of poly(ADP-ribose) polymerase (PARP) inhibitors. Docking of ligands and quantitative structure-activity relationship analysis. J Med Chem 44:3794

Culmsee C, Zhu C, Landshamer S, Becattini B, Wagner E, Pellechia M, Blomgren K, Plesnila N (2005) Apoptosis-inducing factor triggered by poly(ADP-Ribose) polymerase and bid mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia. J Neurosci 25:10262–10272

D'Amours D, Desnoyers S, Poirier GG (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem J 342:249–268

de Murcia G, Huletsky A, Lamarre D, Gaudreau A, Poujet J, Daune M, Poirier GG (1986) Modulation of chromatin superstructure induced by poly(ADP-ribose) synthesis and degradation. Proc Natl Acad Sci 261:7011–7017

de Murcia G, Huletsky A, Poirier GG (1988) Modulation of chromatin structure by poly(ADP-ribosyl)ation. Biochem Cell Biol 66:626–635

Ditsworth D, Zong WX, Thompson CB (2007) Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus. J Biol Chem 282:17845–17854

Dumitriu IE, Voll RE, Kolowos W, Gaipl US, Heyder P, Kalden JR, Herrmann M (2004) UV irradiation inhibits ABC transporters via generation of ADP-ribose by concerted action of poly(ADP-ribose) polymerase-1 and glycohydrolase. Cell Death Differ 11:314–320

Eliasson MJ, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang ZQ, Dawson TM, Snyder SH, Dawson VL (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. Nat Med 3:1089–1095

Endres M, Wang ZQ, Namura S, Waeber C, Moskowitz MA (1997) Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. J Cereb Blood Flow Metab 17:1143–1151

Faraco G, Fossati S, Bianchi ME, Patrone M, Pedrazzi M, Sparatore B, Moroni F, Chiarugi A (2007) High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration in vitro and in vivo. J Neurochem 103:590–603

Ferraris D, Ficco RP, Dain D, Ginski M, Lautar S, Lee-Wisdom K, Liang S, Lin Q, Lu MX, Morgan L, Thomas B, Williams LR, Zhang J, Zhou Y, Kalish VJ (2003a) Design and synthesis of poly(ADP-ribose) polymerase-1 (PARP-1) inhibitors. Part 4: biological evaluation of imidazobenzodiazepines as potent PARP-1 inhibitors for treatment of ischemic injuries. Bioorg Med Chem 11:3695–3707

Ferraris D, Ficco RP, Pahutski T, Lautar S, Huang S, Zhang J, Kalish V (2003b) Design and synthesis of poly(ADP-ribose)polymerase-1 (PARP-1) inhibitors. Part 3: In vitro evaluation of 1, 3, 4, 5-tetrahydro-benzo[c][1, 6]- and [c][1, 7]-naphthyridin-6-ones. Bioorg Med Chem Lett 13:2513–2518

Ferraris D, Ko YS, Pahutski T, Ficco RP, Serdyuk L, Alemu C, Bradford C, Chiou T, Hoover R, Huang S, Lautar S, Liang S, Lin Q, Lu MX, Mooney M, Morgan L, Qian Y, Tran S, Williams LR, Wu QY, Zhang J, Zou Y, Kalish V (2003c) Design and synthesis of poly ADP-ribose polymerase-1 inhibitors. 2. Biological evaluation of aza-5[H]-phenanthridin-6-ones as potent, aqueous-soluble compounds for the treatment of ischemic injuries. J Med Chem 46:3138–3151

Feuerstain GZ, Wang X, Barone FC (1998) The role of cytokine in the pathology of stroke and neurotrauma. Neuroimmunomodul 5:143–159

Gan L, Anton KE, Masterson BA, Vincent VA, Ye S, Gonzalez-Zulueta M (2002) Specific interference with gene expression and gene function mediated by long dsRNA in neural cells. J Neurosci Methods 121:151–157

Gao G, Dou QP (2000) N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. J Cell Biochem 80:53–72

Goto S, Xue R, Sugo N, Sawada M, Blizzard KK, Poitras MF, Johns DC, Dawson TM, Dawson VL, Crain BJ, Traistman RG, Mori S, Hurn PD (2002) Poly(ADP-ribose) polymerase impairs early and long-term experimental stroke recovery. Stroke 33:1101–1106

Ha HC, Hester LD, Snyder SH (2002) Poly(ADP-ribose) polymerase-1 dependence of stress-induced transcription factors and associated gene expression in glia. Proc Natl Acad Sci 99:3270–3275

Ha HC, Snyder SH (2000) Poly (ADP-ribose) polymerase-1 in the nervous system. Neurobiol Dis 7:225–239

Haddad M, Rhinn H, Bloquel C, Coqueran B, Szabo C, Plotkine M, Scherman D, Margaill I (2006) Anti-inflammatory effects of PJ34, a poly(ADP-ribose) polymerase inhibitor, in transient focal cerebral ischemia in mice. Br J Pharmacol 149:23–30

Hassa PO, Covic M, Hasan S, Imhof R, Hottinger MO (2001) The enzymatic and DNA binding activity of PARP-1 are not required for NF-kappaB coactivator function. J Biol Chem 276:45588–45597

Hassa PO, Hottinger MO (1999) A role of poly(ADP-ribose) polymerase in NF-kB transcriptional activation. Biol Chem 380:953–959

Herceg Z, Wang ZQ (2001) Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. Mutat Res 477:97–110

Homburg S, Visochek L, Moran N, Dantzer F, Priel E, Asculai E, Schwartz D, Rotter V, Dekel N, Cohen-Armon M (2000) A fast signal induced activation of poly(ADP-ribose)polymerase: a downstream target of phospholipase C. J Cell Biol 150:293–307

Hong SJ, Dawson TM, Dawson VL (2004) Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. Trends Pharmacol Sci 25:259–264

Iwashita A, Tojo N, Matsuura S, Yamazaki S, Kamijo K, Ishida J, Yamamoto H, Hattori K, Matsuoka N, Mutoh S (2004) A novel and potent poly(ADP-ribose) polymerase-1 inhibitor, FR247304 (5-chloro-2-[3-(4-phenyl-3, 6-dihydro-1(2H)-pyridinyl)propyl]-4(3H)-quinazolinone), attenuates neuronal damage in in vitro and in vivo models of cerebral ischemia. J Pharmacol Exp Ther 310:425–436

Kim JB, Sig CJ, Yu YM, Nam K, Piao CS, Kim SW, Lee MH, Han PL, Park JS, Lee JK (2006) HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J Neurosci 26:6413–6421

Koedel U, Pfister HW (1999) Oxidative stress in bacterial meningitis. Brain Pathol 9:57–67

Kofler J, Otsuka T, Zhang Z, Noppens R, Grafe MR, Koh DW, Dawson VL, de Murcia JM, Hurn PD, Traystman RJ (2006) Differential effect of PARP-2 deletion on brain injury after focal and global cerebral ischemia. J Cereb Blood Flow Metab 26:135–141

Koh SH, Park Y, Song CW, Kim JG, Kim K, Kim J, Kim MH, Lee SR, Kim DW, Yu HJ, Chang DI, Hwang SJ, Kim SH (2004) The effect of PARP inhibitor on ischaemic cell death, its related inflammation and survival signals. Eur J Neurosci 20:1461–1472

Komjati K, Mabley JG, Virag L, Southan GJ, Salzman AL, Szabo C (2004) Poly(ADP-ribose) polymerase inhibition protect neurons and the white matter and regulates the translocation of apoptosis-inducing factor in stroke. Int J Mol Med 13:373–382

Kraus WL, Lis JT (2003) PARP goes transcription. Cell 113:677–683

Kun E, Kirsten E, Ordahl CP (2002) Coenzymatic activity of randomly broken or intact double-stranded DNAs in auto and histone H1 trans-poly(ADP-ribosylation), catalyzed by poly(ADP-ribose) polymerase (PARP I). J Biol Chem 277:39066–39069

Le Page C, Sanceau J, Drapier JC, Wietzerbin J (1998) Inhibitors of ADP-ribosylation impair inducible nitric oxide synthase gene transcription through inhibition of NF-kB activation. Biochem Biophys Res Comm 243:451–457

Lee JM, Grabb MC, Zipfel GJ, Choi DW (2000) Brain tissue responses to ischemia. J Clin Invest 106:723–731

Lenzser G, Kis B, Snipes JA, Gaspar T, Sandor P, Komjati K, Szabo C, Busija DW (2007) Contribution of poly(ADP-ribose) polymerase to postischemic blood-brain barrier damage in rats. J Cereb Blood Flow Metab 27:1318–1326

Lipton P (1999) Ischemic cell death in brain neurons. Physiol Rev 79:1431–1568

Liu J, Ying W, Massa S, Duriez PJ, Swanson RA, Poirier GG, Sharp FR (2000) Effects of transient global ischemia and kainate on poly(ADP-ribose) polymerase (PARP) gene expression and proteolytic cleavage in gerbil and rat brains. Brain Res Mol Brain Res 80:7–16

Lo EH, Bosque-Hamilton P, Meng W (1998) Inhibition of poly(ADP-ribose) polymerase: reduction of ischemic injury and attenuation of N-methyl-D-aspartate-induced neurotransmitter dysregulation. Stroke 29:830–836

Lo EH, Dalkara T, Moskowitz MA (2003) Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci 4:399–415

Love S, Barber R, Wilcock GK (2000) Neuronal death in brain infarcts in man. Neuropathol Appl Neurobiol 26:55–66

Mandir AS, Poitras MF, Berliner AR, Herring WJ, Guastella DB, Feldman A, Poirier GG, Wang ZQ, Dawson TM, Dawson VL (2000) NMDA but not non-NMDA excitotoxicity is mediated by Poly(ADP-ribose) polymerase. J Neurosci 20:8005–8011

Mandir AS, Przedborski S, Jackson-Lewis V, Wang ZQ, Simbulan-Rosenthal CM, Smulson ME, Hoffman BE, Guastella DB, Dawson VL, Dawson TM (1999) Poly(ADP-ribose)polymerase activation mediates 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced parkynsonism. Proc Natl Acad Sci 96:5774–5779

Mattson MP, Camandola S (2001) NF-kB in neuronal plasticity and neurodegenerative disorders. J Clin Invest 107:247–254

Mattson MP, Culmsee C, Yu Z, Camandola S (2000) Roles of nuclear factor kappaB in neuronal survival and plasticity. J Neurochem 74:443–456

Moroni F, Meli E, Peruginelli F, Chiarugi A, Cozzi A, Picca R, Romagnoli P, Pellicciari R, Pellegrini-Giampietro DE (2001) Poly(ADP-ribose) polymerase inhibitors attenuate necrotic but not apoptotic neuronal death in experimental models of cerebral ischemia. Cell Death Diff 8:921–932

Moubarak RS, Yuste VJ, Artus C, Bouharrour A, Greer PA, Menissier-de Murcia J, Susin SA (2007) Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. Mol Cell Biol 27:4844–4862

Nagayama T, Simon RP, Chen D, Henshall DC, Pei W, Stetler RA, Chen J (2000) Activation of poly(ADP-ribose) polymerase in the rat hippocampus may contribute to cellular recovery following sublethal transient global ischemia. J Neurochem 74:1636–1645

Nakajima H, Kakui N, Ohkuma K, Ishikawa M, Hasegawa T (2004a) A newly synthesized poly(ADP-ribose) polymerase inhibitor, 2-methyl-3,5,7,8-tetrahydrothiopyrano[4,3-d]pyrimidine-4-one (DR2313): pharmacological profiles, neuroprotective effects, and therapeutic time window in cerebral ischemia in rats. J Pharmacol Exp Ther 312:472–481

Nakajima H, Nagaso H, Kakui N, Ishikawa M, Hiranuma T, Hoshiko S (2004b) Critical role of the automodification of poly(ADP-ribose) polymerase-1 in nuclear factor-kappaB-dependent gene expression in primary cultured mouse glial cells. J Biol Chem 279:42774–42786

Nirodi C, NagDas S, Gygi SP, Olson G, Ruedi A, Richmond A (2001) A role for poly(ADP-ribose) polymerase (PARP) in the transcriptional regulation of the melanoma growth stimulatory activity (CXCL1) gene expression. J Biol Chem 276:9366–9374

Oliver FJ, Menissier-de Murcia J, Nacci C, Decker P, Andriantsitohania R, Muller S, de la Rubia G, Stoclet JC, de Murcia G (1999) Resistance to endotoxic shock as a consequence of defective NF-kB activation in poly(ADP-ribose) polymerase-1 deficient mice. EMBO J 18:4446–4454

Paschen W, Olah L, Mies G (2000) Effect of transient focal ischemia of mouse brain on energy state and NAD levels: no evidence that NAD depletion plays a major role in secondary disturbances of energy metabolism. J Neurochem 75:1675–1680

Plaschke K, Kopitz J, Weigand MA, Martin E, Bardenheuer HJ (2000) The neuroprotective effect of cerebral poly (ADP-ribose) polymerase inhibition in a rat model of global ischemia. Neurosci Lett 284:109–112

Plesnila N, Zhu C, Culmsee C, Groger M, Moskowitz MA, Blomgren K (2004) Nuclear translocation of apoptosis-inducing factor after focal cerebral ischemia. J Cereb Blood Flow Metab 24:458–466

Poirier GG, de Murcia G, Jongstra-Bilen J, Mandel P (1982) Poly(ADP-ribosyl)ation of polynucleosomes causes relaxation of chromatin structure. Proc Natl Acad Sci 79:3423–3427

Polster BM, Basanez G, Etxebarria A, Hardwick JM, Nicholls DG (2005) Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. J Biol Chem 280:6447–6454

Potaman VN, Shlyakhtenko LS, Oussatcheva EA, Lyubchenko YL, Soldatenkov VA (2005) Specific binding of poly(ADP-ribose) polymerase-1 to cruciform hairpins. J Mol Biol 348:609–615

Satoh M, Date I, Nakajima M, Takahashi K, Iseda K, Tamiya T, Ohmoto T, Ninomiya Y, Asari S (2001) Inhibition of poly(ADP-ribose) polymerase attenuates cerebral vasospasm after subarachnoid hemorrhage in rabbits. Stroke 32:225–231

Scaffidi P, Misteli T, Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191–195

Schmid G, Wang ZQ, Wesierska-Gadek J (1999) Compensatory expression of p73 in PARP-deficient mouse fibroblast as response to reduced level of regularly spliced wild-type p53. Biochem Biophys Res Comm 255:399–405

Schreiber V, Ame JC, Dolle P, Schultz I, Rinaldi B, Fraulob V, Menissier-de Murcia J, de Murcia G (2002) Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. J Biol Chem 277:23028–23036

Schreiber V, Hunting D, Trucco C, Gowans B, Grunwald D, de Murcia G, de Murcia JM (1995) A dominant-negative mutant of human poly(ADP-ribose) polymerase affects cell recovery, apoptosis, and sister chromatid exchange following DNA damage. Proc Natl Acad Sci USA 92:4753–4757

Scott GS, Hake P, Kean RB, Virag L, Szabo C, Hooper DC (2001) Role of poly(ADP-ribose) synthetase activation in the development of experimental allergic encephalomyelitis. J Neuroimm 117:78–86

Simbulan-Rosenthal CM, Rosenthal DS, Iyer S, Boulares AH, Smulson ME (1998) Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. J Biol Chem 273:13703–13712

Skaper SD (2003a) Poly(ADP-Ribose) polymerase-1 in acute neuronal death and inflammation: a strategy for neuroprotection. Ann N Y Acad Sci 993:217–228

Skaper SD (2003b) Poly(ADP-ribosyl)ation enzyme-1 as a target for neuroprotection in acute central nervous system injury. Curr Drug Targets CNS Neurol Disord 2:279–291

Slattery E, Dignam JD, Matsui T, Roeder RG (1983) Purification and analysis of a factor which suppresses nick-induced transcription by RNA polymerase II and its identity with poly(ADP-ribose) polymerase. J Biol Chem 285:5955–5959

Smith S (2001) The world according to PARP. Trends Biochem Sci 26:174–179

Southan GJ, Szabo C (2003) Poly(ADP-ribose) polymerase inhibitors. Curr Med Chem 10:321–340

Strosznajder RP, Gadamski R, Czapski GA, Jesko H, Strosznajder JB (2003) Poly(ADP-ribose) polymerase during reperfusion after transient forebrain ischemia: its role in brain edema and cell death. J Mol Neurosci 20:61–72

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446

Szabo C, Dawson VL (1998) Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. Trends Pharmacol Sci 19:287–298

Takahashi K, Greenberg JH (1999) The effect of reperfusion on neuroprotection using an inhibitor of poly(ADP-ribose) polymerase. NeuroReport 10:1017–1022

Takahashi K, Greenberg JH, Jackson P, Maclin K, Zhang J (1997) Neuroprotective effects of inhibiting poly(ADP-ribose) synthetase on focal cerebral ischemia in rats. J Cereb Blood Flow Metab 17:1137–1142

Takahashi K, Piper AA, Croul SE, Zhang J, Snyder SH, Greenberg JH (1999) Post-treatment with an inhibitor of poly(ADP-ribose) polymerase attenuates cerebral damage in focal ischemia. Brain Res 829(1–2):46–54

Tokime T, Nozaki K, Sugino T, Kikuchi H, Hashimoto N, Ueda K (1998) Enhanced poly(ADP-ribosyl)ation after focal ischemia in rat brain. J Cereb Blood Flow Metab 18:991–997

Tong WM, Cortes U, Wang ZQ (2001) Poly(ADP-ribose) polymerase: a guardian angel protecting the genome and suppressing tumorigenesis. Biochim Biophys Acta 1552:27–37

Ullrich O, Diestel A, Bechmann I, Homberg M, Grune T, Hass R, Nitsch R (2001a) Turnover of oxidatively damaged nuclear proteins in BV-2 microglial cells is linked to their activation state by poly(ADP-ribosyl)ation. FASEB J 15:1460–1462

Ullrich O, Diestel A, Eyupoglu IY, Nitsch R (2001b) Regulation of microglial expression of integrins by poly(ADP-ribose) polymerase-1. Nat Cell Biol 3:1035–1042

Virag L, Szabo C (2002) The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. Pharmacol Rev 54:375–429

Wang H, Yu SW, Poitras MF, Norris J, Poirier GG, Dawson TM, Dawson VL (2003) Poly(ADP-ribose) polymer serves as a death signal by triggering apoptosis-inducing factor release. Soc Neurosci Abstr 34:1232

Wang X, Ohnishi K, Takahashi A, Ohnishi T (1998) Poly(ADP-ribosyl)ation is required for p53-dependent signal transduction induced by radiation. Oncogene 17:2819–2825

Wesierska-Gadek J, Schid G, Cerni C (1996) ADP-ribosylation of wilde-type p53 in vitro: binding of p53 to specific p53-consensus sequence prevents its modification. Biochem Biophys Res Comm 224:96–102

Wesierska-Gadek J, Schmid G (2000) Overexpressed poly(ADP-ribose) polymerase dealys the relase of rat cells from p53-mediated G(1) checkpoint. J Cell Biochem 80:85–103

Whalen MJ, Clark RS, Dixon CE, Robichaud P, Marion DW, Vagni V, Graham SH, Virag L, Hasko G, Stachlewitz R, Szabo C, Kochanek PM (1999) Reduction of cognitive and motor deficits after traumatic brain injury in mice deficient in poly(ADP-ribose) polymerase. J Cereb Blood Flow Metab 19:835–842

Yang J, Klaidman LK, Nalbandian A, Oliver J, Chang ML, Chan PH, Adams JD Jr (2002) The effects of nicotinamide on energy metabolism following transient focal cerebral ischemia in Wistar rats. Neurosci Lett 333:91–94

Ying W, Chen Y, Alano CC, Swanson RA (2002) Tricarboxylic acid cycle substrates prevent PARP-mediated death of neurons and astrocytes. J Cereb Blood Flow Metab 22:774–779

Yu SW, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Zhu C, Qiu L, Wang X, Hallin U, Cande C, Kroemer G, Hagberg H, Blomgren K (2003) Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. J Neurochem 86:306–317

Ziegler M, Oei SL (2001) A cellular survival switch: poly(ADP-ribosyl)ation stimulates DNA repair and silences transcription. BioEssay 23:543–548

Zingarelli B, Hake PW, O'Connor M, Denenberg A, Wong HR, Kong S, Aronow BJ (2004) Differential regulation of activator protein-1 and heat shock factor-1 in myocardial ischemia and reperfusion injury: role of poly(ADP-ribose) polymerase-1. Am J Physiol Heart Circ Physiol 286:H1408–H1415

Part III
Transient Global Ischemia

Chapter 8
Transient Global Cerebral Ischemia Produces Morphologically Necrotic, Not Apoptotic Neurons

Frederick Colbourne and Roland N. Auer

8.1 Introduction

Global cerebral ischemia is a devastating insult resulting in high mortality and frequent neurological impairment in survivors. The common occurrences are cardiac arrest or hypotension, in turn due to arrhythmias (ventricular fibrillation) or myocardial failure as root causes. Prolonged ischemia inevitably leads to death or extensive brain damage (Brierley et al. 1971; Cole and Cowie 1987), except in rare cases where the individual is hypothermic at the time, as during near-drowning in cold water (Siebke et al. 1975). In cases where there is more rapid reperfusion, but no neuroprotective intervention, there is a more selective and delayed injury, often to the hippocampal CA1 sector neurons. Damage largely confined to the CA1 sector pyramidal neurons also occurs in humans after global ischemia (Horn and Schlote 1992; Petito et al. 1987), and it can result in significant anterograde amnesia, as shown in patient R.B., who suffered global ischemia from the consequence of an atrial tear 5 years before death (Zola-Morgan et al. 1986). Such severe impairments are rare clinical events, more common being the subtle hippocampal deficits that preclude functioning at the high levels before cardiac arrest (Longstreth et al. 1983).

Development of effective neuroprotective interventions requires an accurate understanding of the mechanisms of cell death. To that end, many studies have examined the morphological features of ischemic brain injury with electron microscopy, which reveals cell membranes and organelle pathology. Before we delve into ultrastructural findings, however, it is important to keep in mind factors that significantly influence the extent, time course and nature of cell death. For instance, it is well known that the extent and rate of injury will vary with insult severity – the

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so-called “maturation phenomenon” described originally by Ito and colleagues (Ito et al. 1975) and later popularized by Kirino (Kirino 1982). Insult severity depends upon the duration of ischemia, the degree of reduced blood flow (method-dependent or model-dependent) and other factors such as temperature and blood glucose. The extent and features of cell death vary little by brain region and cell type.

8.2 Animal Models of Global Ischemia

While global ischemia in humans typically results from cardiac arrest, investigators infrequently use cardiac arrest to produce brain ischemia in animal models. Instead, investigators commonly induce transient forebrain ischemia by interrupting the blood flow to the brain through the carotid and vertebral arteries. For instance, in rats this is achieved by either 2 or 4 vessel occlusion (2-VO, 4-VO) methods. In the 4-VO model, the two vertebral arteries are cauterized and this is followed a day later by brief bilateral common carotid artery occlusion (Pulsinelli and Brierley 1979). The 2-VO model similarly produces severe ischemia by combining systemic hypotension with common carotid artery occlusion (Smith et al. 1984). In contrast, gerbils usually experience severe forebrain ischemia with common carotid artery occlusion alone (Kirino 1982; Laidley et al. 2005). Global ischemia models exist in other higher species as well (e.g., cats, dogs, primates).

Rodent studies, using the aforementioned models, indicate that the CA1 zone is exquisitely sensitive to brief ischemia, with cell death typically occurring from 2 to 4 days post-ischemia (Kirino 1982; Pulsinelli et al. 1982). Other brain regions are additionally affected after more prolonged ischemia, including other cells within the hippocampal formation (e.g., the dentate hilus or CA4, CA2, CA3), striatum, cortex, and thalamus (Hossmann et al. 2001; Petito and Pulsinelli 1984b). Cell death in these areas occurs more rapidly than in CA1. Despite the importance of extra-hippocampal cell death to the clinical prognosis of cardiac arrest, most animal studies, whether mechanistic or neuroprotective, continue to focus on CA1 zone injury.

The duration of ischemia needed to produce consistent brain injury in any given structure, such as the CA1 zone, varies considerably among studies (range: 3–30 min) in part due to species differences, but also due to differences in intra-ischemic residual blood flow as well as post-ischemic hemodynamic differences (e.g., arterial occlusion models vs. cardiac arrest). Further complicating comparisons among studies are differences in the control of physiological variables (e.g., temperature, blood oxygenation, and glucose), animal age, etc. There is a trend for recent studies to use briefer insults than those used in the early studies. While this has probably resulted in part from improved technique (e.g., better temperature control) giving more consistent results, it may be that investigators are also now deliberately studying milder insults in an effort to maximize the likelihood of finding neuroprotective effects on a particular mode of cell death. The problem with this approach is that findings may be less likely to translate to cardiac arrest-induced injury in humans where injury is often considerably greater than that produced in the mild ischemia models in animals.

8.3 Cell Death Modes

Necrosis is traditionally considered an externally wrought, unregulated cell death of which there are multiple causes and mechanisms of injury (e.g., excitotoxic swelling, energy failure, toxins, and free radical generation). Generally speaking, changes within the cytoplasm, such as organelle swelling, precede the appearance of nuclear alterations. Other prominent features include cell swelling and plasma membrane rupture (Trump et al. 1965). In contrast, apoptosis, described originally by Kerr and colleagues (Kerr 1971; Kerr et al. 1972), is a mode of cell death characterized by early nuclear changes, notably prominent chromatin condensation (crescent or round shape), while cytoplasmic organelles are still intact. In contrast to necrosis, which results in the release of cellular contents into the surrounding tissue, apoptotic cells condense and are broken up into large, membrane-bound vesicles (apoptotic bodies) for degradation by neighboring cells or phagocytes. The apoptotic morphology has been linked to distinct molecular pathways (e.g., caspase activation) and is a programmed cell death.

Cerebral ischemic injury has been traditionally thought to be necrotic with more recent reports claiming that at least some of it results from apoptosis (Brahma et al. 2009; Cao et al. 2007; Carboni et al. 2005; Hayashi et al. 2005; Nitatori et al. 1995; Ostrowski et al. 2008; Ruan et al. 2003; Zeng and Xu 2000). Some suggest that ischemic injury is neither apoptotic nor necrotic (Sheldon et al. 2001), according to the original meaning of these words. Several names for alternative cell death modes have been coined including oncosis (Majno and Joris 1995), paraptosis (Sperandio et al. 2000), parthanatos (Andrabi et al. 2008; David et al. 2009; Wang et al. 2009), and programmed necrosis (Boujrad et al. 2007). Since apoptosis is the biological counterforce of mitosis, one theoretically expects to find apoptosis in highly mitotic areas such as gut epithelium (Ikeda et al. 1998), but not in post-mitotic brain neurons.

Another potential route of cell death in ischemia is via autophagy, which has been more thoroughly described (Chu 2006; Kroemer and Levine 2008; Levine and Yuan 2005). Autophagy is a normal process for removing cellular constituents, including weak and damaged organelles. Like apoptosis, it is highly regulated, and free radicals play a major role in regulating autophagy, which is increased in injurious processes (Lai et al. 2008). Morphological features include the appearance of multi-membrane structures (autophagosomes) that fuse with lysosomes to form autolysosomes (e.g., mitochondrial autolysosomes). While excessive autophagy can cause cell death, it is important to remember that autophagy is a normal process to recycle cellular waste or to generate amino acids, etc. in times of cellular stress. Accordingly, the appearance of autophagy does not mean the cell has died by autophagy, or even that it has died. Indeed, the ischemic neuroprotection by hypothermia (Colbourne et al. 1999b) can cause, in long term surviving neurons, increased autophagy (see Figures 7–9 in that study). While each ‘mode’ of cell death described above has unique biochemical features, ultrastructural morphology remains indispensible to help distinguish between them.

8.4 Morphological Features of Ischemic Neuronal Death

In rodents, mitochondrial swelling is one of the earliest changes to occur after cerebral ischemia and it is this swelling that largely contributes to the microvacuolated appearance of neurons (Brown and Brierley 1972; Kalimo et al. 1977; Petito and Pulsinelli 1984a). Swelling of endoplasmic reticulum and microvesiculation of the Golgi apparatus is also commonly reported along with disaggregation of polyribosomes (Deshpande et al. 1992; Hossmann et al. 2001; Kalimo et al. 1977; Petito and Pulsinelli 1984a; Rafols et al. 1995; Ruan et al. 2003; Yamamoto et al. 1990). These changes are reversible. In CA1, a lamellar alignment and proliferation of endoplasmic reticulum has been subsequently observed (Colbourne et al. 1999b; Kirino and Sano 1984; Kirino et al. 1984; Yamamoto et al. 1990), but not in all cells or studies (Hossmann et al. 2001). Early excitotoxicity-induced dendritic swelling commonly occurs (Johansen et al. 1984). Many reports emphasize alterations in neuronal volume with some neurons swelling up while others show a shrunken appearance (Hossmann et al. 2001; Petito and Pulsinelli 1984b). Slight chromatin condensation (pyknosis) can be found immediately after ischemia (Petito and Pulsinelli 1984b), with moderate to substantial chromatin aggregation occurring later after ischemia in some neurons (Colbourne et al. 1999a, b; Hossmann et al. 2001; Nitatori et al. 1995, Petito and Pulsinelli 1984b, Ruan et al. 2003). Notably, this is distinct from the large rounded or crescent-shaped appearance of chromatin during apoptotic death. While post-ischemic mitochondrial swelling is often reversed, unequivocal signs of mitochondrial damage (Trump et al. 1965) are frequently seen later; these include ruptured mitochondria and mitochondrial flocculent densities (Colbourne et al. 1999a, b). Membranous whorls are frequently observed (Colbourne et al. 1999b; Petito and Pulsinelli 1984b). Some are derived from mitochondria while others are continuous with the plasma membrane. The latter is consistent with plasma membrane breaks, found at the time of cell death. Importantly, damage (breaks) to the nuclear and plasma membranes are found regardless of chromatin condensation. *Unequivocal* electron microscopic evidence of apoptotic bodies from global ischemic neuronal death has not been reported, although several studies explicitly looked for it (Colbourne et al. 1999a, b; Deshpande et al. 1992; Hossmann et al. 2001; Winkelmann et al. 2006).

An important issue is whether these rodent findings can be corroborated in other species. Indeed, similar findings have been reported in cats (Kalimo et al. 1977; Martin et al. 1998) and primates (Yamashima et al. 2003, 2007). Specifically, signs of necrosis (e.g., mitochondrial flocculent densities, membrane breaks), not apoptosis, were found.

It has been long argued that insult severity, regardless of whether it is from ischemia, heat stress, etc., affects the mode of cell death with apoptosis predominating after milder insults whereas necrosis occurs after severe injury (e.g., (Bonfoco et al. 1995; Du et al. 1996; Harmon et al. 1990; Kerr 1971)). While this also makes intuitive sense, the data in global ischemia models do not support the tenet that mild insults produce apoptosis. One study produced global ischemia in gerbils that

ranged from 5 to 30 min (Kirino and Sano 1984). While cellular changes occurred faster following more severe injury, the pathological findings did not otherwise change. Similarly, rats subjected to 5 or 15 min of 4-VO ischemia, which have different rates of CA1 sector death, had similar light and ultrastructural features of cell death (Colbourne et al. 1999a). A subsequent comparison of 10 and 20 min of 4-VO ischemia over three survival times (2–10 days) confirmed these findings (Winkelmann et al. 2006).

Neuroprotective treatments seek to reduce cell death, but they might also influence the mode of cell death in those cells incompletely protected. The most potent neuroprotectant is hypothermia (MacLellan et al. 2009) and two studies examined the use of post-ischemic cooling on the survival and morphological appearance of CA1 neurons. In one study, rats were subjected to a 2-VO insult with 3 h of immediate post-ischemic cooling (Dietrich et al. 1993). While CA1 cell death was postponed by the treatment, the ultrastructural features were comparable and consistent with necrosis. We studied post-ischemic cooling of 48 h duration induced 1, 6 or 12 h after a 5 min ischemic insult in gerbil (Colbourne et al. 1999b) and found that while delayed cooling markedly reduced injury, the protective effect was less with the longest intervention delay. Furthermore, some cell death occurred much later with hypothermic treatment (e.g., at 60 days). Despite this delay, the cell death had similar morphological features to that observed after untreated ischemia (Fig. 8.1), which was complete at 4 days post-ischemia. Interestingly, a few (<1%) rescued neurons did have signs of increased autophagy (e.g., mitochondrial autolysosomes), but these occurred in otherwise healthy-looking neurons. Macroautophagy was not readily found in non-ischemic gerbils that were cooled. Signs of microautophagy (e.g., accumulation of secondary lysosomes), however, were found in untreated ischemic neurons that also suffered from other forms of injury (e.g., dilated mitochondria). Unfortunately, from these findings it cannot be determined whether autophagy contributed to cell death or was activated to help prevent it, as suggested by work in a neonatal hypoxia-ischemia study (Carloni et al. 2008).

The aforementioned studies and comprehensive reviews (Martin et al. 1998) support the view that neuronal death after global ischemia is necrotic, not apoptotic. This has been the general finding regardless of insult severity, brain structure, species, ischemia model, survival time, and the presence of a neuroprotectant (hypothermia) to mitigate and/or slow the injury. Considering the tens of thousands of cells examined in these studies, one would expect some apoptotic neurons and subsequent apoptotic bodies even if only a small percentage of neurons succumbed in that manner, but this has not been unequivocally demonstrated. Several studies have argued that cell death is apoptotic based upon their morphological findings (Nitatori et al. 1995; Pagnussat et al. 2007; Ruan et al. 2003; Zeng and Xu 2000). For instance, one study, which varied the duration of 4-VO ischemia, claimed that apoptosis occurred in <10% of dying striatal neurons after the milder insult (Ruan et al. 2003). However, in none of these studies has definitive morphological evidence been presented, including the crescent or rounded-shaped chromatin followed by the formation of membrane-delimited apoptotic bodies. Instead some have misinterpreted chromatin condensation alone as sufficient evidence for apoptosis

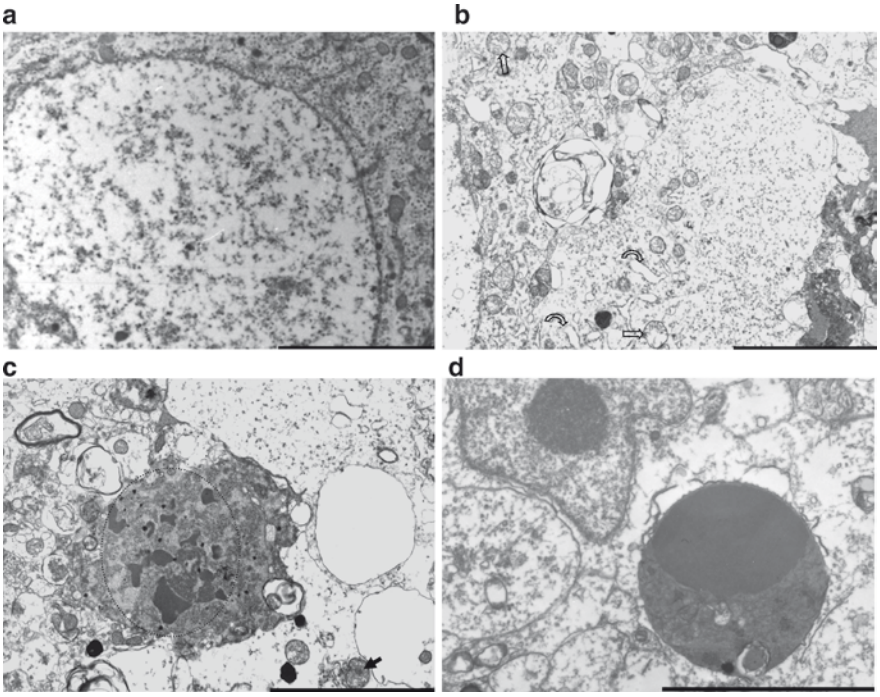

Fig. 8.1 Electron microscopic findings of hippocampal CA1 pyramidal neurons after 5 min of normothermic forebrain ischemia in gerbils (data from Colbourne et al. 1999b). (**a**) Normal CA1 neuron from a sham-operated gerbil. (**b**) Pre-lethal signs of injury include, for example, swollen mitochondria (*hollow straight arrows*) and dilated rough endoplasmic reticulum (*curved hollow arrows*), occur after untreated ischemia. Compare this to the normal looking mitochondria and rough endoplasmic reticulum found in the sham operated animal. (**c**) Neuronal necrosis at 4 days following untreated ischemia illustrating mitochondrial flocculent densities (*solid arrow*) and a mostly amorphous cytoplasm. The irregular, diffuse chromatin condensation (delineated by an oval) is unlike that seen in apoptosis. Although there was less cell death in animals treated with post-ischemic hypothermia, the pre-lethal and lethal signs of injury (necrosis) were comparable to untreated ischemic gerbils. (**d**) For comparison, an apoptotic cell with a distinct round chromatin condensation is illustrated, which was observed in a naïve gerbil brain at postnatal day 6. Apoptotic-looking neurons or apoptotic bodies were not found after ischemia in adult gerbils, regardless of treatment or survival time. Photos reprinted with permission from Highwire Press. Scale bar = 5 μm

despite the presence of signs of necrosis (e.g., membrane breaks, mitochondrial flocculent densities) and differences in the pattern of chromatin condensation. Moreover, dark neurons, a widespread histological problem in neuroscience (Jortner 2006; Kherani and Auer 2008), have been mistakenly identified as apoptotic. Besides, intracellular densities resembling autophagic vacuoles have been mislabeled as apoptotic bodies. Finally, it must also be kept in mind that even if a few neurons have died by apoptosis in these studies, they are in the minority despite use of intentionally mild insults in many studies.

8.5 Summary, Limitations and Future Directions

There are a number of important limitations to the present review. First, our discussion on the morphological features of cell death after global ischemia was restricted to neuronal injury. Death of glia (astrocytes, oligodendroglia and microglia) must also be considered in the quest to limit ischemic injury with cytoprotectants. Indeed, it has been argued that glial but not neuronal apoptosis occurs in post-ischemic brain (Martin et al. 1998; Petito et al. 1998). Furthermore, the death of invading leukocytes (e.g., macrophages) must be considered as their apoptotic remnants might be mislabeled as apoptotic neurons. Second, we have limited our discussion to adult animals. Programmed cell death, specifically apoptosis, is more likely in developing organisms. Third, neurogenesis has been reported after cerebral ischemia, including in the CA1 zone (Bendel et al. 2005; Nakatomi et al. 2002; Schmidt and Reymann 2002), although many reports do not find this. An interesting question is whether the death of these newly-generated neurons (Bueters et al. 2008) occurs via programmed cell death mechanisms and whether this is apoptotic or necrotic in appearance.

While insight into the mechanisms of ischemic injury is gained from morphology studies, such findings must be considered in tandem with biochemical data. Notably, the consistent observation of neuronal necrosis after global ischemia does not exclude a contribution of programmed cell death mechanisms. There are many deleterious mechanisms set in motion by ischemia, and this undoubtedly includes programmed cell death mediators (e.g., apoptosis-inducing factor release from mitochondria (Boujrad et al. 2007). The question is whether such pathways are causal to cell death or are merely activated concomitantly with many other pathways. We expect, certainly in cases of more severe ischemia mimicking cardiac arrest, that the latter situation is true. If so, the activation of programmed cell death pathways is akin to taking a suicide pill just prior to being executed by firing squad. The reliance upon mild global ischemic insults in arterial occlusion models is conceptually similar to the use of very brief focal ischemic insults. In both cases, fewer cells tend to die, they take longer to succumb, and perhaps cells express more markers for programmed cell death. However, is this situation really going to predict clinical outcome? Thus, we encourage investigators to consider using more clinically relevant insults (e.g., cardiac arrest models, permanent focal ischemic insults) in their assessment of treatments targeting programmed cell death. Such studies must also be careful to avoid problems encountered with other neuroprotectants (e.g., NMDA-receptor antagonists). For instance, physiological variables should be controlled (e.g., to prevent unintentional hypothermia) and long-term functional and histological outcome must be assessed.

In summary, the majority of studies examining the ultrastructural morphology of cell death following global ischemia report features more consistent with neuronal necrosis; although some differences exist among studies and brain regions. Only a few studies claim to have observed apoptotic neuronal morphology, but they either do not present convincing evidence or they misinterpret the morphology. Further

work is needed to link the morphology of ischemic cell death with mechanisms of action. Such studies should take into account the pattern and extent of injury commonly found after cardiac arrest and other causes of global ischemia in humans.

References

Andrabi SA, Dawson TM, Dawson VL (2008) Mitochondrial and nuclear cross talk in cell death: parthanatos. Ann N Y Acad Sci 1147:233–241

Bendel O, Bueters T, von Euler M et al (2005) Reappearance of hippocampal CA1 neurons after ischemia is associated with recovery of learning and memory. J Cereb Blood Flow Metab 25(12):1586–1595

Bonfoco E, Krainc D, Ankarcrona M et al (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults after *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci USA 92:7162–7166

Boujrad H, Gubkina O, Robert N et al (2007) AIF-mediated programmed necrosis: a highly regulated way to die. Cell Cycle 6:2612–2619

Brahma MK, Dohare P, Varma S et al (2009) The neuronal apoptotic death in global cerebral ischemia in gerbil: important role for sodium channel modulator. J Neurosci Res 87:1400–1411

Brierley J, Adams J, Graham D et al (1971) Neocortical death after cardiac arrest. A clinical, neurophysiological, and neuropathological report of two cases. Lancet 2:560–565

Brown A, Brierley J (1972) Anoxic-ischemic cell change in rat brain. Light microscopic and fine-structural observations. J Neurol Sci 16:59–84

Bueters T, von Euler M, Bendel O et al (2008) Degeneration of newly formed CA1 neurons following global ischemia in the rat. Exp Neurol 209:114–124

Cao G, Xing J, Xiao X et al (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. J Neurosci 27:9278–9293

Carboni S, Antonsson B, Gaillard P et al (2005) Control of death receptor and mitochondrial-dependent apoptosis by c-Jun N-terminal kinase in hippocampal CA1 neurones following global transient ischaemia. J Neurochem 92:1054–1060

Carloni S, Buonocore G, Balduini W (2008) Protective role of autophagy in neonatal hypoxia-ischemia induced brain injury. Neurobiol Dis 32:329–339

Chu CT (2006) Autophagic stress in neuronal injury and disease. J Neuropathol Exp Neurol 65:423–432

Colbourne F, Li H, Buchan AM (1999a) Continuing postischemic neuronal death in CA1: influence of ischemia duration and cytoprotective doses of NBQX and SNX-111 in rats. Stroke 30:662–668

Colbourne F, Sutherland GR, Auer RN (1999b) Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J. Neurosci. 19:4200–4210

Cole G, Cowie VA (1987) Long survival after cardiac arrest: case report and neuropathological findings. Clin Neuropathol 6:104–109

David KK, Andrabi SA, Dawson TM et al (2009) Parthanatos, a messenger of death. Front Biosci 14:1116–1128

Deshpande J, Bergstedt K, Lindén T et al (1992) Ultrastructural changes in the hippocampal CA1 region following transient cerebral ischemia: evidence against programmed cell death. Exp Brain Res 88:91–105

Dietrich WD, Busto R, Alonso O et al (1993) Intraischemic but not postischemic brain hypothermia protects chronically following global forebrain ischemia in rats. J Cereb Blood Flow and Metab 13:541–549

Du C, Hu R, Csernansky CA et al (1996) Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? J Cereb Blood Flow and Metab 16:195–201

Harmon BV, Corder AM, Collins RJ et al (1990) Cell death induced in a murine mastocytoma by 42–47 degrees C heating in vitro: evidence that the form of death changes from apoptosis to necrosis above a critical heat load. Int J Radiat Biol 58:845–858
Hayashi T, Saito A, Okuno S et al (2005) Damage to the endoplasmic reticulum and activation of apoptotic machinery by oxidative stress in ischemic neurons. J Cereb Blood Flow Metab 25:41–53
Horn M, Schlote W (1992) Delayed neuronal death and delayed neuronal recovery in the human brain following global ischemia. Acta Neuropathol 85:79–87
Hossmann KA, Oschlies U, Schwindt W et al (2001) Electron microscopic investigation of rat brain after brief cardiac arrest. Acta Neuropathol (Berl) 101:101–113
Ikeda H, Suzuki Y, Suzuki M et al (1998) Apoptosis is a major mode of cell death caused by ischaemia and ischaemia/reperfusion injury to the rat intestinal epithelium. Gut 42:530–537
Ito U, Spatz M, Walker JT Jr et al (1975) Experimental cerebral ischemia in mongolian gerbils. I. Light microscopic observations. Acta Neuropathol (Berl) 32:209–223
Johansen FF, Jorgensen MB, Ekstrom von Lubitz DK et al (1984) Selective dendrite damage in hippocampal CA1 stratum radiatum with unchanged axon ultrastructure and glutamate uptake after transient cerebral ischaemia in the rat. Brain Res 291:373–377
Jortner BS (2006) The return of the dark neuron. A histological artifact complicating contemporary neurotoxicologic evaluation. Neurotoxicology 27:628–634
Kalimo H, Garcia JH, Kamijyo Y et al (1977) The ultrastructure of "brain death". II. Electron microscopy of feline cortex after complete ischemia. Virchows Arch B Cell Pathol 25:207–220
Kerr JF (1971) Shrinkage necrosis: a distinct mode of cellular death. J Pathol 105:13–20
Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257
Kherani ZS, Auer RN (2008) Pharmacologic analysis of the mechanism of dark neuron production in cerebral cortex. Acta Neuropathol 116:447–452
Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 239:57–69
Kirino T, Sano K (1984) Fine structural nature of delayed neuronal death following ischemia in the gerbil hippocampus. Acta Neuropathol (Berl) 62:209–218
Kirino T, Tamura A, Sano K (1984) Delayed neuronal death in the rat hippocampus following transient forebrain ischemia. Acta Neuropathol (Berl) 64:139–147
Kroemer G, Levine B (2008) Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol 9:1004–1010
Lai Y, Hickey RW, Chen Y et al (2008) Autophagy is increased after traumatic brain injury in mice and is partially inhibited by the antioxidant gamma-glutamylcysteinyl ethyl ester. J Cereb Blood Flow Metab 28:540–550
Laidley DT, Colbourne F, Corbett D (2005) Increased behavioral and histological variability arising from changes in cerebrovascular anatomy of the mongolian gerbil. Curr Neurovasc Res 2:401–407
Levine B, Yuan J (2005) Autophagy in cell death: an innocent convict? J Clin Invest 115:2679–2688
Longstreth WT Jr, Inui TS, Cobb LA et al (1983) Neurologic recovery after out-of-hospital cardiac arrest. Ann Intern Med 98:588–592
MacLellan CL, Clark DL, Silasi G et al (2009) Use of prolonged hypothermia to treat ischemic and hemorrhagic stroke. J Neurotrauma 26:313–323
Majno G, Joris I (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 146:3–15
Martin LJ, Al-Abdulla NA, Brambrink AM et al (1998) Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A perspective on the contributions of apoptosis and necrosis. Brain Res Bull 46:281–309
Nakatomi H, Kuriu T, Okabe S et al (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 110:429–441
Nitatori T, Sato N, Waguri S et al (1995) Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. J Neurosci 15:1001–1011

Ostrowski RP, Graupner G, Titova E et al (2008) The hyperbaric oxygen preconditioning-induced brain protection is mediated by a reduction of early apoptosis after transient global cerebral ischemia. Neurobiol Dis 29:1–13

Pagnussat AS, Faccioni-Heuser MC, Netto CA et al (2007) An ultrastructural study of cell death in the CA1 pyramidal field of the hippocapmus in rats submitted to transient global ischemia followed by reperfusion. J Anat 211:589–599

Petito CK, Feldmann E, Pulsinelli WA et al (1987) Delayed hippocampal damage in humans following cardiorespiratory arrest. Neurology 37:1281–1286

Petito CK, Olarte JP, Roberts B et al (1998) Selective glial vulnerability following transient global ischemia in rat brain. J Neuropathol Exp Neurol 57:231–238

Petito CK, Pulsinelli WA (1984a) Delayed neuronal recovery and neuronal death in rat hippocampus following severe cerebral ischemia: possible relationship to abnormalities in neuronal processes. J Cereb Blood Flow Metab 4:194–205

Petito CK, Pulsinelli WA (1984b) Sequential development of reversible and irreversible neuronal damage following cerebral ischemia. J Neuropathol Exp Neurol 43:141–153

Pulsinelli WA, Brierley JB (1979) A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 10:267–272

Pulsinelli WA, Brierley JB, Plum F (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 11:491–498

Rafols JA, Daya AM, O'Neil BJ et al (1995) Global brain ischemia and reperfusion: Golgi apparatus ultrastructure in neurons selectively vulnerable to death. Acta Neuropathol 90:17–30

Ruan YW, Ling GY, Zhang JL et al (2003) Apoptosis in the adult striatum after transient forebrain ischemia and the effects of ischemic severity. Brain Res 982:228–240

Schmidt W, Reymann KG (2002) Proliferating cells differentiate into neurons in the hippocampal CA1 region of gerbils after global cerebral ischemia. Neurosci Lett 334:153–156

Sheldon RA, Hall JJ, Noble LJ et al (2001) Delayed cell death in neonatal mouse hippocampus from hypoxia-ischemia is neither apoptotic nor necrotic. Neurosci Lett 304:165–168

Siebke H, Rod T, Breivik H et al (1975) Survival after 40 minutes; submersion without cerebral sequeae. Lancet 1:1275–1277

Smith ML, Bendek G, Dahlgren N et al (1984) Models for studying long-term recovery following forebrain ischemia in the rat. 2. A 2-vessel occlusion model. Acta Neurol Scand 69:385–401

Sperandio S, de Belle I, Bredesen DE (2000) An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci U S A 97:14376–14381

Trump BF, Goldblatt PJ, Stowell RE (1965) Studies of mouse liver necrosis in vitro. Ultrastructural and cytochemical alterations in hepatic parenchymal cell nuclei. Lab Invest 14:1969–1999

Wang Y, Dawson VL, and Dawson TM, 2009. Poly(ADP-ribose) signals to mitochondrial AIF: A key event in parthanatos. Exp Neurol 218:193–202

Winkelmann ER, Charcansky A, Faccioni-Heuser MC et al (2006) An ultrastructural analysis of cellular death in the CA1 field in the rat hippocampus after transient forebrain ischemia followed by 2, 4 and 10 days of reperfusion. Anat Embryol (Berl) 211:423–434

Yamamoto K, Hayakawa T, Mogami H et al (1990) Ultrastructural investigation of the CA1 region of the hippocampus after transient cerebral ischemia in gerbils. Acta Neuropathol (Berl) 80:487–492

Yamashima T, Tonchev AB, Borlongan CV (2007) Differential response to ischemia in adjacent hippocampalsectors: neuronal death in CA1 versus neurogenesis in dentate gyrus. Biotechnol J 2:596–607

Yamashima T, Tonchev AB, Tsukada T et al (2003) Sustained calpain activation associated with lysosomal rupture executes necrosis of the postischemic CA1 neurons in primates. Hippocampus 13:791–800

Zeng YS, Xu ZC (2000) Co-existence of necrosis and apoptosis in rat hippocampus following transient forebrain ischemia. Neurosci Res 37:113–125

Zola-Morgan S, Squire LR, Amaral DG (1986) Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. J Neurosci 6:2950–2967

Chapter 9
Apoptosis-Inducing Factor Translocation to Nuclei After Transient Global Ischemia

Can Liu, Armando P. Signore, Guodong Cao, and Jun Chen

9.1 Introduction

Global cerebral ischemia produces both acute and delayed neuronal cell death. The loss of mitochondrial membrane integrity and the subsequent release of apoptogenic factors are critical in mediating the intrinsic, or mitochondrial, neurodegenerative pathway (Fujimura et al. 1999; Sugawara et al. 1999; Cao et al. 2003). Both caspase-dependent and -independent prodeath effector pathways can be initiated by the intrinsic pathway (Graham and Chen 2001). The key signaling molecule, released by mitochondria that initiates the caspase-independent route is apoptosis-inducing factor (AIF), a mitochondrial-specific flavoprotein that normally resides in the intermembrane space. Following global ischemia, AIF is truncated by calpain, allowing it to translocate from compromised mitochondria to the nucleus, where it degrades the nuclear genome (Fig. 9.1a–f). Along with freed AIF, endonuclease G (EndoG) is also released from mitochondria and mediates early chromatinolysis in neurons (Susin et al. 1999).

Transient global ischemia can be produced in rodents using several models. The essential feature of these models is a delayed (24–48 h) but selective loss of neurons in the CA1 region of the hippocampus (for review, see Zhang et al. 2004). Cell death in these models occurs at least in part via an apoptotic mechanism (Jin et al. 1999). Current knowledge of programmed cell death signaling pathways has resulted in a revised definition and classification of ischemia-induced cell death that now recognizes contributions from both the necrotic and apoptotic pathways. Understanding the relative contribution of the signaling pathways activated by each of the cell death mechanisms in ischemia-induced neurodegeneration, is therefore critical for identifying potential therapeutic targets and developing successful treatments.

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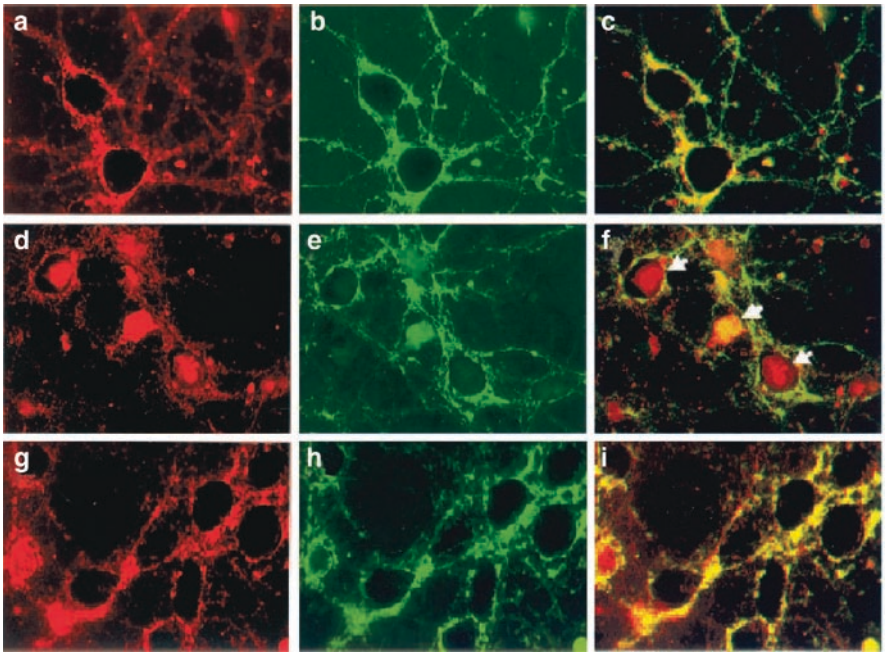

Fig. 9.1 AIF is translocated to the nucleus following oxygen glucose deprivation (OGD). Immunofluorescent staining of AIF in cultured primary cortical neurons after OGD. Cortical cultures were subjected to 60 min of OGD and then cultured under normal conditions for 6 h. The cells were incubated in the mitochondrial marker Mitotracker Green (*green*) and then immunostained for AIF (*red*). In the control neurons (**a–c**), AIF and Mitotracker fluorescence are largely overlaid, showing a cytoplasmic pattern. After OGD (**d–f**), AIF shows a nuclear localization in many neurons (*arrows* in f). In contrast, the addition of PTD-Bcl-xl protein (50 g/ml) inhibits OGD-induced nuclear translocation of AIF (**g–i**) (From Cao et al. 2007)

The "classic" view of cell death in mammalian cells largely envisioned two distinct processes with mechanisms that shared little in common: apoptosis and necrosis. Apoptosis is the process whereby cell death results from a controlled, energy-dependent and well-orchestrated degradation of cellular structures from within the cell itself. It was first described in development where apoptosis induces the loss of extraneous tissue in the molding of body and organ structures, and is exemplified by the morphological condensation of the nucleus, followed by the active dismantling of cellular components. The second broadly defined process is necrosis, usually referred to as an uncontrolled or nonregulated death of cells because of sudden and accidental irreversible damage. The distinction between these modes of death has recently been blurred, and cell death is now described as a continuum of programmed cell death pathways that show characteristics from each type of cell death (Bredesen et al. 2006; Boujrad et al. 2007; Golstein and Kroemer 2007).

In cerebral ischemia and reperfusion, features of both apoptosis and necrosis occur (Muller et al. 2007; Pagnussat et al. 2007). The pathological process that an

individual neuron follows depends on the type, intensity and duration of the cell death stimuli (Pagnussat et al. 2007). The harmful release of AIF depends in large part upon the health status of the mitochondria. Therefore, the greater the mitochondrial injury, the more likely AIF will be actively involved in producing cell death. This chapter now focuses on AIF and the mechanisms by which AIF activation occurs in the global cerebral ischemia model.

9.1.1 Physiological Functions of AIF

The discovery of many of the normal functions of AIF was aided a great deal by a fortunate mutation in the mouse. The AIF-deficient mouse, called Harlequin, was found to harbor a viral insertion in AIF, which diminished AIF expression to 20% or less in mutant *Hq/Hq* mice compared to wild type mice (Klein et al. 2002). Neuronal degeneration, particularly in the cerebellum and retina, are hallmarks of the *Hq/Hq* mouse. Study of *Hq/Hq* mice was useful in determining several physiological functions of AIF, including an important NAD oxidase activity that uses nicotinamide adenine dinucleotide (NAD^+) as a cofactor (Susin et al. 1999). A deficit in AIF expression causes Complex I dysfunction and impaired oxidative phosphorylation, evidenced by increased dependence on glucose metabolism, and followed by progressive multifocal neuropathology (Vahsen et al. 2004; El Ghouzzi et al. 2007). Loss of central neurons was due to both reduced neuronal survival during brain development and increased oxidative radical activity (Cheung et al. 2006). Increased sensitivity to oxidative stress occurs in *Hq/Hq* neurons to both endogenous and exogenous peroxides, and neurons can aberrantly re-enter the cell cycle (Klein et al. 2002). The loss of cells in Harlequin mice is specific to the brain and retina, and do not appear to occur in the heart or liver, despite little AIF expression in these tissues as well (Vahsen et al. 2004). In addition, AIF plays an important role in maintaining the integrity of mitochondrial structure via preserving Complex I and III expression, most likely via post-translational mechanisms (Vahsen et al. 2004).

Given that caspase-independent cell death requires AIF activation, several studies have shown neuroprotective effects by either neutralizing intracellular AIF or genetically reducing the expression of AIF (Yu et al. 2002; Culmsee et al. 2005; Cao et al. 2007). In transient global ischemia, total AIF expression levels per se are not significantly altered (Cao et al. 2003). Instead, regulation of AIF activation occurs via its *N*-terminal truncation and translocation from the mitochondria to the nucleus. The mature mitochondrial AIF is known to be 62 kDa, whereas AIF that is released into the cytosol is about 57 kDa, indicating a specific truncation occurs before release from mitochondria (Cao et al. 2007). The discharge of AIF from mitochondria is also dependent on the death-promoting Bcl-2 family members, Bax and Bid (Cregan et al. 2002; van Loo et al. 2002; Culmsee et al. 2005). Through direct interaction with genomic DNA along with the activity of EndoG, AIF leads to chromatin condensation (Cande et al. 2002; Ye et al. 2002).

9.2 AIF Translocation Mechanism and Therapeutic Targets

9.2.1 The Time Course of AIF Translocation

AIF-mediated cell death is an energy-dependent process. After the ischemic brain has passed through the period of greatest energy depletion, the delivery of energy by reperfusion allows neuronal death to occur (Pagnussat et al. 2007). The time course for nuclear translocation of AIF to occur after experimental stroke, varies with the severity of injury. AIF translocation into the nucleus did not occur until after 6 h of reperfusion following short (30 min) MCAO in mice, but was seen following as little as 20 min of reperfusion after longer (1 h) MCAO. When 2 h of MCAO was used instead, AIF translocation was again delayed until 6 h reperfusion (Plesnila et al. 2004; Li et al. 2007). There appears to be, at least in rodents, a level of ischemic insult that maximally induces the nuclear translocation of AIF. This variability may contribute to the inconsistency seen in brain damage following global ischemic events between animals and even humans.

9.2.2 Mechanism of AIF Release

9.2.2.1 PARP-1 and AIF

The activation and release of AIF by global ischemia occurs amidst a variety of stimuli, most of which promote neuronal death. As expected, several cascades contribute to AIF neurotoxicity, including the inappropriate activation of DNA reparative and proteolytic enzymes.

Our previous work showed that DNA single-strand breaks (SSBs) are a form of DNA damage induced early in neurons following cerebral ischemia and reperfusion (Chen et al. 1997). Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant and very active chromatin-associated enzyme involved in DNA repair and histone and other nuclear protein modifications. The enzyme relies on consumption of NAD^+ to form ADP-ribose polymers [for reviews see (Ame et al. 2004; Ha 2004)]. Enzyme activity is in fact activated by DNA strand breaks, and thus PARP-1 is a detector of DNA damage (Demurcia and Menissier 1994). Reperfusion following cerebral ischemia induces oxidative stimuli such as reactive oxygen species (ROS), which can lead to DNA damage that activate PARP-1 (Demurcia and Menissier 1994; Eliasson et al. 1997). PARP-1 is a crucial part of both apoptosis and necrosis for, while it is a reparative enzyme, its pathological over-activation can lead to cell death, generally by depletion of cellular NAD^+ and ATP (Szabo and Dawson 1998; Ha and Snyder 1999; Herceg and Wang 1999; Shall and de Murcia 2000; Yu et al. 2002). For example, in *N*-methyl-D-aspartic acid (NMDA)-mediated excitotoxicity, DNA damage and PARP-1 activity are nitric oxide (NO) dependent, which is activated at a very high level in this insult.

AIF activation and translocation to the nucleus also increases PARP-1 toxicity in a caspase-independent manner (Wang et al. 2004). Inhibition of PARP-1 overactivation should ameliorate cell death under these toxic conditions, and not surprisingly, PARP-1 knockout animals show protection from stroke (Eliasson et al. 1997; Endres et al. 1997; Goto et al. 2002). It is possible to prevent the death of even highly sensitive hippocampal CA1 neurons after transient ischemia by administering the PARP inhibitor PJ34 as late as 8 h after ischemia (Hamby et al. 2007). The PARP-1 inhibitor 3-AB protects neurons against necrosis and is dependent on the duration of the ischemic-reperfusion episode (Strosznajder and Walski 2004). In contrast, our previous work showed that when adequate cellular NAD^+ levels were maintained, inhibition of PARP-1 using 3-AB, diminished neuronal survival in the transient global ischemia model (Nagayama et al. 2000). Since caspase-3-mediated cleavage of PARP-1 blocks DNA repair and concomitantly prevents a depletion of cellular NAD^+ stores by PARP activity (Boulares et al. 1999; Herceg and Wang 1999), the precise role PARP-1 plays in cell death remains contentious, and may depend on which pathway is preferentially activated in individual neurons.

There is evidence demonstrating that the release of AIF from mitochondria is also dependent on PARP-1-initiated nuclear signals (Yu et al. 2002; Cipriani et al. 2005). Reperfusion accelerates the appearance of nuclear AIF after 1 h of MCAO compared with permanent MCAO, consistent with the possibility that early oxidant stress triggers the signaling pathways that stimulate AIF translocation (Li et al. 2007). Inhibition of PARP-1 and Bid reduces nuclear AIF translocation (Culmsee et al. 2005).

The signaling pathway from PARP to mitochondria requires receptor interaction protein 1 (RIP1), TNFR-associated factor 2 (TRAF2) and c-Jun *N*-terminal protein kinase-1 (JNK1) (Xu et al. 2006). The JNKs are a subfamily of mitogen-activated protein kinases (MAPKs). JNK1 has been reported to facilitate motoneuron health by maintaining the membrane potential of mitochondria (Newbern et al. 2007). The protective effect of JNK and the mitochondrial membrane potential however remains unclear (Newbern et al. 2007). In the gerbil transient global ischemia model, substantially increased amounts of active P-JNK can be found with the mitochondrial fraction isolated from ischemia-injured hippocampus (Zablocka et al. 2003).

The activation of PARP-1 in AIF-mediated neuronal death may not occur uniformly throughout the brain. Distinct from neurons in the caudate-putamen, reticular thalamus, thalamus and cortex, DNA SSBs are absent in CA1 neurons until 72 h after reperfusion, and occur simultaneously with DNA double strand breaks (DBSs) (Jin et al. 1999). AIF translocation has already occurred at this time. Thus, the mechanism of neuronal death in CA1 may be DNA strand break-independent and thus, not likely to involve PARP-1.

PARP-1 might also contribute to the gender differences found in cerebral ischemia that are not directly attributable to the neuroprotective effects of the female hormones. In the immature male brain, neurons display greater caspase-independent translocation of AIF after hypoxic ischemia, whereas female-derived neurons exhibit stronger activation of caspase-3 (Zhu et al. 2006). In addition, while male PARP-1 knockout

mice were protected from ischemia, female brain showed exacerbated histological injury after MCAO (McCullough et al. 2005).

The activation of AIF by DNA damage via PARP-1 mediated signals, thus occur with a significant number of variables due to length of insult, moment to moment levels of cell energy levels, cell-specific expression and gender. The final activation due to cellular damage can thus be highly variable and may spare some neurons for reasons not readily apparent.

9.2.2.2 Direct Activation of AIF: Truncation by Calpain

A number of cysteine proteases, including the caspases, cathepsins, and calpain, are activated in neurons after ischemic injury (Graham and Chen 2001). In AIF-mediated neuronal death, the key enzyme that activates AIF are the calpains. Calpain I, also known as μ-calpain, requires micromolar concentrations of calcium to be activated, while calpain II or m-calpain requires millimolar calcium, as measured in vitro. The cellular distribution of mRNA for calpain I and calpastatin, the endogenous calpain inhibitor, are relatively uniform throughout the mouse brain. On the other hand, calpain II gene expression is selectively higher in specific neuronal populations, including pyramidal neurons of the hippocampus (Li et al. 1996), which are the most sensitive population of neurons to global ischemia.

While calpain is a normal complement of cellular enzymatic activity, inappropriate- or over-activation will lead to pathophysiological activity and contribute to cell death following a number of different stressors. A major target for calpain is now known to be AIF. Calpain I cleaves AIF in a caspase-independent cell death manner in liver mitochondria (Polster et al. 2005) and the PC12 cell line (Liou et al. 2005), and our recent work shows that calpain I induces AIF release in the nervous system after both oxygen-glucose deprivation (OGD) in vitro and transient global ischemia (Fig. 9.2) (Cao et al. 2007). *N*-terminal truncation by calpain was in fact found to be a requirement for AIF activation. When neurons expressing a mutant form of AIF that could not be cleaved by calpain after OGD or global ischemia, AIF was not released from mitochondria and was not found in the nucleus (Fig. 9.3). This important finding shows that calpain is a direct activator of AIF release, and is specifically involved in caspase-independent cell death.

Calpain activity was previously thought to be limited to the cytosol; however, a new form of calpain, calpain-10, is a recently discovered calpain that is targeted at mitochondria. Calpain-10 is an important mediator of mitochondrial dysfunction via the cleavage of Complex I subunits and activation of the mitochondrial permeability transition pore (Arrington et al. 2006). There is ample evidence that a mitochondrial calpain exists and that it cleaves AIF, thus allowing AIF to leave the mitochondria (Garcia et al. 2005). The mitochondrial calpain most closely resembles μ-calpain, as anti-μ-calpain antibodies can also stain the mitochondrial calpain, and the calcium dependency of μ-calpain and mitochondria calpain are similar (Ozaki et al. 2007). Thus, the discovery of calpain activity in the mitochondria itself

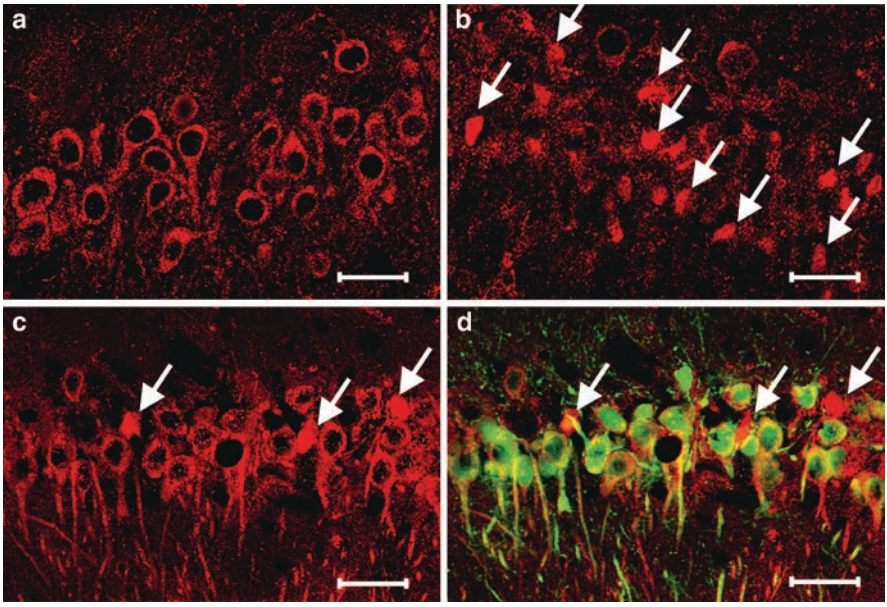

Fig. 9.2 AIF translocation in vivo following global ischemia is prevented by overexpression of calpastatin. Immunofluorescent images showing AIF (*red*) from nonischemic CA1 (**a**) or 72 h after global ischemia (**b–d**). AAV-calpastatin with an HA tag (c, d) or the empty vector (b) was infused 14 days before ischemia, and brain sections were double-label immunostained for AIF (*red*) and HA (*green*, d). Note that majority of CA1 neurons lost normal localization of AIF after ischemia (b, *arrows*), but AIF translocation was rare in calpastatin-overexpressed CA1 (c, d, *arrows*). Scale bars, 50 μm (From Cao et al. 2007)

shows that there is a direct link between mitochondrial dysfunction, elevated calcium levels and protease activity.

Calpains also cleave a number of other substrates crucial in the cell death process, illustrating how intertwined the different cell death pathways are in neurons. For example, calpain II can trigger the ischemia-induced lysosomal release of cathepsins in brain (Windelborn and Lipton 2008), and bring about the truncation of procaspase-3 into its active form (McGinnis et al. 1999; Blomgren et al. 2001). This latter pathway is particularly interesting, as caspase-3 in turn reduces calpastatin activity, the endogenous calpain inhibitor, to form a positive feedback loop, which can result in the further activation of calpain and subsequently, AIF release (Porn-Ares et al. 1998; Wang et al. 1998; Kato et al. 2000).

9.2.2.3 Release of AIF from Mitochondria: Formation of the Mitochondrial Outer Membrane Pore

Mitochondria can release a variety of death-promoting molecules in addition to AIF and EndoG, including cytochrome c, Smac/Diablo and Omi/HtrA2. The ability of

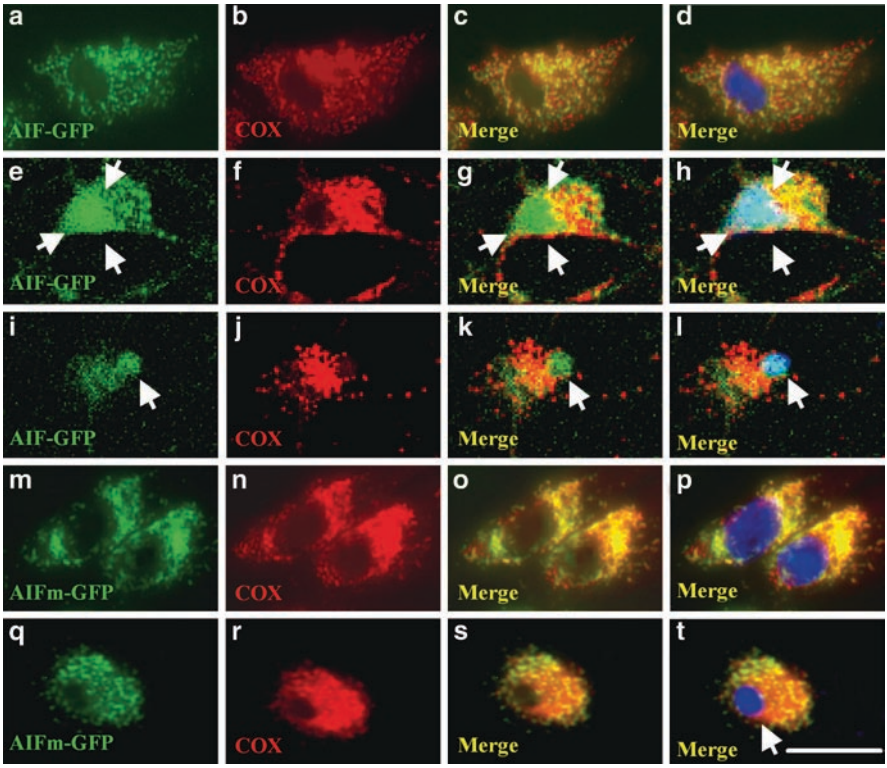

Fig. 9.3 AIF translocation following oxygen glucose deprivation (OGD) requires calpain-mediated truncation. Cultured primary cortical neurons were infected for 3 days with AAV carrying either an AIF-green fluorescent protein (GFP) fusion construct (**a–l**) or a calpain-resistant mutant (L101/103G) AIFm-GFP construct (**m–t**) and then subjected to OGD for 60 min. Confocal images were taken on triple-label immunofluorescent neurons (GFP, *green*; mitochondrial-specific protein COX IV, *red*; nuclear stain Hoechst 33258, *blue*) in non-OGD cultures (a–d, m–p) or at 2 h (e–h, q–t) and 6 h (i–l) after OGD. In non-OGD neurons, AIF-GFP and AIFm-GFP are localized in mitochondria; after OGD, AIF-GFP is translocated into the nucleus (e–l), whereas AIFm-GFP remains in the mitochondria (q–t). Scale bar, 25 μm (From Cao et al. 2007)

mitochondria to release these molecules depends largely on the formation of large and nonselective pores or channels through the two sets of membrane systems found in the mitochondria. The inner membrane forms the mitochondrial permeability transition pore, which is a calcium-dependent process and uses the proteins cyclophilin D, voltage-dependent anion channel and adenylate nucleotide translocase. The Bcl-2 family proteins including Bid, Bax and Bak and very likely other as yet unidentified proteins, form the outer membrane permeabilization pore, also called the mitochondrial apoptosis channel (for complete review, see Belizario et al. 2007). Many of the proteins involved must undergo proteolytic cleavage before they can form any type of functional channel, which is usually induced by stressors, including ischemia. For example, Bid is truncated by caspase-8 and translocates to

the outer mitochondrial membrane (Gross et al. 1999). The understanding of the mechanisms behind the formation of the mitochondrial death channels is still incomplete and under debate. The prevention of unwanted mitochondrial channels during normal physiological conditions is critical for maintaining normal organelle function and health. Regulation occurs via several antiapoptogenic members of the Bcl-2 protein family, including Bcl-2 and Bcl-xL (Breckenridge and Xue 2004). AIF translocation and cleavage is in fact inhibited by Bcl-2 and Bcl-xL overexpression (Fig. 9.1g–i) (Cao et al. 2003; Otera et al. 2005).

9.2.3 Regulation of AIF Activity in the Cytoplasm

9.2.3.1 AIF Function is Inhibited by Hsp70

Previous studies have shown that heat shock protein 70 (Hsp70) over-expression protects cells from death induced by various insults that cause either necrosis or apoptosis, including hypoxia and ischemia and reperfusion, by inhibiting multiple cell death pathways (Giffard and Yenari 2004). One of the mechanisms by which Hsp70 may be neuroprotective is evidenced by Hsp70 overexpression, resulting in the sequestration of AIF by Hsp70 (Ravagnan et al. 2001; Gurbuxani et al. 2003). The mechanisms include increased cytosolic retention of AIF when bound to Hsp70, thus limiting the entry of activated and cytotoxic AIF into the nucleus (Gurbuxani et al. 2003).

9.2.3.2 Ubiquitination of AIF via XIAP

X-linked inhibitor of apoptosis (XIAP) is an inhibitor of caspases and apoptosis (Suzuki et al. 2001). XIAP is also involved in the signal transduction and regulation of ubiquitin-ligase activity in the cell (Yamaguchi et al. 1999; Yang et al. 2000; Reffey et al. 2001). Recent work shows that XIAP may participate in the ubiquitinization of AIF (Wilkinson et al. 2008), and thus target AIF for proteosomal degradation.

9.2.4 AIF-Induced DNA Fragmentation

Although AIF is involved in the breakdown of neuronal DNA, AIF itself is devoid of any nuclease activity (Susin et al. 1999; Susin et al. 2000). AIF translocates to the nucleus where it directly interacts with DNA by virtue of positive charges, which are clustered on the surface of AIF. DNA binding is therefore required for the death-promoting action of AIF (Ye et al. 2002). The binding of AIF to DNA induces chromatin condensation by interacting directly with DNA and possibly

displacing chromatin-associated proteins. AIF could then disrupt normal chromatin structure, leading to the appearance of nuclear condensation. The remodeling of chromatin upon AIF interaction may increase the susceptibility of DNA to nucleases (Ye et al. 2002). The binding site within AIF is the same for distinct nucleic acid species, with no clear sequence specificity (Vahsen et al. 2006).

Endonuclease G (endoG) is another death-promoting element released from mitochondria, specifically along with AIF (Susin et al. 1999). In the cerebral cortex, 4 h after transient focal cerebral ischemia, endoG localization is significantly increased in the nucleus, corresponding with decreased mitochondrial endoG content. EndoG may also interact with AIF in the nucleus after transient focal cerebral ischemia (Lee et al. 2005). Thus, AIF truncation and release leads to disruption of neuronal genetic material via both direct (DNA binding) and indirect (EndoG) mechanisms.

9.2.4.1 Cyclophilins

Cyclophilins were first identified as the intracellular receptors for the immunosuppressant drug cyclosporin A (Handschumacher et al. 1984). Previous studies have shown that cyclophilins are involved in degradation of the genome during apoptosis (Montague et al. 1997). Cyclophilin A co-operates with AIF during apoptosis-associated chromatinolysis in vitro (Cande et al. 2004). Elimination of cyclophilin A affords neuroprotection in vivo, suggesting that the lethal translocation of AIF to the nucleus requires interaction with cyclophilin A (Zhu et al. 2007). Cyclophilin D on the other hand, is thought to be one of the components that forms the inner permeability transition pore, and is thus involved in the release of death-promoting factors from the mitochondria (see above). A clearer understanding of how cyclophilin D participates in the formation of the mitochondrial permeability transition pore will provide important answers to the role these proteins play in caspase-independent cell death.

9.3 Conclusions

Our knowledge about caspase-independent, AIF-induced cell death is far from complete. Although a large proportion of programmed cell death pathways involve the activation of caspases, inhibition of caspases alone as a therapeutic strategy is not sufficient to rescue damaged neurons. The caspase-independent pathway occurring via AIF elicits a complex network of signaling cascades that in and of themselves, can account for some of the specific cell death seen in the hippocampus following global ischemia. In vivo, the caspase-dependent and caspase-independent death pathways are, however, highly interconnected and often not easily distinguished from each other. The over-activation of calpains, the specific activator of AIF, may, however, be a viable target for drug intervention using calpain-specific inhibitors.

References

Ame JC, Spenlehauer C, de Murcia G (2004) The PARP superfamily. Bioessays 26:882–893

Arrington DD, Van Vleet TR, Schnellmann RG (2006) Calpain 10: a mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction. Am J Physiol Cell Physiol 291:C1159–C1171

Belizario JE, Alves J, Occhiucci JM, Garay-Malpartida M, Sesso A (2007) A mechanistic view of mitochondrial death decision pores. Braz J Med Biol Res 40:1011–1024

Blomgren K, Zhu CL, Wang XY, Karlsson JO, Leverin AL, Bahr BA, Mallard C, Hagberg H (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia – A mechanism of "pathological apoptosis"? J Biol Chem 276:10191–10198

Boujrad H, Gubkina O, Robert N, Krantic S, Susin SA (2007) AIF-mediated programmed necrosis – A highly regulated way to die. Cell Cycle 6:2612–2619

Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang GP, Iyer S, Smulson M (1999) Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis – Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem 274:22932–22940

Breckenridge DG, Xue D (2004) Regulation of mitochondrial membrane premeabilization by BCL-2 family proteins and caspases. Curr Opin Cell Biol 16:647–652

Bredesen DE, Rao RV, Mehlen P (2006) Cell death in the nervous system. Nature 443:796–802

Cande C, Cohen I, Daugas E, Ravagnan L, Larochette N, Zamzami N, Kroemer G (2002) Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie 84:215–222

Cande C, Vahsen N, Kouranti I, Schmitt E, Daugas E, Spahr C, Luban J, Kroemer RT, Giordanetto F, Garrido C, Penninger JM, Kroemer G (2004) AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. Oncogene 23:1514–1521

Cao GD, Clark RSB, Pei W, Yin W, Zhang F, Sun FY, Graham SH, Chen J (2003) Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation. J Cereb Blood Flow Metab 23:1137–1150

Cao GD, Xing J, Xiao X, Liou AKF, Gao YQ, Yin XM, Clark RSB, Graham SH, Chen J (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. J Neurosci 27:9278–9293

Chen J, Jin KL, Chen MZ, Pei W, Kawaguchi K, Greenberg DA, Simon RP (1997) Early detection of DNA strand breaks in the brain after transient focal ischemia: Implications for the role of DNA damage in apoptosis and neuronal cell death. J Neurochem 69:232–245

Cheung ECC, Joza N, Steenaart NAE, McClellan KA, Neuspiel M, McNamara S, MacLaurin JG, Rippstein P, Park DS, Shore GC, McBride HM, Penninger JM, Slack RS (2006) Dissociating the dual roles of apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis. EMBO J 25:4061–4073

Cipriani G, Rapizzi E, Vannacci A, Rizzuto R, Moroni F, Chiarugi A (2005) Nuclear poly(ADP-ribose) polymerase-1 rapidly triggers mitochondrial dysfunction. J Biol Chem 280:17227–17234

Cregan SP, Fortin A, MacLaurin JG, Callaghan SM, Cecconi F, Yu SW, Dawson TM, Dawson VL, Park DS, Kroemer G, Slack RS (2002) Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. J Cell Biol 158:507–517

Culmsee C, Zhu CL, Landshamer S, Becattini B, Wagner E, Pellecchia M, Blomgren K, Plesnila N (2005) Apoptosis-inducing factor triggered by poly(ADP-ribose) polymerase and bid mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia. J Neurosci 25:10262–10272

Demurcia G, Menissier J (1994) Poly(ADP-ribose) polymerase – a molecular nick-sensor (1994;19:172). Trends Biochem Sci 19:250–250

El Ghouzzi V, Csaba Z, Olivier P, Lelouvier B, Schwendimann L, Dournaud P, Verney C, Rustin P, Gressens P (2007) Apoptosis-inducing factor deficiency induces early mitochondrial degeneration in brain followed by progressive Multifocal neuropathology. J Neuropathol Exp Neurol 66:838–847

Eliasson MJL, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang ZQ, Dawson TM, Snyder SH, Dawson VL (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. Nat Med 3:1089–1095

Endres M, Wang ZQ, Namura S, Waeber C, Moskowitz MA (1997) Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. J Cereb Blood Flow Metab 17:1143–1151

Fujimura M, Morita-Fujimura Y, Kawase M, Copin JC, Calagui B, Epstein CJ, Chan PH (1999) Manganese superoxide dismutase mediates the early release of mitochondrial cytochrome C and subsequent DNA fragmentation after permanent focal cerebral ischemia in mice. J Neurosci 19:3414–3422

Garcia M, Bondada V, Geddes JW (2005) Mitochondrial localization of mu-calpain. Biochem Biophys Res Commun 338:1241–1247

Giffard RG, Yenari MA (2004) Many mechanisms for Hsp70 protection from cerebral ischemia. J Neurosurg Anesthesiol 16:53–61

Golstein P, Kroemer G (2007) Cell death by necrosis: towards a molecular definition. Trends Biochem Sci 32:37–43

Goto S, Xue R, Sugo N, Sawada M, Blizzard KK, Poitras MF, Johns DC, Dawson TM, Dawson VL, Crain BJ, Traystman RJ, Mori S, Hurn PD (2002) Poly(ADP-ribose) polymerase impairs early and long-term experimental stroke recovery. Stroke 33:1101–1106

Graham SH, Chen J (2001) Programmed cell death in cerebral ischemia. J Cereb Blood Flow Metab 21:99–109

Gross A, Yin XM, Wang K, Wei MC, Jockel J, Millman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-X-L prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 274:1156–1163

Gurbuxani S, Schmitt E, Cande C, Parcellier A, Hammann A, Daugas E, Kouranti I, Spahr C, Pance A, Kroemer G, Garrido C (2003) Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. Oncogene 22:6669–6678

Ha HC (2004) Defective transcription factor activation for proinflammatory gene expression in poly(ADP-ribose) polymerase 1-deficient glia. Proc Natl Acad Sci U S A 101:5087–5092

Ha HC, Snyder SH (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci U S A 96:13978–13982

Hamby AM, Suh SW, Kauppinen TM, Swanson RA (2007) Use of a poly (ADP-ribose) polymerase inhibitor to suppress inflammation and neuronal death after cerebral ischemia-reperfusion. Stroke 38:632–636

Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW (1984) Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 226:544–547

Herceg Z, Wang ZQ (1999) Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis. Mol Cell Biol 19:5124–5133

Jin KL, Chen J, Nagayama T, Chen MZ, Sinclair J, Graham SH, Simon RP (1999) In situ detection of neuronal DNA strand breaks using the Klenow fragment of DNA polymerase I reveals different mechanisms of neuron death after global cerebral ischemia. J Neurochem 72:1204–1214

Kato M, Nonaka T, Maki M, Kikuchi H, Imajoh-Ohmi S (2000) Caspases cleave the amino-terminal calpain inhibitory unit of calpastatin during apoptosis in human Jurkat T cells. J Biochem 127:297–305

Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, Bronson RT, Ackerman SL (2002) The harlequin mouse mutation down-regulates apoptosis-inducing factor. Nature 419:367–374

Lee BI, Lee DJ, Cho KJ, Kim GW (2005) Early nuclear translocation of endonuclease G and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. Neurosci Lett 386:23–27

Li JH, Grynspan F, Berman S, Nixon R, Bursztajn S (1996) Regional differences in gene expression for calcium activated neutral proteases (calpains) and their endogenous inhibitor calpastatin in mouse brain and spinal cord. J Neurobiol 30:177–191

Li X, Nemoto M, Xu Z, Yu SW, Shimoji M, Andrabi SA, Haince JF, Poirier GG, Dawson TM, Dawson VL, Koehler RC (2007) Influence of duration of focal cerebral ischemia and neuronal nitric oxide synthase on translocation of apoptosis-inducing factor to the nucleus. Neuroscience 144:56–65

Liou AKF, Zhou ZG, Pei W, Lim TM, Yin XM, Chen J (2005) BimEL up-regulation potentiates AIF translocation and cell death in response to MPTP. FASEB J 19:1350–1352

McCullough LD, Zeng ZY, Blizzard KK, Debchoudhury I, Hurn PD (2005) Ischemic nitric oxide and poly (ADP-ribose) polymerase-1 in cerebral ischemia: male toxicity, female protection. J Cereb Blood Flow Metab 25:502–512

McGinnis KM, Gnegy ME, Park YH, Mukerjee N, Wang KKW (1999) Procaspase-3 and poly(ADP)ribose polymerase (PARP) are calpain substrates. Biochem Biophys Res Commun 263:94–99

Montague JW, Hughes FM, Cidlowski JA (1997) Native recombinant cyclophilins A, B, and C degrade DNA independently of peptidylprolyl cis-trans-isomerase activity – Potential roles of cyclophilins in apoptosis. J Biol Chem 272:6677–6684

Muller GJ, Lassmann H, Johansen FF (2007) Anti-apoptotic signaling and failure of apoptosis in the ischemic rat hippocampus. Neurobiol Dis 25:582–593

Nagayama T, Simon RP, Chen DX, Henshall DC, Pei W, Stetler RA, Chen J (2000) Activation of poly(ADP-Ribose) polymerase in the rat hippocampus may contribute to cellular recovery following sublethal transient global ischemia. J Neurochem 74:1636–1645

Newbern J, Taylor A, Robinson M, Lively MO, Milligan CE (2007) c-Jun N-terminal kinase signaling regulates events associated with both health and degeneration in motoneurons. Neuroscience 147:680–692

Otera H, Ohsakaya S, Nagaura ZI, Ishihara N, Mihara K (2005) Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. EMBO J 24:1375–1386

Ozaki T, Tomita H, Tamai M, Ishiguro SI (2007) Characteristics of mitochondrial calpains. J Biochem 142:365–376

Pagnussat AD, Faccioni-Heuser MC, Netto CA, Achaval M (2007) An ultrastructural study of cell death in the CA1 pyramidal field of the hippocapmus in rats submitted to transient global ischemia followed by reperfusion. J Anat 211:589–599

Plesnila N, Zhu CL, Culmsee C, Groger M, Moskowitz MA, Blomgren K (2004) Nuclear translocation of apoptosis-inducing factor after focal cerebral ischemia. J Cereb Blood Flow Metab 24:458–466

Polster BM, Basanez G, Etxebarria A, Hardwick JM, Nicholls DG (2005) Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. J Biol Chem 280:6447–6454

Porn-Ares MI, Samali A, Orrenius S (1998) Cleavage of the calpain inhibitor, calpastatin, during apoptosis. Cell Death Differ 5:1028–1033

Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C, Kroemer G (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3:839–843

Reffey SB, Wurthner JU, Parks WT, Roberts AB, Duckett CS (2001) X-linked inhibitor of apoptosis protein functions as a cofactor in transforming growth factor-beta signaling. J Biol Chem 276:26542–26549

Shall S, de Murcia G (2000) Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? Mutat Res 460:1–15

Strosznajder RP, Walski M (2004) Effects 3-aminobenzamide on ultrastructure of hippocampal CA1 layer after global ischemia in gerbils. J Physiol Pharmacol 55(Suppl 3):S127–S133

Sugawara T, Fujimura M, Morita-Fujimura Y, Kawase M, Chan PH (1999) Mitochondrial release of cytochrome C corresponds to the selective vulnerability of hippocampal CA1 neurons in rats after transient global cerebral ischemia. J Neurosci 19:RC39

Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M, Costantini P, Ferri KF, Irinopoulou T, Prevost MC, Brothers G, Mak TW, Penninger J, Earnshaw WC, Kroemer G (2000) Two distinct pathways leading to nuclear apoptosis. J Exp Med 192:571–579

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446

Suzuki Y, Nakabayashi Y, Nakata K, Reed JC, Takahashi R (2001) X-linked inhibitor of apoptosis protein (XIAP) inhibits caspase-3 and-7 in distinct modes. J Biol Chem 276:27058–27063

Szabo C, Dawson VL (1998) Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. Trends Pharmacol Sci 19:287–298

Vahsen N, Cande C, Briere JJ, Benit P, Joza N, Larochette N, Mastroberardino PG, Pequignot MO, Casares N, Lazar V, Feraud O, Debili N, Wissing S, Engelhardt S, Madeo F, Piacentini M, Penninger JM, Schagger H, Rustin P, Kroemer G (2004) AIF deficiency compromises oxidative phosphorylation. EMBO J 23:4679–4689

Vahsen N, Cande C, Dupaigne P, Giordanetto F, Kroemer RT, Herker E, Scholz S, Modjtahedi N, Madeo F, Le Cam E, Kroemer G (2006) Physical interaction of apoptosis-inducing factor with DNA and RNA. Oncogene 25:1763–1774

van Loo G, Saelens X, van Gurp M, MacFarlane M, Martin SJ, Vandenabeele P (2002) The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. Cell Death Differ 9:1031–1042

Wang HM, Yu SW, Koh DW, Lew J, Coombs C, Bowers W, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2004) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. J Neurosci 24:10963–10973

Wang KKW, Posmantur R, Nadimpalli R, Nath R, Mohan P, Nixon RA, Talanian RV, Keegan M, Herzog L, Allen H (1998) Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. Arch Biochem Biophys 356:187–196

Wilkinson JC, Wilkinson AS, Galban S, Csomos RA, Duckett CS (2008) Apoptosis-inducing factor is a target for ubiquitination through interaction with XIAP. Mol Cell Biol 28:237–247

Windelborn JA, Lipton P (2008) Lysosomal release of cathepsins causes ischemic damage in the rat hippocampal slice and depends on NMDA-mediated calcium influx, arachidonic acid metabolism, and free radical production. J Neurochem 106:56–69. doi:10.1111/j.1471-4159.2008.05349.x

Xu Y, Huang S, Liu ZG, Han JH (2006) Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. J Biol Chem 281:8788–8795

Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K (1999) XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. EMBO J 18:179–187

Yang Y, Fang SY, Jensen JP, Weissman AM, Ashwell JD (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 288:874–877

Ye H, Cande C, Stephanou NC, Jiang SL, Gurbuxani S, Larochette N, Daugas E, Garrido C, Kroemer G, Wu H (2002) DNA binding is required for the apoptogenic action of apoptosis inducing factor. Nat Struct Biol 9:680–684

Yu SW, Wang HM, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Zablocka B, Dluzniewska J, Zajac H, Domanska-Janik K (2003) Opposite reaction of ERK and JNK in ischemia vulnerable and resistant regions of hippocampus: involvement of mitochondria (vol 110, pg 245, 2003). Mol Brain Res 113:143–143

Zhang F, Yin W, Chen J (2004) Apoptosis in cerebral ischemia: executional and regulatory signaling mechanisms. Neurol Res 26:835–845

Zhu CL, Wang XY, Deinum J, Huang ZH, Gao JF, Modjtahedi N, Neagu MR, Nilsson M, Eriksson PS, Hagberg H, Luban J, Kroemer G, Blomgren K (2007) Cyclophilin A participates in the nuclear translocation of apoptosis-inducing factor in neurons after cerebral hypoxia-ischemia. J Exp Med 204:1741–1748

Zhu CL, Xu FL, Wang XY, Shibata M, Uchiyama Y, Blomgren K, Hagberg H (2006) Different apoptotic mechanisms are activated in male and female brains after neonatal hypoxia-ischaemia. J Neurochem 96:1016–1027

Chapter 10
Role of μ-Calpain I and Lysosomal Cathepsins in Hippocampal Neuronal Necrosis After Transient Global Ischemia in Primates

Anton B. Tonchev and Tetsumori Yamashima

10.1 The Actors: Calpain I and Cathepsins B and L

Calpain (EC 3.4.22.17) and cathepsins B (EC 3.4.22.1) and L (EC 3.4.22.15) belong to the papain superfamily of cysteine proteases (Chapman et al. 1997). Calpain has cytosolic localization and is ubiquitously and constitutively expressed the mammalian cells (Sorimachi et al. 1997). Its two forms (μ-calpain; calpain I, and m-calpain; calpain II) have a different Ca^{2+} sensitivity, as μ-calpain is activated at micromolar while m-calpain is activated at millimolar Ca^{2+} concentrations. Calpains are heterodimers, consisting of 28 and 80 kDa subunits. Calpastatin, a selective endogenous calpain inhibitor, interacts with the Ca^{2+}-binding domains of both large and small subunits (Croall and Demartino 1991). Recently, a second calpain inhibitor has been identified that specifically inactivates m-calpain (Benetti et al. 2001). The 28-kDa subunit is identical in the μ-calpain and m-calpain molecules, while the 80-kDa subunits share 55–65% sequence homology within a given species (Goll et al. 2003). The large subunits are composed of four domains: domains II and IV are the cysteine protease and Ca^{2+}-binding domains, respectively. In contrast, the small subunits are divided into two domains: N-terminal glycine-clustering hydrophobic domain V- and C-terminal Ca^{2+}-binding domain VI, similar to domain IV of the large subunit. Each of domains IV and VI contains four EF-hand structures, similar to calmodulin (Sorimachi et al. 1997; Goll et al. 2003). The calpain/calpastatin system associates with the membranes of the endoplasmic reticulum, Golgi apparatus, and plasma membrane through hydrophobic interactions. Such specific compartmentalization is perhaps involved in providing access of calpain to Ca^{2+}-rich microdomains and to membrane-associated phosphoinositides, both involved in the activation of calpain enzymatic activity. Calpain is activated not only in physiological states but also during various pathological phenomena, such

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as free radical activation, brain ischemia-reperfusion, apoptosis, degenerative processes such as cataractogenesis, muscular dystrophy, Alzheimer's and Parkinson's diseases (reviewed by Yamashima 2004), and recently in autophagy (reviewed by Demarchi et al. 2007).

Cathepsins are localized predominantly to the lysosomes (cathepsins B, H, L, S, C and K), but can be found also in the nucleus (cathepsins B and L) and cytosol (cathepsins B and E) (Keppler et al. 1996). They are synthesized as inactive precursors and require proteolytic activation that generally proceeds in an acidic pH. Unlike calpains, they lack Ca^{2+}-binding domains. Various cathepsin inhibitors exist, the most abundant of which are cystatins. Cathepsin B is the most abundant lysosomal protease of the papain family, being required for the house-keeping function of lysosomes in protein turnover by cells. Cathepsin B is capable of gradually digesting cell proteins, nucleic acids, complex carbohydrates and lipids, and is related to the physiological turnover of cell proteins in neurons (Pope and Nixon 1984).

Numerous calpain and cathepsin substrates have been demonstrated, including proteins related to the cytoskeleton, extracellular matrix, growth factor signal transduction, transcription and cell cycle regulation, and apoptotic cascade proteins (reviewed in Tonchev and Yamashima 1999; Rami 2003; Stoka et al. 2007). It is worth noting that calpastatin itself is also a target of calpain, potentially leading to persistent calpain activation (Porn-Ares et al. 1998).

10.2 The Scene: Modes of Neuronal Demise in DND

A transient ischemic insult with a duration of 5–20 min (variable among species) is sufficient to cause a selective degeneration to certain sensitive brain regions. The CA1 sector of the hippocampus is considered to be the most sensitive region, and its postischemic neuronal loss is considered a hallmark of global brain ischemia. This phenomenon occurs a few days after the ischemic insult and is thus designated delayed neuronal death (DND) (Fig. 10.1). It can be induced experimentally in low-species animals in which it was first described (Kirino 1982; Pulsinelli et al. 1982), or can arise in humans as a consequence of cardiac arrest or cardiac surgery (Zola-Morgan et al. 1986; Petito et al. 1987). DND kills bilaterally selective neuronal populations, particularly the pyramidal neurons in the hippocampal CA1 sector.

An initial molecular event in the development of DND is the mobilization of Ca^{2+}. An excessive postischemic Ca^{2+} mobilization occurs in the CA1 sector (Deshpande et al. 1987; Martins et al. 1988; Mitani et al. 1994; Nakamura et al. 1993; Yamashima et al. 1996) via membrane ion channel (e.g., glutamate receptor) opening or from the intracellular stores via inositol 1,4,5-trisphosphate (IP_3) receptor. Elevated intracellular Ca^{2+} concentration in turn activates various molecular cascades leading to cell death. The type of cell demise in DND has been and still is a subject of debate, because since the time of the discovery of DND both apoptotic and necrotic types of cell death have been put forward to occur in postischemic CA1 neurons.

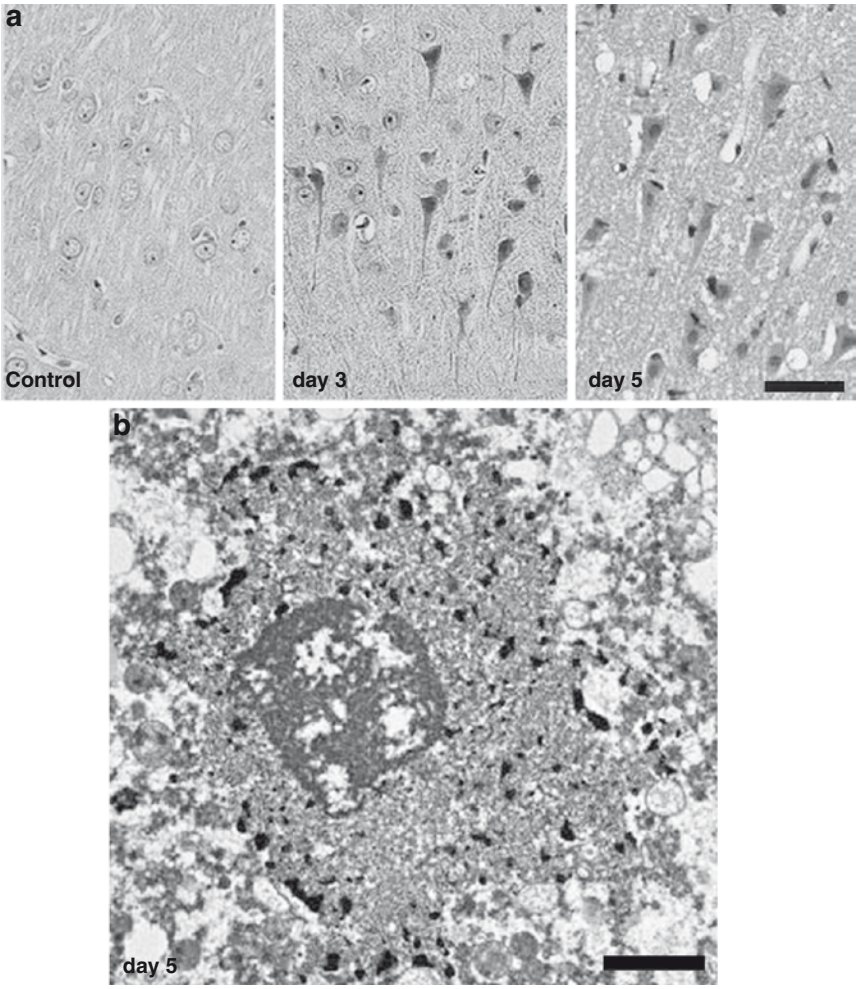

Fig. 10.1 Morphological features of necrotic DND in adult primates. Adult macaque monkeys were subjected to transient global cerebral ischemia for 20 min. (**a**) A series of light micrographs show hematoxylin and eosin staining of hippocampal CA1 sector in a control, and on postischemic days 3 and 5. Note the occurrence of eosinophilic coagulation necrosis on day 3. On day 5 the CA1 sector shows a complete neuronal death and secondary glial proliferation. Scale bar = 50 μm. (**b**) Ultrastructural features of neuronal degeneration on day 5. A CA1 neuron shows a large nucleolus and small, electron-dense chromatin clumps in the nudens, while the cytoplasm shows disruption of organelles and cell membranes. Note that coarse chromatin condensation called apoptotic body is not observed. Scale bar = 2 μm

Apoptosis is a programmed ATP-dependent process characterized by plasma membrane blebbing, retraction of cellular processes, chromatin condensation, fragmentation of DNA, and the formation of apoptotic bodies. It does not provoke inflammation and damage to the tissue. Its most recognized molecular markers are the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick

end-labeling (TUNEL) assay, detecting a specific type of DNA fragmentation and the caspase family of cysteine proteases, which are at the core of the initiation and execution of apoptosis. Necrosis begins with cellular and mitochondrial swelling, dilatation of the endoplasmic reticulum, and chromatin clumping into poorly defined masses (Fig. 10.1b). In contrast to apoptosis, necrosis is classically considered a poorly orchestrated and regulated process ending with organelle destruction and spillage of cellular constituents in the extracellular space, resulting in inflammation and tissue damage.

Necrosis and apoptosis exhibit different morphological, particularly ultrastructural, features. Typical light microscopic characteristic of necrosis is eosinophilic degeneration (shrunken eosinophilic cytoplasm; Fig. 10.1a) while at the electron microscopical level, the nucleus appears with a punctate chromatin distribution (Fig. 10.1b). Ultrastructurally, apoptosis is characterized by the formation of large round or crescent-shaped chromatin masses, while its other typical signs are detected primarily using molecular methods. It was mainly with such methods that various research groups reported that DND occurs primarily via the apoptotic pathway (MacManus et al. 1993; Nitatori et al. 1995; Li et al. 1997). However, careful ultrastructural investigation of postischemic CA1 neurons provided evidence against apoptosis in DND (Colbourne et al. 1999; see chapter 8 by Colbourne and Auer in this book for more details on apoptosis vs. necrosis in DND).

As the evidence on the mechanisms of cell death is accumulating, it is becoming evident that necrosis and apoptosis share some common mechanisms, at least in their early stages. On one hand, there is evidence for activation of apoptosis cascade players such as caspase-3 and the caspase-activated nuclease in necrosis (Tsukada et al. 2001) as well as for necrotic cell death by cell surface death receptors such as Fas and tumor necrosis factor receptor (reviewed by Golstein and Kroemer 2007). On the other hand, cathepsins, presumed necrotic pathway executioners, are activated in certain cases of apoptotic cell death (reviewed by Stoka et al. 2007). The picture is further complicated by data showing that necrosis is not always "accidental" and that it can occur in response to the activation of specific cellular signal transduction pathways, thus being "programmed" similarly to apoptosis (reviewed by Proskuryakov et al. 2003; Edinger and Thompson 2004). Thus, both caspase-dependent and caspase-independent cell death events can occur in DND (see the chapters "Caspase-Independent Cell Death Mechanisms in Simple Animal Models" by Rieckher and Tavernarakis, "Programmed Necrosis: A "New" Cell Death Outcome for Injured Adult Neurons?" by Krantic and Susin, and "Excitotoxic Programmed Cell Death Involves Caspase-Independent Mechanisms" by Kang et al. in this book for more details on programmed non-apoptotic cell death).

In addition to apoptosis and necrosis, a third type of cell death appears to be involved in postischemic neuropathology – autophagy (Kundu and Thompson 2008). Two major proteolytic systems contribute to the continuous removal of intracellular components: the ubiquitin/proteasome system and the autophagic/lysosomal system. Autophagy is a programmed, evolutionally conserved catabolic process by which the cytoplasmic contents of a cell are sequestered within double

membrane vacuoles, called autophagosomes, and subsequently delivered to the lysosome for degradation. The fact that autophagy is commonly observed in dying cells has suggested it as an alternative form of programmed cell death (reviewed by Debnath et al. 2005; Rubinsztein 2006). Constitutive ("basal") autophagy is an important protein quality control system, particularly in the nervous system. Its suppression in the nervous system leads to accumulation of abnormal proteins, followed by the generation of aggregates and inclusions, and ultimately to neurodegeneration (Hara et al. 2006; Komatsu et al. 2006). Autophagy was recently implicated in ischemic injury. It was shown to be induced under conditions of ischemia in the brain (Adhami et al. 2006). Further, Beclin1 and LC3, the important activators of autophagosome formation, are upregulated in neurons after stroke (Rami et al. 2008). Importantly, excessive autophagosome formation is induced in the early phase of necrotic cell death, and autophagy is required for necrotic cell death (Samara et al. 2008). Thus, interplay of apoptotic, necrotic and autophagic mechanisms appears to orchestrate the cellular fate toward demise or survival, and identifying how these molecular players are balanced may provide valuable therapeutic strategies for neuroprotection.

10.3 The Play: Calpain–Cathepsin Cascade in Primate DND

The "calpain–cathepsin hypothesis" of DND has been formulated using a primate model of global cerebral ischemia, in which the blood flow to the brain is transiently interrupted for 20 min in adult Japanese monkeys (*Macaca fuscata*) (reviewed by Yamashima 2000, 2004; Yamashima et al. 2007). It is characterized morphologically by eosinophilic degeneration starting at day 1 and progresses to nearly complete neuronal loss in the hippocampal CA1 sector at day 5 after injury (Fig. 10.1a). Ultrastructurally, formation of large round or crescent-shaped chromatin masses typical for apoptosis is not observed. In contrast, the CA1 neurons exhibit a necrotic pattern (Fig. 10.1b). The hypothesis is based on documentation of the following sequence of molecular events:

1. The maximal elevation of intracellular Ca^{2+} in monkey hippocampal organotypic slices was selectively localized to the CA1 sector, the area of DND in vivo (Yamashima et al. 1996).
2. The Ca^{2+}-dependent μ-calpain was found to be subsequently and selectively activated in CA1 neurons (Yamashima et al. 1996). Notably, calpain activation in vivo persisted long after Ca^{2+} levels have returned to the normal values (Yamashima et al. 2003).
3. Immunoelectron microscopy detected activated μ-calpain at the vacuolated or disrupted lysosomal membranes (Yamashima et al. 1996). Taken together, these data suggested that excessive calpain activation in postischemic CA1 neurons may cause lysosomal membrane disruption with the resultant release of lysosomal enzymes triggering the cascade of DND.

4. Both expression and enzyme activity of cathepsins B and L were increased in the CA1 sector after ischemia, concomitantly with the development of DND (Kohda et al. 1996). Importantly, the cathepsin B immunosignal showed an extralysosomal distribution in postischemic CA1 neurons (Yamashima et al. 1998), as did a lysosomal marker protein, lysosome-associated membrane protein-1 (LAMP-1) (Yamashima 2004).
5. The early postischemic application of a selective cathepsin B inhibitor reduced the immunoreactivity of cathepsin B in the lysosomal granules of CA1 neurons and rescued approximately two thirds of these cells even by the postictal administration (Yamashima et al. 1998). Further, the application of a combined (calpain plus cathepsin) inhibitor rescued nearly all CA1 neurons (Tsuchiya et al. 1999).

In summary (Fig. 10.2), this hypothesis states that Ca^{2+}-induced μ-calpain overactivation after transient global brain ischemia in primates leads to disruption of lysosomes. The resultant release of cathepsins contributes to the development of DND via degrading cell constituents (Yamashima et al. 1998, 2007; Yamashima 2000, 2004).

The role of lysosomes and cathepsins in necrosis and neurodegeneration received attention by the scientific community after the elegant studies by Tavernarakis and co-workers in *C. elegans*. They showed that the nematode analogs of calpain and two cathepsins are required for neurodegeneration and that altered lysosomal biogenesis and function markedly affect neuronal necrosis (Syntichaki et al. 2002;

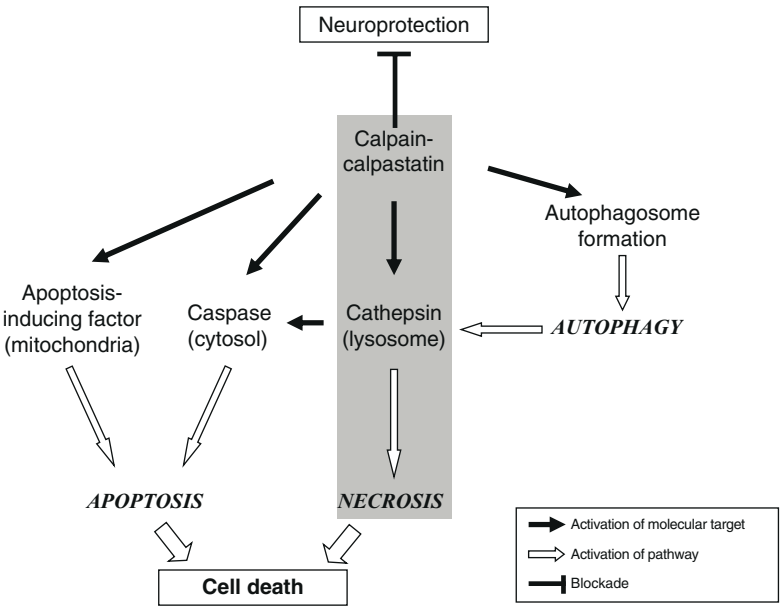

Fig. 10.2 A hypothetical model proposing a central role of the calpain–cathepsin cascade (in *gray*) in the regulation of four cell death programs. Further, the cascade may contribute to neuronal death also by blocking the intrinsic neuroprotective systems in the cell

Artal-Sanz et al. 2006). The same group also identified that autophagy contributes to cellular destruction during necrosis by synergizing with lysosomal catabolic mechanisms (Samara et al. 2008). Thus, initially proposed cascade for necrotic cell death, the "calpain–cathepsin hypothesis" appears to be involved also in the autophagic program of cell demise.

10.4 The Future: Calpain–Cathepsin Cascade at the Core of Cell Death Machinery?

Enzymatic proteolysis appears to be crucially implicated in diverse neuropathological conditions including acute and chronic neurodegenerative diseases. Caspase and calpain–cathepsin cascades appear to be central for these conditions. However, the two systems are not independent and autonomous. Numerous sites of interaction between them have been identified, such as calpain or cathepsin activation working upstream of caspases or caspase-mediated calpastatin degradation that in turn leads to persistent calpain activation (reviewed in Tonchev and Yamashima 1999; Yamashima 2000; Rami 2003; Stoka et al. 2007). Interestingly, calpain is capable of triggering mitochondrial-dependent neuronal death program in CA1 neurons via a caspase-independent pathway involving a direct activation by calpain of apoptosis-inducing factor (AIF) (Cao et al. 2007). Further, calpain is required for autophagosome formation, and autophagy is severely compromised in calpain I-deficient cells (Demarchi et al. 2006, 2007). These data place calpain (and possibly cathepsin) in the center of a regulatory network involving at least four cell death programs: (i) necrosis, (ii) apoptosis, (iii) autophagy and (iv) AIF-mediated caspase-independent cell death (Fig. 10.2). Last but not least, a poorly investigated aspect of calpain–cathepsin involvement in the proteolytic neurodegeneration is the degradation of potentially neuroprotective factors such as glutamic acid decarboxylase (Monnerie and Le Roux 2007).

Cerebral ischemia is a leading cause of disability and mortality, but there is no effective treatment at present. Identification of novel effective therapeutic regimens requires a precise identification of the variety of pathways contributing to ischemic neuronal death, as well as their relative weight and interplay in this pathological condition. Current evidence suggests that members of the calpain–cathepsin cascade are suitable molecular targets for neuroprotection, whose modulation may become a powerful tool for counteracting ischemic neuronal death in humans.

References

Adhami F, Liao G, Morozov YM et al (2006) Cerebral ischemia-hypoxia induces intravascular coagulation and autophagy. Am J Pathol 169:566–583

Artal-Sanz M, Samara C, Syntichaki P et al (2006) Lysosomal biogenesis and function is critical for necrotic cell death in Caenorhabditis elegans. J Cell Biol 173:231–239

Benetti R, Del Sal G, Monte M et al (2001) The death substrate Gas2 binds m-calpain and increases susceptibility to p53-dependent apoptosis. EMBO J 20:2702–2714
Cao G, Xing J, Xiao X et al (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. J Neurosci 29:9278–9293
Chapman HA, Riese RJ, Shi GP (1997) Emerging roles for cysteine proteases in human biology. Annu Rev Physiol 59:63–88
Croall DE, Demartino GN (1991) Calcium-activated neutral protease (calpain) system: structure, function, and regulation. Physiol Rev 81:813–847
Colbourne F, Sutherland GR, Auer RN (1999) Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J Neurosci 19:4200–4210
Debnath J, Baehrecke EH, Kroemer G (2005) Does autophagy contribute to cell death? Autophagy 1:66–74
Demarchi F, Bertoli C, Copetti T et al (2006) Calpain is required for macroautophagy in mammalian cells. J Cell Biol 175:595–605
Demarchi F, Bertoli C, Copetti T et al (2007) Calpain as a novel regulator of autophagosome formation. Autophagy 3:235–237
Deshpande JK, Siesjö BK, Wieloch T (1987) Calcium accumulation and neuronal damage in the rat hippocampus following cerebral ischemia. J Cereb Blood Flow Metab 7:89–95
Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16:663–669
Goll DE, Thompson VF, Li H et al (2003) The calpain system. Physiol Rev 83:731–801
Golstein P, Kroemer G (2007) Cell death by necrosis: towards a molecular definition. Trends Biochem Sci 32:37–43
Hara T, Nakamura K, Matsui M et al (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441:885–889
Keppler D, Sameni M, Moin K et al (1996) Tumor progression and angiogenesis: cathepsin & Co. Biochem Cell Biol 74:799–810
Kirino T (1982) Delayed neuronal death in the gebril hippocampus following ischemia. Brain Res 239:57–69
Kohda Y, Yamashima T, Sakuda K et al (1996) Dynamic changes of cathepsin B and L expression in the monkey hippocampus after transient ischemia. Biochem Biophys Res Commun 228:616–622
Komatsu M, Waguri S, Chiba T et al (2006) Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 441:880–884
Kundu M, Thompson CB (2008) Autophagy: basic principles and relevance to disease. Annu Rev Pathol 3:427–455. doi: 10.1146/annurev.pathol.2.010506.091842
Li Y, Chopp M, Powers C (1997) Granule cell apoptosis and protein expression in hippocampal dentate gyrus after forebrain ischemia in the rat. J Neurol Sci 150:93–102
MacManus JP, Buchan AM, Hill IE et al (1993) Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. Neurosci Lett 164:89–92
Martins E, Yanase H, Sakai K et al (1988) Accumulation of calcium and loss of potassium in the hippocampus following transient cerebral ischemia: a proton microprobe study. J Cereb Blood Flow Metab 8:531–538
Monnerie H, Le Roux PD (2008) Glutamate alteration of glutamic acid decarboxylase (GAD) in GABAergic neurons: the role of cysteine proteases. Exp Neurol 213:145–153
Mitani A, Takeyasu S, Yanase H et al (1994) Changes in intracellular Ca^{2+} and energy levels during in vitro ischemia in the gebril hippocampal slice. J Neurochem 62:626–634
Nakamura K, Hatakeyama T, Furuta S et al (1993) The role of early Ca^{2+} influx in the pathogenesis of delayed neuronal death after brief forebrain ischemia in gerbils. Brain Res 613:181–192
Nitatori T, Sato N, Waguri S et al (1995) Delayed neuronal death in the CA1 pyramidal cell layer of the gebril hippocampus following cerebral ischemia is apoptosis. J Neurosci 15:1001–1011
Pope A, Nixon RA (1984) Proteases of human brain. Neurochem Res 9:291–323
Porn-Ares MI, Samali A, Orrenius S (1998) Cleavage of the calpain inhibitor, calpastatin, during apoptosis. Cell Death Differ 5:1028–1033

Petito CK, Feldmann E, Pulsinelli WA et al (1987) Delayed hippocampal damage in humans following cardiorespiratory arrest. Neurology 37:1281–1286

Pulsinelli WA, Brierly JB, Plum F (1982) Temporal profile of neuronal damage in a model of transient brain ischemia. Ann Neurol 11:491–498

Proskuryakov SY, Konoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? Exp Cell Res 283:1–16

Rami A (2003) Ischemic neuronal death in the rat hippocampus: the calpain–calpastatin–caspase hypothesis. Neurobiol Dis 13:75–88

Rami A, Langhagen A, Steiger S (2008) Focal cerebral ischemia induces upregulation of Beclin 1 and autophagy-like cell death. Neurobiol Dis 29(1):132–141. doi:10.1016/j.nbd.2007.08.005

Rubinsztein DC (2006) The roles of intracellular protein-degradation pathways in neurodegeneration. Nature 443:780–786

Samara C, Syntichaki P, Tavernarakis N (2008) Autophagy is required for necrotic cell death in Caenorhabditis elegans. Cell Death Differ 15:105–112

Sorimachi H, Ishiura S, Suzuki K (1997) Structure and physiological function of calpains. Biochem J 328:721–732

Stoka V, Turk V, Turk B (2007) Lysosomal cysteine cathepsins: signaling pathways in apoptosis. Biol Chem 388:555–560

Syntichaki P, Xu K, Driscoll M et al (2002) Specific aspartyl and calpain proteases are required for neurodegeneration in C. elegans. Nature 419:939–944

Tonchev AB, Yamashima T (1999) Ischemic delayed neuronal death: role of the cysteine proteases calpain and cathepsins. Neuropathology 19:356–365

Tsuchiya K, Kohda Y, Yoshida M et al (1999) Postical blockade of ischemic hippocampal neuronal death in primates using selective cathepsin inhibitors. Exp Neurol 155:187–194

Tsukada T, Watanabe M, Yamashima T (2001) Implications of CAD and DNase II in ischemic neuronal necrosis specific for the primate hippocampus. J Neurochem 79:1196–1206

Yamashima T, Saido TC, Takita M et al (1996) Transient brain ischemia provokes Ca^{2+}, PIP_2 and calpain responses prior to delayed neuronal death in monkeys. Eur J Neurosci 8:1932–1944

Yamashima T, Kohda Y, Tsuchiya K et al (1998) Inhibition of ischemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074: a novel strategy for neuroprotection based on "calpain–cathepsin hypothesis". Eur J Neurosci 10:1723–1733

Yamashima T (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. Prog Neurobiol 62:273–295

Yamashima T, Tonchev AB, Tsukada T et al (2003) Sustained calpain activation associated with lysosomal rupture executes necrosis of the postischemic CA1 neurons in primates. Hippocampus 13:791–800

Yamashima T (2004) Ca^{2+}-dependent proteases in ischemic neuronal death: a conserved 'calpain–cathepsin cascade' from nematodes to primates. Cell Calcium 36:285–293

Yamashima T, Tonchev AB, Borlongan CV (2007) Differential response to ischemia in adjacent hippocampal sectors: neuronal death in CA1 versus neurogenesis in dentate gyrus. Biotechnol J 2:596–607

Zola-Morgan S, Squire LR, Amaral DG (1986) Human amnesia and the medical temporal region: Enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. J Neurosci 6:2950–2967

Part IV
Traumatic Central Nervous System (CNS) Injury

Chapter 11
Mitochondrial Damage in Traumatic CNS Injury

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11.1 Traumatic Brain Injury

With over 1.5 million injuries every year, traumatic brain injury (TBI) has become an almost ubiquitous phenomenon in our country. As this disorder can be present without any outward sign of physical damage and the victims are usually cognitively impaired (i.e., not vocal), it has become a largely "silent epidemic" (Jennett 1998; Thurman et al. 1999; Jager et al. 2000; CDC 2003; Langlois et al. 2006). Current advancements in medicine have allowed patients who would have previously succumbed to their wounds, to survive long after their initial injury. Also, there is a growing population of individuals who sustain a mild to moderate TBI who do not seek treatment (~25%), and often develop prolonged and chronic neurological symptoms (CDC 2003). The growing injured population has presented our society with an enormous economic and social burden, as these patients are commonly unable to properly reintegrate in to their previous professional and social networks. They become exceedingly dependent on family and social outreach programs to live their daily lives which cause their health care costs to total tens of billion dollars per year (Langlois et al. 2006). Although there are limited treatment options designed to allow people to survive their injuries, consisting of minimizing acute brain edema, decreasing intracranial pressure, and the prevention of peripheral complications, there is no current treatment to attenuate or recover the loss of neural tissue (Hatton 2001).

Perhaps the most insidious aspect of TBI is that it can occur without obvious signs of injury to the patient's body. There have been recorded medical incidences of mysterious neurological disorders dating back to World War I (WWI). Physicians in the British armed forces had then given it the somewhat enigmatic label of "shell shock" (SS) (Jones et al. 2007). Although some cases could be attributed to psychosis, by 1917 SS was responsible for 14% of all discharges from the armed forces, and accounted for 33% of all discharges of non-wounded soldiers. It had become

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so prevalent throughout the armed forces and had such a wide array of presenting symptoms that it was highly debated whether or not it was a real condition, and the etiology and management was highly disputed during the early twentieth century. By the end of WWI the prevalence of SS began to incur a large financial burden upon the British armed forces, primarily due to the 32,000 pensions that had been awarded to "neurasthenic" soldiers suffering from SS with no obvious cerebral injury. The controversial definition of the disorder and its method of treatment, in addition to the development of public controversy and stigma over diagnosis, delayed the development of a treatment protocol and even caused the British army to ban the use of the term "shell shock" from medical reports.

During World War II (WWII) the British army banned the SS terminology in hopes of avoiding another epidemic of these cases, which they may or may not have viewed as physical disorders. However, with the start of the war it became readily apparent that disavowing the existence of this disorder did not prevent another epidemic. In response to the army regulations regarding this disorder, alternative terminology arose in its place, such as post-concussion syndrome (PS) or post-trauma concussion state (coined by Shaller). Eventually, physicians began to realize that many of the soldiers that suffered from this concussed state had been in close proximity of an explosion during battle. This led them to speculate that some force, that had no perceptible outward affect on the body, had a substantial effect on fragile neural tissues. In an attempt to, once again, clarify the etiology of this disorder, Denny-Brown suggested that it was the timeline of symptom presentation within the individual patient instead of the symptom type that was the key factor between severe head injury and PS. His etiological account indicated that severe head injury would present with immediate neurologic symptoms that would trend toward recovery, whereas PS would have delayed onset of neurologic symptoms with a trend toward worsening symptoms (Jones et al. 2007). It has been estimated that 50% of patients with a mild TBI can develop PS, consisting of dizziness, headaches, cognitive dysfunction, sleep disorders, and depression (CDC 2003; Langlois et al. 2006; Rapoport et al. 2006). This delayed development of symptoms in the mild to moderate patient populations is perhaps the most unfortunate aspect of this condition, as soldiers and civilians can often suffer immense psychiatric morbidity without realizing that they require medical treatment for a physical injury. A recent online polling study indicated that 42% of their respondents who suffered from TBI failed to seek medical care, which is considerably higher than the CDC estimate of 25%. It has been observed clinically that even mild or moderate TBI can require neurosurgical intervention, and any delay in treatment could prove to be costly in terms of cognitive and functional recovery (Setnik and Bazarian 2007).

Of the more than 1.5 million military personnel deployed since 2001 to the Middle East, approximately 25% of the injured service members have reported brain injury. Unpublished data from the Department of Defense indicates that blast injuries are the leading cause of TBI in war zones; and has been labeled as a signature injury of the current Middle Eastern conflicts (Hoge et al. 2008). Recently, the ravages of TBI has been documented through the unfortunate injury of network news anchor Bob Woodruff, which showed the grim reality of the recovery process

of this disorder and its effects on patients' lives in terms of cognitive dysfunction and its impact on the family dynamics (Woodruff and Woodruff 2007). Although the TBI sustained by Woodruff was severe, mild and moderate injury have also become a long-term problem coupled with prolonged cognitive dysfunction within the armed forces population, with approximately 18% prevalence in various reports (Hoge et al. 2008). In addition to prolonged cognitive deficits, the injured population also has an increased predisposition to the development of post-traumatic stress disorder (PTSD). There is also a problem of failure to report due to a perceived stigma concerning psychological problems within the armed forces population, which could contribute to the development of chronic neurologic dysfunction due to physical injury within this population of injured patients (Hoge et al. 2004).

Within the civilian population of the United States ~2% of the population (5.3 million) is currently living with long-term disabilities resulting from TBI (Langlois et al. 2006). The leading causes of TBI (Fig. 11.1) are falls and motor vehicle accidents, followed closely by assault and "struck by/against" incidents (Langlois et al. 2006). There has also been an increasing population of pediatric (5–18 years) TBI cases resulting from sports-related injuries, which can often be misdiagnosed as the symptoms manifest as lethargy, irritability or fatigue (CDC 2003; Yang et al. 2008). TBI has a biphasic age distribution of incidence; occurring in young (<25) and elderly (>75) populations (Langlois et al. 2006; Rutland-Brown et al. 2006). Over 50,000 deaths are attributed to TBI each year, as well as 235,000 hospitalizations and 1,111,000 emergency department visits (Fig. 11.2). With such a high incidence and great propensity for the development of chronic symptoms, the total medical costs incurred by individuals currently living with TBI within the USA can reach $50 billion dollars per year. This figure increases to $60 billion when lost societal

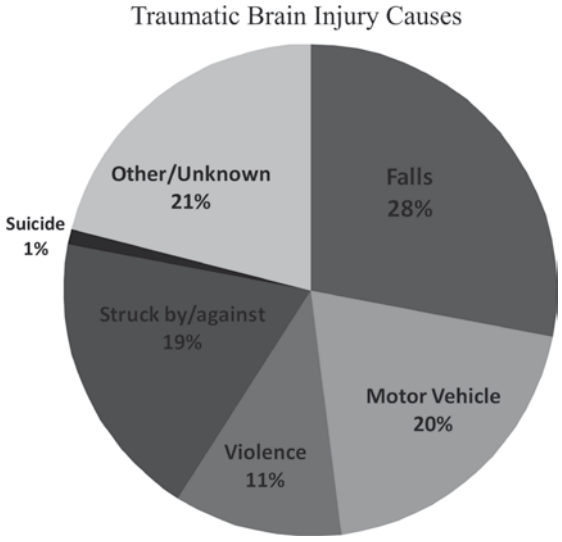

Fig. 11.1 Traumatic brain injury causes

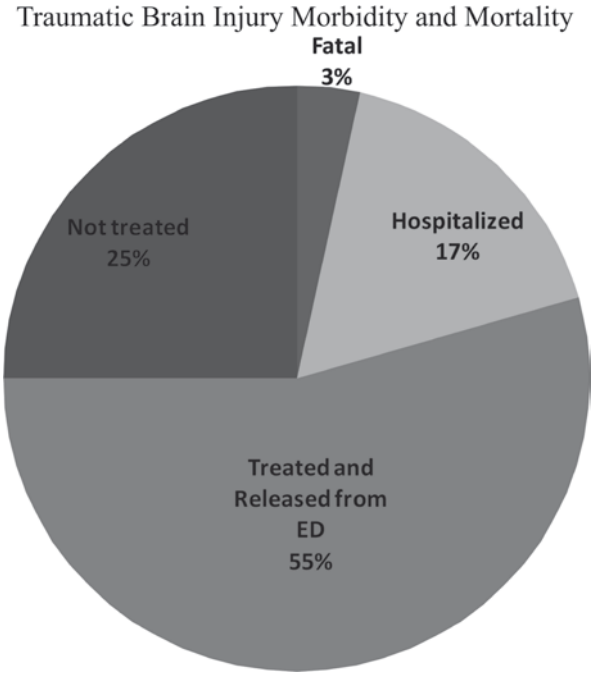

Fig. 11.2 Traumatic brain injury morbidity and mortality

productivity of these individuals is factored in; however, these figures do not factor in how this disorder impacts social and family dynamics (Langlois et al. 2006; Rutland-Brown et al. 2006). As such, there is a clear need for the development of neuroprotective therapies and effective protocols for the treatment of TBI.

11.2 Mitochondria

The development of mitochondrial function was the basis of the development of multi-cellular organisms. It was at this evolutionary crossroads that the cell was able to produce enough energy, in the form of ATP, to form highly complex interconnected networks that developed into the organ systems we see in the human body as well as all other organisms (Lane 2006). Underscoring the dependence on mitochondrial ATP production is the evolutionary development of all multicellular organisms upon this planet to require oxygen utilization through some sort of respiration. It is essential that mitochondria are provided with adequate oxygen in order for the cell to maintain homeostatic regulation of its intercellular processes (Lane 2006). The importance of oxygen consumption is highly evident when we

examine any pathological disease in which tissues become oxygen-deprived (ischemic) for even the shortest time period. These regions undergo massive cellular loss as a result of mitochondrial damage and dysfunction, leading to the initiation of cell death pathways, such as necrosis and apoptosis (Obrenovitch 2008).

Although we have only been studying mitochondria since the turn of the century, these organelles have proven to be one of the most important discoveries in the history of cellular research. Ever since Kolliker (1850), Altman (1890), and Benda (1898) described their presence in cells, we have been fascinated with their function. The first Nobel Prize for mitochondrial research was awarded to Meyerhof in 1922 for the discovery of the connection between substrate oxidation and oxygen consumption in relation to glycolysis. Next to be awarded in 1931, was the work done by Warburg on the nature and mode of action of the respiratory enzyme, indicating that ATP production was coupled with enzymatic oxidation of glyceraldehyde phosphate. Szent-Gyorgyi was awarded the Nobel Prize in 1937 for the discovery of the connection with biological combustion process of dicarboxylic acids within respiration. The most recent Nobel Prize was awarded in 1997 to Boyer and Walker for their discovery of the underlying enzymatic mechanism underlying the synthesis of ATP. Although we have learned much over the past century about mitochondrial bioenergetics, there remains a great deal to be discovered.

In order to discuss mitochondrial dysfunction, we must first discuss normal mitochondrial function (Fig. 11.3). Mitochondria are intracellular organelles with a dual (inner and outer) membrane system, each of which is responsible for specific functions. The outer membrane (OM) contains many transporter proteins which import and export many ions and proteins necessary for mitochondrial function (Nicholls and Ferguson 2002). The inner membrane (IM) exhibits many folds, termed cristae, which increase the surface area available for mitochondrial respiration. The space which is enclosed by the IM is called the matrix and contains enzymes involved in cellular metabolism and calcium regulation. Within the IM there are a series of five protein complexes that comprise the electron transport chain (ETC), which is the primary site of ATP production within the cell (Fig. 11.3). Complex I (NADH-Ubiquinone Oxidoreductase), which is embedded within the IM, converts NADH to NAD^+ by accepting an electron into the Fe-S center of the protein. As a byproduct of this electron donation, a proton is translocated from the matrix to the intermembrane space (IMS), which is located between the inner and outer membranes. Complex II (Succinate Dehydrogenase), in addition to its function as an ETC protein, is also a key component of the Krebs Cycle, which converts the glycolytic product pyruvate into different molecules in order to produce substrates for the ETC. This complex utilizes the conversion of succinate to accept electrons from $FADH_2$ into the ETC and as it is only anchored to the inner half of the IM, there is no translocation of protons from the matrix to the IMS. Complex I and II transfer their electrons to ubiquinone (CoEnzyme Q_{10}) located within the IM (Fig. 11.3). These electrons are then passed to Complex III (Ubiquinone-Cytochrome-C Oxidoreductase) via the Q-cycle, resulting in proton translocation to the IMS. Another electron transfer protein, Cytochrome c, accepts

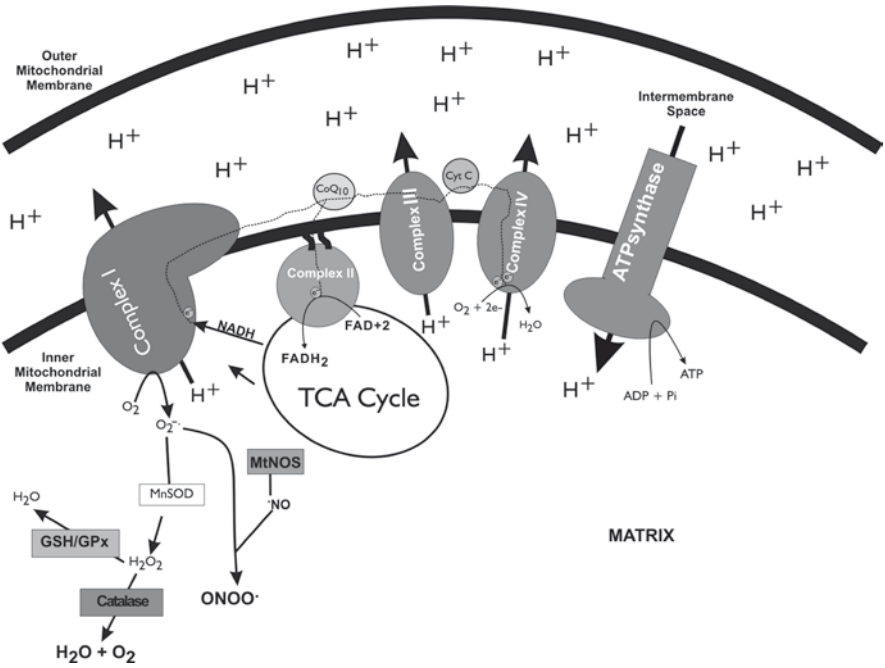

Fig. 11.3

this electron and transports it to Complex IV (Cytochrome-C Oxidase); again translocating a proton across into the IMS via complex IV (Fig. 11.3). It is at Complex IV that oxygen plays its vital role as the final electron acceptor for the ECT, where it is combined with electrons to form H_2O (Fig. 11.3). Without the presence of the oxygen molecule, electrons become backed up within the ETC. resulting in damage to the surrounding structures. Meanwhile, all of the protons that have been pumped into the IMS create a proton concentration gradient ($\Delta\psi$) which is utilized by Complex V (ATP synthase) to facilitate phosphorylation of ADP into ATP for use as an energy source for cellular processes (Fig. 11.3).

11.3 Role of Ca^{2+} in Neurons and Mitochondrial Ca^{2+} Sequestration

Although the complex mechanisms of secondary neuronal injury following TBI are poorly understood, it is clear that excitatory amino acid (EAA) neurotoxicity plays an important upstream role (Faden et al. 1989). Elevated EAA increase the levels of intracellular Ca^{2+} ($[Ca^{2+}]_i$) by activation of *N*-methyl-D-asparate (NMDA) receptor/ion channels, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and voltage-gated Ca^{2+} channels. This results in excessive entry of Ca^{2+},

leading to a loss of cellular homeostasis and subsequent neuronal Ca^{2+} overload. Calcium is the most common signal transduction element in cells, but unlike other second-messenger molecules, it is required for life. Paradoxically, prolonged high levels of $[Ca^{2+}]_i$ leads to cell death. Excessive $[Ca^{2+}]_i$ can damage the structure of nucleic acids and proteins and interfere with kinase activity as well as activating proteases or phosholipases, causing cellular damage. Therefore, maintenance of low $[Ca^{2+}]_i$ is necessary for proper cell function and to allow brief pulses of it to initiate second-messenger pathways, allowing intracellular communication. Since Ca^{2+} cannot be metabolized like other second-messenger molecules, it must be tightly regulated by cells. Numerous intracellular proteins and some organelles have adapted to bind or sequester Ca^{2+} to ensure that homeostasis is maintained. Mitochondria are one such organelle.

The mitochondrial membrane potential ($\Delta\psi$) is generated by the translocation of protons across the inner mitochondria membrane via the ETC, culminating in the reduction of O_2 to H_2O. This store of potential energy (the electrochemical gradient) can then be coupled to ATP production as protons flow back through ATP synthase and complete the proton circuit. The potential can also be used to drive Ca^{2+} into the mitochondrial matrix via the electrogenic uniporter when cytosolic levels increase. When cytosolic levels of Ca^{2+} decrease, mitochondria pump Ca^{2+} out to precisely regulate cytosolic Ca^{2+} homeostasis. During excitotoxic insult, Ca^{2+} uptake into mitochondria has been shown to increase ROS production, inhibit ATP synthesis, and induce mitochondrial permeability transition. It is also important to note that inhibition of mitochondrial Ca^{2+} uptake by reducing $\Delta\psi$ (chemical uncoupling) following excitotoxic insults is neuroprotective, emphasizing the pivotal role of mitochondrial Ca^{2+} uptake in EAA-mediated neuronal cell death (Sullivan et al. 2004b; Pandya et al. 2007).

11.4 Free Radicals, $\Delta\psi$, Mitochondria and Neuronal Cell Death

Free radical production is a byproduct of ATP generation in mitochondria via the ETC. Electrons escape from the chain and reduce oxygen (O_2) to superoxide ($^{\bullet}O_2^-$). Normally, cells convert $^{\bullet}O_2^-$ to hydrogen peroxide (H_2O_2), utilizing both manganese superoxide dismutase (MnSOD), which is localized to the mitochondria, and copper-zinc superoxide dismutase (Cu-ZnSOD), found in the cytosol. H_2O_2 is rapidly converted to H_2O via catalase and gluathione peroxidase, but has the potential to be converted to the highly reactive hydroxyl radical ($^{\bullet}OH$) via the Fenton reaction, underlying ROS neurotoxicity. $^{\bullet}OH$ rapidly attacks unsaturated fatty acids in membranes, causing lipid peroxidation and the production of 4-hydroxynonenal (HNE) that conjugates to membrane proteins, impairing their function. Such oxidative injury results in significant alterations in cellular function. In particular, ROS induction of lipid peroxidation and protein oxidation products may be particularly important in neurodegeneration and TBI (Hall and Sullivan 2004; Lifshitz et al. 2004).

Mitochondrial ROS production is intimately linked to $\Delta\psi$ such that hyperpolarization (high $\Delta\psi$) increases and promotes ROS production. The underlying mechanism

is the altered redox potential of ETC carriers (reduced) and an increase in semiquinone anion half-life time (high $\Delta\psi$ prevents oxidation of cytochrome b1 in the Q cycle). In other words, at a high $\Delta\psi$, protons can no longer be pumped out of the matrix (against the electrochemical proton gradient) by the chain, so electron transport slows/stalls, resulting in intermediates staying reduced longer and increasing the chance of the electrons' escape from these intermediates, reduced oxygen and increased ROS production. Since the magnitude of ROS production is largely dependent on – and correlates with – $\Delta\psi$, even a modest reduction via increased proton conductance (decreases $\Delta\psi$, the electrochemical proton gradient) across the mitochondrial inner membrane (uncoupling) reduces ROS formation (Sullivan et al. 2004b; Pandya et al. 2007).

11.5 Mitochondria & TBI

It has become increasingly clear that TBI, as well as other neurological disorders, either cause or are the result of mitochondrial dysfunction (Hovda et al. 1992; Sullivan et al. 1998; Nicholls and Budd 2000; Hatton 2001; Pellock et al. 2001; Schurr 2002; Sullivan et al. 2002; Tieu et al. 2003; Lifshitz et al. 2004; Sullivan 2005; Sullivan et al. 2005). Unfortunately there is very little we can do to prevent the initial blunt force trauma that is caused by TBI; however we may be able to intervene within the massive secondary signaling cascade that can last for hours to weeks following the primary insult (Fig. 11.4). Secondary injury is initiated by a massive depolarization of the plasma membrane by voltage-dependent Na^+ channels. Along with glutamate release, this depolarization causes the removal of the Mg^+

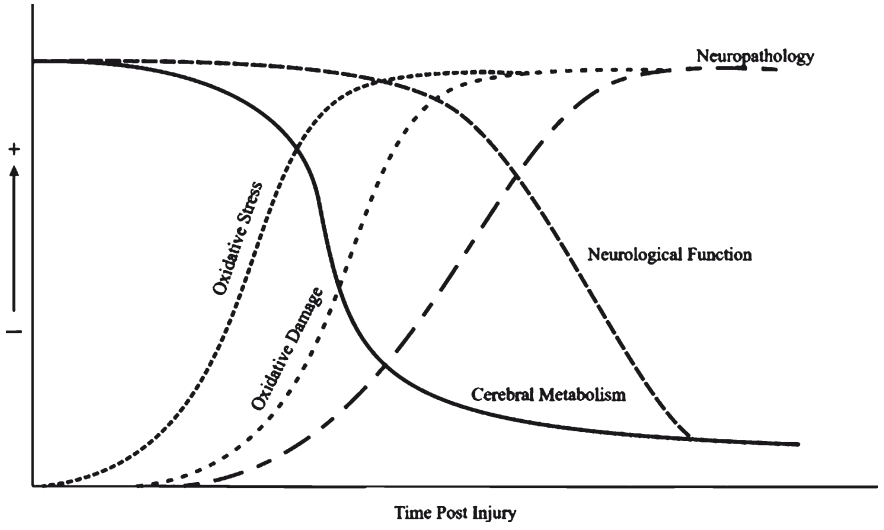

Fig. 11.4

block within NMDA channels, causing a massive Ca^{2+} influx into the cell (Nicholls et al. 1999; Nicholls and Budd 2000; Gunter et al. 2004). This Ca^{2+} can activate many damaging cellular enzymes within the cytosol, and as such must be sequestered by intracellular organelles, mainly the mitochondria. After Ca^{2+} is imported into mitochondria via membrane potential-driven transporters, it is stored as a Ca^{2+} phosphate compound within the matrix, causing the matrix to have an almost gel-like consistency (Nicholls and Budd 2000).

However, the Ca^{2+} buffering capacity of mitochondria is finite and eventually the Ca^{2+} influx becomes too great, resulting in mitochondrial dysfunction and subsequent initiation of cell death pathways (Brookes et al. 2004; Lifshitz et al. 2004; Sullivan et al. 2004a; Sullivan et al. 2005). Calcium seems to affect primarily complex-driven respiration, and causes damage to this major site of electron acceptance that can significantly hinder the ability of the mitochondria to produce ATP (Tieu et al. 2003; Gunter et al. 2004; Sleven et al. 2006; Maalouf et al. 2007). The loss of adequate membrane potential will cause the ATP synthase to run in reverse, thereby dephosphorylating ATP and pumping protons into the IMS in an attempt to restore membrane potential and preserve mitochondrial homeostasis. However, by depleting ATP stores, membrane channels that require energy to maintain ionic balances will be unable to sustain operations. This causes the mitochondria and the cell to swell and eventually burst, which are characteristic signs of necrotic cell death (Nicholls and Budd 2000; Sullivan et al. 2005). Calcium overload can also activate intramitochondrial proteins, such as μ-calpain (calpain I), that contribute to the formation of the mitochondrial permeability transition pore (mPTP) and release of IM proteins (Scheff and Sullivan 1999; Nicholls and Budd 2000; Garcia et al. 2005; Sullivan et al. 2005).

The formation of the mPTP results in mitochondrial dysfunction and has been shown to occur after acute TBI (Nicholls and Budd 2000; Sullivan et al. 2000; Sullivan et al. 2005). This structure spans both inner and outer membrane, and causes a massive efflux of calcium into the cytosol and the release of death-inducing proteins, ultimately leading to cellular loss and cognitive dysfunction. The mPTP is a pore comprising multiple mitochondrial proteins within the inner and outer membranes, including the adenine nucleotide translocase (ANT), inner and outer protein transporters (Tim/Tom), voltage-dependent anion channel (VDAC), and cyclophilin D. It allows nonspecific conductance of matrix and intermembrane space components to the cytosol, where they can activate detrimental signaling cascades leading to cell death.

One protein that is highly involved in both normal mitochondrial respiration and cell death cascades is cytochrome *c*. It is normally found in the IMS, electrostatically attached to the inner membrane where it shuttles electrons from complex III to complex IV. However, in the presence of increased Ca^{2+} levels it is cleaved from the inner membrane by the Ca^{2+}-activated cysteine protease μ-calpain (Sullivan et al. 2002; Nasr et al. 2003; Garcia et al. 2005). After mPTP opening it is released into the cytosol where it binds to apoptosis activation factor-1 (Apaf-1), which is also bound to pro-caspase 9. This complex, known as the apoptosome, initiates the activation of caspase 3 and subsequent cleavage of apoptotic substrates, ultimately

resulting in cellular loss. The opening of the mPTP also releases apoptosis inducing factor (AIF) and endonuclase G (Endo G), both of which are responsible for nuclear DNA degradation.

As described previously, a common byproduct of normal mitochondrial function is the production of ROS. However, TBI damage to the mitochondria and Ca^{2+} loading significantly increases mitochondrial ROS production, primarily at complex I and III of the ETC. Recently, it has become increasingly apparent that the key to maintaining cellular and mitochondrial function is to decrease the levels of oxidative stress-induced damage after this TBI-induced excitotoxic Ca^{2+} influx (Hatton 2001; Sullivan et al. 2005; Singh et al. 2006). However, because the production of oxidative stress molecules is a normal byproduct of mitochondrial function and mitochondrial function is required for proper cellular function, there must be a balance between preserving mitochondrial function and reducing oxidative damage. Therefore, the metabolic pathways involving mitochondria can become a critical component of the treatment of TBI (Robertson et al. 1991; Davis et al. 2008; Prins 2008).

11.6 Closing Remarks

Strategies that target specific mitochondrial events may prove beneficial as therapeutic interventions following central nervous system (CNS) injury. This is supported by a wealth of experimental data demonstrating that mitochondrial function is severely impaired following TBI and that this dysfunction is related to cell death pathways known to be activated in these distinct models. The loss of mitochondrial homeostasis that occurs following CNS injury implies that mPTP activation may be a common link in several models of TBI and spinal cord injury (SCI). The pathophysiological role of mPT in CNS injury is also supported by several lines of scientific work that have utilized inhibitors (e.g., cyclosporin A [CsA] and its derivatives) of the mitochondrial permeability transition (mPT) in vivo, to test this hypothesis. The development of new pharmacological tools that specifically target the mPT and exhibit minimal toxicity will provide further support for its role in CNS injury and may prove beneficial as possible treatments for this and other neurodegenerative conditions.

References

Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol 287:C817–C833

CDC (2003) Report to Congress on Mild Traumatic Brain Injury in the United States: Steps to Prevent a Serious Public Health Problem. Centers for Disease Control and Prevention, Atlanta, GA

Davis LM, Pauly JR, Readnower RD, Rho JM, Sullivan PG (2008) Fasting is neuroprotective following traumatic brain injury. J Neurosci Res 86(8):1812–1822

Faden AI, Demediuk P, Panter SS, Vink R (1989) The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science 244:798–800

Garcia M, Bondada V, Geddes JW (2005) Mitochondrial localization of mu-calpain. Biochem Biophys Res Commun 338:1241–1247

Gunter TE, Yule DI, Gunter KK, Eliseev RA, Salter JD (2004) Calcium and mitochondria. FEBS Lett 567:96–102

Hall ED, Sullivan PG (2005) Preserving function in acute nervous system injury. In: From Neuroscience to Neurology: Neuroscience, Molecular Medicine, and the Theraputic Translation of Neurology. Waxman, S (ed) Elsevier/Academic Press, pp. 35–39

Hatton J (2001) Pharmacological treatment of traumatic brain injury: a review of agents in development. CNS Drugs 15:553–581

Hoge CW, Castro CA, Messer SC, McGurk D, Cotting DI, Koffman RL (2004) Combat duty in Iraq and Afghanistan, mental health problems, and barriers to care. N Engl J Med 351:13–22

Hoge CW, McGurk D, Thomas JL, Cox AL, Engel CC, Castro CA (2008) Mild traumatic brain injury in U.S. Soldiers returning from Iraq. N Engl J Med 358:453–463

Hovda DA, Becker DP, Katayama Y (1992) Secondary injury and acidosis. J Neurotrauma 9(Suppl 1):S47–S60

Jager TE, Weiss HB, Coben JH, Pepe PE (2000) Traumatic brain injuries evaluated in U.S. emergency departments, 1992–1994. Acad Emerg Med 7:134–140

Jennett B (1998) Epidemiology of head injury. Arch Dis Child 78:403–406

Jones E, Fear NT, Wessely S (2007) Shell shock and mild traumatic brain injury: a historical review. Am J Psychiatry 164:1641–1645

Lane N (2006) Power, sex, suicide : mitochondria and the meaning of life. Oxford University Press, Oxford, New York

Langlois JA, Rutland-Brown W, Wald MM (2006) The epidemiology and impact of traumatic brain injury: a brief overview. J Head Trauma Rehabil 21:375–378

Lifshitz J, Sullivan PG, Hovda DA, Wieloch T, McIntosh TK (2004) Mitochondrial damage and dysfunction in traumatic brain injury. Mitochondria 1:705–713

Maalouf M, Sullivan PG, Davis L, Kim DY, Rho JM (2007) Ketones inhibit mitochondrial production of reactive oxygen species production following glutamate excitotoxicity by increasing NADH oxidation. Neuroscience 145:256–264

Nasr P, Gursahani HI, Pang Z, Bondada V, Lee J, Hadley RW, Geddes JW (2003) Influence of cytosolic and mitochondrial Ca^{2+}, ATP, mitochondrial membrane potential, and calpain activity on the mechanism of neuron death induced by 3-nitropropionic acid. Neurochem Int 43:89–99

Nicholls DG, Budd SL (2000) Mitochondria and neuronal survival. Physiol Rev 80:315–360

Nicholls DG, Budd SL, Castilho RF, Ward MW (1999) Glutamate excitotoxicity and neuronal energy metabolism. Ann N Y Acad Sci 893:1–12

Nicholls DG, Ferguson SJ (2002) Bioenergetics3. Academic Press, London

Obrenovitch TP (2008) Molecular physiology of preconditioning-induced brain tolerance to ischemia. Physiol Rev 88:211–247

Pandya JD, Pauly JR, Nukala VN, Sebastian AH, Day KM, Korde AS, Maragos WF, Hall ED, Sullivan PG (2007) Post-injury administration of mitochondrial uncouplers increases tissue sparing and improves behavioral outcome following traumatic brain injury in rodents. J Neurotrauma 24:798–811

Pellock JM, Dodson WE, Bourgeois BFD (2001) Pediatric epilepsy: diagnosis and therapy. DEMOS, New York

Prins ML (2008) Cerebral metabolic adaptation and ketone metabolism after brain injury. J Cereb Blood Flow Metab 28:1–16

Rapoport MJ, Kiss A, Feinstein A (2006) The impact of major depression on outcome following mild-to-moderate traumatic brain injury in older adults. J Affect Disord 92:273–276

Robertson CS, Goodman JC, Narayan RK, Contant CF, Grossman RG (1991) The effect of glucose administration on carbohydrate metabolism after head injury. J Neurosurg 74:43–50

Rutland-Brown W, Langlois JA, Thomas KE, Xi YL (2006) Incidence of traumatic brain injury in the United States, 2003. J Head Trauma Rehabil 21:544–548

Scheff SW, Sullivan PG (1999) Cyclosporin A significantly ameliorates cortical damage following experimental traumatic brain injury in rodents. J Neurotrauma 16:783–792

Schurr A (2002) Energy metabolism, stress hormones and neural recovery from cerebral ischemia/hypoxia. Neurochem Int 41:1–8

Setnik L, Bazarian JJ (2007) The characteristics of patients who do not seek medical treatment for traumatic brain injury. Brain Inj 21:1–9

Singh IN, Sullivan PG, Deng Y, Mbye LH, Hall ED (2006) Time course of post-traumatic mitochondrial oxidative damage and dysfunction in a mouse model of focal traumatic brain injury: implications for neuroprotective therapy. J Cereb Blood Flow Metab 26:1407–1418

Sleven H, Gibbs JE, Heales S, Thom M, Cock HR (2006) Depletion of reduced glutathione precedes inactivation of mitochondrial enzymes following limbic status epilepticus in the rat hippocampus. Neurochem Int 48:75–82

Sullivan PG (2005) Interventions with neuroprotective agents: novel targets and opportunities. Epilepsy Behav 7(Suppl 3):S12–S17

Sullivan PG, Keller JN, Bussen WL, Scheff SW (2002) Cytochrome c release and caspase activation after traumatic brain injury. Brain Res 949:88–96

Sullivan PG, Keller JN, Mattson MP, Scheff SW (1998) Traumatic brain injury alters synaptic homeostasis: implications for impaired mitochondrial and transport function. J Neurotrauma 15:789–798

Sullivan PG, Rabchevsky AG, Keller JN, Lovell M, Sodhi A, Hart RP, Scheff SW (2004a) Intrinsic differences in brain and spinal cord mitochondria: implication for therapeutic interventions. J Comp Neurol 474:524–534

Sullivan PG, Rabchevsky AG, Waldmeier PC, Springer JE (2005) Mitochondrial permeability transition in CNS trauma: cause or effect of neuronal cell death? J Neurosci Res 79:231–239

Sullivan PG, Springer JE, Hall ED, Scheff SW (2004b) Mitochondrial uncoupling as a therapeutic target following neuronal injury. J Bioenerg Biomembr 36:353–356

Sullivan PG, Thompson M, Scheff SW (2000) Continuous infusion of cyclosporin A postinjury significantly ameliorates cortical damage following traumatic brain injury. Exp Neurol 161:631–637

Thurman DJ, Alverson C, Dunn KA, Guerrero J, Sniezek JE (1999) Traumatic brain injury in the United States: a public health perspective. J Head Trauma Rehabil 14:602–615

Tieu K, Perier C, Caspersen C, Teismann P, Wu DC, Yan SD, Naini A, Vila M, Jackson-Lewis V, Ramasamy R, Przedborski S (2003) D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. J Clin Invest 112:892–901

Woodruff L, Woodruff B (2007) In an instant: a family's journey of love and healing. Random House, New York

Yang J, Phillips G, Xiang H, Allareddy V, Heiden E, Peek-Asa C (2008) Hospitalizations for sport-related concussions in US children aged 5 to 18 years during 2000–2004. Br J Sports Med 42(8):664–669

Chapter 12
Programmed Neuronal Cell Death Mechanisms in CNS Injury

Bogdan A. Stoica and Alan I. Faden

12.1 Introduction

Neuronal cell death is a critical physiological process necessary for the normal development of the CNS, as well as essential for removing dysfunctional cells after injury or other various pathological conditions. However, excessive or inappropriate neuronal cell loss is also a hallmark of acute or chronic neurodegeneration (Eldadah and Faden 2000; Snider et al. 1999; Graeber and Moran 2002; Honig and Rosenberg 2000). Many cell death effector pathways are common to both physiological and pathophysiological processes. Elucidating such mechanisms and initiating signals is essential for understanding neurodegeneration and designing effective therapies.

Cell loss following CNS injuries results in part from secondary biochemical changes initiated by the primary insult (Panter and Faden 1992a). Secondary injury factors involved in such delayed autodestructive cascades within the CNS include the loss of ionic homeostasis, lipid degradation products, free radicals, excitatory amino acids, compromised bioenergetics and blood flow, mitochondrial changes, inflammation, and/or immune responses among others (Yakovlev and Faden 2004; Panter and Faden 1992b; McIntosh 1994). Acting sequentially or simultaneously, over periods ranging from seconds (e.g., lipid, cation changes) to days (e.g., inflammatory, immune responses) after the primary insult, the secondary injury factors trigger various molecular mechanisms, depending on the specific developmental age, injury severity or cell type, and cause delayed or progressive cell death (Panter and Faden 1992b; McIntosh 1994; Pohl et al. 1999).

Cell death phenotypes after CNS injury include both apoptosis and necrosis (Yakovlev et al. 2001). Although distinguishable by histological features, these two patterns of cell death often coexist and intermediate morphological forms have been identified, leading some to propose the term "aponecrosis" (Formigli et al. 2000). Until quite recently, cell death has been simplistically divided into a passive form

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associated with the loss of ionic homeostasis and membrane integrity (necrosis) and an energy requiring form characterized by cytoplasmic and nuclear condensation, diminished cell volume, relative preservation of organellar structure, and nuclear fragmentation (apoptosis) (Bredesen 2000). On the basis of known cell death mechanisms in *C. elegans*, apoptosis observed after CNS injury has been assumed to be initiated by the activation of a class of cysteine proteases called caspases (Eldadah and Faden 2000). Such caspase-dependent apoptosis appears to result largely from the release and translocation of cytochrome c from mitochondria to cytosol and subsequent activation of a caspase-9/caspase-3 pathway ("intrinsic pathway") (Yakovlev et al. 2001). In certain models, there also may be activation of the "extrinsic pathway," in which "death receptors" on the cell surface related to the TNF (Tumor necrosis factor) superfamily mediate a signal transduction cascade that involves caspase-8, and ultimately caspase-3 (Eldadah and Faden 2000).

During the past several years, this simple classification of necrotic versus apoptotic cell death has been undergoing significant revision. In addition to the classically defined form of "necrotic cell death," there are examples of programmed cell death (PCD) with necrosis-like morphological features (Yu et al. 2006). Furthermore, it is now recognized that a number of mitochondrial proteins are released in response to various apoptotic stimuli and contribute to cell death.

12.2 Cell Death Phenotypes: Morphological-Based Classification

Although substantial progress has been achieved in the last three decades in deciphering the molecular pathways of cell death, a clear definition of fundamental concepts such as "apoptosis" or "necrosis" remains to be developed (Farber 1994; Roy and Sapolsky 1999) despite the efforts of many authors (Clarke 1990; Majno and Joris 1995; Levin et al. 1999; Dikranian et al. 2001; Fujikawa 2000; Sloviter 2002).

As reviewed by Majno and Joris (1995), the term "necrosis" has been used since the time of Galen 2000 years ago. The word "necrosis" comes from the ancient Greek word *nekros* which meant dead body. In the modern era, "necrosis" was mentioned in the works of the renowned pathologists C. Rokitansky (1846) and R. Virchow (1859). Virchow understood necrosis as a phenomenon at the macroscopic level representing an advanced phase of tissue breakdown, with the necrotic tissue still in place. The use of microscopy techniques toward the end of the nineteenth century served to link necrosis to cell death (Majno and Joris 1995). Early studies focused on the cellular changes in tissues following impairment of blood supply (artery ligation) or placing tissues in peritoneal cavity of the experimental animal. Nuclear transformations were observed in the cells of the necrotic tissue (Weigert 1878), leading to the creation of terms such as "pyknosis" (chromatin condensation), "karyolysis" (nuclear disappearance), "karyorhexis" (nuclear fragmentation), and "chromatin margination" (chromatin condensation inside the nuclear membrane) (Schmaus and Albrecht 1894; Arnheim 1890; Klebs 1889) – which are still in the use today. Importantly, it was recognized that macroscopic

necrosis had a microscopic counterpart, a cellular event reflecting cell death. At this time, physiological conditions were also recognized to be associated with spontaneous cell death. W. Flemming presented a careful description of dying cells in the regressing ovarian follicles in mammals (Flemming 1885). These cells manifested various stages of pyknotic chromatin starting with margination and condensation (into shapes described as half-moons and chromatin balls) as well as cell fractionation into smaller bodies; although consistent with what is now termed apoptosis, Flemming named this process "chromatolysis" (describing the eventual disappearance of the nuclear fragments).Approximately at the same time, F. Nissen described chromatolysis in the lactating mammary gland (Nissen 1886) and H. Strobe described very similar cellular changes in breast cancer tissue (Strobe 1892). On the basis of these studies, Graper (1914) argued that an organism requires a process to counterbalance mitosis; he proposed that chromatolysis fulfilled this physiological role and was involved in cell elimination by various organs during development. Graper's conceptualization not only described what we now call apoptosis, but his interpretation of its role is also consistent with our contemporary understanding. As described in a recent review, A. Weissmann as early as the end of the nineteenth century also proposed that death has an important physiological role in higher organisms (Rich et al. 1999; Weissmann 1889) and described how fragments of the dying cell are engulfed by neighboring cells-another feature consistent with apoptotic cell death. Unfortunately, these early and important contributions were soon forgotten in all but the embryology field. In the early 1950s, Glucksmann (1951) detailed the cellular changes of chromatolysis in physiological cell death during embryo development, adding the important observation that mitochondria appeared largely unaffected during this process. Embryologists, such as Glucksmann, saw chromatolysis only as a form of physiologic cell death specific to development, ignoring the role of chromatolysis in various adult conditions (Strobe 1892). The next major contribution was made by Kerr, Wyllie, and colleagues in a seminal series of papers (Kerr et al. 1972; Kerr 1971; Kerr 2002; Wyllie 1974). They identified a series of conserved ultrastructural features – condensation of the cytoplasm and chromatin, cell shrinkage, formation of chromatin balls, normal organelles, and fragmentation of cells by budding of membrane enclosed fragments-apoptotic bodies – that appeared in dying cells not only during development but also in physiological tissue turnover as well as various pathological conditions. Initially, they called this form of cell death "shrinkage necrosis" but later created the name "apoptosis" (from Greek words *apo*-from and *ptosis*-falling) denoting falling of leaves from trees in the autumn. The authors were apparently not familiar with older publications that would have shown that apoptosis was essentially identical to chromatolysis. The work of Kerr and colleagues, as well as other 1960s developmental studies, based cell death phenotypes on ultrastructural changes visualized by electron microscopy (Clarke 1990; Schweichel and Merker 1973).

Schweichel and Merker (1973) were among the first to propose a classification of developmental cell death in terms of a conserved set of ultrastructural features. On the basis of the role of lysosomes in the death process, they described three cell death models: type 1, with no involvement of the dying cell's own lysosomes but

involving lysosomes of another cell after phagocytosis–heterophagocytosis; type 2, with major involvement of the dying cells own lysosomes–autophagocytosis; and type 3, with no lysosomal involvement. Clarke (1990) revised the Schweichel and Merker classification based on a wider range of ultrastructural changes that included not only lysosomes but also nuclei/chromatin, cytoplasm, organelles, and cell budding/blebbing. Clarke maintained the three main developmental cell death paradigms (type 1, 2 and 3) but subdivided type 3 in A and B components; each of these types shared a conserved set of ultrastructural features and were seen as reflecting distinct mechanisms of cell destruction (Clarke 1990). Type 1 is apoptosis-characterized by early chromatin condensation and cell shrinkage with largely normal organelles, followed by the detachment of membrane-enclosed fragments (budding) and the eventual cell fragmentation into membrane bound components that are engulfed/destroyed by lysosomes of neighboring cells and/or macrophages. Type 2 is autophagic degeneration, characterized by presence of "autophagic vacuoles" – which in conjunction with the cell's own lysosomes destroy the cytosol and organelles of the cell. In contrast to apoptosis, with autophagic degeneration nuclear pyknosis is less obvious, and dilation of organelles is more often found. Moreover, unlike apoptosis that usually occurs in isolated cells, type 2 cell death tends to be observed in regions undergoing massive degeneration (Schweichel and Merker 1973), though exceptions are known in each case (Clarke 1990). The vast majority of cell death in development can be classified as type 1 or 2. Type 3A (nonlysosomal vesiculate degradation, corresponding to type 3 in Schweichel and Merker classification) is characterized by swelling of the organelles, presence of empty spaces in the cytosol and cellular disintegration without phagocytosis; one of the few sites showing type 3A is in cartilage during mineralization (Schweichel and Merker 1973). Type 3B (cytoplasmic degeneration) is characterized by early dilation of organelles, vacuolation of the cytosol and later by nuclear changes ending in nuclear degeneration by karyolysis. The cell is phagocytosed without fragmentation. Type 1 and 3B correspond to the "nuclear" and "cytoplasmic" cell death (in reference to the location of the earliest and most pronounced changes), respectively, in the classification of neuronal cell death by Pilar and Landmesser (1976). Most developmental cell death reflects one of the discrete types enumerated. However, some dying cells may share features of more than one type or may be missing a critical component of a given type (Clarke 1990). In the classifications of Schweichel and Merker, as well as Clarke, type 3B cell death shows ultrastructural characteristics (mitochondrial swelling, dilation of organelles, late karyolysis) that are very similar to the "necrotic cell death" phenotype described by pathologists in severely injured tissues (Wyllie 1981). Kerr, Wyllie, and colleagues have suggested that cell death is either apoptotic (involved in physiological situations and some pathological conditions) or "necrotic" (occurring in severe pathological situations and characterized by early cell swelling and organelle dilation leading to rupture of the cellular membranes) (Wyllie 1981). In contrast, Clarke suggested that a necrotic phenotype might be found in development (type 3). Clarke also proposed that his classification applies to both physiological

and pathological cell death, arguing that review of the literature confirms the presence of the three death types in both situations.

Majno and Joris also object to the simple apoptosis versus necrosis dichotomy (Majno and Joris 1995) and underscore the importance of identifying the point of "no return" in cell death (irreversible). They propose that the cell can be considered dead once it reached this point and that "death processes" are those mechanisms that take the cell to the point of "no return," even if active processes such as cell dismantling continue. Others define cell death as the irreversible loss of integrated cellular activity (Trump and Mergner 1974); this occurs past the point of "no return," but may include the rupture of cell membranes and lysis of the cell, cell fragmentation in separate bodies, or cell engulfment by other cells (Kroemer et al. 2005). For Majno and Joris, necrosis has been traditionally understood as the visible presence of dead tissue surrounded by live tissue, a condition that occurs very late in the cell death process and well past the point of no return (Majno and Joris 1995). Therefore, necrosis is not a form of cell death, but rather can be understood as secondary changes that occur after cell death, more like the cellular equivalent of postmortem decay (Majno and Joris 1999). These authors observed that one of the earliest cellular changes during ischemia is cellular swelling, and suggested the term "oncosis" to describe these prelethal processes leading to cell death. Oncosis (from the Greek word *onkos*-to swell) was first used by von Recklinghausen to describe the osteocyte cell death characterized by swelling during rickets as well as cell death observed during cartilage growth (von Recklinghausen 1910). The cellular changes which characterize oncosis as described by Majno and Joris are very similar to the features of type 3 cell death as defined by Clarke. According to Majno and Joris and others (Van Cruchten and Van Den Broeck 2002), the term necrosis should denote the late phase of degradation of the cell irrespective of the mechanisms of the cell death, for example, karyolysis at the end of oncosis or breakup of apoptotic bodies in apoptosis. Levin and colleagues (Levin et al. 1999; Levin 1998) similarly proposed to apply the term necrosis not to a specific cell death phenotype but to any dead cells detected in living tissue regardless of the cell death mechanisms; they also recommended adding a prefix to mark the type of cell death, for example, apoptotic necrosis or oncotic necrosis. Furthermore, they insisted that cell death type can only be determined by morphologic/ultrastructural analysis and pointing that other frequently used criteria for separation are in fact nonspecific; for example, apoptosis can affect groups of cells in large numbers, oncosis can occur in single cells and that apoptosis can be accompanied by inflammation (Zychlinsky and Sansonetti 1997).

PCD is not equivalent to apoptosis; rather it was described in development, even before apoptosis, as cell death occurring at a predetermined time as a result of a genetic clock (Lockshin and Williams 1964). Others have interpreted PCD as a cell death process where dying cells follow a fixed molecular pathway (Schwartz et al. 1993). All developmental cell death types meet one or both criteria, and therefore can be considered versions of PCD. We propose a more practical definition of PCD inspired in part by a review by R. Sloviter (2002): PCD encompasses all "active cell deaths" where cellular processes are required for the cell to reach the point of

"no return" and the cell's demise occurs only with their participation. PCD requires time to develop, and its mechanisms are thus potentially amenable to therapeutic intervention. In contrast, "passive cell death" is the result of overwhelming cell injuries (e.g., catastrophic cell damage) that instantaneously push the cell past the point of "no return" with no involvement of any active cell process. There is no therapeutic window associated with this type of cell death.

It is unfortunate that these important efforts to define, organize, and classify cell death in a rigorous manner based on ultrastructural changes and distinctions between prelethal cell death processes (i.e., apoptosis, autophagy or oncosis) and postlethal degradation (i.e., necrosis) have not been sufficiently recognized or reflected in most recent publications on cell death. There are several possible explanations, including the fact that the gold standard of electron microcopy is difficult to implement or that responses in different organs/cell types may differ somewhat morphologically. Thus, many authors use the simplistic apoptosis versus necrosis dichotomy, employ nonultrastructural methods that cannot reliably determine the precise cell death phenotype, or focus on mechanisms rather than morphology to establish cell death. Rather than making definitive pronouncements regarding cell death type, authors should instead use descriptive terms when presenting results (e.g., "caspase-3-positive cell death" instead of "apoptosis") (Kroemer et al. 2005).

The study of neuronal cell death in the central nervous system (CNS) is complicated by the fact that in brightfield microscopy all dead neurons appear as shrunken, eosinophilic, and with pyknotic nuclei regardless of how they died (Levin et al. 1999). Furthermore, even using electron microscopy, the ultrastructure of dying neurons does not exactly match the cell death paradigms established in other tissues and/or development. For example, dying neurons in mouse models of Huntington's disease or amyotrophic lateral sclerosis demonstrate neither classical necrosis nor apoptosis morphology (Turmaine et al. 2000; Dal Canto and Gurney 1994) but rather appear more similar to a separate type of neuronal cell death called paraptosis (Sperandio et al. 2000) – characterized by a requirement for gene expression, nonapoptotic morphology marked by vacuolization, and absence of caspase activation. It is uncertain whether this form of cell death is more similar to type 2 or 3B (Castro-Obregon et al. 2002), but in general nonapoptotic cell death showing cytoplasmic vacuolization appears to be most closely related to type 3B (Sperandio et al. 2000). Paraptosis is only one of the forms of apoptosis with nonclassical morphological features and biochemical markers that have been identified (Ravagnan et al. 2002).

In spite of these challenges, groups such as those led by J.W. Olney and D.G. Fujikawa have made important contributions over the years in describing at ultrastructural level neuronal cell death in the brain after various injuries. Olney and colleagues detected two types of neuronal cell death in the brain in vivo (Dikranian et al. 2001), physiologic cell death (PCD) and excitotoxic cell death (ECD). Using rat pups, they characterized the developmental death of neurons in the mammalian brain, which they called PCD and confirmed that it was very similar to type 1/apoptosis (Ishimaru et al. 1999). The earliest changes are seen in the nucleus before any cytosolic abnormalities are apparent and consist of clumping of the nuclear

chromatin followed by intense condensation of chromatin in several large, smooth, and round electron-dense chromatin masses (balls) randomly distributed; these chromatin balls differ from the nonspherical chromatin clumps that form at the nuclear periphery – margination – in apoptosis in other tissues (Dikranian et al. 2001). In the next phase, the nuclear membrane is disrupted (a detail not mentioned in any other previous descriptions of apoptosis) followed by mixing of nucleoplasm and chromatin bodies with the cytosol. The cells then divide into separate membrane-bound bodies (containing cytosol and chromatin balls) that would either be digested after phagocytosis or degraded in the extracellular compartment with glial participation (Dikranian et al. 2001). Initially, organelles appear normal except for mild mitochondrial swelling (Ishimaru et al. 1999) associated with defects in the outer mitochondrial membrane, but after rupture of the nuclear membrane mitochondrial degeneration and endoplasmic reticulum vacuolization become prominent (Dikranian et al. 2001) and the cell appears shrunken and condensed (Bayly et al. 2006). This orderly sequence of morphological changes occurs in a similar fashion during apoptosis of neurons from various brain regions, in response to various inducers; it occurs in neonates and adults, as well as in neurons from the peripheral nervous system (Dikranian et al. 2001). Morphologically, apoptotic neuronal cell death appears similar during cell death during development (PCD), following ethanol (Young et al. 2005) or in response to TBI (Bayly et al. 2006). In contrast, ECD induced by exposure to glutamate is characterized by early cytoplasmic changes, including intense cell swelling, as well as dilation of mitochondria and endoplasmic reticulum followed by their rupture. Nuclear changes only occur later and include chromatin condensation forming small chromatin clumps that later consolidate in a large irregular mass at the center of the nucleus, unlike the large smooth chromatin bodies seen in PCD (Bayly et al. 2006). Eventually, the cell membrane ruptures, but the nuclear membrane still remains intact (Bayly et al. 2006). ECD significantly resembles type 3b/oncosis cell death with the major exception of the ECD-specific formation of large masses of clumped chromatin which are more intense and long lasting than the chromatin clumps in type 3b cell death. Interestingly, although none of the three separate ECD patterns that were seen in different brain regions (Ishimaru et al. 1999) resembles apoptosis under the electron microscope, they could be mistaken with apoptosis if light microscopy is used. On the basis of their TBI experiments that showed that ECD occurred in the first several hours, mostly at the local lesion site whereas the PCD occurred later, around 24 h and at a distance (Dikranian et al. 2001), Olney's group interpreted PCD and ECD as two fundamentally distinct neuronal cell death types triggered in an either/or manner by specific manipulations and cell conditions. Their data contradict the hypothesis that ECD and PCD are extremes of the same process-the continuum theory which holds that the same stimuli acting on the same cells can initiate both pathways or various in between phenotypes containing features from both (Portera-Cailliau et al. 1997). Importantly, their experiments show that the immature brain is much more susceptible to PCD after TBI compared to the adult brain (Bittigau et al. 1999).

Fujikawa and colleagues also describe two distinct neuronal cell death phenotypes, one apoptotic (cell shrinkage with normal organelles/intact plasma membrane

and early nuclear membrane disruption with mixing of nuclear and cytoplasmic content and chromatin condensation in large well-defined bodies) and ECD (necrotic, with intact nuclear membrane and swollen organelles, small chromatin clumps and plasma membrane breaks, similar to type 3b/oncosis), as opposed to a continuum between apoptosis and necrosis. They also stress the importance of ultrastructural studies to correctly identify the type of cell death (Fujikawa 2000; Fujikawa et al. 2007). These authors highlighted some differences between neuronal ECD and oncosis, for example, unlike type 3b/oncotic cell death in other tissues, neuronal ECD is characterized by a shrunken cell body and condensed cytoplasm and nucleus (pyknosis) (Fujikawa et al. 2000, 2002); they also indicated that there are some minor differences between immature and adult excitotoxic morphology (Fujikawa et al. 2000). Fujikawa also indicates that most studies showing incontrovertible ultrastructural proof of apoptosis in vivo are limited to neonatal animals (Fujikawa 2000), that ECD is always of "necrotic" morphology irrespective of age, and that TUNEL or internucleosomal DNA cleavage are nonspecific tests with positive cells present in both apoptosis and ECD (Fujikawa et al. 2000). This latter observation as well as the protective effect of caspase and protein synthesis inhibitors against some instances of "necrotic" neuronal cell death are indicative of an active cell participation (program) in excitotoxicity (Fujikawa 2000).

In many publications, the term "necrosis" is not used as suggested here, but rather is used to reflect type 3/oncotic cell death being associated with the loss of membrane integrity, cellular swelling, damage to organelles, lysosomal disruption, and uncontrolled cell lysis that often leads to tissue inflammation (Bredesen 1995).

There is a sharp contrast between the large number of signaling pathways that participate in cell death and the few well defined morphological types that describe neuronal cell death. The fact that none of the molecular pathways are specific for apoptosis suggests that cell death diagnosis should not be based on mechanisms but rather on morphology, which reflects the summation of participating factors. It is probable that in each cell depending on its status (type, age, location) and inducing agent, multiple pathways will be activated and their interplay will determine how the cell will advance toward one or another of the several discrete types of cell death, each with its unique set of ultrastructural features.

Some reports claim that apoptosis and necrosis may have common signal transduction pathways, and under some circumstances might even participate together in the development of cell death (Formigli et al. 2000). Other groups have shown that apoptosis and necrosis can be initiated by the same trigger events, and that low intensity of the insult favors apoptosis whereas high intensity of the insult pushes cells toward necrosis (Bonfoco et al. 1995). It has been also suggested that high intracellular ATP levels favor initiation of apoptosis whereas low intracellular ATP levels favor the necrotic execution programs (Turmaine et al. 2000; Eguchi et al. 1997). Furthermore, some reports indicate that inhibition of one cell death pathway may redirect the cell toward other mechanisms and phenotypes of cell death (Pohl et al. 1999; Fiskum 2000).

12.3 Cell Death Programs: Molecular Pathways that Participate in Cell Death

As discussed, PCD is a cell death that is executed by activation/inactivation of various molecular mechanisms in the cell that is destined to die. Over the past few years, scientists have identified a large number of molecular changes affecting various pathways associated with cell death. These mechanisms can be divided in three categories: (1) required for cell death (no cell death in their absence); (2) required for a particular characteristic of cell death (e.g., "apoptotic" chromatin condensation) but not required for cell death, which can occur in their absence, possibly after a delay and/or with a changed morphology/type; and (3) bystander with no impact on cell death. Several of the most important molecular mechanisms belonging to the first two categories and their upstream regulators, especially with regard to neuronal cell death in the context of CNS injury, are discussed.

Cell cycle activation induces neuronal apoptosis (Kruman et al. 2004) both in vitro (Otsuka et al. 2004; Strazza et al. 2004; Park et al. 2000a; Giovanni et al. 1999) and in vivo (Nishioka and Arnold 2004; Wen et al. 2004; Love 2003). For example, induction of cell cycle proteins plays a role in neuronal cell death in vitro caused by excitotoxins (Park et al. 2000b), ceramide (Strazza et al. 2004), β-amyloid (Giovanni et al. 1999), KCl withdrawal (Padmanabhan et al. 1999), trophic factor deprivation (Park et al. 1997), or DNA damage (Kruman et al. 2004; Otsuka et al. 2004; Park et al. 2000a). Cyclin-dependent kinases also appear involved in neuronal apoptosis in the developing brain, as well as in neuronal degeneration in the adult brain following cerebral ischemia (Love 2003; Wang et al. 2002a), Alzheimer disease (Wen et al. 2004; Swerdlow and Khan 2004), Parkinson disease (Smith et al. 2003), or amyotrophic lateral sclerosis (Nguyen et al. 2003). Moreover, inhibition of cell cycle pathways is neuroprotective in vitro (Verdaguer et al. 2003) and in vivo (Wang et al. 2002a).

Apoptosis and cell cycle pathways share several common regulatory elements, such as retinoblastoma protein (Rb), E2F, and p53. Generally, postmitotic cells such as neurons do not engage in cell cycle progression once they differentiate and cell cycle proteins are usually downregulated in them (Okano et al. 1993). However, mature neurons have the capacity to re-enter the cell cycle, which results in apoptosis rather than neuronal proliferation (Nguyen et al. 2002; Wartiovaara et al. 2002). Whereas in many types of neuronal injury, the neurons re-enter the cell cycle, but do not advance past the G1/S checkpoint before dying, recent reports show that in some models of cerebral ischemia, a significant proportion of neurons enter S phase and begin DNA synthesis (BrdU positive) before ultimately dying (Kuan et al. 2004; Wen et al. 2005).

Progression through the cell cycle is controlled by the interaction of numerous molecules (Boonstra 2003) including cyclins (Sherr 1993), cyclin-dependent kinases (CDKs) (Nishitani and Lygerou 2002; Obaya and Sedivy 2002), and cyclin-dependent kinase inhibitors (CDKIs) (Fischer et al. 2003) such as p21 and p27 (Coqueret 2003). In response to mitogenic stimulation, D-type cyclins are

synthesized and bind/activate CDK4 and CDK6, which prompt cells to enter the G1 phase (Sherr 1995). Cyclin E is induced later in G1 phase, and its association with CDK2 is required for the transition into S phase, during which DNA synthesis is associated with active cyclin A/CDK2 complex (Li et al. 1993). Cyclin A is degraded at the G2/M transition, whereas the released CDK2 forms a complex with newly synthesized cyclin B. The cyclin B/CDK2 complex is necessary for entry and progression through mitosis (Guadagno and Newport 1996). This complex is inactivated in late M phase by the anaphase-promoting complex (Thornton and Toczyski 2003). The progress through cell cycle is strictly controlled by checkpoints, and cells stopped at any of the checkpoints will either return to G0 phase or die by apoptosis (Pietenpol and Stewart 2002; Nagy 2000). Increased levels of G1 cyclins D and E, and their involvement in neuronal cell death, have been demonstrated both in in vitro (Verdaguer et al. 2002) and in vivo (Park et al. 2000b) models of excitotoxicity. Moreover, upregulation of cyclins D, A, and B has been associated with toxin-induced hippocampal damage (McPherson et al. 2003) and increased protein levels of cyclin D1 and CDK4 are critical for excitotoxin-induced neuronal cell death (Ino and Chiba 2001). Increased protein levels of cyclin D1 and CDK4 in motor neurons have been linked to the PCD of these neurons in a model of transient spinal cord ischemia (Sakurai et al. 2000). Translocation of cyclin D and CDK4/6 from the cytosol to the nucleus is also part of the mechanism responsible for the activation of CDK4/6 and apoptosis in neurons (Padmanabhan et al. 1999; Ino and Chiba 2001; Sumrejkanchanakij et al. 2003). In the nucleus, CDK4/6 phosphorylates the Rb on specific residues including Ser780 and Ser795 (Kitagawa et al. 1996; Riedel et al. 1995). Unphosphorylated Rb forms a complex with the E2F transcription factors, resulting in repression of their transcriptional activity (Liu and Greene 2001), but the phosphorylation of Rb results in the dissociation of Rb from the Rb/E2F complex and resulting activation of E2F (Kitagawa et al. 1996; Sears and Nevins 2002). Therefore, the cascade of molecular events linking activation of cell cycle to neuronal apoptosis involves formation of the cyclin D1-CDK4/6 complex, activation of CDK4/6 followed by phosphorylation of Rb, dissociation of Rb-E2F complex and activation of E2F transcriptional activity (Liu and Greene 2001; Freeman et al. 1994; Herrup and Busser 1995; Osuga et al. 2000). E2F induces apoptosis by the upregulation of the cell death machinery at multiple levels: (1) activation of B- and C-myb genes (Liu et al. 2004), (2) increased expression of crucial components of caspase pathway such as caspases 3, 9, and 8 and Apaf-1(Nahle et al. 2002), (3) activation of p53 and p73 (Sears and Nevins 2002; Greene et al. 2004). p53 and p73 lead to the activation of proapoptotic Bcl-2 family members, resulting in the release of mitochondrial proapoptotic proteins such as cytochrome *c*, a critical component of the intrinsic caspase activation pathway (Nguyen et al. 2003; Nahle et al. 2002). Recent reports have also established that in some models of neuronal cell death, activation of CDK5 is upstream of CDK4/6 (Nguyen et al. 2002, 2003). Furthermore, CDK5 can directly phosphorylate Rb on specific residues Ser807/811 and initiate neuronal cell death (Hamdane et al. 2005). The described molecular interactions are illustrated in Fig. 12.1.

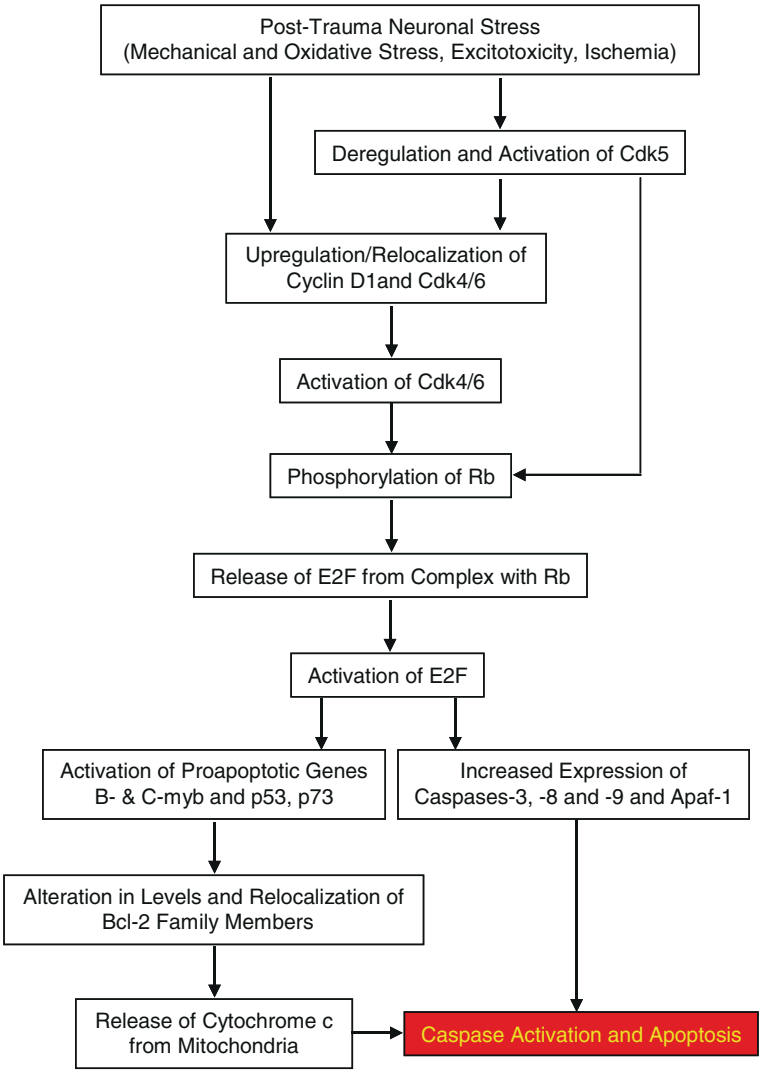

Fig. 12.1 Key mechanisms responsible for the cell cycle-apoptosis connection in neurons

12.3.1 Caspase-Dependent Pathways

Caspases are a family of cysteinyl aspartic acid-proteases that are activated by proteolytic cleavage (Alnemri et al. 1996). After activation, caspases may cleave their own precursors or other procaspases, initiating the caspase activation cascade. The earliest indication of the role played by caspases came from the study of *ced-3*,

a caspase ortholog found in *Caenorhabditis elegans* (Hengartner 1999), where ced-3 was shown to be required for developmental PCD. Caspases can be divided into two large groups, some involved in cell death (caspases-2, -3, -6, -7, -8, -9) and others involved with proinflammatory pathways (caspases-1, -4, -5, -11, -12, -13). The cell death caspases can be further classified as "initiator caspases" (-8 and -9) responsible for initiating the caspase cascade by proteolytic cleavage and activation of the "effector caspases" (-3, -6, -7), which in turn cleave a selective group of molecules. These caspase substrates upon cleavage undergo changes in their activity profiles causing the cellular morphological changes that are hallmarks of apoptosis, such as membrane budding, chromatin condensation, and nuclear fragmentation (Cohen 1997). Therefore, the apoptotic cell death depends on caspase activation for the full development of its unique characteristics.

Caspase-3 appears to be the major effector caspase in neuronal apoptosis. Studies using the caspase-3 knockout mice have shown profound abnormalities in brain development (Kuida et al. 1996). Furthermore, caspase-3 plays a major role in injury-induced neuronal loss, as shown by investigations using peptide-based caspase inhibitors in vivo and in vitro (Yakovlev and Faden 2001).

Cells have two major mechanisms to activate caspase-3. The extrinsic pathway (receptor-mediated apoptosis) involves oligomerization of surface receptors such as the TNF and FAS receptors after binding TNFα and FAS ligand, respectively, resulting in the formation of death-inducing signaling complex, which recruits initiator procaspase-8 and triggers its activation (Yakovlev and Faden 2001). Caspase-8 then cleaves and activates the effector (executioner) caspase-3. The intrinsic pathway (mitochondria-mediated apoptosis) involves mitochondrial outer membrane permeabilization (MOMP), followed by the release of cytochrome *c* from mitochondria intermembrane space to the cytosol, wherein the presence of dATP, it binds to Apaf-1, forming the apoptosome, to which procaspase-9 is recruited and activated (Yakovlev and Faden 2001; Aravind et al. 1999; Luo et al. 1998). Active caspase-9 will then cleave and activate caspase-3.

Activation of the intrinsic pathway, unlike the extrinsic pathway, requires an active transcription/translation to increase the expression of proapoptotic members of the Bcl-2 family that mediates the release of cytochrome *c* from the mitochondria (Yakovlev and Faden 2001; Cory and Adams 2002).

Multiple publications support a key role for the intrinsic pathway as initiator of caspase activation and determinant of neuronal cell death. Caspase-9 as well as Apaf-1 knockout mice suffers from severe brain development abnormalities linked to a reduction in developmental PCD in the brain (Kuida et al. 1998). The phenotype of caspase-$9^{-/-}$ and Apaf-$1^{-/-}$ is similar to that of caspase-$3^{-/-}$, suggesting that the intrinsic pathway plays an important role in neuronal apoptosis at early stages of brain development (Kuida et al. 1998). Furthermore, the intrinsic caspase pathway appears to be required for neuronal apoptosis after various type of pathologic condition in adult animals, including cerebral ischemia, traumatic brain injury, spinal cord injury, Huntington's disease, Alzheimer's disease, and seizures (Eldadah and Faden 2000; Honig and Rosenberg 2000; Yakovlev et al. 2001; Yakovlev and Faden 2001).

Caspases-3, -8, and -9 are not the only caspases responsible for cell death, and receptors and mitochondria are not the only sites that initiate neuronal apoptosis. Recently, the endoplasmic reticulum was found to be the origin of a new cell death pathway (Nakagawa et al. 2000). Nakagawa et al. showed that caspase-12 could be induced and activated by the unfolded protein response after endoplasmic reticulum (ER) stress. In turn, caspase-12 serves as an initiator of the caspase cascade. The significance of this pathway in the nervous system is illustrated by the resistance of neurons from caspase-12-knockout mice to β-amyloid induced apoptosis. In ER-mediated apoptosis, caspase-12 is upstream of caspase-3 (Bitko and Barik 2001). Caspase-12 is induced in the brain after TBI at both mRNA and protein levels (proform and processed form), suggesting a role for the ER apoptotic pathway in the injury-dependent pathology (Larner et al. 2004).

Caspase-11, which has a proinflammatory effect acting as an upstream activator of caspase-1 (Wang et al. 1998), is also able to activate caspase-3. This latter role has a physiological relevance, as caspase-11-deficient mice show reduced caspase-3 activity and cell death after brain ischemia (Kang et al. 2000). In a mouse model of Parkinson's disease, it was shown that MPTP-dependent neurotoxicity involves caspase-11 both in a noncell-autonomous role as an activator of inflammation and its cell-autonomous role as an inducer of neuronal apoptosis by activating caspase-3 (Furuya et al. 2004).

Caspase-2 may also play important albeit divergent regulatory roles in some types of neuronal apoptosis. In a model of serum-deprivation-induced apoptosis in primary cortical neurons, inhibition of caspase-2 results in attenuation of Bax-dependent mitochondrial permeabilization, caspase-3 activation and ultimately in decreased neuronal cell death (Chauvier et al. 2005). Increased expression and activation of caspase-2 parallels the seizure-induced degenerative process in experimental and human temporal lobe epilepsy, suggesting a role for caspase-2 in the neuronal cell death process associated with these conditions (Narkilahti et al. 2007). In contrast, motor neurons undergo more intense cell death during development in caspase-2-deficient mice compared to control, and sympathetic neurons lacking caspase-2 expression are more prone to nerve growth factor deprivation-induced apoptosis compared to wild-type neurons (Bergeron et al. 1998).

12.3.1.1 Regulation of Caspase Activation

Bcl-2 family includes many proteins; some are proapoptotic and others are antiapoptotic. Studies of human patients who have suffered traumatic cerebral contusion have shown that the presence of caspase-3 or of the antiapoptotic bcl-2 in the contused brain are good albeit opposite predictors of neurological outcome, with caspase-3-positive/bcl-2 negative status being associated with a poor outcome and caspase-3-positive/bcl-2-positive status being associated with a more favorable prognosis (Nathoo et al. 2004). In animal models of traumatic brain injury, the expression of the antiapoptotic genes bcl-2 and bcl-xL was downregulated for at least 3 days in the injured cortex and hippocampus, whereas the expression of

the proapoptotic gene bax was elevated in the same areas, suggesting a switch inside the bcl-2 family to promote cell death (Strauss et al. 2004). Other studies have confirmed the TBI-induced increase in the injured cortex at mRNA and protein levels of not just bax but also of another proapoptotic bcl-2 family member, bad. It was hypothesized that the neuroprotective effects of progesterone after experimental TBI can be explained by its ability to push the apoptotic balance of power toward the survival side. This hypothesis is supported by findings such as the progesterone-induced decrease in post-TBI expression of proapoptotic proteins caspase-3 and bax (Djebaili et al. 2005), or in the progesterone-dependent attenuation of the post-TBI levels of bax and bad in parallel with an increase in the levels of bcl-2 and bcl-xL (Yao et al. 2005). The neuroprotective effects of estrogen after experimental TBI may also result from its ability to upregulate bcl-2 expression (Soustiel et al. 2005). Estrogen had no significant impact on bax levels (Soustiel et al. 2005). In contrast, beneficial effects of hyperbaric oxygen therapy after TBI are associated with increased levels of bcl-2 and bcl-xL without any significant effect on bax levels (Vlodavsky et al. 2005). Such data suggest that the cumulative effect on proapoptotic/antiapoptotic balance may be more important for cell survival than the exact protein changes.

In cells under normal conditions, Bax has a cytosolic localization, but in response to various injuries/stresses it translocates to the mitochondria where it forms homo-oligomers that cause MOMP, cytochrome *c* release, formation of the apoptosome complex, and caspase activation (Hsu et al. 1997). Although traditionally caspase-activation by the intrinsic pathway is downstream of cytochrome *c* release following MOMP, recent data paint a more complex picture in which MOMP itself is, at least in some circumstances, induced by activation of some members of the caspase family (Bossy-Wetzel and Green 1999; Lassus et al. 2002). On one hand, some reports suggest that caspase-2 is required for MOMP and promotes cytochrome *c* release (Lassus et al. 2002; Paroni et al. 2002; Robertson et al. 2002; Enoksson et al. 2004; Robertson et al. 2004), whereas another recent study documents the involvement of caspases-3 and -7 in MOMP (Lakhani et al. 2006). On the other hand, there is evidence that Apaf-1, a key apoptosome component, is required for the activation of all apoptotic caspases, seeming to reestablish the prominence of the apoptosome as the initiator of the caspase cascade (Franklin and Robertson 2007). Surprisingly, in the same work, Franklin and Robertson show that Apaf-1 is also required for MOMP and cytochrome *c* release. The interpretation proposed by the same authors is that there is a loop rather than a linear relationship between mitochondrial events and caspase activation with MOMP often the initiating event but with caspases representing a critical mechanism that creates a feed-forward amplification loop driving the efficient release of cytochrome *c*. The inference is that in cells, MOMP is not an all or nothing event but one that starts slowly and requires caspase activation to achieve full power and result in cell death (Franklin and Robertson 2007).

Inhibitors of apoptosis (IAPs) are a family of proteins that keep in check the apoptotic machinery at several molecular levels, the best known being their ability

to inhibit caspase activity (Deveraux and Reed 1999). The antiapoptotic activity of IAPs is regulated by caspase-dependent cleavage or by interaction with other proteins (Deveraux and Reed 1999). Smac/DIABLO (second mitochondrial activator of caspases) is a mitochondrial protein that is released to the cytosol upon MOMP, where it binds and abrogates the caspase-inhibiting activities of IAPs such as XIAP (Srinivasula et al. 2000). Recent data suggest that Smac/DIABLO levels are increased, and there are mitochondria to cytosol translocation of Smac/DIABLO after brain ischemia in a manner that correlates with caspase-3 activity (Saito et al. 2003, 2004a, 2005a, b).

Omi/HtrA2 is a member of a novel family of serine proteases homologous to the *Escherichia coli* chaperone HtrA and in vitro studies have shown that Omi/HtA2 can cleave and inactivate IAPs independent of caspase activity (Nakka et al. 2008). Omi/HtrA2, like Smac/DIABLO, resides in the mitochondria inter-membrane space and is released to the cytosol after MOMP (Saito et al. 2004b). Studies have shown that after cerebral ischemia Omi/HtrA2 expression is increased and that Omi/HtrA2 is released to the cytosol where it interacts with XIAP, suggesting that Omi/HtrA2 may play a role in promoting cell death under these conditions (Saito et al. 2004b). Omi/HtrA2 has other cellular functions, some of which might be neuroprotective, as indicated by findings showing that animals which are deficient in Omi/HtrA2 expression are more prone to neurodegeneration and early death (Martins et al. 2004).

12.3.2 Caspase-Independent Cell Death and Its Regulators

Caspase-independent mechanisms are important mediators of neuronal cell death (Ravagnan et al. 2002; van Loo et al. 2002). It has been suggested that in some situations the beneficial effects resulting from the inhibition of just one cell death pathway such as the caspase cascade are more modest than expected as other mechanisms such as caspase-independent mechanisms progress unimpeded (Volbracht et al. 2001). The balance between caspase-dependent versus caspase-independent mechanisms changes dependent on brain region, cell type, and age (Oppenheim et al. 2001), as suggested, for example, by differential benefit from caspase inhibition seen in various neuronal populations in experimental models of stroke (Zhan et al. 2001).

It is now recognized that there are a number of mitochondrial proteins that may be released by various death-promoting stimuli and contribute to cell death. In addition to cytochrome *c*, these include Smac/DIABLO, Omi/HtrA$_2$, AIF, and endonuclease G (Ravagnan et al. 2002; Srinivasula et al. 2000; van Loo et al. 2002; Daugas et al. 2000; Suzuki et al. 2001; Li et al. 2001). Smac/DIABLO and Omi/HtrA$_2$ may cause apoptosis through either caspase-dependent or caspase-independent mechanisms (Ravagnan et al. 2002; van Loo et al. 2002; Suzuki et al. 2001), whereas AIF and EndoG appear to cause cell death through caspase-independent mechanisms (Daugas et al. 2000; Li et al. 2001).

AIF is one of the most important agents of caspase-independent cell death, and the study of the role and regulation of this pathway by PARP-1, cyclophilin A (CypA), and HSP-70 are active fields of research. AIF is a phylogenetically ancient flavoprotein NADH oxidase contained in the mitochondrial inter-membrane space, where its local redox function is essential for efficient oxidative phosphorylation and for antioxidant defense (Lipton and Bossy-Wetzel 2002; Vahsen et al. 2004). Upon induction of the death-promoting stimulus, AIF translocates from the mitochondria to the cytosol and then to the nucleus, where it associates with chromatin and causes peripheral chromatin condensation and high molecular weight DNA fragmentation (Ravagnan et al. 2002; van Loo et al. 2002; Daugas et al. 2000) Susin et al. 1999. AIF is essential for PCD during cavitation of embryoid bodies, as indicated by studies with AIF knockout mice (Joza et al. 2001). Translocation of AIF to the nucleus in neurons in vitro can be induced by *N*-methyl-D-aspartate (NMDA) excitoxicity (Cheung et al. 2005) or oxygen-glucose deprivation (Culmsee et al. 2005). Other models such as hypoglycemic coma (Ferrand-Drake et al. 2003), global cerebral ischemia (Cao et al. 2003), focal cerebral ischemia (Ferrer et al. 2003), postnatal hypoxia-ischemia (Zhu et al. 2003), and TBI (Zhang et al. 2002) have also been associated with neuronal cell death in the brain characterized by AIF nuclear translocation.

The role of AIF in secondary injury has been somewhat controversial because, like other mitochondrial proteins implicated in apoptosis (e.g., cytochrome *c*), it appears to have beneficial physiological activity under normal conditions (Garrido and Kroemer 2004). Thus, within mitochondria, AIF plays an important role in oxidative phosphorylation (Vahsen et al. 2004), and depletion of AIF may make certain cells (retinal, cerebellar) more sensitive to neurotoxicity. Nonetheless, Harlequin mutant (Hq) mice, which express low levels of AIF (20% of normal AIF expression), show less cell death after cerebral ischemia despite reduced capability to use glucose by oxidative phosphorylation (Culmsee et al. 2005). This suggests that the effect of AIF depletion on cell death outweighs potential deleterious effects of altered oxidative phosphorylation (Culmsee et al. 2005). Neurons with low AIF expression, or in which AIF has been neutralized with antibodies, show resistance to NMDA or glutamate excitotoxicity (Wang et al. 2004; Culmsee et al. 2005). Excitotoxic studies using kainic acid have shown that Hq mice are less susceptible to hippocampal damage compared to wild-type (WT) littermates (Cheung et al. 2005). A recent report by Cheung et al. underscored this dual role of AIF as important for both the maintenance of mitochondrial structure as well as for cell death (Cheung et al. 2006). They also confirmed that the death-promoting effects of AIF from mitochondria are twofold: an indirect mechanism reflecting dysfunction of mitochondria depleted of AIF; and a direct mechanisms resulting from AIF-dependent chromatin digestion and condensation in the nucleus. The latter component appears to play the primary role because preservation of an AIF subpopulation in the mitochondria only transiently protects againt cell death (Cheung et al. 2006). A preponderance of evidence indicates that translocation of AIF, like cytochrome *c*, causes cell death through both BAX-dependent and BAX-independent mechanisms (Cheung et al. 2005). Although some studies indicate that AIF release may occur

downstream of caspase-3, most studies and models show that AIF-mediated cell death is caspase-independent and can occur before cytochrome *c* release (Cregan et al. 2004; Cande et al. 2004a) and/or in the presence of caspase inhibitors (Cao et al. 2003; Cregan et al. 2002).

AIF translocation and its association with cell death have been demonstrated after both experimental cerebral ischemia and TBI (Zhang et al. 2002; Plesnila et al. 2004; Culmsee et al. 2005). Recent work also supports a causal role for AIF in excitotoxic (Cheung et al. 2005) and ischemic neuronal cell death in vitro and in vivo (Culmsee et al. 2005). Whether inhibition of AIF-mediated neuronal cell death can serve to enhance caspase-dependent death has been debated (Culmsee et al. 2005). However, in neonatal cerebral hypoxia-ischemia, AIF and caspases act through parallel pathways, with caspase inhibition and low AIF expression (Hq mice) having potentially additive therapeutic effects (Zhu et al. 2007).

In contrast to caspase-mediated apoptosis and similar to necrosis, AIF-mediated cell death can occur under low bioenergetic conditions and is prominent in the lesion core region after cerebral ischemia (Cao et al. 2003; Cregan et al. 2004). Of note is the observation that AIF translocation can occur in conditions with ATP depletion, which leads to inhibition of caspase-dependent apoptosis and promotion of necrotic cell death (Zhang et al. 2002). Moreover, AIF-induced cell death has been shown in cells lacking Apaf-1 or caspase-3 (Loeffler et al. 2001). Redistribution of AIF has been shown in neuronal cultures exposed to peroxynitrite and after brain injury (Zhang et al. 2002). Given the fact that both reduced blood flow and bioenergetic declines occur to a greater degree after severe versus mild brain injury, we propose that AIF may play a larger pathophysiological role after more severe brain injuries, in contrast to caspase-mediated apoptosis. Consistent with this interpretation is the observation that AIF translocation contributes to neuronal cell death after excitotoxic insults mediated by NMDA receptors or induced by kainic acid (Cheung et al. 2005; Wang et al. 2004). NMDA receptor-mediated excitotoxicity contributes to both cell death and neurological dysfunction after experimental TBI in an injury dose-dependent manner (Faden et al. 1989). Indeed, such excitotoxicity is correlated with changes in cellular bioenergetics (Faden et al. 1989). However, it should be underscored that the pathobiology of TBI differs substantively from ischemic injury with regard to the levels of blood flow and bioenergetic compromise after the insult (Kochanek et al. 1995; Yamakami and McIntosh 1991; Yuan et al. 1988; Lewelt et al. 1982; Vink et al. 1988).

There is now substantial experimental support that AIF release is mediated by the activation of PARP-1(Hong et al. 2004) and that the absence or inhibition of PARP-1 provides considerable neuroprotection in experimental stroke (Takahashi et al. 1997; Endres et al. 1997; Eliasson et al. 1997) as well as to some degree after TBI (Whalen et al. 1999). The effect of PARP inhibition preventing AIF release directly results from decreased PARP activity (Culmsee et al. 2005). PARP inhibitors also attenuate death of cerebral endothelial cells after ischemia by limiting AIF nuclear translocation (Zhang et al. 2005). AIF translocation is inhibited in cells from PARP-1 knockouts or WT cells administered PARP inhibitors (Hong et al. 2004; Yu et al. 2002, 2003). Moreover, absence or inhibition of PARP-1 provides

striking protection against excitotoxic insults (Yu et al. 2003). Mechanisms linking these various phenomena may involve NMDA receptor-mediated Ca^{2+} influx, leading to the activation of nitric oxide synthase (NOS) with generation of nitric oxide (NO) and peroxynitrite; the latter is a potent oxidant that causes DNA damage and PARP activation (Wang et al. 2004), resulting in decreased mitochondrial NAD^+ and AIF release (Yu et al. 2002). Swanson and colleagues have suggested that PARP-1 activation depletes cytosolic $NAD,^+$ which by inhibiting glycolysis causes mitochondrial dysfunction and mitochondrial permeability transition; the subsequent release of death-promoting proteins, including AIF, leads to cell death (Alano et al. 2004; Ying et al. 2002, 2005). The importance of NO production as inducer of PAR polymer formation and AIF release during focal cerebral ischemia has been demonstrated by showing that inhibition of neuronal NOS attenuated both accumulation of PAR polymer and AIF translocation to the nucleus (Li et al. 2007). Recent papers from the Dawson group (Yu et al. 2006; Andrabi et al. 2006) demonstrate that poly(ADP-ribose) (PAR) polymer, a product of PARP-1 activity, is neurotoxic both in vitro and in vivo and is responsible for PARP-1-dependent AIF release from mitochondria. Thus, in NMDA-induced neuronal cell death, the PAR-polymer appears to be the death signal and AIF the executioner (Yu et al. 2006). Multiple reports indicate that AIF is a membrane-integrated mitochondrial protein that becomes soluble and able to exert its proapoptotic role only after cleavage by enzymes such as the calpains (Polster et al. 2005; Sanges et al. 2006; Otera et al. 2005; Yuste et al. 2005). Recent work also implicates calpains in the PARP-1 pathway, resulting in AIF release (Moubarak et al. 2007). Thus, specific PARP-1 activation (via an unspecified mechanism involving PAR polymers) may result in calpain activation, which leads to BAX activation and specific cleavage of AIF – both of which are required for AIF release from the mitochondria, translocation to the nucleus and cell death (Moubarak et al. 2007). Susin's group found that in PARP-1-dependent cell death, mitochondria release not only AIF but also cytochrome *c*, yet there is no caspase activation (Moubarak et al. 2007). In their model, as in other PARP-1 activation paradigms (Alano et al. 2004), a severe drop in ATP occurs as a result of NAD^+ depletion; this might explain the failure of cytochrome *c* release to cause caspase activation, as the cytochrome *c*-Apaf-1 pathway of caspase activation is ATP-dependent (Eguchi et al. 1999).

Cyclophilins are a subgroup of peptidylprolyl cis/trans isomerases (PPIases, immunophilins) and the human genome contains at least 16 proteins that possess the cyclosporin-A (CsA) binding domain (CLD) (Galat 2003); they are found in organisms ranging from bacteria to mammals and are structurally conserved throughout evolution (Wang and Heitman 2005). The archetype of the cyclophilin protein family is a cytosolic protein, cyclophilin A (cyclophilin-18, CypA). CypA is best known for its immunosuppressive function when complexed with CsA. The physiologic functions of cyclophilins are not yet completely understood. CypA may be involved in the folding of certain neuronal receptors (Helekar et al. 1994), and recent data have suggested a role for CypA as a CsA-independent immune regulator (Kim et al. 2005). CypA can also promote G1-S transition by upregulating expression and/or activity of cyclin D1 and cdk4 (Semba and Huebner 2006);

considering the death-promoting effects of cyclin D1/cdk4 and cell cycle activation in neurons (Di Giovanni et al. 2005), it is possible that the absence of CypA might have some neuroprotective effects. In contrast, studies using cortical neuronal cultures have shown a neuroprotective effect for both extracellular and intracellular CypA in models of oxidative stress and ischemia (Boulos et al. 2007); the authors speculate that binding of CypA to its newly described putative receptor, CD147 (expressed on neurons), followed by activation of ERK1/2 prosurvival pathways explains the neuroprotection afforded by exogenous CypA. Neuroprotective effects for CypA have also been found in neurons expressing the mutant Cu/Zn superoxide dismutase (Lee et al. 1999). However, two recent papers have demonstrated a link between AIF and CypA in mammals (Cande et al. 2004b) and yeast (Wissing et al. 2004), resulting in their collaboration during chromatinolysis. AIF and CypA interact directly and are colocalized in the nucleus during caspase-independent cell death. Furthermore, AIF and CypA act synergistically to degrade plasmid and nuclear DNA. The AIF-interacting site of CypA is separate from the catalytic site, and this cooperation between AIF and CypA does not require the PPIase activity (Cande et al. 2004b; Wissing et al. 2004). Cyclosporin A, which inhibits the peptidyl-prolyl *cis–trans* isomerase activity of CypA, had no effect on the AIF/CypA interaction, which thus occurs independently of its chaperone function (Cande et al. 2004b; Wissing et al. 2004). Importantly, AIF function is enhanced by CypA overexpression and attenuated by absence of CypA (Cande et al. 2004b). Unlike the AIF null embryos, CypA null animals are viable (Colgan et al. 2000). More recent data by the Blomgren group show that CypA is required for AIF translocation to the nucleus and that CypA knockout mice have reduced infarct volume after cerebral hypoxia-ischemia compared to WT (Zhu et al. 2007). Together, these observations indicate that AIF-mediated cell death plays an important role in cerebral ischemia and that CypA is a critical cofactor. Some of the described molecular interactions are illustrated in Fig. 12.2.

12.3.2.1 Heat Shock Proteins

The heat shock proteins such as the HSP70 family of stress-inducible proteins function normally as ATP-dependent chaperones, assisting the folding of newly synthesized proteins (Beckmann et al. 1990; Murakami et al. 1988). When HSP70 proteins are upregulated in response to cellular stress they provide strong cytoprotection against a large variety of insults (Parcellier et al. 2003; Li et al. 2002; Garrido et al. 2001). More specifically, they are upregulated after brain injury due to ischemia or trauma, including humans (Lee et al. 2004; Seidberg et al. 2003). Overexpression of HSP70 in transgenic mice or drug-induced upregulation provide strong neuroprotection against brain ischemia (Yasuda et al. 2005), whereas targeted knockouts show larger lesions after ischemic injury (Lee et al. 2004). Several recent studies have indicated that two structurally distinct HSP70-inducing drug families have strong neuroprotective potential. Geranylgeranylacetone (GGA) attenuates neuronal damage after cerebral ischemia (Yasuda et al. 2005; Nagai et al.

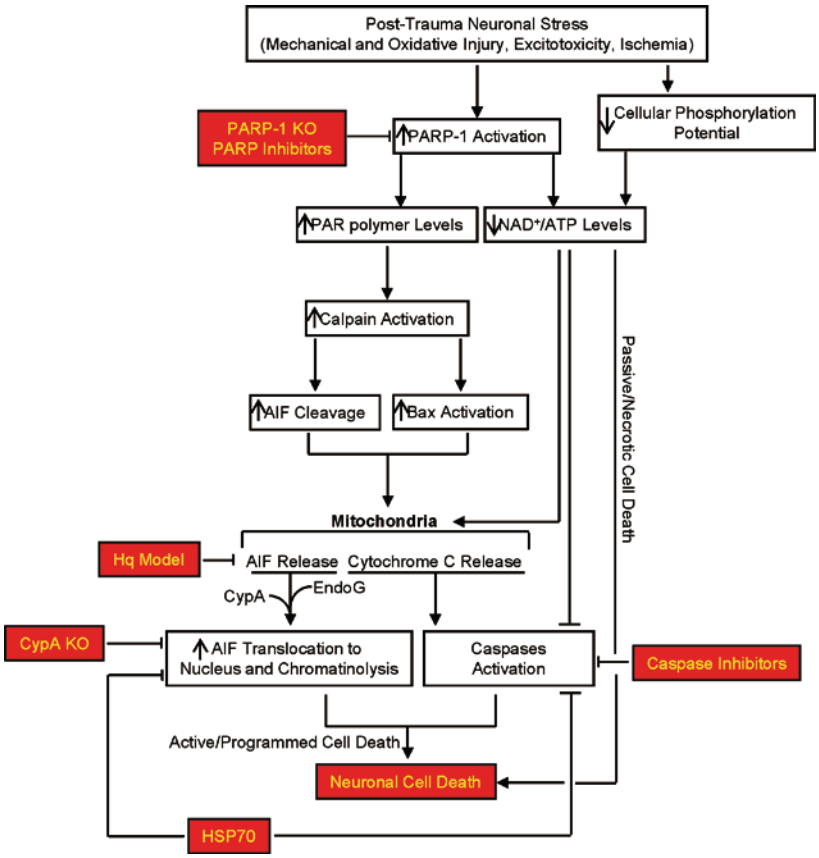

Fig. 12.2 Key mechanisms responsible for neuronal cell death

2005), alleviates polyglutamine-mediated motor neuron disease (Katsuno et al. 2005), and prevents acoustic damage after noise trauma (Mikuriya et al. 2005). Geldanamycin and/or its derivatives, such as 17-allylamino-17-demethoxygeldanamycin (17-AAG), ameliorates polyglutamine-mediated motor neuron degeneration (Waza et al. 2005) and protects the brain against focal ischemia (Lu et al. 2002) and MPTP-induced neurotoxicity (Shen et al. 2005). Importantly, doses of these agents shown to be neuroprotective after the pretreatment in cerebral ischemia are associated with upregulation of HSPs (Yasuda et al. 2005; Nagai et al. 2005). HSP70 has been shown to bind Apaf-1 and AIF, neutralizing their death-promoting functions by preventing the formation of the apoptosome (Beere et al. 2000) and attenuating nuclear translocation (Gurbuxani et al. 2003), respectively. Initial studies indicated that separate HSP70 structural domains are required for Apaf-1 and AIF interaction

(Ravagnan et al. 2001); the binding of Apaf-1 is dependent on the HSP70 ATPase domain, whereas this domain is not required for AIF binding (Ravagnan et al. 2001). Interestingly, more recent studies suggest that the ATPase domain is in fact involved in AIF binding and limits its nuclear uptake (Ruchalski et al. 2006). In vivo data obtained using transgenic models have demonstrated that HSP70 overexpression attenuates ischemic brain injury by sequestering AIF in complexes with HSP70 and preventing ischemia-induced AIF nuclear translocation and resulting cell death (Matsumori et al. 2005). Overexpression of HSP70 reduces both cytochrome *c* release and caspase-mediated apoptosis, as well as caspase-independent cell death associated with AIF translocation (Parcellier et al. 2003; Gurbuxani et al. 2003; Ravagnan et al. 2001) and attenuates neurological injury after brain ischemia (Tsuchiya et al. 2003). Conversely, disruption of the HSP70.1 gene results in increased infarction and cell death after cerebral ischemia and associated release of cytochrome *c* and activation of caspase-3 (Lee et al. 2004).

12.3.2.2 Endonuclease G

Endonuclease G (EndoG) is a mitochondrial endonuclease that is released from mitochondria during MOMP and participates in caspase-independent DNA fragmentation during cell death (Li et al. 2001). Studies in *C. elegans* have suggested that the AIF and EndoG homologs cooperate in DNA fragmentation (Wang et al. 2002b). It was shown that decreased EndoG expression is associated with increased resistance to excitotoxicity (Wu et al. 2004) and that early translocation of EndoG to the nucleus, where it colocalizes with AIF and is associated with DNA fragmentation, is detected after cerebral ischemia (Lee et al. 2005). EndoG translocation to the neuronal nucleus and its role in neuronal death pathways is also supported using models of stroke and hypoxia (Zhang et al. 2007). In contrast, other studies using EndoG null mice have shown that EndoG is not essential for normal embryogenesis or in various apoptotic paradigms (David et al. 2006), and although they do not deny a role for EndoG in cell death, they imply the existence of redundant mechanisms.

12.4 Cooperation of Cell Death Programs

Support is growing for the concept that in various stress-induced cell death models there is more than a single pathway promoting cell death that is activated (Proskuryakov et al. 2003). The relative balance that ultimately decides which pathway is dominant probably depends not as much on the trigger as on the cellular bioenergetic status. For example, AIF translocation can occur under low energetic conditions, in association with activation of PARP-1 and reduction of NAD^+. In contrast, caspase activation is generally associated with a more preserved bioenergetic state and requires adenosine 5′-triphosphate (ATP) (Eguchi et al. 1999; Leist et al. 1997). Thus, caspase-independent cell death may play a greater role than caspase-

mediated apoptosis at sites at which cellular bioenergetic state is substantially compromised. AIF-mediated cell death may be initiated either by the same mechanisms responsible for intrinsic caspase activation or through PARP-1 activation. In the former, the role played by AIF becomes visible only when caspase activation has been blocked. In the latter, AIF is the main death-inducing factor. In certain conditions, it was observed that inhibition of the dominant pathways did not stop and only delayed cell death. Cellular demise still occurred, often with a different morphology, accounted for by continuation and perhaps even activation of other cell death mechanisms (Pohl et al. 1999; Oppenheim et al. 2001; Chautan et al. 1999).

12.5 Cell Death Mechanisms: Implications for Treatment

There are several major challenges to a successful treatment of neuronal cell death caused by CNS injury. First, recent studies have uncovered a multitude of cell death pathways which have both overlapping and distinct molecular mechanisms. Second, as shown by some data, inhibition of one pathway may initiate/enhance alternative pathways. For example, there is evidence that inhibition of apoptosis may enhance necrotic cell death (Formigli et al. 2000). Third, some types of cell death, such as oncosis, occur so quickly after injury that by the time a treatment is attempted it is too late for an effective intervention (Faden 2002). These facts might explain why so many of the neuroprotective treatment trials in stroke or traumatic brain injury have failed (Faden 2002; Maas 2001). There are several potential solutions to this challenge: treatment strategies should optimally be directed at multiple targets/mechanisms, using either a combination of therapeutic agents directed at individual mechanisms or using single compounds that modulate multiple cell death mechanisms (multipotential agents). Ideally, the focus should be directed toward cell death pathways that are more delayed and that therefore have longer therapeutic windows. These include caspase-dependent and caspase-independent cell death with apoptotic and necrotic morphology, respectively. Some studies have already shown that caspase inhibitors are effective in experimental stroke, head injury, and spinal cord injury (Yakovlev and Faden 2001); caspase-independent mechanisms such as AIF-dependent cell death should also offer promising avenues for therapy.

References

Alano CC, Ying W, Swanson RA (2004) Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD+depletion and mitochondrial permeability transition. J Biol Chem 279:18895–18902

Alnemri ES, Livingston DJ, Nicholson DW et al (1996) Human ICE/CED-3 protease nomenclature. Cell 87:171

Andrabi SA, Kim NS, Yu SW et al (2006) Poly(ADP-ribose) (PAR) polymer is a death signal. Proc Natl Acad Sci U S A 103:18308–18313

Aravind L, Dixit VM, Koonin EV (1999) The domains of death: evolution of the apoptosis machinery. Trends Biochem Sci 24:47–53

Arnheim G (1890) Coagulationsnekrose und Kernschwund. Virchows Arch Pathol Anat 120:367–383

Bayly PV, Dikranian KT, Black EE et al (2006) Spatiotemporal evolution of apoptotic neurodegeneration following traumatic injury to the developing rat brain. Brain Res 1107:70–81

Beckmann RP, Mizzen LE, Welch WJ (1990) Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. Science 248:850–854

Beere HM, Wolf BB, Cain K et al (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat Cell Biol 2:469–475

Bergeron L, Perez GI, Macdonald G et al (1998) Defects in regulation of apoptosis in caspase-2-deficient mice. Genes Dev 12:1304–1314

Bittigau P, Sifringer M, Pohl D et al (1999) Apoptotic neurodegeneration following trauma is markedly enhanced in the immature brain. Ann Neurol 45:724–735

Bitko V, Barik S (2001) An endoplasmic reticulum-specific stress-activated caspase (caspase-12) is implicated in the apoptosis of A549 epithelial cells by respiratory syncytial virus. J Cell Biochem 80:441–454

Boonstra J (2003) Progression through the G1-phase of the on-going cell cycle. J Cell Biochem 90:244–252

Bonfoco E, Krainc D, Ankarcrona M et al (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-d-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 92:7162–7166

Bossy-Wetzel E, Green DR (1999) Caspases induce cytochrome c release from mitochondria by activating cytosolic factors. J Biol Chem 274:17484–17490

Boulos S, Meloni BP, Arthur PG et al (2007) Evidence that intracellular cyclophilin A and cyclophilin A/CD147 receptor-mediated ERK1/2 signalling can protect neurons against in vitro oxidative and ischemic injury. Neurobiol Dis 25:54–64

Bredesen DE (1995) Neural apoptosis. Ann Neurol 38:839–851

Bredesen DE (2000) Apoptosis: overview and signal transduction pathways. J Neurotrauma 17:801–810

Cande C, Vahsen N, Garrido C, Kroemer G (2004a) Apoptosis-inducing factor (AIF): caspase-independent after all. Cell Death Differ 11:591–595

Cande C, Vahsen N, Kouranti I et al (2004b) AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. Oncogene 23:1514–1521

Cao G, Clark RS, Pei W et al (2003) Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation. J Cereb Blood Flow Metab 23:1137–1150

Castro-Obregon S, Del Rio G, Chen SF et al (2002) A ligand-receptor pair that triggers a non-apoptotic form of programmed cell death. Cell Death Differ 9:807–817

Chauvier D, Lecoeur H, Langonne A et al (2005) Upstream control of apoptosis by caspase-2 in serum-deprived primary neurons. Apoptosis 10:1243–1259

Chautan M, Chazal G, Cecconi F et al (1999) Interdigital cell death can occur through a necrotic and caspase- independent pathway. Curr Biol 9:967–970

Cheung EC, Melanson-Drapeau L, Cregan SP et al (2005) Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms. J Neurosci 25:1324–1334

Cheung EC, Joza N, Steenaart NA et al (2006) Dissociating the dual roles of apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis. EMBO J 25:4061–4073

Clarke PG (1990) Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol (Berl) 181:195–213

Coqueret O (2003) New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol 13:65–70

Cohen GM (1997) Caspases: the executioners of apoptosis. Biochem J 326(Pt 1):1–16

Cory S, Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2:647–656

Colgan J, Asmal M, Luban J (2000) Isolation, characterization and targeted disruption of mouse ppia: cyclophilin A is not essential for mammalian cell viability. Genomics 68:167–178

Cregan SP, Fortin A, MacLaurin JG et al (2002) Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. J Cell Biol 158:507–517

Cregan SP, Dawson VL, Slack RS (2004) Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 23:2785–2796

Culmsee C, Zhu C, Landshamer S et al (2005) Apoptosis-inducing factor triggered by poly(ADP-Ribose) polymerase and bid mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia. J Neurosci 25:10262–10272

Dal Canto MC, Gurney ME (1994) Development of central nervous system pathology in a murine transgenic model of human amyotrophic lateral sclerosis. Am J Pathol 145:1271–1279

Daugas E, Nochy D, Ravagnan L et al (2000) Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. FEBS Lett 476:118–123

David KK, Sasaki M, Yu SW et al (2006) EndoG is dispensable in embryogenesis and apoptosis. Cell Death Differ 13:1147–1155

Deveraux QL, Reed JC (1999) IAP family proteins–suppressors of apoptosis. Genes Dev 13:239–252

Di Giovanni S, Movsesyan V, Ahmed F et al (2005) Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury. Proc Natl Acad Sci U S A 102:8333–8338

Dikranian K, Ishimaru MJ, Tenkova T et al (2001) Apoptosis in the in vivo mammalian forebrain. Neurobiol Dis 8:359–379

Djebaili M, Guo Q, Pettus EH et al (2005) The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats. J Neurotrauma 22:106–118

Eguchi Y, Shimizu S, Tsujimoto Y (1997) Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res 57:1835–1840

Eguchi Y, Srinivasan A, Tomaselli KJ et al (1999) ATP-dependent steps in apoptotic signal transduction. Cancer Res 59:2174–2181

Eldadah BA, Faden AI (2000) Caspase pathways, neuronal apoptosis, and CNS injury. J Neurotrauma 17:811–829

Eliasson MJ, Sampei K, Mandir AS et al (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. Nat Med 3:1089–1095

Endres M, Wang ZQ, Namura S et al (1997) Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. J Cereb Blood Flow Metab 17:1143–1151

Enoksson M, Robertson JD, Gogvadze V et al (2004) Caspase-2 permeabilizes the outer mitochondrial membrane and disrupts the binding of cytochrome c to anionic phospholipids. J Biol Chem 279:49575–49578

Faden AI, Demediuk P, Panter SS, Vink R (1989) The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science 244:798–800

Faden AI (2002) Neuroprotection and traumatic brain injury: theoretical option or realistic proposition. Curr Opin Neurol 15:707–712

Farber E (1994) Programmed cell death: necrosis versus apoptosis. Mod Pathol 7:605–609

Ferrand-Drake M, Zhu C, Gido G et al (2003) Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome c and caspase-3 activation in neurons exposed to transient hypoglycemia. J Neurochem 85:1431–1442

Ferrer I, Friguls B, Dalfo E et al (2003) Caspase-dependent and caspase-independent signalling of apoptosis in the penumbra following middle cerebral artery occlusion in the adult rat. Neuropathol Appl Neurobiol 29:472–481

Fischer PM, Endicott J, Meijer L (2003) Cyclin-dependent kinase inhibitors. Prog Cell Cycle Res 5:235–248

Fiskum G (2000) Mitochondrial participation in ischemic and traumatic neural cell death. J Neurotrauma 17:843–855

Flemming W (1885) Uber die Bildung von Richtungsfiguren in Saugethiereiern beim Untergang Graaf'scher Follikel. Arch Anat EntwGesch 221–244

Formigli L, Papucci L, Tani A et al (2000) Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J Cell Physiol 182:41–49

Franklin EE, Robertson JD (2007) Requirement of Apaf-1 for mitochondrial events and the cleavage or activation of all procaspases during genotoxic stress-induced apoptosis. Biochem J 405:115–122

Freeman RS, Estus S, Johnson EM Jr (1994) Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of Cyclin D1 during programmed cell death. Neuron 12:343–355

Fujikawa DG (2000) Confusion between neuronal apoptosis and activation of programmed cell death mechanisms in acute necrotic insults. Trends Neurosci 23:410–411

Fujikawa DG, Shinmei SS, Zhao S, Aviles ER Jr (2007) Caspase-dependent programmed cell death pathways are not activated in generalized seizure-induced neuronal death. Brain Res 1135:206–218

Fujikawa DG, Ke X, Trinidad RB et al (2002) Caspase-3 is not activated in seizure-induced neuronal necrosis with internucleosomal DNA cleavage. J Neurochem 83:229–240

Fujikawa DG, Shinmei SS, Cai B (2000) Kainic acid-induced seizures produce necrotic, not apoptotic, neurons with internucleosomal DNA cleavage: implications for programmed cell death mechanisms. Neuroscience 98:41–53

Furuya T, Hayakawa H, Yamada M et al (2004) Caspase-11 mediates inflammatory dopaminergic cell death in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine mouse model of Parkinson's disease. J Neurosci 24:1865–1872

Galat A (2003) Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity–targets–functions. Curr Top Med Chem 3:1315–1347

Garrido C, Gurbuxani S, Ravagnan L, Kroemer G (2001) Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem Biophys Res Commun 286:433–442

Garrido C, Kroemer G (2004) Life's smile, death's grin: vital functions of apoptosis-executing proteins. Curr Opin Cell Biol 16:639–646

Giovanni A, Wirtz-Brugger F, Keramaris E et al (1999) Involvement of cell cycle elements, cyclin-dependent kinases, pRb, and E2F x DP, in B-amyloid-induced neuronal death. J Biol Chem 274:19011–19016

Glucksmann A (1951) Cell deaths in normal vertebrate ontogeny. Biol Rev Camb Philos Soc 26:59–86

Graeber MB, Moran LB (2002) Mechanisms of cell death in neurodegenerative diseases: fashion, fiction, and facts. Brain Pathol 12:385–390

Graper L (1914) Eine neue Anschauung uber physiologische Zellausschaltung. Arch Zellforsch 12:373–394

Greene LA, Biswas SC, Liu DX (2004) Cell cycle molecules and vertebrate neuron death: E2F at the hub. Cell Death Differ 11:49–60

Guadagno TM, Newport JW (1996) Cdk2 kinase is required for entry into mitosis as a positive regulator of Cdc2-cyclin B kinase activity. Cell 84:73–82

Gurbuxani S, Schmitt E, Cande C et al (2003) Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. Oncogene 22:6669–6678

Hamdane M, Bretteville A, Sambo AV et al (2005) p25/Cdk5-mediated retinoblastoma phosphorylation is an early event in neuronal cell death. J Cell Sci 118:1291–1298

Helekar SA, Char D, Neff S, Patrick J (1994) Prolyl isomerase requirement for the expression of functional homo-oligomeric ligand-gated ion channels. Neuron 12:179–189

Hengartner MO (1999) Programmed cell death in the nematode *C. elegans*. Recent Prog Horm Res 54:213–222 discussion 222–214

Herrup K, Busser JC (1995) The induction of multiple cell cycle events precedes target-related neuronal death. Development 121:2385–2395

Hong SJ, Dawson TM, Dawson VL (2004) Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. Trends Pharmacol Sci 25:259–264
Honig LS, Rosenberg RN (2000) Apoptosis and neurologic disease. Am J Med 108:317–330
Hsu YT, Wolter KG, Youle RJ (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A 94:3668–3672
Ino H, Chiba T (2001) Cyclin-dependent kinase 4 and cyclin D1 are required for excitotoxin-induced neuronal cell death in vivo. J Neurosci 21:6086–6094
Ishimaru MJ, Ikonomidou C, Tenkova TI et al (1999) Distinguishing excitotoxic from apoptotic neurodegeneration in the developing rat brain. J Comp Neurol 408:461–476
Joza N, Susin SA, Daugas E et al (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 410:549–554
Kang SJ, Wang S, Hara H et al (2000) Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. J Cell Biol 149:613–622
Katsuno M, Sang C, Adachi H et al (2005) Pharmacological induction of heat-shock proteins alleviates polyglutamine-mediated motor neuron disease. Proc Natl Acad Sci U S A 102:16801–16806
Kerr JF (1971) Shrinkage necrosis: a distinct mode of cellular death. J Pathol 105:13–20
Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257
Kerr JF (2002) History of the events leading to the formulation of the apoptosis concept. Toxicology 181–182:471–474
Kim H, Kim WJ, Jeon ST et al (2005) Cyclophilin A may contribute to the inflammatory processes in rheumatoid arthritis through induction of matrix degrading enzymes and inflammatory cytokines from macrophages. Clin Immunol 116:217–224
Kitagawa M, Higashi H, Jung HK et al (1996) The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. EMBO J 15:7060–7069
Klebs E (1889) Die Allgemeine Pathologie. Verlag Gustav Fisher, Jena
Kochanek PM, Marion DW, Zhang W et al (1995) Severe controlled cortical impact in rats: assessment of cerebral edema, blood flow, and contusion volume. J Neurotrauma 12:1015–1025
Kroemer G, El-Deiry WS, Golstein P et al (2005) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ 12(Suppl 2):1463–1467
Kruman II, Wersto RP, Cardozo-Pelaez F et al (2004) Cell cycle activation linked to neuronal cell death initiated by DNA damage. Neuron 41:549–561
Kuan CY, Schloemer AJ, Lu A et al (2004) Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain. J Neurosci 24:10763–10772
Kuida K, Zheng TS, Na S et al (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384:368–372
Kuida K, Haydar TF, Kuan CY et al (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell 94:325–337
Lakhani SA, Masud A, Kuida K et al (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science 311:847–851
Larner SF, Hayes RL, McKinsey DM et al (2004) Increased expression and processing of caspase-12 after traumatic brain injury in rats. J Neurochem 88:78–90
Lassus P, Opitz-Araya X, Lazebnik Y (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. Science 297:1352–1354
Lee JP, Palfrey HC, Bindokas VP et al (1999) The role of immunophilins in mutant superoxide dismutase-1linked familial amyotrophic lateral sclerosis. Proc Natl Acad Sci U S A 96: 3251–3256
Lee SH, Kwon HM, Kim YJ et al (2004) Effects of hsp70.1 gene knockout on the mitochondrial apoptotic pathway after focal cerebral ischemia. Stroke 35:2195–2199
Lee BI, Lee DJ, Cho KJ, Kim GW (2005) Early nuclear translocation of endonuclease G and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. Neurosci Lett 386:23–27
Leist M, Single B, Castoldi AF et al (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 185:1481–1486

Levin S (1998) Apoptosis, necrosis, or oncosis: what is your diagnosis? A report from the Cell Death Nomenclature Committee of the Society of Toxicologic Pathologists. Toxicol Sci 41: 155–156

Levin S, Bucci TJ, Cohen SM et al (1999) The nomenclature of cell death: recommendations of an ad hoc Committee of the Society of Toxicologic Pathologists. Toxicol Pathol 27:484–490

Lewelt W, Jenkins LW, Miller JD (1982) Effects of experimental fluid-percussion injury of the brain on cerebrovascular reactivity of hypoxia and to hypercapnia. J Neurosurg 56:332–338

Li LJ, Naeve GS, Lee AS (1993) Temporal regulation of cyclin A-p107 and p33cdk2 complexes binding to a human thymidine kinase promoter element important for G1-S phase transcriptional regulation. Proc Natl Acad Sci U S A 90:3554–3558

Li LY, Luo X, Wang X (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412:95–99

Li X, Nemoto M, Xu Z et al (2007) Influence of duration of focal cerebral ischemia and neuronal nitric oxide synthase on translocation of apoptosis-inducing factor to the nucleus. Neuroscience 144:56–65

Li F, Mao HP, Ruchalski KL et al (2002) Heat stress prevents mitochondrial injury in ATP-depleted renal epithelial cells. Am J Physiol Cell Physiol 283:C917–C926

Lipton SA, Bossy-Wetzel E (2002) Dueling activities of AIF in cell death versus survival: DNA binding and redox activity. Cell 111:147–150

Liu DX, Greene LA (2001) Neuronal apoptosis at the G1/S cell cycle checkpoint. Cell Tissue Res 305:217–228

Liu DX, Biswas SC, Greene LA (2004) B-myb and C-myb play required roles in neuronal apoptosis evoked by nerve growth factor deprivation and DNA damage. J Neurosci 24:8720–8725

Lockshin RA, Williams CM (1964) Programmed cell death. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. J Insect Physiol 10:643–649

Loeffler M, Daugas E, Susin SA et al (2001) Dominant cell death induction by extramitochondrially targeted apoptosis-inducing factor. FASEB J 15:758–767

Love S (2003) Neuronal expression of cell cycle-related proteins after brain ischaemia in man. Neurosci Lett 353:29–32

Lu A, Ran R, Parmentier-Batteur S et al (2002) Geldanamycin induces heat shock proteins in brain and protects against focal cerebral ischemia. J Neurochem 81:355–364

Luo X, Budihardjo I, Zou H et al (1998) Bid, a Bcl2 interacting protein, mediates cytochrome release from mitochondria in response to activation of cell surface death receptors. Cell 94:481–490

Majno G, Joris I (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 146:3–15

Majno G, Joris I (1999) Commentary: on the misuse of the term “necrosis”: a step in the right direction. Toxicol Pathol 27:494

Martins LM, Morrison A, Klupsch K et al (2004) Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. Mol Cell Biol 24:9848–9862

Matsumori Y, Hong SM, Aoyama K et al (2005) Hsp70 overexpression sequesters AIF and reduces neonatal hypoxic/ischemic brain injury. J Cereb Blood Flow Metab 25(7):899–910

Maas AI (2001) Neuroprotective agents in traumatic brain injury. Expert Opin Investig Drugs 10:753–767

McIntosh TK (1994) Neurochemical sequelae of traumatic brain injury: therapeutic implications. Cerebrovasc Brain Metab Rev 6:109–162

McPherson CA, Kubik J, Wine RN et al (2003) Alterations in cyclin A, B, and D1 in mouse dentate gyrus following TMT-induced hippocampal damage. Neurotox Res 5:339–354

Mikuriya T, Sugahara K, Takemoto T et al (2005) Geranylgeranylacetone, a heat shock protein inducer, prevents acoustic injury in the guinea pig. Brain Res 1065:107–114

Moubarak RS, Yuste VJ, Artus C et al (2007) Sequential Activation of PARP-1, Calpains, and Bax is Essential in AIF-Mediated Programmed Necrosis. Mol Cell Biol 27(13):4844–4862

Murakami H, Pain D, Blobel G (1988) 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. J Cell Biol 107:2051–2057

Nagai Y, Fujiki M, Inoue R et al (2005) Neuroprotective effect of geranylgeranylacetone, a noninvasive heat shock protein inducer, on cerebral infarction in rats. Neurosci Lett 374:183–188
Nagy Z (2000) Cell cycle regulatory failure in neurones: causes and consequences. Neurobiol Aging 21:761–769
Nahle Z, Polakoff J, Davuluri RV et al (2002) Direct coupling of the cell cycle and cell death machinery by E2F. Nat Cell Biol 4:859–864
Nakagawa T, Zhu H, Morishima N et al (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 403:98–103
Nakka VP, Gusain A, Mehta SL, Raghubir R (2008) Molecular mechanisms of apoptosis in cerebral ischemia: multiple neuroprotective opportunities. Mol Neurobiol 37(1):7–38
Narkilahti S, Jutila L, Alafuzoff I et al (2007) Increased expression of caspase 2 in experimental and human temporal lobe epilepsy. Neuromolecular Med 9:129–144
Nathoo N, Narotam PK, Agrawal DK et al (2004) Influence of apoptosis on neurological outcome following traumatic cerebral contusion. J Neurosurg 101:233–240
Nguyen MD, Mushynski WE, Julien JP (2002) Cycling at the interface between neurodevelopment and neurodegeneration. Cell Death Differ 9:1294–1306
Nguyen MD, Boudreau M, Kriz J et al (2003) Cell cycle regulators in the neuronal death pathway of amyotrophic lateral sclerosis caused by mutant superoxide dismutase 1. J Neurosci 23:2131–2140
Nishioka N, Arnold SE (2004) Evidence for oxidative DNA damage in the hippocampus of elderly patients with chronic schizophrenia. Am J Geriatr Psychiatry 12:167–175
Nishitani H, Lygerou Z (2002) Control of DNA replication licensing in a cell cycle. Genes Cells 7:523–534
Nissen F (1886) Uber das Verhalten der Kerne in den Mich-drusenzellen bei der Absonderung. Arch Mikroskop Anat 26:337–342
Obaya AJ, Sedivy JM (2002) Regulation of cyclin-Cdk activity in mammalian cells. Cell Mol Life Sci 59:126–142
Okano HJ, Pfaff DW, Gibbs RB (1993) RB and Cdc2 expression in brain: correlations with 3H-thymidine incorporation and neurogenesis. J Neurosci 13:2930–2938
Oppenheim RW, Flavell RA, Vinsant S et al (2001) Programmed cell death of developing mammalian neurons after genetic deletion of caspases. J Neurosci 21:4752–4760
Osuga H, Osuga S, Wang F et al (2000) Cyclin-dependent kinases as a therapeutic target for stroke. Proc Natl Acad Sci U S A 97:10254–10259
Otera H, Ohsakaya S, Nagaura Z et al (2005) Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. EMBO J 24:1375–1386
Otsuka Y, Tanaka T, Uchida D et al (2004) Roles of cyclin-dependent kinase 4 and p53 in neuronal cell death induced by doxorubicin on cerebellar granule neurons in mouse. Neurosci Lett 365:180–185
Padmanabhan J, Park DS, Greene LA, Shelanski ML (1999) Role of cell cycle regulatory proteins in cerebellar granule neuron apoptosis. J Neurosci 19:8747–8756
Panter SS, Faden AI (1992a) Pretreatment with NMDA antagonists limits release of excitatory amino acids following traumatic brain injury. Neurosci Lett 136:165–168
Panter SS, Faden AI (1992b) Biochemical changes and secondary injury from stroke and trauma, vol Chapter 4, Principles and Practice of Restorative Neurology. Butterworth-Heinemann, New York, pp 32–52
Parcellier A, Gurbuxani S, Schmitt E et al (2003) Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. Biochem Biophys Res Commun 304:505–512
Park DS, Levine B, Ferrari G, Greene LA (1997) Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons. J Neurosci 17:8975–8983
Park DS, Morris EJ, Bremner R et al (2000a) Involvement of retinoblastoma family members and E2F/DP complexes in the death of neurons evoked by DNA damage. J Neurosci 20:3104–3114
Park DS, Obeidat A, Giovanni A, Greene LA (2000b) Cell cycle regulators in neuronal death evoked by excitotoxic stress: implications for neurodegeneration and its treatment. Neurobiol Aging 21:771–781

Paroni G, Henderson C, Schneider C, Brancolini C (2002) Caspase-2 can trigger cytochrome C release and apoptosis from the nucleus. J Biol Chem 277:15147–15161
Pietenpol JA, Stewart ZA (2002) Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. Toxicology 181–182:475–481
Pilar G, Landmesser L (1976) Ultrastructural differences during embryonic cell death in normal and peripherally deprived ciliary ganglia. J Cell Biol 68:339–356
Plesnila N, Zhu C, Culmsee C et al (2004) Nuclear translocation of apoptosis-inducing factor after focal cerebral ischemia. J Cereb Blood Flow Metab 24:458–466
Pohl D, Bittigau P, Ishimaru MJ et al (1999) *N*-methyl-d-aspartate antagonists and apoptotic cell death triggered by head trauma in developing rat brain. Proc Natl Acad Sci U S A 96:2508–2513
Polster BM, Basanez G, Etxebarria A et al (2005) Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. J Biol Chem 280:6447–6454
Portera-Cailliau C, Price DL, Martin LJ (1997) Non-NMDA and NMDA receptor-mediated excitotoxic neuronal deaths in adult brain are morphologically distinct: further evidence for an apoptosis-necrosis continuum. J Comp Neurol 378:88–104
Proskuryakov SY, Konoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? Exp Cell Res 283:1–16
Ravagnan L, Roumier T, Kroemer G (2002) Mitochondria, the killer organelles and their weapons. J Cell Physiol 192:131–137
Ravagnan L, Gurbuxani S, Susin SA et al (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3:839–843
Rich T, Watson CJ, Wyllie A (1999) Apoptosis: the germs of death. Nat Cell Biol 1:E69–E71
Riedel G, Wetzel W, Kozikowski AP, Reymann KG (1995) Block of spatial learning by mGluR agonist tADA in rats. Neuropharmacology 34:559–561
Robertson JD, Enoksson M, Suomela M et al (2002) Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. J Biol Chem 277:29803–29809
Robertson JD, Gogvadze V, Kropotov A et al (2004) Processed caspase-2 can induce mitochondria-mediated apoptosis independently of its enzymatic activity. EMBO Rep 5:643–648
Rokitansky C (1846) Handbuch der allgemeinen pathologischen Anatomie. Braumuller & Seidel, Vienna
Roy M, Sapolsky R (1999) Neuronal apoptosis in acute necrotic insults: why is this subject such a mess? Trends Neurosci 22:419–422
Ruchalski KL, Mao H, Li Z et al (2006) Distinct Hsp70 domains mediate apoptosis inducing factor release and nuclear accumulation. J Biol Chem 281(12):7873–7880
Saito A, Hayashi T, Okuno S et al (2003) Interaction between XIAP and Smac/DIABLO in the mouse brain after transient focal cerebral ischemia. J Cereb Blood Flow Metab 23:1010–1019
Saito A, Hayashi T, Okuno S et al (2004a) Oxidative stress is associated with XIAP and Smac/DIABLO signaling pathways in mouse brains after transient focal cerebral ischemia. Stroke 35:1443–1448
Saito A, Hayashi T, Okuno S et al (2004b) Modulation of the Omi/HtrA2 signaling pathway after transient focal cerebral ischemia in mouse brains that overexpress SOD1. Brain Res Mol Brain Res 127:89–95
Sakurai M, Hayashi T, Abe K et al (2000) Cyclin D1 and Cdk4 protein induction in motor neurons after transient spinal cord ischemia in rabbits. Stroke 31:200–207
Sanges D, Comitato A, Tammaro R, Marigo V (2006) Apoptosis in retinal degeneration involves cross-talk between apoptosis-inducing factor (AIF) and caspase-12 and is blocked by calpain inhibitors. Proc Natl Acad Sci U S A 103:17366–17371
Schmaus H, Albrecht E (1894) Uber Karyorrhexis. Virchows Arch Pathol Anat 1894:138
Schweichel JU, Merker HJ (1973) The morphology of various types of cell death in prenatal tissues. Teratology 7:253–266
Schwartz LM, Smith SW, Jones ME, Osborne BA (1993) Do all programmed cell deaths occur via apoptosis? Proc Natl Acad Sci U S A 90:980–984
Sears RC, Nevins JR (2002) Signaling networks that link cell proliferation and cell fate. J Biol Chem. 277:11617–11620

Seidberg NA, Clark RS, Zhang X et al (2003) Alterations in inducible 72-kDa heat shock protein and the chaperone cofactor BAG-1 in human brain after head injury. J Neurochem 84:514–521
Semba S, Huebner K (2006) Protein expression profiling identifies cyclophilin A as a molecular target in Fhit-mediated tumor suppression. Mol Cancer Res 4:529–538
Shen HY, He JC, Wang Y et al (2005) Geldanamycin induces heat shock protein 70 and protects against MPTP-induced dopaminergic neurotoxicity in mice. J Biol Chem 280:39962–39969
Sherr CJ (1993) Mammalian G1 cyclins. Cell 73:1059–1065
Sherr CJ (1995) D-type cyclins. Trends Biochem Sci 20:187–190
Siegelin M, Touzani O, Toutain J et al (2005a) Induction and redistribution of XAF1, a new antagonist of XIAP in the rat brain after transient focal ischemia. Neurobiol Dis 20:509–518
Siegelin MD, Kossatz LS, Winckler J, Rami A (2005b) Regulation of XIAP and Smac/DIABLO in the rat hippocampus following transient forebrain ischemia. Neurochem Int 46:41–51
Sloviter RS (2002) Apoptosis: a guide for the perplexed. Trends Pharmacol Sci 23:19–24
Smith PD, Crocker SJ, Jackson-Lewis V et al (2003) Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci U S A 100:13650–13655
Snider BJ, Gottron FJ, Choi DW (1999) Apoptosis and necrosis in cerebrovascular disease. Ann N Y Acad Sci 893:243–253
Soustiel JF, Palzur E, Nevo O et al (2005) Neuroprotective anti-apoptosis effect of estrogens in traumatic brain injury. J Neurotrauma 22:345–352
Sperandio S, de Belle I, Bredesen DE (2000) An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci U S A 97:14376–14381
Srinivasula SM, Datta P, Fan XJ et al (2000) Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. J Biol Chem 275:36152–36157
Strauss KI, Narayan RK, Raghupathi R (2004) Common patterns of bcl-2 family gene expression in two traumatic brain injury models. Neurotox Res 6:333–342
Strazza M, Luddi A, Brogi A et al (2004) Activation of cell cycle regulatory proteins in the apoptosis of terminally differentiated oligodendrocytes. Neurochem Res 29:923–931
Strobe H (1892) Zur Kenntnis verschiedener cellularer Vorgange und Erscheinungen in Geschwulsten. Beitr Pathol 11:1–38
Sumrejkanchanakij P, Tamamori-Adachi M, Matsunaga Y et al (2003) Role of cyclin D1 cytoplasmic sequestration in the survival of postmitotic neurons. Oncogene 22:8723–8730
Susin SA, Lorenzo HK, Zamzami N et al (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446
Suzuki S, Chuang LF, Doi RH et al (2001) Kappa-opioid receptors on lymphocytes of a human lymphocytic cell line: morphine-induced up-regulation as evidenced by competitive RT-PCR and indirect immunofluorescence. Int Immunopharmacol 1:1733–1742
Swerdlow RH, Khan SM (2004) A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. Med Hypotheses 63:8–20
Takahashi K, Greenberg JH, Jackson P et al (1997) Neuroprotective effects of inhibiting poly(ADP-ribose) synthetase on focal cerebral ischemia in rats. J Cereb Blood Flow Metab 17:1137–1142
Thornton BR, Toczyski DP (2003) Securin and B-cyclin/CDK are the only essential targets of the APC. Nat Cell Biol 5:1090–1094
Trump BF, Mergner WJ (1974) Cell Injury. In: GL ZBW, McCluskey RT (eds) The inflammatory process. Academic Press, New York, pp 115–257
Tsuchiya D, Hong S, Matsumori Y et al (2003) Overexpression of rat heat shock protein 70 reduces neuronal injury after transient focal ischemia, transient global ischemia, or kainic acid-induced seizures. Neurosurgery 53:1179–1187 discussion 1187-1178
Turmaine M, Raza A, Mahal A et al (2000) Nonapoptotic neurodegeneration in a transgenic mouse model of Huntington's disease. Proc Natl Acad Sci U S A 97:8093–8097
Vahsen N, Cande C, Briere JJ et al (2004) AIF deficiency compromises oxidative phosphorylation. EMBO J 23:4679–4689

Van Cruchten S, Van Den Broeck W (2002) Morphological and biochemical aspects of apoptosis, oncosis and necrosis. Anat Histol Embryol 31:214–223
van Loo G, Saelens X, van Gurp M et al (2002) The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. Cell Death Differ 9:1031–1042
Verdaguer E, Garcia-Jorda E, Canudas AM et al (2002) Kainic acid-induced apoptosis in cerebellar granule neurons: an attempt at cell cycle re-entry. NeuroReport 13:413–416
Verdaguer E, Jorda EG, Stranges A et al (2003) Inhibition of CDKs: a strategy for preventing kainic acid-induced apoptosis in neurons. Ann N Y Acad Sci 1010:671–674
Vink R, McIntosh TK, Yamakami I, Faden AI (1988) 31P NMR characterization of graded traumatic brain injury in rats. Magn Reson Med 6:37–48
Virchow R (1859) Cellular pathology as based upon physiological and pathological histology. Dover Publications, New York Chance B, Translator
Vlodavsky E, Palzur E, Feinsod M, Soustiel JF (2005) Evaluation of the apoptosis-related proteins of the BCL-2 family in the traumatic penumbra area of the rat model of cerebral contusion, treated by hyperbaric oxygen therapy: a quantitative immunohistochemical study. Acta Neuropathol 110:120–126
von Recklinghausen F (1910) Untersuchungen uber Rachitis und Osteomalacie. Verlag Gustav Fisher, Jena
Volbracht C, Leist M, Kolb SA, Nicotera P (2001) Apoptosis in caspase-inhibited neurons. Mol Med 7:36–48
Wang F, Corbett D, Osuga H et al (2002a) Inhibition of cyclin-dependent kinases improves CA1 neuronal survival and behavioral performance after global ischemia in the rat. J Cereb Blood Flow Metab 22:171–182
Wang X, Yang C, Chai J et al (2002b) Mechanisms of AIF-mediated apoptotic DNA degradation in *Caenorhabditis elegans*. Science 298:1587–1592
Wang H, Yu SW, Koh DW et al (2004) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. J Neurosci 24:10963–10973
Wang S, Miura M, Jung YK et al (1998) Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell 92:501–509
Wang P, Heitman J (2005) The cyclophilins. Genome Biol 6:226
Wartiovaara K, Barnabe-Heider F, Miller FD, Kaplan DR (2002) N-myc promotes survival and induces S-phase entry of postmitotic sympathetic neurons. J Neurosci 22:815–824
Waza M, Adachi H, Katsuno M et al (2005) 17-AAG, an Hsp90 inhibitor, ameliorates polyglutamine-mediated motor neuron degeneration. Nat Med 11:1088–1095
Weissmann A (1889) Essays upon hereditary and kindred biological problems. Oxford Press, Oxford
Wissing S, Ludovico P, Herker E et al (2004) An AIF orthologue regulates apoptosis in yeast. J Cell Biol 166:969–974
Weigert C (1878) Ein Experimeteller und anatomischer Beitrag zur Pathologie der specifischen Eintzundungsformen. Virchows Arch Pathol Anat 72:461–501
Wen Y, Yang S, Liu R et al (2004) Transient cerebral ischemia induces aberrant neuronal cell cycle re-entry and Alzheimer's disease-like tauopathy in female rats. J Biol Chem 279:22684–22692
Wen Y, Yang S, Liu R, Simpkins JW (2005) Cell-cycle regulators are involved in transient cerebral ischemia induced neuronal apoptosis in female rats. FEBS Lett 579:4591–4599
Whalen MJ, Clark RS, Dixon CE et al (1999) Reduction of cognitive and motor deficits after traumatic brain injury in mice deficient in poly(ADP-ribose) polymerase. J Cereb Blood Flow Metab 19:835–842
Wu Y, Dong M, Toepfer NJ et al (2004) Role of endonuclease G in neuronal excitotoxicity in mice. Neurosci Lett 364:203–207
Wyllie AH (1974) Death in normal and neoplastic cells. J Clin Pathol Suppl (R Coll Pathol) 7:35–42
Wyllie AH (1981) Cell death: a new classification separating apoptosis from necrosis. Chapman & Hall, New York
Yakovlev AG, Faden AI (2001) Caspase-dependent apoptotic pathways in CNS injury. Mol Neurobiol 24:131–144

Yakovlev AG, Ota K, Wang G et al (2001) Differential expression of apoptotic protease-activating factor-1 and caspase-3 genes and susceptibility to apoptosis during brain development and after traumatic brain injury. J Neurosci 21:7439–7446

Yakovlev AG, Faden AI (2004) Mechanisms of neural cell death: implications for development of neuroprotective treatment strategies. NeuroRX 1:5–16

Yamakami I, McIntosh TK (1991) Alterations in regional cerebral blood flow following brain injury in the rat. J Cereb Blood Flow Metab 11:655–660

Yao XL, Liu J, Lee E et al (2005) Progesterone differentially regulates pro- and anti-apoptotic gene expression in cerebral cortex following traumatic brain injury in rats. J Neurotrauma 22:656–668

Yasuda H, Shichinohe H, Kuroda S et al (2005) Neuroprotective effect of a heat shock protein inducer, geranylgeranylacetone in permanent focal cerebral ischemia. Brain Res 1032:176–182

Ying W, Alano CC, Garnier P, Swanson RA (2005) NAD+as a metabolic link between DNA damage and cell death. J Neurosci Res 79:216–223

Ying W, Chen Y, Alano CC, Swanson RA (2002) Tricarboxylic acid cycle substrates prevent PARP-mediated death of neurons and astrocytes. J Cereb Blood Flow Metab 22:774–779

Young C, Roth KA, Klocke BJ et al (2005) Role of caspase-3 in ethanol-induced developmental neurodegeneration. Neurobiol Dis 20:608–614

Yu SW, Wang H, Poitras MF et al (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Yu SW, Wang H, Dawson TM, Dawson VL (2003) Poly(ADP-ribose) polymerase-1 and apoptosis inducing factor in neurotoxicity. Neurobiol Dis 14:303–317

Yu SW, Andrabi SA, Wang H et al (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Proc Natl Acad Sci U S A 103:18314–18319

Yuan XQ, Prough DS, Smith TL, Dewitt DS (1988) The effects of traumatic brain injury on regional cerebral blood flow in rats. J Neurotrauma 5:289–301

Yuste VJ, Moubarak RS, Delettre C et al (2005) Cysteine protease inhibition prevents mitochondrial apoptosis-inducing factor (AIF) release. Cell Death Differ 12:1445–1448

Zhang X, Chen J, Graham SH et al (2002) Intranuclear localization of apoptosis-inducing factor (AIF) and large scale DNA fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. J Neurochem 82:181–191

Zhang Y, Zhang X, Park TS, Gidday JM (2005) Cerebral endothelial cell apoptosis after ischemia-reperfusion: role of PARP activation and AIF translocation. J Cereb Blood Flow Metab 25:868–877

Zhang Z, Yang X, Zhang S et al (2007) BNIP3 upregulation and EndoG translocation in delayed neuronal death in stroke and in hypoxia. Stroke 38:1606–1613

Zhan RZ, Wu C, Fujihara H et al (2001) Both caspase-dependent and caspase-independent pathways may be involved in hippocampal CA1 neuronal death because of loss of cytochrome *c* from mitochondria in a rat forebrain ischemia model. J Cereb Blood Flow Metab 21:529–540

Zhu C, Qiu L, Wang X et al (2003) Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. J Neurochem 86:306–317

Zhu C, Wang X, Huang Z et al (2007) Apoptosis-inducing factor is a major contributor to neuronal loss induced by neonatal cerebral hypoxia-ischemia. Cell Death Differ 14:775–784

Zychlinsky A, Sansonetti PJ (1997) Apoptosis as a proinflammatory event: what can we learn from bacteria-induced cell death? Trends Microbiol 5:201–204

Part V
Hypoglycemic Neuronal Death

Chapter 13
Hypoglycemic Brain Damage

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13.1 Historical and Clinical Aspects

Before the discovery of insulin, sources of insulin exogenous to the body were not available and hypoglycemic brain damage occurred only in the context of endogenous tumors of the islets of Langerhans of the pancreas (Auer 1986). Human consumption of food had reached the point where diabetes was becoming more common, but it was then only treatable through starvation. Indeed, diabetes can metabolically be considered "starvation in the midst of plenty" due to the inability of glucose to enter cells, in the face of high blood glucose levels. Once insulin was discovered, it was tested in diabetes and was found to allow improved survival over the unpalatable treatment option of starvation: the latter could reduce glucose loss through the urine but only slowed the diabetic process and could not stave off eventual death.

When insulin was discovered in 1921, islet cell adenoma was joined by diabetic insulin treatment in reports of hypoglycemic brain damage (Bodechtel 1933; Baker 1938). Suicide or homicide with insulin also occurred regularly, and together with medication error, provided a steady source of material with hypoglycemic brain damage. Tumors secreting insulin or congener molecules were later recognized to cause hypoglycemia (Le Roith 1999).

The effects of insulin on inhibiting hepatic gluconeogenesis and more importantly, allowing cellular entry of glucose, account for the capacity of insulin to produce hypoglycemia. Not widely appreciated is the fact that other molecules causing hypoglycemia are not powerful enough to produce the degree of blood sugar drop that is a prerequisite for EEG silence, the harbinger of neuronal death in hypoglycemia (Auer et al. 1984). An exception to this may be IGF-II, secreted by some tumors (Le Roith 1999). Situations of glucose imbalance, such as a new cause

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of hypoglycemia in the modern era – gastric bypass surgery (Patti et al. 2005) lead to symptomatic hypoglycemia but not to brain damage.

The morphology of hypoglycemic brain damage has been the focus of two rounds of controversy, one played out in the time period extending from the late 1920s to roughly 1950 (Auer 1986) and the second controversy still raging today. The first controversy surrounded the very existence of hypoglycemic brain damage, the second, present controversy whether hypoglycemic cell death constitutes apoptosis or necrosis. Since a 30-min period of hypoglycemia was therapeutically used for the treatment of schizophrenia, the question naturally arose as to whether this could cause brain damage. Some patients did not awaken after therapeutic insulin coma (Cammermeyer 1938; Döring 1938; MacKeith and Meyer 1939). In the early days of research on hypoglycemia, the association of insulin with hypoglycemia did not establish that hypoglycemia was the culprit in producing brain damage, indeed, insulin itself was thought to perhaps play a role. The hotly debated topic was an important one at the time, since insulin overdose resulted in catastrophic clinical situations of permanent coma also in the context of diabetes treatment, not only iatrogenically in the context of psychiatric treatment.

When insulin was used to treat diabetes, the longer acting formulations usually led to slow fluctuations in blood glucose level that generally avoided hypoglycemic brain damage. Its methodical use in the clinical context of treatment of schizophrenia (Sakel 1937) involved the intentional use of a defined duration of hypoglycemic coma to produce an effect. Although easily considered outmoded and even barbaric by today's standards, such treatment for severe psychiatric illness was the only treatment measure available at the time. And hypoglycemic coma of 30-min duration did indeed have a salubrious effect on the psychiatric condition (Dr. John Menkes, personal communication), perhaps as a result of cortical depolarisation and coma itself, rather than the sparse neuronal necrosis that occurs after 30 min of hypoglycemic coma. Nevertheless, criticism mounted (Mayer-Gross 1951) and the development of phenothiazines in the 1950s sealed the fate of insulin shock therapy for schizophrenia.

The desired duration of insulin coma in iatrogenic hypoglycemia was 30 min (Sakel 1937). It was well known that a patient in coma for 1 h would never awaken from the coma. Clearly, even in this early historical period, it was already known that something happened between 30 and 60 min of coma, which was devastating to the brain. We now know that this time period is on the cusp of accelerating hypoglycemic brain damage in the form of neuronal necrosis (Auer et al. 1984). The sparse neuronal death after only 30 min explains clinical patient survival with relatively intact neurological function after periods of hypoglycemic coma of 30 min or less.

The debate over the nature of hypoglycemic neuronal death, apoptosis, or necrosis, will be entered into when we examine dying neurons using the electron microscope. First, we describe the light and electron microscopic findings in hypoglycemic brain damage, drawing on human as well as animal material.

13.2 Gross Features

Since hypoglycemia leads to neither vascular occlusion nor lactic acidosis, infarction (pan-necrosis, with death of glia as well as neurons) is not seen in hypoglycemic brain damage. Instead, hypoglycemia leads to selective neuronal necrosis, meaning death of neurons but not glia and other supporting cells of the nervous system. Thus, depending on the degree of selective neuronal necrosis, the brain in hypoglycemic brain damage can be either normal or can show variable degrees of gross atrophy (Fig. 13.1). Cysts are not seen.

The location of the gross atrophy is dictated by the brain areas that are vulnerable in hypoglycemic brain damage. These constitute the cerebral cortex, the striatum, the hippocampus and the thalamus. A metabolic release of aspartate underlies the selective neuronal death in hypoglycemia (Sandberg et al. 1986). Glucose transport is apparently better in the brainstem and cerebellum (LaManna and Harik 1985). Sparing of the brainstem and cerebellum from even a metabolic insult during hypoglycemia precludes the development of necrosis in these structures in durations of hypoglycemic coma less than one hour. Protein synthesis, a high-level function utilizing considerable cellular energy, continues unabated in the brainstem even during hypoglycemic coma (Kiessling et al. 1986). The brainstem and cerebellum are thus normal in pure primary insults of hypoglycemic brain damage to the nervous system.

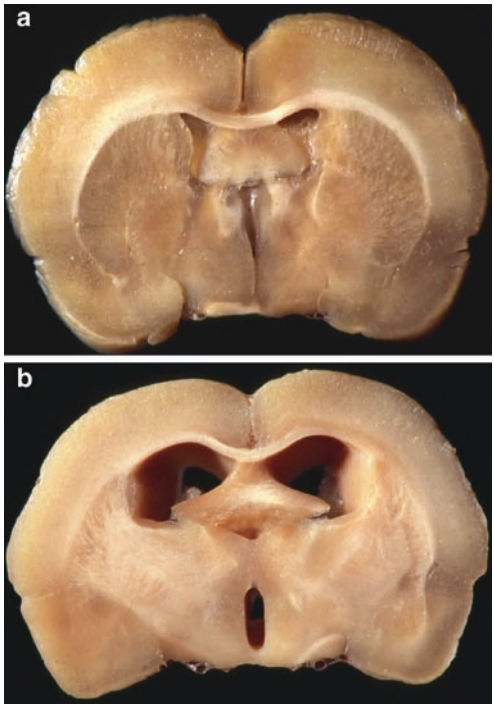

Fig. 13.1 Gross atrophy after 60 min of hypoglycemic coma followed by 1 week of recovery (**b**) as compared to a normal rat brain (**a**). Ventricular dilatation is seen, but no cysts, as the missing tissue is due to selective death of neurons, sparing glia and the neuropil. Lateral and third ventricles are dilated, indicating tissue loss throughout the hemisphere

13.3 Light Microscopy

Within the hippocampus, the CA1 pyramidal cells are vulnerable, in common with ischemia. However, the signature lesion of dentate gyrus necrosis in the absence of severe necrosis of the CA1 and especially CA3 pyramidal cells, indicates hypoglycemic brain damage as the etiology of the lesion in the hippocampus of the human. In rats, the lesion of the dentate is seen early, already after 10–20 minutes of coma, a comparably mild insult. The dentate gyrus lesions of rats and humans are compared (Fig. 13.2), both showing dentate necrosis as a feature of hypoglycemia, and CA1 necrosis, a feature in common with cerebral ischemia.

13.4 Electron Microscopy

The discovery by John Olney of the phenomenon of excitotoxicity in the late 1960s and early 1970s was a signal discovery (Olney 1969, 1971), a harbinger of our understanding of the nature of hypoglycemic neuronal death. Apparently unrelated to hypoglycemic brain damage at the time, the phenomenon of amino acid neurotoxicity to neurons, when the excitatory amino acid is present in the extracellular space in high concentrations, proved to be an essential key to our understanding of how nerve cells die after hypoglycemia. This connection was not initially obvious since hypoglycemia is a deficiency disorder where not enough of a substrate is present in the brain. How a molecular deficiency of glucose could lead to an excess of another metabolite was only apparent after the biochemistry of hypoglycemic brain damage was worked out by Siesjö and colleagues (Agardh et al. 1978). Already in 1951, aspartate was known to increase massively in hypoglycemic brains (Cravioto et al. 1951).

We now know that aspartate is released in massive quantities into the extracellular space in hypoglycemic brain damage (Sandberg et al. 1986). But the first clue that hypoglycemic neuronal death may have a connection to excitotoxicity emerged in 1985. Remarkably, the notion that a molecule in the extracellular space, perhaps a toxin, could kill nerve cells in hypoglycemic brain damage, was mentioned already in 1938 by Arthur Weil, based on dentate gyrus necrosis he observed in a geographic distribution in rabbits (Weil et al. 1938). Electron microscopy of hypoglycemic neuronal death showed a striking similarity to excitotoxic neuronal necrosis (Fig. 13.3). This similarity was quite specific: dendritic swelling, sparing intermediate axons, was known to occur in glutamate neurotoxicity. The appearance was due to the selective dendritic location of receptors, sparing axons. Thus, ion fluxes and water fluxes would lead to swelling of dendrites but not axons when examined under the electron microscope. The appearance of axon-sparing dendritic swelling is pathognomonic of the tissue action of excitotoxins. This axon-sparing dendritic lesion is also seen in hypoglycemia (Fig. 13.3). Neuronal death can be thought of as a brush fire in the dendritic tree (Auer et al. 1985), beginning where synaptic receptors are located and spreading towards the cell body or perikaryon. This is not characteristic of apoptotic cell death.

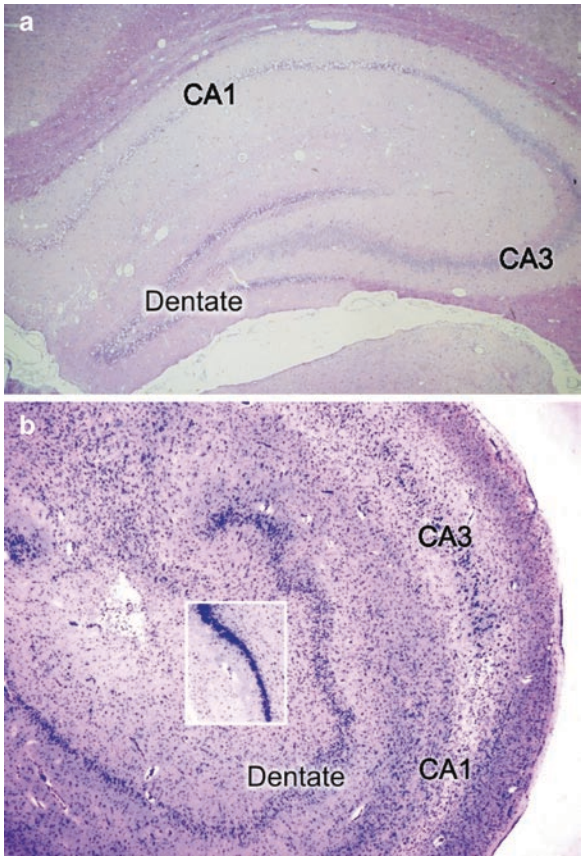

Fig. 13.2 Dentate gyrus necrosis compared in rats and humans. In the rat (**a**) the necrosis is extensive after 60 min of EEG silence. In a human with hypoglycemic coma (**b**), dentate granule cell necrosis is sparse as compared to normal control (*inset*). Both human and rat show selective vulnerability of CA1 pyramidal neurons, unlike the dentate necrosis, a feature shared in both species with global ischemia

The perikaryon of the neuron in hypoglycemic neuronal death shows features of necrosis, not apoptosis. The nucleus shows a characteristic coarse chromatin having a tigroid appearance (Fig. 13.4), which does not at all resemble the nuclear homogenization of apoptosis. The cytoplasm also shows changes characteristic of necrosis, not apoptosis. By this we mean mitochondrial flocculent densities and cell membrane breaks (Fig. 13.4), features of necrosis first described by Benjamin Trump in liver cells undergoing necrosis (Trump et al. 1965).

13.5 Biochemical Features

The biochemistry of hypoglycemic brain damage has been reviewed previously (Auer and Siesjö 1993; Suh et al. 2007b). Essentially, the brain maintains adequate energy by running a truncated version of the Krebs cycle in a new metabolic homeostasis (Sutherland et al. 2008) that becomes established with the onset of

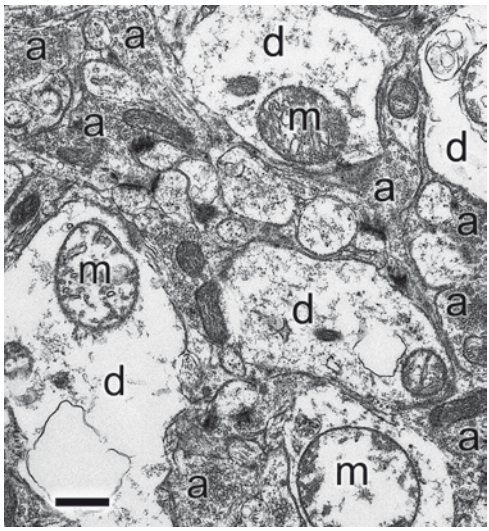

Fig. 13.3 Axon-sparing, dendritic lesion in hypoglycemia, analogous to that first described by Olney in glutamate excitotoxicity. The dendrites (d) and contained mitochondria (m) are swollen, sparing intermediate axons (a). Synapses are seen as *dark densities*. Dentate gyrus, hypoglycemia with 10 min electrocerebral silence. Bar = 1 μm

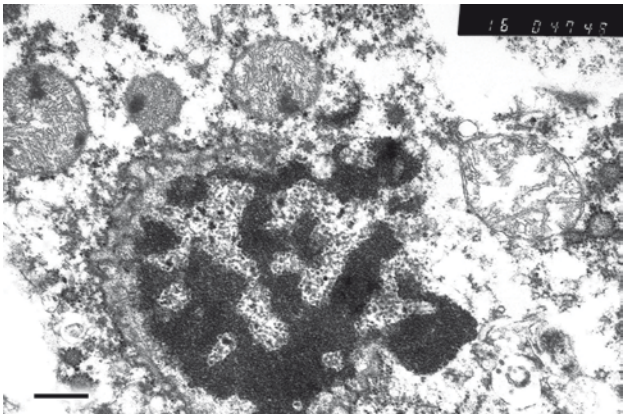

Fig. 13.4 Mitochondrial flocculent densities are seen in the three mitochondria in the *upper* and *upper left* part of the micrograph, but the mitochondrion on the *right* shows only swelling. Cell membrane breaks are everywhere and the nucleus has very coarse nuclear clumps of chromatin.

EEG silence during profound hypoglycemia. Aspartate is increased as part of the new metabolic homeostasis, being produced by a shift in the aspartate-glutamate transaminase reaction to favor aspartate. Cellular leakage of this metabolically derived aspartate leads to its flooding the extracellular space, producing the axon-sparing lesions described above by binding to dendritic receptors on neurons. The respective α-keto acids to aspartate and glutamate, oxaloacetate and α-ketoglutarate, are both part of the Krebs cycle, and hence this reaction may be written across the

Krebs cycle. It then becomes clear that the Krebs cycle can turn, albeit in an altered form, during hypoglycemia (Sutherland et al. 2008).

Hypoglycemic neuronal death has been shown to activate programmed cell death pathways, possibly as a non-specific part of the necrotic process described here. There is release of cytochrome *c*, Bax activation and caspase activation (Ouyang et al. 2000), and activation of poly(ADP-ribose) polymerase 1 (PARP-1) (Suh et al. 2005, 2007a), usually considered a necrotic pathway (Endres et al. 1997; Ha and Snyder 1999; Pieper et al. 1999). The evidence for hypoglycemic cell death being due to apoptosis may constitute guilt by association. It seems that the biochemical pathways for apoptosis are not specific for apoptosis. This is supported by the finding of apoptotic mechanisms, such as double-stranded DNA breaks, in necrosis and autophagy, as well as in apoptosis (Grasl-Kraupp et al. 1995). Hypoglycemic neuronal death shows very specific features not seen in apoptosis, such as early dendritic lesions, spreading to the perikaryon of the neuron over time. Cell membrane breaks are early in the dendritic tree, while the cell body is still intact (Auer et al. 1985). Early appearance of mitochondrial flocculent densities and cell membrane breaks contrast with the late appearance of these features in apoptosis.

References

Agardh C-D, Folbergrová J, Siesjö BK (1978) Cerebral metabolic changes in profound insulin-induced hypoglycemia, and in the recovery period following glucose administration. J Neurochem 31:1135–1142

Auer RN (1986) Progress review: hypoglycemic brain damage. Stroke 17:699–708

Auer RN, Siesjö BK (1993) Hypoglycaemia: brain neurochemistry and neuropathology. Baillières Clin Endocrinol Metab 7:611–625

Auer RN, Olsson Y, Siesjö BK (1984) Hypoglycemic brain injury in the rat. Correlation of density of brain damage with the EEG isoelectric time: A quantitative study. Diabetes 33:1090–1098

Auer R, Kalimo H, Olsson Y, Wieloch T (1985) The dentate gyrus in hypoglycemia: Pathology implicating excitotoxin-mediated neuronal necrosis. Acta Neuropathol (Berl) 67:279–288

Baker AB (1938) Cerebral lesions in hypoglycemia. II. Some possibilities of irrevocable damage from insulin shock. Arch Pathol 26:765–776

Bodechtel G (1933) Der Hypoglykämische Shock und seine Wirkung auf das Zentralnervensystem. Deutsches Arch f klin Med 175:188–201

Cammermeyer J (1938) Über Gehirnveränderungen, entstanden unter Sakelscher Insulintherapie bei einem Schizophrenen. Z ges Neurol Psychiat 163:617–633

Cravioto RO, Massieu H, Izquierdo JJ (1951) Free amino acids in rat brain during insulin shock. Proc Soc Exp Biol Med 78:856–858

Döring G (1938) Zur Histopathologie und Pathogenese des tödlichen Insulinshocks. Deutsche Ztschr Nervenheilkunde 147:217–227

Endres M, Wang ZQ, Namura S, Waeber C, Moskowitz MA (1997) Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. J Cereb Blood Flow Metab 17:1143–1151

Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R (1995) In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 21:1465–1468

Ha HC, Snyder SH (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci USA 96:13978–13982

Kiessling M, Auer RN, Kleihues P, Siesjö BK (1986) Cerebral protein synthesis during long-term recovery from severe hypoglycemia. J Cereb Blood Flow Metab 6:42–51
LaManna JC, Harik SI (1985) Regional comparisons of brain glucose influx. Brain Res 326:299–305
Le Roith D (1999) Tumor-induced hypoglycemia. N Engl J Med 341:757–758
MacKeith SA, Meyer A (1939) A death during insulin treatment of schizophrenia; with pathological report. J Ment Sci 85:96–105
Mayer-Gross W (1951) Insulin coma therapy of schizophrenia: Some critical remarks on Dr. Sakel's report. J Ment Sci 97:132–135
Olney JW (1969) Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science 164:719–721
Olney JW (1971) Glutamate-induced neuronal necrosis in the infant mouse hypothalamus. J Neuropathol Exp Neurol 30:75–90
Ouyang YB, He QP, Li PA, Janelidze S, Wang GX, Siesjö BK (2000) Is neuronal injury caused by hypoglycemic coma of the necrotic or apoptotic type? Neurochem Res 25:661–667
Patti ME, McMahon G, Mun EC, Bitton A, Holst JJ, Goldsmith J, Hanto DW, Callery M, Arky R, Nose V, Bonner-Weir S, Goldfine AB (2005) Severe hypoglycaemia post-gastric bypass requiring partial pancreatectomy: Evidence for inappropriate insulin secretion and pancreatic islet hyperplasia. Diabetologia 48:2236–2240
Pieper AA, Verma A, Zhang J, Snyder SH (1999) Poly (ADP-ribose) polymerase, nitric oxide and cell death. Trends Pharmacol Sci 20:171–181
Sakel M (1937) The methodical use of hypoglycemia in the treatment of psychoses. Am J Psychiatry 94:111–129
Sandberg M, Butcher SP, Hagberg H (1986) Extracellular overflow of neuroactive amino acids during severe insulin-induced hypoglycemia: in vivo dialysis of the rat hippocampus. J Neurochem 47:178–184
Suh SW, Aoyama K, Matsumori Y, Liu J, Swanson RA (2005) Pyruvate administered after severe hypoglycemia reduces neuronal death and cognitive impairment. Diabetes 54:1452–1458
Suh SW, Aoyama K, Alano CC, Anderson CM, Hamby AM, Swanson RA (2007a) Zinc inhibits astrocyte glutamate uptake by activation of poly(ADP-ribose) polymerase-1. Mol Med 13:344–349
Suh SW, Hamby AM, Swanson RA (2007b) Hypoglycemia, brain energetics, and hypoglycemic neuronal death. Glia 55:1280–1286
Sutherland GR, Tyson RL, Auer RN (2008) Truncation of the Krebs cycle during hypoglycemic coma. Med Chem 4:379–385
Trump BF, Goldblatt PJ, Stowell RE (1965) Studies on necrosis of mouse liver in vitro. Ultrastructural alterations in the mitochondria of hepatic parenchymal cells. Lab Invest 14:343–371
Weil A, Liebert E, Heilbrunn G (1938) Histopathologic changes in the brain in experimental hyperinsulinism. Arch Neurol Psychiatry (Chic) 39:467–481

Chapter 14
Hypoglycemic Neuronal Death

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14.1 Hypoglycemic Brain Injury: Epidemiology and Background

The normal range for blood glucose concentrations is 3.9–7.1 mM (1 mM = ~18 mg/dL), and hypoglycemia is broadly defined as blood glucose concentrations below this range. Studies using mice and rats indicate that brain injury does not generally occur unless blood glucose concentrations fall below 1 mM and the cortical electroencephalogram (EEG) is isoelectric (silent) for at least 30 min (Auer et al. 1984a, b; Auer et al. 1985a, b; Suh et al. 2003). Case series suggest that a similar degree of hypoglycemia is required to induce brain injury in humans, although this may vary in infants and children, susceptible individuals, with repeated hypoglycemia, and with co-morbid factors (Kalimo and Olsson 1980; Malouf and Brust 1985; Auer et al. 1989; Langan et al. 1991; Ben-Ami et al. 1999; de Courten-Myers et al. 2000; Ennis et al. 2008).

Reduced food intake does not reduce blood glucose levels below 2.8 mM, even with prolonged fasting (Auer 2004); however, reductions below 1 mM can be induced by the administration of insulin, glucagon, and drugs such as sulfonylurea derivatives. Not surprisingly, hypoglycemic brain injury occurs most frequently in diabetic patients attempting tight glucose control (Lincoln et al. 1996; Davis and Jones 1998), and consequently the risk (or fear) of hypoglycemic brain injury is the major factor limiting tight glucose control. While most diabetic patients experience moderate to severe hypoglycemia at times, very few experience hypoglycemia to the degree causing coma or brain injury. This said, the prevalence of diabetes is so high that hypoglycemic brain injury is not rare; in a case series from a single hospital emergency room, there were 125 patients visits for symptomatic hypoglycemia in 1 year. Of these, 23 were comatose, 1 died, and 4 survivors had permanent neurological sequelae (Malouf and Brust 1985).

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The brain is uniquely vulnerable to hypoglycemia. Reductions in blood glucose sufficient to cause coma and neuronal death have no significant effect on the heart, kidney, or other organ systems. The brain has a greater metabolic demand than other tissues, and has a unique demand for glucose as a metabolic substrate (Siesjö 1978). It was initially thought that hypoglycemic brain injury was simply a result of insufficient metabolic substrate for this high demand, but seminal work by Auer and colleagues showed this not to be the case. Work by these authors and subsequent studies showed that hypoglycemic neuronal death can be completely blocked by eliminating afferent glutamatergic projections (Wieloch et al. 1985; Butcher et al. 1987a, b) or by blocking glutamate receptors (Wieloch 1985; Papagapiou and Auer 1990; Nellgard and Wieloch 1992), thus establishing hypoglycemic neuronal death as excitotoxic in origin. Subsequent studies have identified several other key steps in the hypoglycemic pathway, and these are reviewed here. As would be expected, this cell death pathway shares many cardinal features of direct glutamate-induced excitotoxicity, but there are also features that may be unique to hypoglycemia.

14.2 Hypoglycemia and Brain Energetics

Glucose concentration in the brain normally has a linear relationship to blood concentrations (Gruetter et al. 1998; Gruetter 2003), with normal brain concentrations ranging from roughly 0.8 to 2.3 mM (Gruetter et al. 1998). However, brain glucose consumption outstrips transport capacity at reduced blood glucose concentrations, resulting in brain glucose concentrations approaching zero when blood glucose concentrations fall below 2 mM (Feise et al. 1977; Choi et al. 2001). Although glucose is a necessary fuel for brain energy metabolism, it is not the only fuel that can be utilized. The brain can also utilize ketone bodies, particularly during early development and during starvation (Robinson and Williamson 1980), but ketone bodies cannot fully substitute for glucose.

A small reserve of glucose is stored in the brain, as in other tissues, in the form of glycogen (Koizumi 1974). This glycogen store is localized primarily to astrocytes. In contrast to ischemia, hypoglycemia causes a gradual consumption of brain glycogen (Feise et al. 1977; Agardh et al. 1978; Ratcheson et al. 1981; Choi et al. 2003). ATP depletion and onset of EEG isoelectricity were found to occur simultaneously with brain glycogen depletion (Lewis et al. 1974), suggesting a special role for this astrocyte energy store in maintaining brain function. More recent studies show that increasing brain glycogen stores can prolong brain activity during severe hypoglycemia (Suh et al. 2007a, b, c). Observations using an in vitro mouse optic nerve preparation provide insight into the means by which astrocyte glycogen stores may exert this effect (Wender et al. 2000; Brown et al. 2005; Tekkok et al. 2005). Under conditions of complete glucose deprivation, the mouse optic nerve is able to maintain an electrical activity for roughly 30 min. This time interval is

extended by increasing optic nerve glycogen stores or by supplying exogenous L-lactate. Conversely, the effect of increased glycogen stores is blocked by the concurrent administration of a lactate transport inhibitor. These results indicate that, at least under conditions of glucose deprivation, transfer of glycogen-derived lactate from astrocytes to neurons can support neuronal activity.

It has been recognized for several decades that impairment of brain function during hypoglycemia begins prior to any detectable change in overall brain ATP or phosphocreatine concentrations (Lewis et al. 1974; Norberg and Siesjö 1976). The reason for this impairment in brain function has not been established, but some evidence suggests that neuronal activity is suppressed during hypoglycemia as a protective, energy-conserving strategy, thereby maintaining ATP levels in the face of decreased substrate. Adenosine release may be a key factor leading to the suppression of neuronal activity prior to ATP depletion. Studies using brain slice preparations show that adenosine release is induced by glucose deprivation (Fowler 1993; Zhu and Krnjevic 1993), and that adenosine suppresses excitatory postsynaptic potentials through action at pre-synaptic sites (Zhu and Krnjevic 1993; Calabresi et al., 1997a, b).

An alternative explanation for brain dysfunction in hypoglycemia prior to energy failure is the possibility of a special role for ATP produced by glycolysis, as opposed to net cellular ATP levels, in maintaining glutamatergic neurotransmission. In neuronal cultures, glucose is necessary for preventing depolarization-induced reversal of glutamate uptake (Bak et al. 2006). Similarly, glucose is uniquely required to support some aspects of neuronal function in brain slices (Arakawa et al. 1991; Izumi et al. 1998; Ikemoto et al. 2003). Glutamate uptake into vesicles is an active, ATP-dependent process, and the membranes of glutamate vesicles contain enzymes responsible for the ATP-generating steps of glycolysis (Ikemoto et al. 2003), such that the rate of glutamate refilling into these vesicles is reduced in the absence of glucose. Studies in vitro have suggested a special role for glycolysis in the uptake of glutamate by astrocytes (Pellerin and Magistretti 1994), although this remains a controversial point; others have found no effect of glutamate on astrocytic glycolytic rate (Hertz et al. 1998; Peng et al. 2001) and no reduction in astrocytic glutamate uptake in the absence of glucose (Swanson and Benington 1996).

A third possibility is that the initial suppression of neuronal activity during hypoglycemia results from ammonia accumulation resulting from oxidative metabolism of amino acids during hypoglycemia. In support of this concept, ammonia levels in a hypoglycemic brain have been found to be in excess of those required to induce coma in normoglycemic animals (Hindfelt and Siesjö 1971; Siesjö 1978). However, with profound hypoglycemia, ATP levels do eventually decline. The fall in ATP occurs abruptly and almost simultaneously with the onset of EEG isoelectricity and massive dissipation of the intracellular: extracellular potassium gradient (Feise et al. 1977; Ratcheson et al. 1981). Studies using animal models have identified little or no neuronal death with hypoglycemia that does not induce this energetic crisis (Auer et al. 1984a, b; 1985a, b; Suh et al. 2003).

14.3 Anatomical Distribution of Neuronal Injury After Severe Hypoglycemia

Not all neurons are equally sensitive to hypoglycemic injury, and studies using rodent models indicate a reproducible pattern of vulnerability. The most sensitive neuronal populations are, in order, those of the subiculum, the small and medium-size neurons of the caudate, CA1 hippocampus, dentate gyrus of the hippocampus, and superficial layers of the cortex (Auer et al. 1984a; 1985a, b). This rank order of vulnerability is similar to that seen with global ischemia-reperfusion, but with some notable differences; in ischemia, the dentate gyrus is much less vulnerable, and the deeper rather than the superficial layers of cortex are more vulnerable (Auer 1986). Electron microscopy studies of hippocampal neurons destined to undergo necrosis reveal early dendritic, axon-sparing lesions (Auer et al. 1985a, b). Swollen dendrites appear after 10 min of isoelectric EEG. After 30 min, swollen mitochondria and abnormal plasma membranes are seen in the cell body. One hour after the onset of isoelectric EEG, the cytoplasm of these cells stain acidophilic when imaged by light microscopy. The characteristic ultrastructural appearance is that of axon-sparing, dendro-somal pathology.

Human autopsy studies of patients dying after severe hypoglycemia show the same general pattern of neuronal injury as observed in the animal models. Neurons in the cerebral cortex and the hippocampus are affected preferentially, followed by neurons in the basal ganglia and the thalamus (Baker and Lufkin 1937; Auer et al. 1989). Neurons in the brain stem, cerebellum, and spinal cord are generally spared, as are glial cells and white matter tracts. It should be noted, however, that the resistance of many neuronal populations to hypoglycemia is relative; with hypoglycemia of sufficient severity and duration, virtually all neuronal populations can be affected. Conversely, recent studies indicate that mild, recurrent hypoglycemia can cause persisting synaptic dysfunction in hippocampus in the absence of neuron death (Yamada et al. 2004; McNay et al. 2006).

14.4 The Hypoglycemic Neuronal Cell Death Pathway

Hypoglycemic neuronal death is not a direct result of energy failure, but results instead from a multi-step process (Fig. 14.1). Key steps identified in this process include glutamate receptor stimulation (Wieloch 1985; Wieloch et al. 1985; Butcher et al. 1987a, b; Papagapiou and Auer 1990; Nellgard and Wieloch 1992), glucose re-perfusion (Suh et al. 2007a, b), production of reactive oxygen species (Patockova et al. 2003; McGowan et al. 2006; Suh et al. 2007a, b, 2008), Zn^{2+} release (Suh et al. 2004, 2008), mitochondrial permeability transition (Ferrand-Drake et al. 1999), and activation of poly(ADP-ribose) polymerase-1 (PARP-1) (Suh et al. 2003). Although the cause-effect relationships between some of these events remain uncertain, interceding at any one of these steps can prevent hypoglycemia-induced neuronal death.

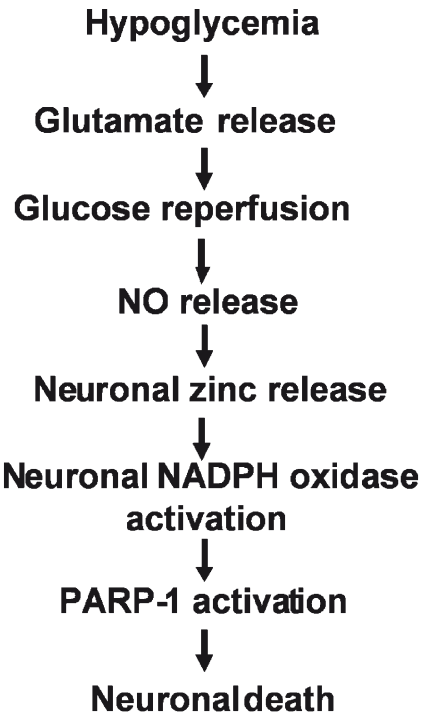

Fig. 14.1 Key events in hypoglycemia-induced neuronal death. Prolonged, severe hypoglycemia leads to the release and impaired re-uptake of glutamate and aspartate. With glucose reperfusion, there is neuronal release of nitric oxide and vesicular zinc, both of which may contribute to further glutamate release and impaired re-uptake. Neuronal NADPH oxidase is activated in a zinc- and glutamate receptor-dependent manner, and utilizes glucose-derived NADPH to generate superoxide. Superoxide, in conjunction with nitric oxide, causes DNA damage and PARP-1 activation. Extensive PARP-1 activation leads to neuronal death. Blocking any one of these steps blocks hypoglycemic neuronal death

14.4.1 Glutamate Receptor Activation

Hypoglycemia causes several-fold elevations in brain extracellular glutamate concentrations (Feise et al. 1977; Engelsen et al. 1986), and ablation of pre-synaptic glutamatergic terminals prevents both the rise of glutamate in the extracellular space and hypoglycemic neuronal death (Wieloch et al. 1985; Butcher et al. 1987a, b). Antagonists to both NMDA and non-NMDA glutamate receptors can also prevent hypoglycemic neuronal death (Wieloch 1985; Papagapiou and Auer 1990; Nellgard and Wieloch 1992). A unique feature of hypoglycemia is that, in addition to glutamate release, aspartate production and release result from the aspartate aminotransferase reaction: glutamate + oxaloacetate ↔ aspartate + α-ketoglutarate (Sutherland et al. 2008). The absence of glucose carbon entry into the tricarboxylic acid (TCA) cycle during hypoglycemia leads to a net conversion of α-ketoglutarate to oxaloacetic acid. The result is a rise in tissue aspartate (Cravioto et al. 1951), and roughly 15-fold elevations in extracellular aspartate (Sandberg et al. 1985; Auer and Siesjö 1993). Aspartate is a potent ligand at some glutamate receptor subtypes, notably the NMDA receptor subtype. A second characteristic feature of hypoglycemic excitotoxicity is that reduced ATP levels associated with glucose deprivation increase neuronal vulnerability to glutamate receptor agonists (Vergun et al. 2003). Thus, the elevation in extracellular aspartate and energy compromise are factors that may confer distinctive aspects to excitotoxicity in hypoglycemia.

Glutamate and aspartate are normally cleared very rapidly from the extracellular space by the action of the astrocyte Na^+-dependent transporters, EAAT1 and EAAT2 (Anderson and Swanson 2000). The fact that excitotoxicity occurs in hypoglycemia thus indicates that there must be a failure of this astrocytic uptake mechanism. Energy depletion is presumably the cause of this uptake failure, because microdialysis studies have shown that brain extracellular glutamate and aspartate levels do not rise until ATP levels fall and ionic gradients dissipate (Feise et al. 1977; Wieloch et al. 1984; Butcher et al. 1987a, b). However, Zn^{2+} released into the extracellular space during hypoglycemia may also contribute to impaired uptake of glutamate and aspartate (Suh et al. 2007a, b), particularly during glucose re-perfusion.

14.4.2 Superoxide Production is Triggered by Glucose Reperfusion

Superoxide production has been identified as a necessary event in excitotoxic neuronal death (Lafon-Cazal et al. 1993; Patel et al. 1996). Studies of hypoglycemic neuronal injury have identified two important features of this aspect of excitotoxicity (Suh et al. 2007a, b). First, studies using cultured neurons placed in glucose-free medium show that superoxide production is triggered when glucose is returned to the medium, and rather than during the glucose-free period itself (Fig. 14.2). Studies of hypoglycemia in the rat brain show the same pattern; superoxide production is negligible during hypoglycemia, but occurs in vulnerable neuronal populations during the 30–60 min interval following normalization of plasma glucose concentrations (Fig. 14.2). A point of potential clinical importance is that the rate of superoxide formation, along with neuronal death, is substantially influenced by the blood glucose concentration achieved during this immediate post-hypoglycemic interval (Suh et al. 2007a, b).

How might glucose reperfusion trigger superoxide production? One potential mechanism is by fueling mitochondria, which can generate superoxide from the electron transport chain and from several dehydrogenase enzymes (Andreyev et al. 2005). Several reports have suggested mitochondria as the primary source of superoxide production during excitotoxicity (Dugan et al. 1995; Bindokas et al. 1996; Duan, Gross and Sheu 2007). However, studies using mitochondrial inhibitors showed no attenuation of the hypoglycemia-induced superoxide production by these agents (Suh et al. 2007a, b). By contrast, inhibitors of NADPH oxidase showed a complete suppression of superoxide production, both in neuronal cultures and in neurons in vivo after hypoglycemia (Fig. 14.3). Glucose is the requisite substrate for regeneration of NADPH by the heoxose monophosphate shunt, and inhibition of the hexose monophosphate shunt with 6-aminonicotinamide likewise blocks superoxide production in hypoglycemia – glucose reperfusion. Inhibition of NADPH oxidase activity with either these pharmacological agents, or with genetic disruption of NADPH oxidase, prevents hypoglycemic neuronal death (Fig. 14.3). Thus, studies of hypoglycemia indicate that (1) hypoglycemia-induced superoxide production occurs at the time of glucose reperfusion, and (2) this occurs by glucose fueling superoxide production by NADPH oxidase.

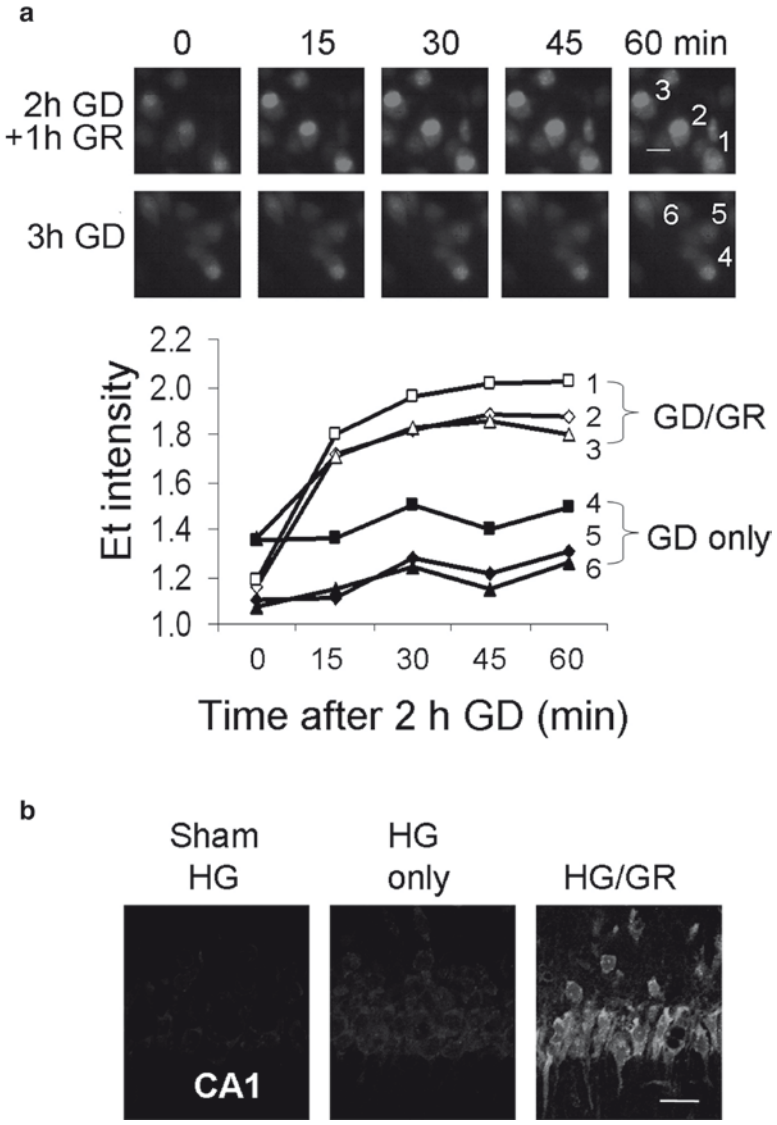

Fig. 14.2 Neuronal superoxide production occurs during glucose reperfusion (**a**) Panels show superoxide production in cultured mouse neurons as evidenced by ethidium (Et) fluorescence at time points after glucose deprivation (GD) and glucose reperfusion (GR). Top row shows neurons subjected to 2 h of GD followed by GR; bottom row shows neurons subjected to GD during the entire 3 h interval. Scale bar = 30 μm. Line graph shows the change in Et fluorescence over time in each of the labeled neurons, with values normalized to the background signal. (**b**) Neuronal superoxide production in rat hippocampus after sham hypoglycemia (HG), 60 min of HG without glucose reperfusion (HG only), or 30 min of HG plus 30 min of glucose reperfusion (GR). Reprinted with modifications from (Suh et al. 2007a, b, c)

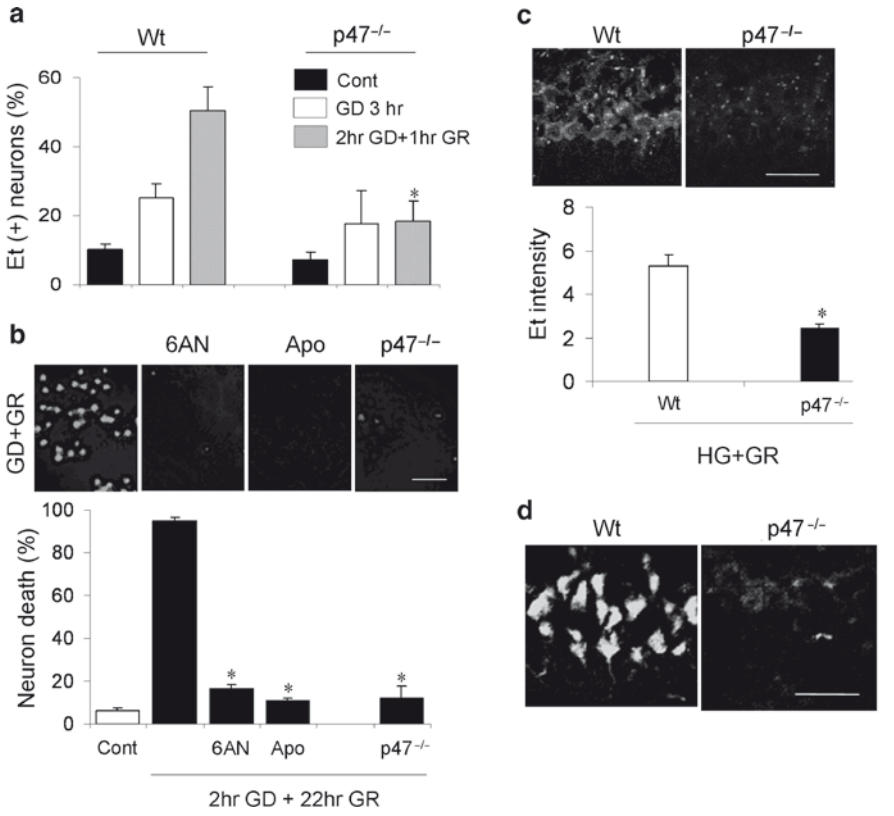

Fig. 14.3 Inhibition of NADPH oxidase prevents hypoglycemia-induced superoxide formation and neuronal death. (**a**) The superoxide production in cortical neuron cultures (Et (+) neurons) induced by glucose deprivation/glucose reperfusion (GD/GR) in wild-type (Wt) neurons is attenuated in neurons deficient in the NADPH oxidase subunit, $p47^{phox}$ ($p47^{-/-}$). Data are means ± s.e.m; $n = 3$, $*p < 0.05$ vs. Wt**.** (**b**) Photomicrographs show propidium iodide staining of dead neurons 22 h after GD in wild-type neurons, $p47^{phox}$ deficient neurons ($p47^{-/-}$), or wild-type neurons treated with pharmacological agents; 6-aminonicotinamide (6AN), which blocks NADPH synthesis by the hexose monophosphate shunt, and apocynin (Apo), which blocks NADPH oxidase assembly. Neuronal death is blocked by both $p47^{phox}$ gene deletion and by the pharmacological inhibitors of NADPH oxidase activity. Scale bar = 100 μm. Graph shows means ± s.e.m; $n = 4–6$, $*p < 0.05$. (**c**) Superoxide production induced by hypoglycemia/glucose reperfusion (HG/GR), as evidence by ethidium fluorescence, was attenuated in neurons of $p47^{phox}$ deficient mice. Scale bar = 100 μm; data are means ± s.e.m; $n = 3–4$, $*p < 0.05$. (**d**) Photomicrographs show dead neurons stained by Fluoro-Jade B in the hippocampal CA1 region of wild-type and $p47^{phox}$ deficient mice 7 days after hypoglycemia/glucose reperfusion. Reprinted with modifications from (Suh et al. 2007a, b, c)

These findings raise the question as to whether these aspects of superoxide production are unique to hypoglycemia, or may also pertain to excitotoxicity in other settings. Additional studies support the latter scenario. Cultured neurons exposed to the glutamate receptor agonist NMDA exhibit a robust production of superoxide, and this production is blocked in glucose-free medium, by the inhibitor

of NADPH synthesis, 6-aminonicotinamide, and by inhibitors or genetic disruption of NADPH oxidase. These interventions also block the neuronal death that otherwise results from NMDA receptor activation (Brennan et al. 2009).

14.4.3 PARP-1 Activation

Superoxide formation leads to DNA damage, particularly in concert with nitric oxide and resultant peroxynitrite formation (Beckman and Koppenol 1996). The nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1), is activated by DNA damage and uses the ADP-ribose group of NAD^+ to form branched ADP-ribose polymers on specific acceptor proteins in the vicinity of DNA strand breaks or kinks (Burzio et al. 1979; D'Amours et al. 1999). Formation of these polymers facilitates DNA repair and prevents chromatid exchange. However, extensive PARP-1 activation promotes cell death through a process involving cytosolic NAD^+ depletion, mitochondrial permeability transition and release of apoptosis-inducing factor (Ha and Snyder 1999; Yu et al. 2002; Alano, Ying and Swanson 2004; Ying et al. 2005). Sustained action of glutamate at neuronal NMDA receptors leads to the production of superoxide and other reactive oxygen species that damage DNA and activate PARP-1 (Dugan et al. 1995; Beckman and Koppenol 1996; Bindokas et al. 1996; Mandir et al. 2000), and PARP-1 inhibitors have been shown to markedly reduce excitotoxic neuronal death (Cosi et al. 1994; Mandir et al. 2000).

Rats subjected to severe hypoglycemia exhibit neuronal accumulation of poly(ADP-ribose), the metabolic product of PARP-1, during the first 3 h following glucose reperfusion (Suh et al. 2003). The accumulation of poly(ADP-ribose) occurs after the formation of nitrotyrosine, a marker of peroxynitrite formation (Fig. 14.4). Rats treated with PARP-1 inhibitors show a striking reduction in neuronal death, even with drug delivery delayed until 3 h after hypoglycemia (Suh et al. 2003). This reduction in cell death is associated with reduced cognitive impairment as assessed by the Morris water maze test of spatial learning and memory (Suh et al. 2003). Although PARP-1 activation can lead to mitochondrial permeability transition (Alano et al. 2004; Cipriani et al. 2005), it has also been reported that mitochondrial permeability transition can lead to DNA damage during hypoglycemia (Ferrand-Drake et al. 1999; Ferrand-Drake et al. 2003), which would place mitochondrial permeability transition upstream of PARP-1 activation. Both scenarios may be operative, forming a positive feed-forward loop.

Activated PARP-1 consumes cytosolic NAD^+, and since NAD^+ is required for glycolysis, hypoglycemia-induced PARP-1 activation may render cells unable to utilize glucose as an energy substrate even when glucose availability is restored. This may contribute to the PARP-1 – mediated cell death in the brain because, as noted previously, the brain has a unique requirement for glucose as a metabolic substrate. Pyruvate, however, can be metabolized in the absence of cytosolic NAD^+, and cell culture studies demonstrate that pyruvate and other non-glucose substrates can rescue cells from PARP-1-induced cell death (Ying et al. 2003; Zong

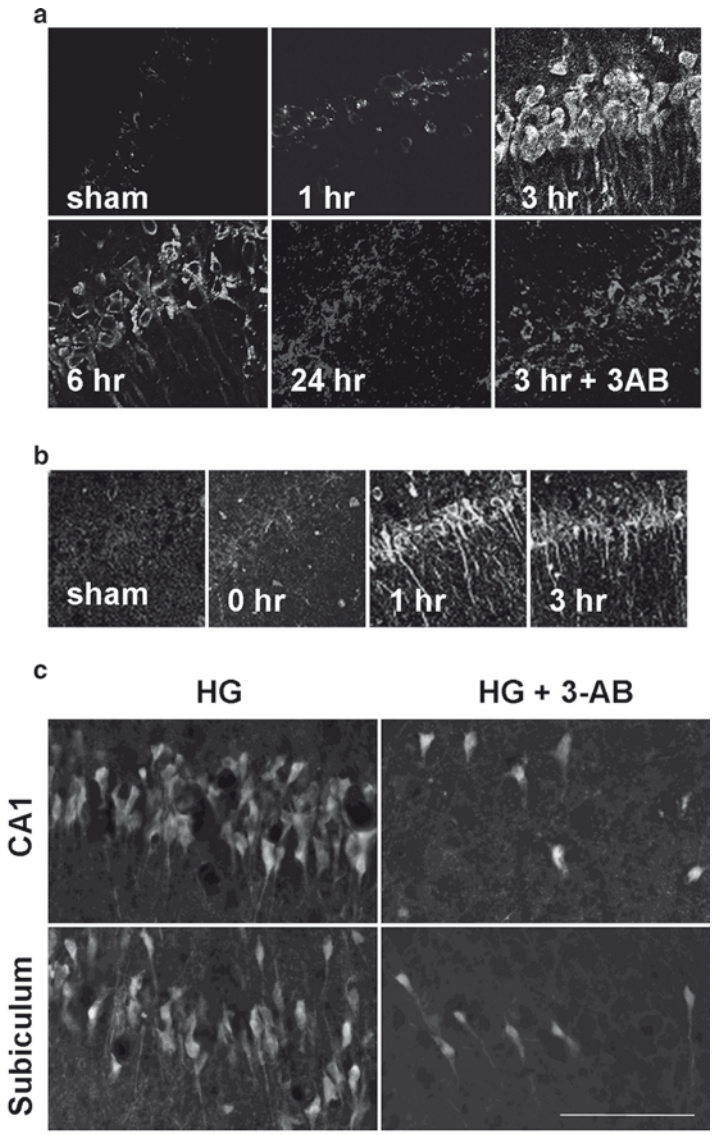

Fig. 14.4 Hypoglycemia/reperfusion – induced poly(ADP-ribose) and nitrotyrosine formation in rat hippocampus. (**a**) Immunoreactivity for poly(ADP-ribose), the enzymatic product of PARP-1, was only modestly increased at termination severe hypoglycemia (0 h), but was markedly increased at 3 h after glucose reperfusion and slowly declined after that point. Poly(ADP-ribose) formation was reduced by administration of the PARP inhibitor 3-aminobenzamide (3-AB) at the time of glucose reperfusion (last panel). (**b**) Nitrotyrosine formation in CA1 neurons was also negligible immediately after 30 min hypoglycemia, but was robust by 1 h after glucose correction. Photomicrographs are representative of three rats under each condition. Scale bar = 100 μm. (**c**) Hypoglycemic damage to hippocampal CA1 and subiculum is reduced by 3-AB. Photomicrographs of hippocampal CA1 and subiculum area from rats subjected to 30 min of isoelectric hypoglycemia show numerous degenerating neurons, identified as fluorescent cells with Fluoro-Jade B staining. Rats treated with 3-AB at the time of glucose correction sustained less neuronal death in both the CA1 (*upper panels*) and subiculum (*lower panels*). Scale bar = 100 μm. Reprinted with modifications from (Suh et al. 2003)

et al. 2004). Pyruvate administered to rats after hypoglycemia prevented neuronal death and preserved spatial memory (Suh et al. 2005). This is a somewhat surprising result, given the relatively low rate of transport of pyruvate across the blood-brain barrier. Presumably, the very high blood levels achieved in this study (estimated at 5 mM, 500 times higher than normal circulating levels of pyruvate) allowed significant brain penetration.

14.4.4 Role of Zinc and Nitric Oxide

Chelatable zinc (weakly bound, histochemically stainable zinc) is present in a subset of glutamatergic axon terminals throughout the mammalian forebrain, especially in the hippocampus and in the cerebral cortex layers II and III (Danscher and Rytter Norgaard 1985; Frederickson 1989). The chelatable zinc is localized in synaptic vesicles (Perez-Clausell and Danscher 1985) and is released into the extracellular space during paroxysmal neuronal activity or membrane depolarization (Assaf and Chung 1984; Howell et al. 1984). Zinc release has been causally linked to neuronal death in several disease conditions, including prolonged seizures (Frederickson et al. 1988; Suh et al. 2001), ischemia (Tonder et al. 1990; Koh et al. 1996) and brain trauma (Suh et al. 2000). Zinc can induce the production of reactive oxygen species (ROS) and resultant PARP-1 activation in cell cultures (Kim et al. 1999; Sensi et al. 1999; Sheline et al. 2000). The mechanism by which elevated extracellular zinc levels lead to ROS production has not yet been established; although some findings suggest that zinc induces superoxide production from mitochondria (Sensi et al. 1999). Zinc has also been identified as an activator of NADPH oxidase (Noh and Koh 2000; Kim and Koh 2002).

In rats subjected to hypoglycemia, zinc staining with *N*-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ) showed depletion of presynaptic vesicular zinc from hippocampal mossy fiber terminals and accumulation of weakly bound zinc in hippocampal CA1 cell bodies induced at the time of glucose reperfusion (Fig. 14.5). The depletion in presynaptic, vesicular zinc was followed by accumulation of TSQ-stainable zinc in post-synaptic neuronal bodies (Suh et al. 2004). Intracerebroventricular injection of the zinc chelator, calcium ethylene-diamine tetraacetic acid (CaEDTA) blocked the zinc accumulation (Fig. 14.5) and reduced hypoglycemia-induced neuronal death. CaEDTA also attenuated the accumulation of poly(ADP-ribose), the enzymatic product of PARP-1 in hippocampal neurons (Suh et al. 2004). These results suggest that zinc translocation is an intermediary step linking hypoglycemia to PARP-1 activation and neuronal death. A key role for the vesicular zinc pool in this process is further suggested by reduced superoxide formation and neuronal death in hypoglycemia in mice deficient in zinc transporter 3 (Suh et al. 2008).

Nitric oxide formation has been shown to play a key role in excitotoxic neuronal death (Sattler et al. 1999; Mandir et al. 2000), and likewise plays a role in hypoglycemic neuronal death. In rats exposed to hypoglycemia-glucose reperfusion, an

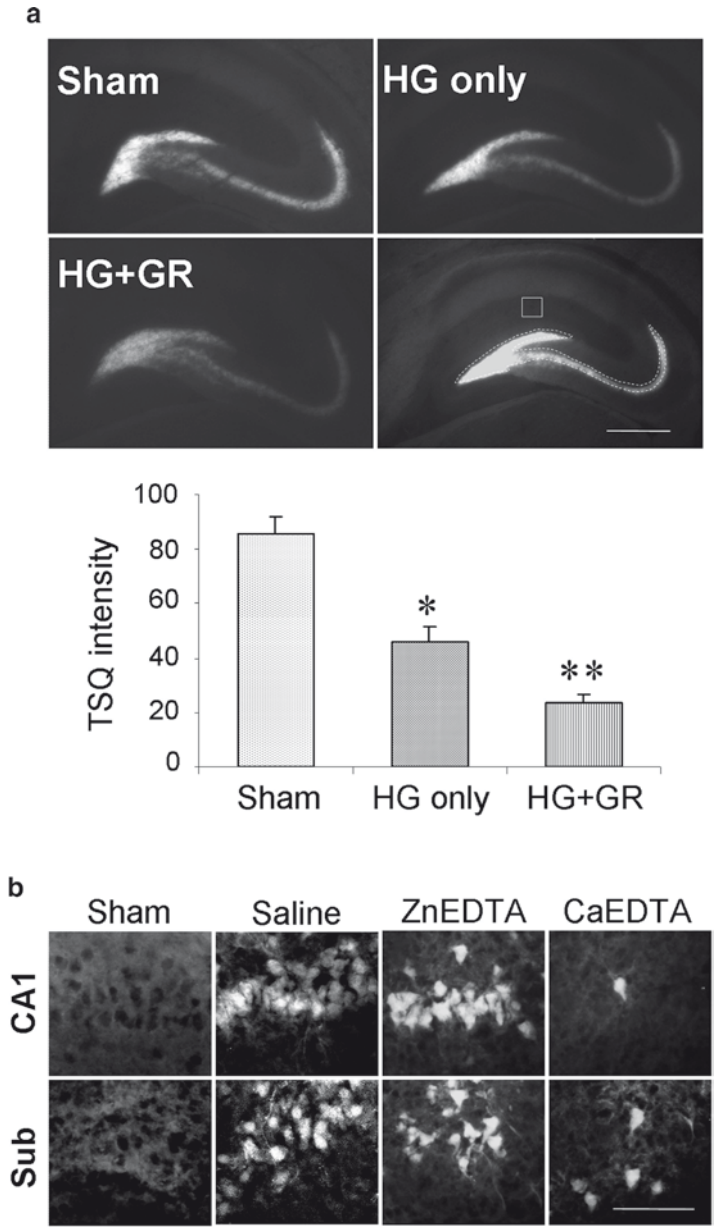

Fig. 14.5 Vesicular zinc release and translocation into postsynaptic neurons after hypoglycemia. (**a**) Chelatable zinc was measured by TSQ fluorescence in the mossy fiber area of hippocampus, as designated by the dotted line. Sham; no HG; HG only, 30 min of HG; HG+GR, 30 min HG and 30 min GR (60 min total elapsed time). Background fluorescence intensity was measured in the molecular layer (box). Bar represents 500 μm. Graph shows quantified fluorescence intensity from the hippocampus mossy fiber area. *$p<0.05$, **$p<0.01$ vs. sham; $n = 10$. (**b**) Zinc accumulation in hippocampal neurons after hypoglycemia. Photomicrographs show TSQ fluorescence in the

increase in protein nitrotyrosine formation is seen during the hour following glucose reperfusion (Suh et al. 2003). Treatment with the nitric oxide synthase inhibitor 7-nitroindazole blocks the formation of nitrotyrosine, blocks PARP-1 activation, and reduces neuronal death. Inhibition of nitric oxide synthase also prevents hypoglycemia-induced zinc translocation and superoxide production after hypoglycemia (Suh et al. 2003), suggesting that nitric oxide acts at both upstream and downstream steps in the hypoglycemia-induced neuronal cell death process.

14.5 Summary of Hypoglycemia-Induced Neuronal Cell Death

Severe hypoglycemia triggers the release and impaired uptake of glutamate and aspartate, which then activate NMDA and non-NMDA glutamate receptor subtypes. With glucose reperfusion, there is additional release of Zn^{2+} and nitric oxide and the production of superoxide. The superoxide production is mediated by NADPH oxidase and requires glucose for NADPH synthesis. Superoxide and nitric oxide together cause DNA damage, PARP-1 activation, and PARP-1-mediated neuronal death.

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References

Agardh CD, Folbergrova J, Siesjö BK (1978) Cerebral metabolic changes in profound, insulin-induced hypoglycemia, and in the recovery period following glucose administration. J Neurochem 31:1135–1142

Alano CC, Ying W, Swanson RA (2004) Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD+ depletion and mitochondrial permeability transition. J Biol Chem 279:18895–18902

Anderson CM, Swanson RA (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32:1–14

Andreyev AY, Kushnareva YE, Starkov AA (2005) Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc) 70:200–214

Arakawa T, Goto T, Okada Y (1991) Effect of ketone body (D-3-hydroxybutyrate) on neural activity and energy metabolism in hippocampal slices of the adult guinea pig. Neurosci Lett 130:53–56

Fig. 14.5 (continued) hippocampal CA1 and subiculum of rat brains harvested 3 days after treatment with sham hypoglycemia or hypoglycemia followed immediately by intracerebroventricular injection of the zinc chelator, CaEDTA, or with saline or ZnEDTA as controls. The dark holes in the sham hypoglycemia sections represent the normal appearance of neuronal cell bodies, and the bright fluorescence in the cell bodies indicates abnormal zinc accumulation. The hypoglycemia-induced zinc accumulation was reduced by CaEDTA but not ZnEDTA. Results are representative of three rats in each treatment group. Reprinted with modifications from (Suh et al. 2004)

Assaf SY, Chung SH (1984) Release of endogenous Zn2+ from brain tissue during activity. Nature 308:734–736
Auer RN (1986) Progress review: hypoglycemic brain damage. Stroke 17:699–708
Auer RN (2004) Hypoglycemic brain damage. Forensic Sci Int 146:105–110
Auer RN, Siesjö BK (1993) Hypoglycaemia: brain neurochemistry and neuropathology. Baillieres Clin Endocrinol Metab 7:611–625
Auer RN, Olsson Y, Siesjö BK (1984a) Hypoglycemic brain injury in the rat. Correlation of density of brain damage with the EEG isoelectric time: a quantitative study. Diabetes 33:1090–1098
Auer RN, Wieloch T, Olsson Y, Siesjö BK (1984b) The distribution of hypoglycemic brain damage. Acta Neuropathol 64:177–191
Auer R, Kalimo H, Olsson Y, Wieloch T (1985a) The dentate gyrus in hypoglycemia: pathology implicating excitotoxin-mediated neuronal necrosis. Acta Neuropathol (Berl) 67:279–288
Auer RN, Kalimo H, Olsson Y, Siesjö BK (1985b) The temporal evolution of hypoglycemic brain damage I. Light- and electron-microscopic findings in the rat cerebral cortex. Acta Neuropathol (Berl) 67:13–24
Auer RN, Hugh J, Cosgrove E, Curry B (1989) Neuropathologic findings in three cases of profound hypoglycemia. Clin Neuropathol 8:63–68
Bak LK, Schousboe A, Sonnewald U, Waagepetersen HS (2006) Glucose is necessary to maintain neurotransmitter homeostasis during synaptic activity in cultured glutamatergic neurons. J Cereb Blood Flow Metab 26(10):1285–1297
Baker A, Lufkin NH (1937) Cerebral lesions in hypoglycemia. Arch Pathol 23:190–201
Beckman JS, Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 271:C1424–C1437
Ben-Ami H, Nagachandran P, Mendelson A, Edoute Y (1999) Drug-induced hypoglycemic coma in 102 diabetic patients. Arch Intern Med 159:281–284
Bindokas VP, Jordan J, Lee CC, Miller RJ (1996) Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. J Neurosci 16:1324–1336
Brennan AM, Suh SW, Won SJ, Narasimhan P, KT M, Lee H, Edling Y, Chan PK, Swanson RA (2009) NADPH oxidase is the primary source of superoxide induced by NMDA receptor activation. Nat Neurosci 12(7):857–863
Brown AM, Sickmann HM, Fosgerau K, Lund TM, Schousboe A, Waagepetersen HS, Ransom BR (2005) Astrocyte glycogen metabolism is required for neural activity during aglycemia or intense stimulation in mouse white matter. J Neurosci Res 79:74–80
Burzio LO, Riquelme PT, Koide SS (1979) ADP ribosylation of rat liver nucleosomal core histones. J Biol Chem 254:3029–3037
Butcher SP, Jacobson I, Sandberg M, Hagberg H, Hamberger A (1987a) 2-Amino-5-phosphonovalerate attenuates the severe hypoglycemia-induced loss of perforant path-evoked field potentials in the rat hippocampus. Neurosci Lett 76:296–300
Butcher SP, Sandberg M, Hagberg H, Hamberger A (1987b) Cellular origins of endogenous amino acids released into the extracellular fluid of the rat striatum during severe insulin-induced hypoglycemia. J Neurochem 48:722–728
Calabresi P, Centonze D, Pisani A, Bernardi G (1997a) Endogenous adenosine mediates the presynaptic inhibition induced by aglycemia at corticostriatal synapses. J Neurosci 17:4509–4516
Calabresi P, Centonze D, Pisani A, Bernardi G (1997b) A possible mechanism for the aglycemia-induced depression of glutamatergic excitation in the striatum. J Cereb Blood Flow Metab 17:1121–1126
Choi IY, Lee SP, Kim SG, Gruetter R (2001) In vivo measurements of brain glucose transport using the reversible Michaelis–Menten model and simultaneous measurements of cerebral blood flow changes during hypoglycemia. J Cereb Blood Flow Metab 21:653–663
Choi IY, Seaquist ER, Gruetter R (2003) Effect of hypoglycemia on brain glycogen metabolism in vivo. J Neurosci Res 72:25–32
Cipriani G, Rapizzi E, Vannacci A, Rizzuto R, Moroni F, Chiarugi A (2005) Nuclear poly(ADP-ribose) polymerase-1 rapidly triggers mitochondrial dysfunction. J Biol Chem 280:17227–17234

Cosi C, Suzuki H, Milani D, Facci L, Menegazzi M, Vantini G, Kanai Y, Skaper SD (1994) Poly(ADP-ribose) polymerase: early involvement in glutamate-induced neurotoxicity in cultured cerebellar granule cells. J Neurosci Res 39:38–46

Cravioto RO, Massieu G, Izquierdo JJ (1951) Free amino-acids in rat brain during insulin shock. Proc Soc Exp Biol Med 78:856–858

D'Amours D, Desnoyers S, D'Silva I, Poirier GG (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem J 342:249–268

Danscher G, Rytter Norgaard JO (1985) Ultrastructural autometallography: a method for silver amplification of catalytic metals. J Histochem Cytochem 33:706–710

Davis EA, Jones TW (1998) Hypoglycemia in children with diabetes: incidence, counterregulation and cognitive dysfunction. J Pediatr Endocrinol Metab 11(Suppl 1):177–182

de Courten-Myers GM, Xi G, Hwang JH, Dunn RS, Mills AS, Holland SK, Wagner KR, Myers RE (2000) Hypoglycemic brain injury: potentiation from respiratory depression and injury aggravation from hyperglycemic treatment overshoots. J Cereb Blood Flow Metab 20:82–92

Duan Y, Gross RA, Sheu SS (2007) Ca2+-dependent generation of mitochondrial reactive oxygen species serves as a signal for poly(ADP-ribose) polymerase-1 activation during glutamate excitotoxicity. J Physiol 585:741–758

Dugan LL, Sensi SL, Canzoniero LM, Handran SD, Rothman SM, Lin TS, Goldberg MP, Choi DW (1995) Mitochondrial production of reactive oxygen species in cortical neurons following exposure to *N*-methyl-D-aspartate. J Neurosci 15:6377–6388

Engelsen B, Westerberg E, Fonnum F, Wieloch T (1986) Effect of insulin-induced hypoglycemia on the concentrations of glutamate and related amino acids and energy metabolites in the intact and decorticated rat neostriatum. J Neurochem 47:1634–1641

Ennis K, Tran PV, Seaquist ER, Rao R (2008) Postnatal age influences hypoglycemia-induced neuronal injury in the rat brain. Brain Res 1224:119–126

Feise G, Kogure K, Busto KR, Scheinberg P, Reinmuth OM (1977) Effect of insulin hypoglycemia upon cerebral energy metabolism and EEG activity in the rat. Brain Res 126:263–280

Ferrand-Drake M, Friberg H, Wieloch T (1999) Mitochondrial permeability transition induced DNA-fragmentation in the rat hippocampus following hypoglycemia. Neuroscience 90:1325–1338

Ferrand-Drake M, Zhu C, Gido G, Hansen AJ, Karlsson JO, Bahr BA, Zamzami N, Kroemer G, Chan PH, Wieloch T, Blomgren K (2003) Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome c and caspase-3 activation in neurons exposed to transient hypoglycemia. J Neurochem 85:1431–1442

Fowler JC (1993) Glucose deprivation results in a lactate preventable increase in adenosine and depression of synaptic transmission in rat hippocampal slices. J Neurochem 60:572–576

Frederickson CJ (1989) Neurobiology of zinc and zinc-containing neurons. Int Rev Neurobiol 31:145–238

Frederickson CJ, Hernandez MD, Goik SA, Morton JD, McGinty JF (1988) Loss of zinc staining from hippocampal mossy fibers during kainic acid induced seizures: a histofluorescence study. Brain Res 446:383–386

Gruetter R (2003) Glycogen: the forgotten cerebral energy store. J Neurosci Res 74:179–183

Gruetter R, Ugurbil K, Seaquist ER (1998) Steady-state cerebral glucose concentrations and transport in the human brain. J Neurochem 70:397–408

Ha HC, Snyder SH (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci U S A 96:13978–13982

Hertz L, Swanson RA, Newman GC, Marrif H, Juurlink BH, Peng L (1998) Can experimental conditions explain the discrepancy over glutamate stimulation of aerobic glycolysis? Dev Neurosci 20:339–347

Hindfelt B, Siesjö BK (1971) Cerebral effects of acute ammonia intoxication II. The effect upon energy metabolism. Scand J Clin Lab Invest 28:365–374

Howell GA, Welch MG, Frederickson CJ (1984) Stimulation-induced uptake and release of zinc in hippocampal slices. Nature 308:736–738

Ikemoto A, Bole DG, Ueda T (2003) Glycolysis and glutamate accumulation into synaptic vesicles. Role of glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase. J Biol Chem 278:5929–5940

Izumi Y, Ishii K, Katsuki H, Benz AM, Zorumski CF (1998) beta-Hydroxybutyrate fuels synaptic function during development. Histological and physiological evidence in rat hippocampal slices. J Clin Invest 101:1121–1132

Kalimo H, Olsson Y (1980) Effects of severe hypoglycemia on the human brain. Neuropathological case reports. Acta Neurol Scand 62:345–356

Kim YH, Koh JY (2002) The role of NADPH oxidase and neuronal nitric oxide synthase in zinc-induced poly(ADP-ribose) polymerase activation and cell death in cortical culture. Exp Neurol 177:407–418

Kim YH, Kim EY, Gwag BJ, Sohn S, Koh JY (1999) Zinc-induced cortical neuronal death with features of apoptosis and necrosis: mediation by free radicals. Neuroscience 89:175–182

Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW (1996) The role of zinc in selective neuronal death after transient global cerebral ischemia. Science 272:1013–1016

Koizumi J (1974) Glycogen in the central nervous system. Prog Histochem Cytochem 6:1–37

Lafon-Cazal M, Pietri S, Culcasi M, Bockaert J (1993) NMDA-dependent superoxide production and neurotoxicity. Nature 364:535–537

Langan SJ, Deary IJ, Hepburn DA, Frier BM (1991) Cumulative cognitive impairment following recurrent severe hypoglycaemia in adult patients with insulin-treated diabetes mellitus. Diabetologia 34:337–344

Lewis LD, Ljunggren B, Ratcheson RA, Siesjö BK (1974) Cerebral energy state in insulin-induced hypoglycemia, related to blood glucose and to EEG. J Neurochem 23:673–679

Lincoln NB, Faleiro RM, Kelly C, Kirk BA, Jeffcoate WJ (1996) Effect of long-term glycemic control on cognitive function. Diabetes Care 19:656–658

Malouf R, Brust JC (1985) Hypoglycemia: causes, neurological manifestations, and outcome. Ann Neurol 17:421–430

Mandir AS, Poitras MF, Berliner AR, Herring WJ, Guastella DB, Feldman A, Poirier GG, Wang ZQ, Dawson TM, Dawson VL (2000) NMDA but not non-NMDA excitotoxicity is mediated by Poly(ADP-ribose) polymerase. J Neurosci 20:8005–8011

McGowan JE, Chen L, Gao D, Trush M, Wei C (2006) Increased mitochondrial reactive oxygen species production in newborn brain during hypoglycemia. Neurosci Lett 399:111–114

McNay EC, Williamson A, McCrimmon RJ, Sherwin RS (2006) Cognitive and neural hippocampal effects of long-term moderate recurrent hypoglycemia. Diabetes 55:1088–1095

Nellgard B, Wieloch T (1992) Cerebral protection by AMPA- and NMDA-receptor antagonists administered after severe insulin-induced hypoglycemia. Exp Brain Res 92:259–266

Noh KM, Koh JY (2000) Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. J Neurosci 20:RC111

Norberg K, Siesjö BK (1976) Oxidative metabolism of the cerebral cortex of the rat in severe insulin-induced hypoglycaemia. J Neurochem 26:345–352

Papagapiou MP, Auer RN (1990) Regional neuroprotective effects of the NMDA receptor antagonist MK-801 (dizocilpine) in hypoglycemic brain damage. J Cereb Blood Flow Metab 10:270–276

Patel M, Day BJ, Crapo JD, Fridovich I, McNamara JO (1996) Requirement for superoxide in excitotoxic cell death. Neuron 16:345–355

Patockova J, Marhol P, Tumova E, Krsiak M, Rokyta R, Stipek S, Crkovska J, Andel M (2003) Oxidative stress in the brain tissue of laboratory mice with acute post insulin hypoglycemia. Physiol Res 52:131–135

Pellerin L, Magistretti PJ (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci U S A 91:10625–10629

Peng L, Swanson RA, Hertz L (2001) Effects of L-glutamate, D-aspartate, and monensin on glycolytic and oxidative glucose metabolism in mouse astrocyte cultures: further evidence that glutamate uptake is metabolically driven by oxidative metabolism. Neurochem Int 38:437–443

Perez-Clausell J, Danscher G (1985) Intravesicular localization of zinc in rat telencephalic boutons. A histochemical study. Brain Res 337:91–98

Ratcheson RA, Blank AC, Ferrendelli JA (1981) Regionally selective metabolic effects of hypoglycemia in brain. J Neurochem 36:1952–1958
Robinson AM, Williamson DH (1980) Physiological roles of ketone bodies as substrates and signals in mammalian tissues. Physiol Rev 60:143–187
Sandberg M, Nystrom B, Hamberger A (1985) Metabolically derived aspartate–elevated extracellular levels in vivo in lodoacetate poisoning. J Neurosci Res 13:489–495
Sattler R, Xiong Z, Lu WY, Hafner M, MacDonald JF, Tymianski M (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science 284:1845–1848
Sensi SL, Yin HZ, Carriedo SG, Rao SS, Weiss JH (1999) Preferential Zn2+ influx through Ca2+-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production. Proc Natl Acad Sci U S A 96:2414–2419
Sheline CT, Behrens MM, Choi DW (2000) Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of $NAD(^+)$ and inhibition of glycolysis. J Neurosci 20:3139–3146
Siesjö BK (1978) Brain Energy Metabolism. John Wiley & Sons, New York
Suh SW, Chen JW, Motamedi M, Bell B, Listiak K, Pons NF, Danscher G, Frederickson CJ (2000) Evidence that synaptically-released zinc contributes to neuronal injury after traumatic brain injury. Brain Res 852:268–273
Suh SW, Thompson RB, Frederickson CJ (2001) Loss of vesicular zinc and appearance of perikaryal zinc after seizures induced by pilocarpine. NeuroReport 12:1523–1525
Suh SW, Aoyama K, Chen Y, Garnier P, Matsumori Y, Gum E, Liu J, Swanson RA (2003) Hypoglycemic neuronal death and cognitive impairment are prevented by poly(ADP-ribose) polymerase inhibitors administered after hypoglycemia. J Neurosci 23:10681–10690
Suh SW, Garnier P, Aoyama K, Chen Y, Swanson RA (2004) Zinc release contributes to hypoglycemia-induced neuronal death. Neurobiol Dis 16:538–545
Suh SW, Aoyama K, Matsumori Y, Liu J, Swanson RA (2005) Pyruvate administered after severe hypoglycemia reduces neuronal death and cognitive impairment. Diabetes 54:1452–1458
Suh SW, Aoyama K, Alano CC, Anderson CM, Hamby AM, Swanson RA (2007a) Zinc inhibits astrocyte glutamate uptake by activation of poly(ADP-ribose) polymerase-1. Mol Med 13:344–349
Suh SW, Bergher JP, Anderson CM, Treadway JL, Fosgerau K, Swanson RA (2007b) Astrocyte glycogen sustains neuronal activity during hypoglycemia: studies with the glycogen phosphorylase inhibitor CP-316, 819 ([R-R*, S*]-5-chloro-N-[2-hydroxy-3- (methoxymethylamino)-3-oxo-1-(phenylmet hyl)propyl]-1H-indole-2-carboxamide). J Pharmacol Exp Ther 321:45–50
Suh SW, Gum ET, Hamby AM, Chan PH, Swanson RA (2007c) Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. J Clin Invest 117:910–918
Suh SW, Hamby AM, Gum ET, Shin BS, Won SJ, Sheline CT, Chan PH, Swanson RA (2008) Sequential release of nitric oxide, zinc, and superoxide in hypoglycemic neuronal death. J Cereb Blood Flow Metab 28:1697–1706
Sutherland GR, Tyson RL, Auer RN (2008) Truncation of the Krebs cycle during hypoglycemic coma. Med Chem 4:379–385
Swanson RA, Benington JH (1996) Astrocyte glucose metabolism under normal and pathological conditions in vitro. Dev Neurosci 18:515–521
Tekkok SB, Brown AM, Westenbroek R, Pellerin L, Ransom BR (2005) Transfer of glycogen-derived lactate from astrocytes to axons via specific monocarboxylate transporters supports mouse optic nerve activity. J Neurosci Res 81:644–652
Tonder N, Johansen FF, Frederickson CJ, Zimmer J, Diemer NH (1990) Possible role of zinc in the selective degeneration of dentate hilar neurons after cerebral ischemia in the adult rat. Neurosci Lett 109:247–252
Vergun O, Han YY, Reynolds IJ (2003) Glucose deprivation produces a prolonged increase in sensitivity to glutamate in cultured rat cortical neurons. Exp Neurol 183:682–694
Wender R, Brown AM, Fern R, Swanson RA, Farrell K, Ransom BR (2000) Astrocytic glycogen influences axon function and survival during glucose deprivation in central white matter. J Neurosci 20:6804–6810

Wieloch T (1985) Hypoglycemia-induced neuronal damage prevented by an *N*-methyl-D-aspartate antagonist. Science 230:681–683

Wieloch T, Harris RJ, Symon L, Siesjö BK (1984) Influence of severe hypoglycemia on brain extracellular calcium and potassium activities, energy, and phospholipid metabolism. J Neurochem 43:160–168

Wieloch T, Engelsen B, Westerberg E, Auer R (1985) Lesions of the glutamatergic cortico-striatal projections in the rat ameliorate hypoglycemic brain damage in the striatum. Neurosci Lett 58:25–30

Yamada KA, Rensing N, Izumi Y, De Erausquin GA, Gazit V, Dorsey DA, Herrera DG (2004) Repetitive hypoglycemia in young rats impairs hippocampal long-term potentiation. Pediatr Res 55:372–379

Ying W, Garnier P, Swanson RA (2003) NAD+ repletion prevents PARP-1-induced glycolytic blockade and cell death in cultured mouse astrocytes. Biochem Biophys Res Commun 308:809–813

Ying W, Alano CC, Garnier P, Swanson RA (2005) NAD+ as a metabolic link between DNA damage and cell death. J Neurosci Res 79:216–223

Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Zhu PJ, Krnjevic K (1993) Adenosine release is a major cause of failure of synaptic transmission during hypoglycaemia in rat hippocampal slices. Neurosci Lett 155:128–131

Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB (2004) Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 18:1272–1282

Part VI
Seizure-Induced Neuronal Death

Chapter 15
Tumor Suppressor p53: A Multifunctional Protein Implicated in Seizure-Induced Neuronal Cell Death

Zhiqun Tan and Steven S. Schreiber

15.1 Tumor Suppressor p53: A Major Regulator of Cell Growth and Death

It is ironic that the p53 "tumor antigen", first discovered more than 20 years ago, was initially thought to play a major role in promoting tumorigenesis (Parada et al. 1984). It was not long after, however, that the p53 protein was found to be a potent tumor suppressor. In fact, since the mid1990s, p53 has been regarded as the "guardian of the genome" on the basis of its ability to block the proliferation of cells with mutated DNA (Lane 1992). For more than two decades, tumor suppressor p53 has been among the most widely studied proteins. Notably, the myriad cellular functions in which p53 is involved continue to increase (Levine et al. 2006; Efeyan and Serrano 2007; Fuster et al. 2007).

The human p53 protein consists of 393 amino acid residues and has a molecular weight of approximately 53 kDa. The p53 gene product is a modular molecule that consists of three well-characterized functional domains: an N-terminal transactivation domain (residues 1–42), a central sequence-specific DNA binding domain (residues 102–292), and a highly basic C-terminal domain that regulates p53 oligomerization and sequence-specific DNA binding (Fig. 15.1) (Prives and Hall 1999; Lavin and Gueven 2006).

It is well-established that p53 functions primarily as a transcription factor that is induced as part of the cellular response to DNA damage (Vousden 2002). The p53 DNA damage response to ionizing radiation and other genotoxic agents occurs in proliferating cells. Following activation, p53 translocates to the nucleus where it either promotes or suppresses the expression of a diverse group of genes, many of which are involved in cell growth control and apoptosis (Morrison et al. 2003; Hughes et al. 1997). Accordingly, p53 activation has been shown to block cell cycle progression at two major cell cycle checkpoints, the G1-S interphase or in G2

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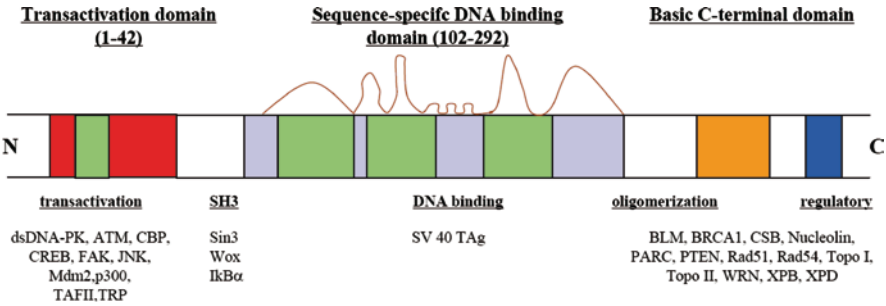

Fig. 15.1 Functional domains of p53 protein. The major domains are *underlined*. The C-terminal regulatory domain modulates sequence-specific DNA binding activity. Approximate locations of oligomerization or binding sites of several protein kinases, viral oncoproteins, and cofactors are shown

before mitosis, or cause apoptotic cell death (Giono and Manfredi 2006). Thus, p53 activation serves to prevent proliferation of cells with damaged DNA and therefore is a major impediment to tumorigenesis. The critical importance of p53 in maintaining genomic stability is underscored by evidence that the loss or inactivation of p53 is associated with more than 50% of human cancers (Ohnishi 2005). Further, patients with Li–Fraumeni syndrome carry germ line mutations in p53 and are at high risk for developing a variety of tumors, while p53 knockout mice display precocious tumor development and premature death (Iwakuma et al. 2005; Donehower et al. 1992). Importantly, most p53 mutations are clustered around the central DNA binding domain, thereby directly affecting transcriptional regulation of p53 target genes, leading to aberrant cell proliferation (Appella and Anderson 2001). Recent evidence suggests that tumorigenesis may depend on the interplay between p53 and its recently identified family members, p63 and p73 (Levrero et al. 2000; Iwakuma et al. 2005).

15.2 p53 Is Regulated Through Multiple Pathways

In addition to genotoxic stress, activation of the p53 pathway can also occur through other types of cellular stress such as hypoxia, nucleotide depletion, and viral infection (Culmsee and Mattson 2005). Although the exact triggering mechanism is unclear, evidence indicates that p53-mediated cell cycle arrest or apoptosis is determined by many factors including cell type, differentiation status, type of stimulus, and extent of DNA damage. In this regard, our laboratory was the first to demonstrate p53 induction in neurons that appeared to be undergoing apoptosis following excitotoxin treatment. In these studies, increased p53 immunoreactivity was present in neurons with DNA damage (Sakhi et al. 1994). Notably, p53 induction in neurons occurred at the transcriptional level, in contrast to nonneural cells in which p53 accumulates posttranscriptionally. These findings support the premise that in fully differentiated

nondividing cells, activation of the p53 pathway causes apoptosis. Induction and activation of p53 have since been documented in a number of animal models as well as human neurodegenerative diseases (Morrison et al. 2003). Despite the growing list of neurological disorders in which p53 activation occurs, the exact mechanism(s) of p53-mediated neuronal cell death remains to be delineated.

In normal cells, p53 protein is maintained at an extremely low level and mainly exists as a latent form that is transcriptionally inactive (Lavin and Gueven 2006). A large body of evidence has shown that stabilization of p53 protein through post-translational modifications such as phosphorylation, acetylation, glycosylation, and sumoylation is required for p53 to mediate either cell cycle arrest or apoptosis in response to stress (Olsson et al. 2007; Meek 1997; Appella and Anderson 2001; Brooks and Gu 2003; Yang et al. 2004). In particular, p53 stabilization is regulated largely by phosphorylation of a number of N- and C-terminal residues. For example, phosphorylation of p53 at serine 15 prevents p53 from interacting with mouse double minute 2 (Mdm2), an E3 ubiquitin ligase that targets p53 for ubiquitination and proteasomal degradation (Lavin and Gueven 2006). Several protein kinases, including Cdk5 and c-Jun N-terminal kinase 1 (JNK1) have been shown to regulate p53 stabilization (Lee et al. 2007; Fogarty et al. 2003). Recently, it has been shown that p53 stabilization and activation are also regulated by acetylation and deacetylation. The Sirt1 protein, a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase implicated in aging, is a negative regulator of p53 that prevents apoptosis (Luo et al. 2001). Both glycosylation and sumoylation have also been implicated in p53 stabilization and cell death (Fiordaliso et al. 2001; Bischof et al. 2006). The importance of these regulatory pathways in neurons remains unclear. The most prominent mechanism by which p53 abundance is regulated is through the ubiquitin-proteasome pathway (Brooks and Gu 2004). In general, polyubiquitination of p53 at specific lysine residues targets p53 for proteasomal degradation. Recent studies indicate that monoubiquitination of p53 may regulate nuclear export without affecting p53 degradation (Li et al. 2003). Conversely, defects in the polyubiquitination cascade may disrupt proteasome-mediated p53 degradation. In this regard, other laboratories as well as ours have demonstrated that decreased free ubiquitin levels may contribute to accumulation of multiple proteins, including p53 (Tan et al. 2001a). Depletion of free ubiquitin in neurons has been found to be responsible, at least in part, for p53 stabilization and neuronal degeneration under a variety of stresses (Tan et al. 2000, 2001a; Yao et al. 2007).

Among all interactions between p53 and other cellular proteins, the most important in terms of regulating p53 abundance and function is the interaction between p53 and Mdm2 (Brooks and Gu 2004). The Mdm2 oncoprotein binds to and inhibits the p53 N-terminal transactivation domain, which promotes tumorigenesis (Deb 2002). As stated earlier, Mdm2 is a ubiquitin ligase that ubiquitinates and targets p53 for proteasomal degradation. Importantly, the *mdm2* gene is a transcriptional target of p53, and it forms part of an inhibitory feedback loop that maintains low intracellular levels of p53 in unstimulated cells, and terminates the p53 response after cellular stress (Brooks and Gu 2004). Accordingly, overexpression of Mdm2 has been shown to block both p53-mediated cell cycle arrest and apoptosis.

15.3 Transcription-Independent Functions of p53

Various lines of evidence have established that the p53 tumor suppressor gene plays a central role as a major effector of either cell cycle arrest or apoptosis following DNA damage or other types of cellular stress (Levine et al. 2006). Following activation, wild-type p53 protein functions primarily as a transcription factor with sequence-specific DNA binding activity to regulate expression of p53 target genes (Vousden 2002). However, in addition to its role as a transcription factor, p53 has recently been found at extra-nuclear locations. In this regard, under stress conditions p53 translocates to mitochondria and interacts with mitochondrial membrane proteins involved in apoptosis (Murphy et al. 2004). Evidence suggests that interactions between p53 and Bcl-2 family members at the mitochondrial membrane promote membrane permeabilization and release of apoptotic factors (Chipuk and Green 2004; Leu et al. 2004; Endo et al. 2006). Evidence for mitochondrial translocation of p53 in neurons has been demonstrated after global ischemia (Endo et al. 2006). In contrast, there was no evidence to support a role for mitochondrial p53 in cultured neurons treated with a variety of cytotoxic agents (Uo et al. 2007). The same authors found that direct targeting of p53 to mitochondria did not induce apoptosis. These results suggest that the role of mitochondrial p53 in neuronal cell death is complex and could be determined, in part, by the type of stimulus or tissue preparation. The interaction between p53 and mitochondria defines a novel p53 transcription-independent cell death pathway that is of potential relevance to seizure-induced neuronal cell death.

Interestingly, a significant increase in p53 abundance occurred at neuronal synapses following exposure to excitotoxic or oxidative insults (Gilman et al. 2003). The synaptic localization of p53 suggests yet another transcription-independent p53 pathway that may contribute to synaptic dysfunction in various neurodegenerative diseases. Moreover, others have demonstrated stabilization of p53 through interactions with nucleolar proteins (Kurki et al. 2004). A connection between p53 and the nucleolus, the locus of rRNA synthesis and ribosome assembly, may be part of a novel cellular stress response (Mayer and Grummt 2005). In this regard, a direct interaction between p53 and the nucleolar protein, SL-1, attenuates RNA polymerase activity (Zhai and Comai 2000). Whether a similar functional relationship between p53 and the nucleolus occurs in neurons remains to be demonstrated.

15.4 p53 and Seizure-Induced Neuronal Cell Death

It is well-established in animal models that prolonged repetitive seizures, i.e., status epilepticus, cause selective neuronal cell death in vulnerable brain regions (Fujikawa 2005). Accordingly, systemic administration of either the glutamate analog, kainic acid (KA), or the potent muscarinic agonist, pilocarpine, in rodents causes a well-described seizure disorder and neuronal loss in the hippocampus, piriform cortex, amygdala, and thalamic nuclei (Wang et al. 2005; Fabene et al. 2007). Recent results

from biochemical and ultrastructural analyses revealed that seizure-induced neuronal cell death bears features consistent with both necrosis and apoptosis (Fujikawa 2005; Bengzon et al. 2002). Notably, seizure-induced neuronal cell death can be attenuated by inhibition of protein synthesis, suggesting that apoptosis plays a prominent role (Sakhi et al. 1994). In this regard, the role of tumor suppressor p53, a major regulator of apoptosis, in seizure-induced neuronal cell death was established more than a decade ago, and has been extensively studied since. Following the administration of either KA or pilocarpine in rats, p53 mRNA is induced within several hours in vulnerable neurons (Sakhi et al. 1994; Tan et al. 2002a). These findings demonstrated that in the central nervous system (CNS), the p53 cellular stress response is activated at the transcriptional level. In these studies, increased p53 mRNA levels were detected within eosinophilic cells, and not in normal appearing cells, up to 24 hours after seizure onset (Fig. 15.2). In addition, other laboratories as well as ours have demonstrated accumulation of p53 protein (Fig. 15.2) along with increased sequence-specific DNA binding activity following prolonged seizures, consistent with the primary role of p53 as a transcription factor (Liu et al. 1999; Sakhi et al. 1996; Yang et al. 2008). Similarly, increased p53 DNA binding activity was found to occur in cultured cerebellar granule neurons following either KA or glutamate treatment (Uberti et al. 1998). Importantly, p53-immunoreactive nuclear aggregates were frequently observed in damaged neurons, suggesting that the p53 response was triggered by DNA damage. This was corroborated by double labeling of p53 and damaged DNA by terminal dUTP nick-end labeling (TUNEL), which indicated that there was a direct link between p53 and DNA damage in the CNS (Fig. 15.2; Tan et al. 2001a, b). The in vivo results described above have been replicated in both organotypic hippocampal slice cultures and dissociated hippocampal or cortical neurons (Tan et al. 2001a; Sakhi et al. 1997). In addition to its pro-apoptotic function, p53 activation in proliferating cells may also result in cell cycle arrest (Giono and Manfredi 2006). In nonproliferating cells, aberrant activation of the cell cycle has been implicated in apoptosis (Byrnes and Faden 2007). Thus, cyclin D1, a G1 phase cell cycle protein encoded by a p53 responsive gene, is upregulated in vulnerable neurons and reactive astrocytes following kainate-induced seizures (Liu et al. 1996). These findings strengthen the connection between p53, a major cell cycle regulator, and neuronal cell death.

Further evidence indicating a more direct role for p53 in seizure-induced neurodegeneration has been provided by studies using p53 null mice. Thus, p53 deficiency has been shown to provide significant neuroprotection from a variety of experimental insults, including status epilepticus (Morrison et al. 2003). Interestingly, there was no difference in the pattern of neuronal injury following subcutaneous administration of KA to heterozygous p53 (+/−) mice compared with wild-type controls, suggesting that a single copy of p53 is sufficient to confer neuronal vulnerability (Morrison et al. 1996). In cultured neurons from p53 null mice, restoration of p53 expression by adenovirus-mediated transduction resulted in neuronal cell death, further demonstrating a direct link between p53 expression and neurodegeneration regardless of genetic background (Xiang et al. 1996; Schauwecker 2003). In addition to genetic manipulation, pharmacological inhibition of p53 activation by the synthetic inhibitor, pifithrin-alpha, or expression of a

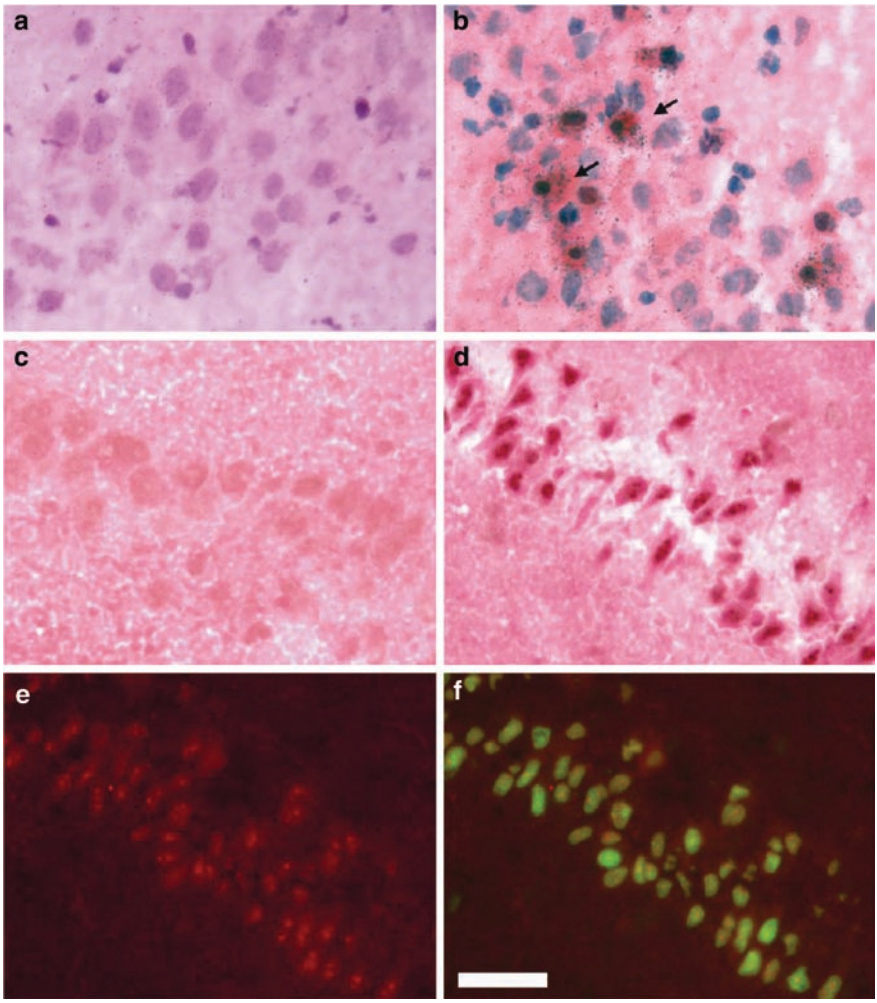

Fig. 15.2 Induction of p53 mRNA and protein in association with neuronal degeneration in rat brain, following pilocarpine-induced seizures. In situ hybridization with a radiolabeled p53 cRNA probe and emulsion autoradiography demonstrated a low level of p53 mRNA expression in a saline-treated control (**a**) and a significant increase in eosinophilic neurons in hippocampal CA1 pyramidal neurons (*arrows*) 24 h after the onset of seizures (**b**). Immunohistochemistry using a p53-specific antibody revealed background staining in the control (**c**) and nuclear accumulation of p53 CA1 pyramidal neurons 8 h after seizure-onset (**d**). (**e**) Nuclear p53 accumulation (*red*) overlaps with damaged DNA shown by TUNEL (*green*) in the same section (**f**) 24 h after the onset of seizures. Scale bar: 30 μm (**a–d**) and 45 μm (**e**, **f**) (Tan et al. 2002a)

dominant-negative p53, protects against a variety of insults including excitotoxic damage (Culmsee et al. 2001; Neema et al. 2005). Thus, there is an impressive body of evidence indicating that cell death signaling mediated by activation of the p53

pathway contributes to neuronal cell death in various pathological conditions, including prolonged seizures.

15.5 Seizures Activate Key Downstream Effectors in the p53 Pathway

Of the many facets comprising the p53 pathway, the most widely studied is the regulation of p53 responsive genes, many of which are involved in cell death (Morrison et al. 2003). Accordingly, Fas protein, apoptosis protease activating factor-1 (Apaf-1) and the Bcl-2 family member Bax are prominent proapoptotic proteins whose genes are transcriptionally activated by p53, while the *bcl-2* gene itself is transcriptionally repressed by p53 (Miyashita et al. 1994; Cregan et al. 1999; Ethell and Buhler 2003; Fortin et al. 2001). In rats treated with either KA or pilocarpine, Fas protein is upregulated and colocalizes with p53 in degenerating neurons (Tan et al. 2001b, 2002b). Prolonged seizures also promote the formation of Apaf-1/cytochrome *c* complexes, a major step prior to caspase-3 activation in the intrinsic apoptosis cascade (Henshall et al. 2001). Bax is also upregulated by seizure activity both in vivo and in vitro (Yang et al. 2008; Tan et al. 2002b; Liu et al. 2001; Djebaïli et al. 2001), while bax-deficient neurons are protected from excitotoxic injury and p53-mediated apoptosis (Xiang et al. 1998; Cregan et al. 1999). Whether p53 mitochondrial translocation plays a role in seizure-induced neuronal cell death through interactions with Bax and other Bcl-2-related proteins remains to be determined.

As mentioned previously, the *mdm2* gene is a p53 response gene and its product is a major regulator of p53 proteasomal degradation. Status epilepticus following treatment with either KA or pilocarpine results in a significant increase in Mdm2 expression that colocalizes with p53 in the nucleus of degenerating neurons (Tan et al. 2001a, 2002b). Interestingly, p53 abundance increased despite the formation of p53-Mdm2 complexes, and was associated with a marked decrease in the abundance of free ubiquitin (Tan et al. 2001a). Normalization of p53 processing by the ubiquitin-proteasome pathway may therefore be protective against seizure-induced neuronal cell death.

In addition to experimental models, recent evidence supporting activation of the p53 pathway has been reported in humans with temporal lobe epilepsy (TLE). Increased expression of bcl-2 family members and caspase cleavage products were demonstrated in resected tissue from patients with intractable TLE (Henshall et al. 2000). More recently, neurons with increased immunoreactivity for bcl-2, p53, Fas, and caspase-3 were identified in sclerotic hippocampi (Xu et al. 2007). In this study, there was a positive correlation between seizure frequency and expression of p53, fas and caspase-3. In addition, increased expression of p53 was found in samples from intractable TLE patients along with significantly lower levels of Mdm2 (Engel et al. 2007). Both the proapoptotic Fas and the antiapoptotic Bcl-2 proteins were proportionately elevated in sera of patients with idiopathic epilepsy, and their levels were correlated with seizure severity and frequency (El-Hodhod

et al. 2006). Additional work will be necessary to firmly establish a causative role for p53 activation in the neuronal loss associated with human epilepsy.

15.6 Summary

For more than two decades, p53 has been at the forefront of investigations relevant to cell death signaling and growth control pathways. In addition to its role as a major transcription factor, the number of ways in which p53 influences cellular physiology is ever-increasing. There is also compelling evidence supporting a role for p53 as a mediator of neuronal cell death in both experimental models and human neurodegenerative conditions, including status epilepticus. Additional work is necessary to fully understand the ramifications of p53 activation in the CNS, which will lead to more effective neuroprotective treatments.

References

Appella E, Anderson CW (2001) Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem 268:2764–2772

Bengzon J, Mohapel P, Ekdahl CT, Lindvall O (2002) Neuronal apoptosis after brief and prolonged seizures. Prog Brain Res 135:111–119

Bischof O, Schwamborn K, Martin N, Werner A, Sustmann C, Grosschedl R, Dejean A (2006) The E3 SUMO ligase PIASy is a regulator of cellular senescence and apoptosis. Mol Cell 22:783–794

Brooks CL, Gu W (2003) Ubiquitination, phosphorylation and acetylation: The molecular basis for p53 regulation. Curr Opin Cell Biol 15:164–171

Brooks CL, Gu W (2004) Dynamics in the p53-Mdm2 ubiquitination pathway. Cell Cycle 3:895–899

Byrnes KR, Faden AI (2007) Role of cell cycle proteins in CNS injury. Neurochem Res 32:1799–1807

Chipuk JE, Green DR (2004) Cytoplasmic p53: Bax and forward. Cell Cycle 3:429–431

Cregan SP, MacLaurin JG, Craig CG, Robertson GS, Nicholson DW, Park DS, Slack RS (1999) Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. J Neurosci 19:7860–7869

Culmsee C, Mattson MP (2005) p53 in neuronal apoptosis. Biochem Biophys Res Commun 331:761–777

Culmsee C, Zhu X, Yu QS, Chan SL, Camandola S, Guo Z, Greig NH, Mattson MP (2001) A synthetic inhibitor of p53 protects neurons against death induced by ischemic and excitotoxic insults, and amyloid beta-peptide. J Neurochem 77:220–228

Deb SP (2002) Function and dysfunction of the human oncoprotein MDM2. Front Biosci 7:d235–d243

Djebaïli M, Lerner-Natoli M, Montpied P, Baille V, Bockaert J, Rondouin G (2001) Molecular events involved in neuronal death induced in the mouse hippocampus by in-vivo injection of kainic acid. Brain Res Mol Brain Res 93:190–198

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 356:215–221

Efeyan A, Serrano M (2007) p53: Guardian of the genome and policeman of the oncogenes. Cell Cycle 6:1006–1010

El-Hodhod MA, Tomoum HY, Abd Al-Aziz MM, Samaan SM (2006) Serum Fas and Bcl-2 in patients with epilepsy. Acta Neurol Scand 113:315–321

Endo H, Saito A, Chan PH (2006) Mitochondrial translocation of p53 underlies the selective death of hippocampal CA1 neurons after global cerebral ischaemia. Biochem Soc Trans 34:1283–1286

Engel T, Murphy BM, Schindler CK, Henshall DC (2007) Elevated p53 and lower MDM2 expression in hippocampus from patients with intractable temporal lobe epilepsy. Epilepsy Res 77:151–156

Ethell DW, Buhler LA (2003) Fas ligand-mediated apoptosis in degenerative disorders of the brain. J Clin Immunol 23:439–446

Fabene PF, Merigo F, Galiè M, Benati D, Bernardi P, Farace P, Nicolato E, Marzola P, Sbarbati A (2007) Pilocarpine-induced status epilepticus in rats involves ischemic and excitotoxic mechanisms. PLoS One 2:e1105

Fiordaliso F, Leri A, Cesselli D, Limana F, Safai B, Nadal-Ginard B, Anversa P, Kajstura J (2001) Hyperglycemia activates p53 and p53-regulated genes leading to myocyte cell death. Diabetes 50:2363–2375

Fogarty MP, Downer EJ, Campbell V (2003) A role for c-Jun N-terminal kinase 1 (JNK1), but not JNK2, in the beta-amyloid-mediated stabilization of protein p53 and induction of the apoptotic cascade in cultured cortical neurons. Biochem J 371:789–798

Fortin A, Cregan SP, MacLaurin JG, Kushwaha N, Hickman ES, Thompson CS, Hakim A, Albert PR, Cecconi F, Helin K, Park DS, Slack RS (2001) APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death. J Cell Biol 155:207–216

Fujikawa DG (2005) Prolonged seizures and cellular injury: Understanding the connection. Epilepsy Behav 7(Suppl 3):S3–S11

Fuster JJ, Sanz-González SM, Moll UM, Andrés V (2007) Classic and novel roles of p53: Prospects for anticancer therapy. Trends Mol Med 13:192–199

Gilman CP, Chan SL, Guo Z, Zhu X, Greig N, Mattson MP (2003) p53 is present in synapses where it mediates mitochondrial dysfunction and synaptic degeneration in response to DNA damage, and oxidative and excitotoxic insults. Neuromol Med 3:159–172

Giono LE, Manfredi JJ (2006) The p53 tumor suppressor participates in multiple cell cycle checkpoints. J Cell Physiol 209:13–20

Henshall DC, Clark RS, Adelson PD, Chen M, Watkins SC, Simon RP (2000) Alterations in bcl-2 and caspase gene family protein expression in human temporal lobe epilepsy. Neurology 55:250–257

Henshall DC, Bonislawski DP, Skradski SL, Araki T, Lan JQ, Schindler CK, Meller R, Simon RP (2001) Formation of the Apaf-1/cytochrome *c* complex precedes activation of caspase-9 during seizure-induced neuronal death. Cell Death Differ 8:1169–1181

Hughes PE, Alexi T, Schreiber SS (1997) Minireview: A role for the tumour-suppressor gene p53 in neuronal apoptosis. NeuroReport 8:v–xi

Iwakuma T, Lozano G, Flores ER (2005) Li-Fraumeni syndrome: A p53 family affair. Cell Cycle 4:865–867

Kurki S, Peltonen K, Laiho M (2004) Nucleophosmin, HDM2 and p53: Players in UV damage incited nucleolar stress response. Cell Cycle 3:976–979

Lane DP (1992) Cancer. p53, guardian of the genome. Nature 358:15–16

Lavin MF, Gueven N (2006) The complexity of p53 stabilization and activation. Cell Death Differ 13:941–950

Lee JH, Kim HS, Lee SJ, Kim KT (2007) Stabilization and activation of p53 induced by Cdk5 contributes to neuronal cell death. J Cell Sci 120:2259–2271

Leu JI, Dumont P, Hafey M, Murphy ME, George DL (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol 6:443–450

Levine AJ, Hu W, Feng Z (2006) The P53 pathway: What questions remain to be explored? Cell Death Differ 13:1027–1036

Levrero M, De Laurenzi V, Costanzo A, Gong J, Wang JY, Melino G (2000) The p53/p63/p73 family of transcription factors: Overlapping and distinct functions. J Cell Sci 113:1661–1670
Li M, Brooks CL, Wu-Baer F, Chen D, Baer R, Gu W (2003) Mono- versus polyubiquitination: Differential control of p53 fate by Mdm2. Science 302:1972–1975
Liu W, Bi X, Tocco G, Baudry M, Schreiber SS (1996) Increased expression of cyclin D1 in the adult rat brain following kainic acid treatment. Neuroreport 7:2785–2789
Liu W, Rong Y, Baudry M, Schreiber SS (1999) Status epilepticus induces p53 sequence-specific DNA binding in mature rat brain. Brain Res Mol Brain Res 63:248–253
Liu W, Liu R, Chun JT, Bi R, Hoe W, Schreiber SS, Baudry M (2001) Kainate excitotoxicity in organotypic hippocampal slice cultures: Evidence for multiple apoptotic pathways. Brain Res 916:239–248
Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W (2001) Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell 107:137–148
Mayer C, Grummt I (2005) Cellular stress and nucleolar function. Cell Cycle 4:1036–1038
Meek DW (1997) Post-translational modification of p53 and the integration of stress signals. Pathol Biol (Paris) 45:804–814
Miyashita T, Harigai M, Hanada M, Reed JC (1994) Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Res 54:3131–3135
Morrison RS, Wenzel HJ, Kinoshita Y, Robbins CA, Donehower LA, Schwartzkroin PA (1996) Loss of the p53 tumor suppressor gene protects neurons from kainate-induced cell death. J Neurosci 16:1337–1345
Morrison RS, Kinoshita Y, Johnson MD, Guo W, Garden GA (2003) p53-dependent cell death signaling in neurons. Neurochem Res 28:15–27
Murphy ME, Leu JI, George DL (2004) p53 moves to mitochondria: a turn on the path to apoptosis. Cell Cycle 3:836–839
Neema M, Navarro-Quiroga I, Chechlacz M, Gilliams-Francis K, Liu J, Lamonica K, Lin SL, Naegele JR (2005) DNA damage and nonhomologous end joining in excitotoxicity: Neuroprotective role of DNA-PKcs in kainic acid-induced seizures. Hippocampus 15:1057–1071
Ohnishi T (2005) The role of the p53 molecule in cancer therapies with radiation and/or hyperthermia. J Cancer Res Ther 1:147–150
Olsson A, Manzl C, Strasser A, Villunger A (2007) How important are post-translational modifications in p53 for selectivity in target-gene transcription and tumour suppression? Cell Death Differ 14:1561–1575
Parada LF, Land H, Weinberg RA, Wolf D, Rotter V (1984) Cooperation between gene encoding p53 tumour antigen and Ras in cellular transformation. Nature 312:649–651
Prives C, Hall PA (1999) The p53 pathway. J Pathol 187:112–126
Sakhi S, Bruce A, Sun N, Tocco G, Baudry M, Schreiber SS (1994) p53 induction is associated with neuronal damage in the central nervous system. Proc Natl Acad Sci USA 91:7525–7529
Sakhi S, Sun N, Wing LL, Mehta P, Schreiber SS (1996) Nuclear accumulation of p53 protein following kainic acid-induced seizures. Neuroreport 7:493–496
Sakhi S, Bruce A, Sun N, Tocco G, Baudry M, Schreiber SS (1997) Induction of tumor suppressor p53 and DNA fragmentation in organotypic hippocampal cultures following excitotoxin treatment. Exp Neurol 145:81–88
Schauwecker PE (2003) Genetic basis of kainate-induced excitotoxicity in mice: Phenotypic modulation of seizure-induced cell death. Epilepsy Res 55:201–210
Tan Z, Qu W, Tu W, Liu W, Baudry M, Schreiber SS (2000) Downregulation of free ubi-quitin: A novel mechanism of p53 stabilization and neuronal cell death. Cell Death Differ 27:675–681
Tan Z, Tu W, Schreiber SS (2001a) Downregulation of free ubiquitin: A novel mechanism of p53 stabilization and neuronal cell death. Brain Res Mol Brain Res 91:179–188
Tan Z, Levid J, Schreiber SS (2001b) Increased expression of Fas (CD95/APO-1) in adult rat brain after kainate-induced seizures. Neuroreport 12:1979–1982
Tan Z, Sankar R, Shin D, Sun N, Liu H, Wasterlain CG, Schreiber SS (2002a) Differential induction of p53 in immature and adult rat brain following lithium-pilocarpine status epilepticus. Brain Res 928:187–193

Tan Z, Sankar R, Tu W, Shin D, Liu H, Wasterlain CG, Schreiber SS (2002b) Immunohistochemical study of p53-associated proteins in rat brain following lithium-pilocarpine status epilepticus. Brain Res 929:129–138

Uberti D, Belloni M, Grilli M, Spano P, Memo M (1998) Induction of tumour-suppressor phosphoprotein p53 in the apoptosis of cultured rat cerebellar neurones triggered by excitatory amino acids. Eur J Neurosci 10:246–254

Uo T, Kinoshita Y, Morrison RS (2007) Apoptotic actions of p53 require transcriptional activation of PUMA and do not involve a direct mitochondrial/cytoplasmic site of action in postnatal cortical neurons. J Neurosci 27:12198–12210

Vousden KH (2002) Activation of the p53 tumor suppressor protein. Biochim Biophys Acta 1602:47–59

Wang Q, Yu S, Simonyi A, Sun GY, Sun AY (2005) Kainic acid-mediated excitotoxicity as a model for neurodegeneration. Mol Neurobiol 31:3–16

Xiang H, Hochman DW, Saya H, Fujiwara T, Schwartzkroin PA, Morrison RS (1996) Evidence for p53-mediated modulation of neuronal viability. J Neurosci 16:6753–6765

Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, Morrison RS (1998) Bax involvement in p53-mediated neuronal cell death. J Neurosci 18:1363–1373

Xu S, Pang Q, Liu Y, Shang W, Zhai G, Ge M (2007) Neuronal apoptosis in the resected sclerotic hippocampus in patients with mesial temporal lobe epilepsy. J Clin Neurosci 14:835–840

Yang Y, Li CC, Weissman AM (2004) Regulating the p53 system through ubiquitination. Oncogene 23:2096–2106

Yang T, Hsu C, Liao W, Chuang JS (2008) Heat shock protein 70 expression in epilepsy suggests stress rather than protection. Acta Neuropathol 115:219–230

Yao X, Liu J, McCabe JT (2007) Ubiquitin and ubiquitin-conjugated protein expression in the rat cerebral cortex and hippocampus following traumatic brain injury (TBI). Brain Res 1182:116–122

Zhai W, Comai L (2000) Repression of RNA polymerase I transcription by the tumor suppressor p53. Mol Cell Biol 20:5930–5938

Chapter 16
DNA Damage and Repair in the Brain: Implications for Seizure-Induced Neuronal Injury, Endangerment, and Neuroprotection

Samantha L. Crowe and Alexei D. Kondratyev

16.1 Introduction

Continuous seizures can be induced in rodents using several methods, including focal or systemic chemoconvulsants or electrical stimulation of particular neural networks. Injurious durations of seizure activity result in neuronal death in vulnerable brain regions, including the hippocampal CA1 and CA3 subfields and hilus; the entorhinal, perirhinal, and piriform cortices; and the amygdala, regardless of the method employed to evoke the seizures (Ben-Ari et al. 1986; Du et al. 1995; Fujikawa 1996; Fujikawa et al. 2000a, b; Henshall et al. 2000; Kondratyev et al. 2001; Motte et al. 1998; Schwob et al. 1980; Sloviter et al. 1996; Sperk et al. 1983). Other neuronal populations, including those located in striatum, in the hippocampal CA2 subfield, and in the hippocampal dentate granule cell layer, are resistant to seizure-evoked injury. It is unclear why particular endangered populations die in the aftermath of injurious seizures, whereas other populations survive. One plausible explanation is that injury-resistant populations are either inherently endowed with or more efficiently engage protective cellular mechanisms.

Although the exact factors mediating the transition from cell endangerment to cell death following seizures are unknown, it is generally accepted that seizure-induced cellular damage significantly contributes to injury (i.e., frank neuronal death). It is well established that the cellular damage caused by continuous seizures increases with seizure duration, and it is generally believed that seizures lasting in excess of 30 min in duration are required to elicit injury (Fujikawa 1996; Henshall et al. 2000; Kondratyev and Gale 2001) (Fig. 16.1a). Such seizures are thus considered to be "injurious." Shorter seizure durations, although likely to evoke some

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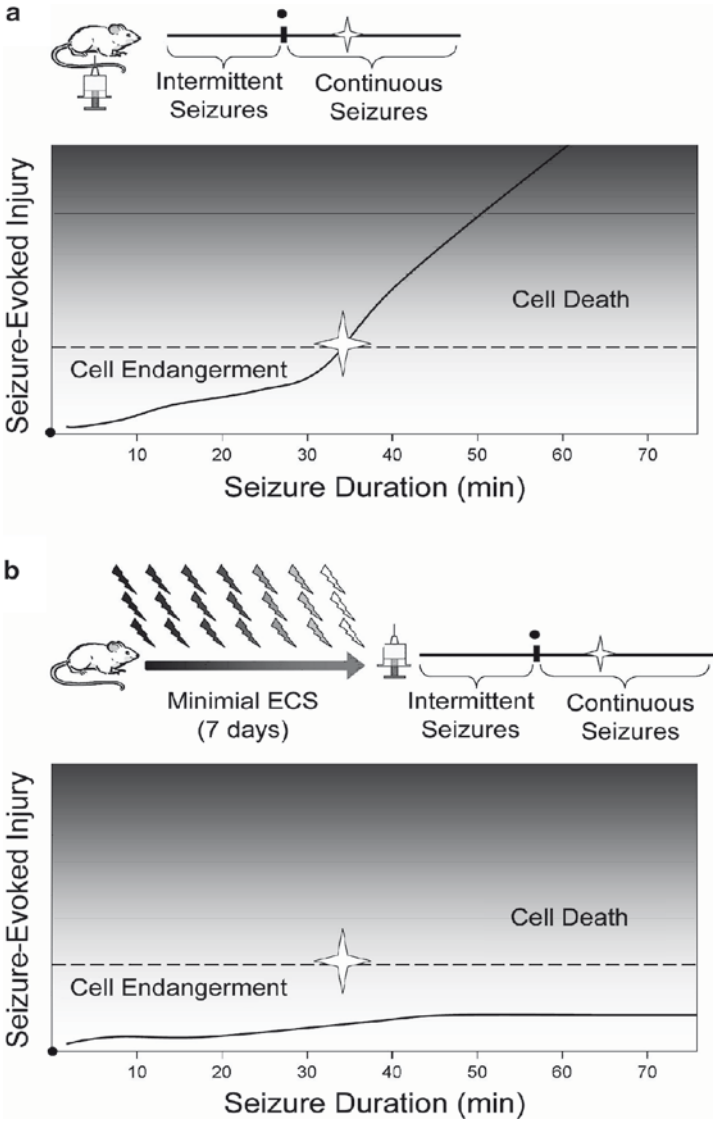

Fig. 16.1 Seizure-induced neuronal injury increases with seizure duration. (**a**) Kainic acid-evoked seizures lasting in excess of 30 min in duration are required to induce significant injury (cell death) in endangered neuronal populations in the brain. (**b**) Preconditioning with noninjurious seizures (e.g., 7 days of ECS) is neuroprotective and prevents the transition from cell endangerment to cell death occurring after injurious seizures. *Black dots* indicate the start of continuous seizure activity after kainic acid injection, and *stars* indicate the point at which the seizure activity becomes injurious (causing cell death in endangered neuronal populations) to the nonprotected brain

degree of cellular damage, are subthreshold for inducing cell death and are thus defined here as being "noninjurious." Given that endangered neurons survive noninjurious seizure durations (i.e., durations not eliciting frank neuronal death), it is plausible that the cellular damage elicited by these seizures does not reach the threshold necessary for triggering cell death and/or compensatory repair processes are both activated in response to and sufficient for survival.

Intriguingly, *preexposure* to noninjurious seizures [e.g., continuous seizures lasting less than 30 min in duration or brief, repeated minimal electroconvulsive seizures (ECS)] prevents the cell death triggered by subsequent injurious seizures (Fig. 16.1b) (Kelly and McIntyre 1994; Kondratyev et al. 2001; Najm et al. 1998; Plamondon et al. 1999; Sasahira et al. 1995; Zhang et al. 2002). This phenomenon may be a form of epileptic "tolerance," akin to the ischemic tolerance facilitated by ischemic preconditioning. Ischemic tolerance has been well documented, whereby a minimal ischemic episode (ischemic preconditioning) protects from a subsequent full insult (Kato et al. 1991). Cross tolerance between both neuroprotective seizure preconditioning and ischemic tolerance, and ischemic preconditioning and epileptic "tolerance" suggests the mechanisms underlying these two types of preconditioning stimuli may be similar (Plamondon et al. 1999). A variety of intracellular mechanisms have been attributed to ischemic tolerance, most falling under the general concept that the preconditioning event causes cellular damage that, in turn, upregulates cellular defense systems (e.g., increased antioxidant levels or cellular repair machinery). Thus, it is plausible that similar *damage*-dependent mechanisms contribute to the tolerance afforded by seizure preconditioning. It is important to note, however, that although seizure preconditioning is, in general, neuroprotective, different models of preconditioning may differ in the mechanisms underlying their neuroprotective effects. As previously mentioned, although 30 min of continuous seizures are noninjurious, they most likely evoke some degree of cellular damage. There is no evidence to suggest, however, that brief, repeated *minimal* ECS results in any cellular damage. Thus, following ECS, there may be *activity*-dependent recruitment of neuroprotective processes that may or may not involve mechanisms distinct from those involved in "tolerance." This distinction between damage-dependent and activity-dependent mechanisms of neuroprotection will be touched upon later in this chapter when we discuss the regulation of DNA repair-related machinery following noninjurious seizure durations versus that occurring following brief, repeated minimal ECS.

In considering the role of cellular damage and compensatory repair in the transition from neuronal endangerment to cell death following seizures, one major question is what types of cellular damage are important to evoking injury? It has been well established that continuous seizures lead to oxidative stress and mitochondrial dysfunction in the brain (Bellissimo et al. 2001; Bruce and Baudry 1995; Chuang et al. 2004; Cock et al. 2002; Dal Pizzol et al. 2007; Patel et al. 2005; Patel 2002). Furthermore, pretreating animals with antioxidants attenuates seizure-induced injury in the brain (Bishnoi et al. 2007; Gupta and Briyal 2006; Kim et al. 2000; MacGregor et al. 1996; Shin et al. 2007), suggesting that oxidative cellular damage may be particularly important in the cellular decision to die in the aftermath of seizures.

Clearly seizures may result in multiple types of oxidative damage, but over the last few decades, increasing attention has been focused on the role of oxidative DNA

damage in neuronal injury. Indeed, oxidative DNA damage is evoked in the brain by seizure activity, as evidenced by increases in 8-hydroxydeoxyguanosine (8-OHdG), (Kajitani et al. 2006; Lan et al. 2000; Liang et al. 2000; Patel and Li 2003; Patel 2002, 2004) and DNA inducible genes such as growth arrest and DNA-damage inducible 45 gene (Gadd45) (Henshall et al. 1999) following seizures in rodents. Given the potential role for DNA damage and repair in neuronal injury (and potentially resilience) following seizures, we will review below the current state of knowledge relevant to DNA damage and repair mechanisms (which has been obtained mostly in nonneural cells) with regard to neural injury and present our own findings relevant to excitotoxic injury in general, and seizure-evoked injury, in particular.

16.2 DNA Damage and Repair in the Brain

To protect genomic integrity, complex cellular response systems have evolved to combat DNA lesions. In proliferating cells, DNA damage is detected by cellular recognition sensors, which in turn, signal downstream to cell-cycle-related proteins, and the cell cycle progression is halted so that the decision can be made to repair the damage or to activate cell-cycle linked cell death processes (Norbury and Zhivotovsky 2004). Repair fidelity and success is important; improper repair can cause replication and mitotic errors, and aberrations in the genetic code can be passed on to daughter cells resulting in transcription errors and, potentially, carcinogenesis. In post-mitotic cells, however, the responses to and consequences of DNA damage are less clear. In neurons, genetic alterations are not passed on to daughter cells, thus minimizing carcinogenic risk, yet the long neuronal life span and heavy requirement for proper gene transcription begets the need for genomic integrity. DNA maintenance may thus be especially critical to neurons following neurological insults or pathologies that result in significant DNA damage.

16.2.1 What Are the Sources of DNA Damage in the Brain?

Nuclear DNA damage arises from a variety of exogenous and endogenous sources, the former including both ionizing and ultraviolet radiation, chemical agents, and heat. The skull and the blood brain barrier are believed to protect the brain from "normal" exposure to exogenous damage sources. Thus, although irradiation for brain tumors and certain medications that target neuronal systems are potential exogenous sources of DNA damage in the central nervous system, DNA damage in the brain is most likely to arise from endogenous sources.

Endogenous DNA damage generally stems from replication stress (semiconservative DNA replication) and normal metabolic processes. Postmitotic cells by definition, however, do not divide, minimizing the contribution of this process to DNA damage in neuronal populations. Thus, oxidative stress, arising from both normal metabolism and

from supraphysiological conditions, is believed to be the key source of endogenous DNA damage in mature neurons. Oxidative stress arises when the production of reactive oxygen species (ROS) surpasses the levels of antioxidant activity. ROS are formed during several cellular processes including during the Krebs cycle (Chinopoulos and Adam-Vizi 2006; Tretter and dam-Vizi 2005), the reduction of oxygen during mitochondrial respiration (oxidative phosphorylation), peroxisomal metabolism, the enzymatic synthesis of nitric oxide, and the metabolism of phagocytic leukocytes (Chinopoulos and Adam-Vizi 2006; Coyle and Puttfarcken 1993).

Under normal physiological conditions, the brain consumes 20% of total body oxygen (Clarke and Sokoloff 1999) and metabolizes high levels of glucose, both processes of which facilitate high respiratory turnover. Furthermore, neurons are almost completely dependent upon oxidative phosphorylation for adenosine triphosphate (ATP) generation, which increases the neuronal consumption of oxygen as neural activity and, in turn, energy demands increase (Coyle and Puttfarcken 1993). The brain also has high levels of iron, which further exacerbates free radical production through mechanisms mentioned above. This suggests that cellular respiration is most likely the greatest source of ROS in the brain under physiological conditions. It also suggests that ROS production in the brain is relatively high as compared with other tissues, especially during periods of intense neuronal activity and/or when energy demands require both the breakdown of glucose and oxygen consumption for ATP generation. Thus, during excessive glutamatergic activity, such as occurs during seizures, increases in metabolic processes may be especially problematic for cellular defense systems (e.g., peroxide detoxification by endogenous antioxidants). Furthermore, during excitatory neurotransmission, calcium and/or zinc entry through permeable glutamate receptors leads to mitochondrial dysfunction and the uncoupling of oxidative phosphorylation, which in turn, further exacerbates ROS production.

In addition to cellular respiration, reactions catalyzed by several enzymes, including xanthine oxidase, cytochrome p450, monoamine oxidase, nitric oxide synthase, cyclooxygenase, and lipoxygenase can all potentially produce ROS (Coyle and Puttfarcken 1993; Phillis et al. 2006). Because peroxides are formed as intermediates of prostaglandin and leukotriene production by cyclooxygenases and lipoxygenases, respectively, increases in the activity of these enzymes, such as occurs during seizures (Bazan et al. 1986; Kawaguchi et al. 2005; Simmet et al. 1987), result in increased ROS production in addition to that produced by cellular respiration.

Finally, radical-mediated lipid peroxidation can also result in the generation of free radicals. Neuronal membranes have high concentrations of polyunsaturated fatty, acids, which are potential substrates for peroxidation by hydroxyl radicals (Coyle and Puttfarcken 1993; Zaleska and Wilson 1989). This creates a type of "ROS begets ROS" cycle following neurological insults that results in lipid peroxidation, such as excessive seizure activity (Bruce and Baudry 1995; Ueda et al. 1997).

The brain is equipped with a diverse endogenous antioxidant system that includes superoxide dismutase and glutathione peroxidase (Dringen and Hirrlinger 2003; Dringen et al. 1998; Vogel et al. 1999; Zoccarato et al. 2004). Although it has been reported that neurons have decreased levels of certain antioxidants as compared with other tissues (Satrustegui and Richter 1984), neurons as well as glia appear to be

efficient at peroxide detoxification and maintaining their oxidative state under normal physiological conditions (Dringen et al. 1998, 2005). The fact that injury induced by neurological insults is alleviated by antioxidant treatments (Gupta et al. 2003; Mishima et al. 2003; Onem et al. 2006; Sumanont et al. 2006; Zhang et al. 2004), however, suggests that neurons may not be entirely efficient in handling pathological increases in oxidative stress levels. Thus, downstream endogenous repair mechanisms may be required to counter oxidative damage in order for endangered cells to survive neurological insults.

16.2.2 Oxidative Stress Yields DNA Damage

Oxidative DNA damage arises when reactive oxygen or nitrogen species (ROS or RNS, respectively) attack DNA at the bases or sugar-phosphates. This results in many forms of DNA damage, including 8-oxoguanine and thymine glycol (Slupphaug et al. 2003). It is more probable that single-strand breaks will occur than double-strand breaks (DSBs) following ROS attack. If located in close proximity, however, multiple single-strand breaks can result in DSBs, the most lethal of all DNA lesions. This can occur either from the induction of two single-strand breaks located across from each other on opposite strands of the DNA or during the repair of clustered single-strand breaks where nicks generated during the repair process can create DNA lesions.

Oxidative damage is not necessarily present in significant amounts in the normal brain (Arnett et al. 2005), presumably because mediators of oxidative stress are balanced by endogenous antioxidant systems. In the rodent, under normal physiological conditions, however, increases in basal levels of oxidative DNA damage have been detected in the brain during aging (Cardozo-Pelaez et al. 1999; Hamilton et al. 2001; Rutten et al. 2003). Furthermore, in the aging human brain, DNA damage has been found in the promoter regions of genes encoding proteins that are downregulated in the aged brain, suggesting that DNA damage accumulates in the brain over time (Lu et al. 2004). Accumulation of DNA damage during the aging process may contribute to age-related neurodegeneration; the extent and consequences, however, of "nonpathological" DNA damage in the brain have yet to be determined.

During pathological states, there is evidence to suggest that increases in oxidative stress and, in turn, DNA damage may significantly impact neuronal fate. Increases in oxidative DNA damage have been detected in several brain-related pathologies and following a variety of neurological insults. For example, DNA damage has been suggested to be elevated in brain-related pathologies characterized by neurodegeneration (Barnham et al. 2004; Simonian and Coyle 1996). Increases in markers of oxidative damage, such as 8-OHdG, have been observed in the brains from patients with Alzheimer's Disease (Gabbita et al. 1998; Lovell et al. 1999; Wang et al. 2006) and Parkinson's Disease (Alam et al. 1997; Hegde et al. 2006). Furthermore, there is evidence to suggest that oxidative DNA damage may contribute to the neurodegeneration occurring in people with amyotrophic lateral sclerosis (Aguirre et al. 2005; Bogdanov et al. 2000; Ferrante et al. 1997; Kisby et al. 1997).

DNA damage is also increased in the brain following experimental models of neurological insults including middle cerebral artery occlusion in rodents (Cui et al. 1999, 2000; Hayashi et al. 1999; Lan et al. 2003; Nagayama et al. 2000a; Zhang et al. 2001), mouse forebrain ischemia and reperfusion (Liu et al. 1996), gerbil global ischemia and reperfusion (An et al. 2002), experimental stroke (Li et al. 2005), traumatic brain injury (Lewen et al. 2001; Mendez et al. 2004), and following hypoxia (Englander et al. 1999; Lee et al. 2002). As previously mentioned, continuous seizures also increase DNA damage in the brain. Thus, diseases and conditions that result in neuronal death have all been linked to elevated levels of DNA damage, suggesting that DNA damage may contribute to neuronal vulnerability.

16.2.3 DNA Repair Counters DNA Damage

Intriguingly, many of the aforementioned neurodegenerative disorders associated with *increased* DNA damage are also associated with *decreased* DNA repair factor expression and/or activity (Davydov et al. 2003; Kisby et al. 1997; Lovell et al. 2000). DNA repair has been reported to decline in the brain during aging (Intano et al. 2003; Krishna et al. 2005; Rao 2007; Ren and de Ortiz 2002; Sharma 2007; Vyjayanti and Rao 2006), and decreases in DNA repair after experimentally-induced injury have been associated with neurodegeneration, implicating deficient DNA repair in cell death processes. Furthermore, the importance of functional DNA-repair pathways in neuronal survival is underscored by the abnormal neuronal development and vulnerability to injury observed in DNA repair-deficient mice (Culmsee et al. 2001; Francisconi et al. 2006; Laposa and Cleaver 2001; Meira et al. 2001; Neema et al. 2005; Vemuri et al. 2001) and humans (Brooks 2002; Rass et al. 2007; Rolig and McKinnon 2000). This suggests that the balance between the induction of DNA damage and compensatory repair processes may influence neuronal survival following neural insults.

Several DNA repair systems have been suggested to affect neuronal function and vulnerability to injury, including base excision, nucleotide excision, and DSB repair pathways. While our major focus is DSB repair, the contribution of other repair pathways to neuronal fate following neurological insults should not be neglected. This is especially true given the fact there is likely a great deal of "cross-talk" and/or redundancy in function among pathways. Thus, a brief discussion of the repair machinery that has been implicated in neuronal vulnerability to injury is warranted.

Base Excision Repair

During base excision repair, DNA glycosylases hydrolyze the *N*-glycosylic bond linking the damaged base to the deoxyribose-phosphate sugar backbone, excising it. This leaves behind an apurinic or apyrimidinic (AP) site. AP endonucleases, such as APE1, then nick the phosphodiester bond usually immediately 5′ to the AP

site, leaving behind a 5′ terminal deoxyribose-phosphate residue. Exonucleases (DNA-deoxyribophosphodiesterases) remove this residue, degrading the DNA ends. Finally, DNA polymerases (pol) fill the nucleotide gap and DNA ligase seals any nicks, completing the repair process. The last step of base excision repair can be broken down into long patch repair, which involves factors also involved in DNA replication such as DNA pol δ/ε/β, proliferating cell nuclear antigen (PCNA), Fen1, DNA ligase I and replication protein A (RPA), (Krokan et al. 2000) and short patch repair, which is mediated by pol β, DNA ligase III, and XRCC1 (Kubota et al. 1996).

Although it has been reported that some base excision repair enzymes (Englander and Ma 2006) and activities (Intano et al. 2003; Rao et al. 2001) decline with aging, the mature brain expresses base excision repair proteins and maintains base excision repair activity (Araneda et al. 2001; Karahalil et al. 2002; Raji et al. 2002). Furthermore, there is evidence to suggest that the activities of this repair pathway are important to neuronal function and viability, especially after injury. Under normal physiological conditions *in vitro*, suppression of uracil-DNA glycosylase using an antisense oligonucleotide directed at the UNG gene increases DNA damage (as measured by COMET), p53, and neuronal apoptosis in hippocampal cultures (Kruman et al. 2004). Additionally, although it was reported that $UNG^{-/-}$ mice have no overt phenotype (Nilsen et al. 2000), cortical neurons from $UNG^{-/-}$ mice are vulnerable to cell death evoked by oxygen–glucose deprivation. Moreover, *in vivo*, infarct size is larger in $UNG^{-/-}$ mice following middle cerebral artery occlusion as compared with the infarct size induced in wild type mice (Endres et al. 2004).

Base excision repair activity is increased in the rodent brain following ischemic preconditioning (Li et al. 2006) and ischemia and reperfusion (Lan et al. 2003; Lin et al. 2000). Furthermore the expression of base excision repair factors is induced in the brain by hypoxia (Lee et al. 2002), hyperoxia (Edwards et al. 1998), middle cerebral artery occlusion (Endres et al. 2004), and kainic acid-evoked seizures (Quach et al. 2005). Intriguingly, following injurious insults, base excision repair factors *decrease* at time points preceding or concurrent with cell death (Fujimura et al. 1999b, c, 2000; Kawase et al. 1999; Lewen et al. 2001; Morita-Fujimura et al. 1999). Whether this decrease in DNA repair factors contributes to the injury or is instead a result of cell death processes is unclear, but it is conceivable that a depletion of energy stores during injury causes repair processes to “shut down,” further exacerbating the cellular damage and injury evoked by the insult.

Nucleotide Excision Repair

Damage not recognized by glycosylases is usually repaired by nucleotide excision repair, which tends to be associated with the repair of “bulky” lesions, chemical bonds between the bases, or damage that distorts the DNA structure. There are two subpathways of nucleotide excision repair: global genome repair and transcription-coupled repair (TCR) (Tijsterman et al. 1999). Overlapping factors mediate both

pathways, including members of the XP complementation group (XPA through G). In nucleotide excision repair, a helicase unwinds the DNA near the damage site and damage-specific endonucleases cut at both sides of the damage, freeing an oligonucleotide fragment. Missing nucleotides are replaced by one or more DNA polymerases, and repair is completed by DNA ligase.

In nondividing cells, it has been suggested that the major nucleotide excision repair pathway is TCR (Nouspikel and Hanawalt 2002). Presumably, stalled RNA polymerases at the site of DNA damage activate repair machinery, resulting in the preferential repair of the transcribed strand in transcriptionally active regions of the genome. TCR is believed to require TFIIH, and, interestingly, some nucleotide excision repair factors are also subunits of the TFIIH complex.

It has been suggested that neurons preferentially repair transcriptionally active regions of the genome, but as opposed to what occurs during TCR, neurons appear to repair the transcribed and nontranscribed strands with equal efficiency. This type of repair has been termed "differentiation-associated repair" (Nouspikel and Hanawalt 2000, 2002). Intriguingly, genes involved in TCR and differentiation associated repair appear to be important to neuronal development and survival in the rodent and human brain. Mutations in XP genes, with the exception of XPC and XPE (which are not thought to be required for TCR) result in a wide range of neurological phenotypes usually characterized by either neuronal loss during development or progressive neurodegeneration in specific neuronal populations (Brooks 2002; Hayashi et al. 2004; Itoh et al. 1999; Kohji et al. 1998; McMurray 2005). Interestingly, astrocytes from $XPA^{-/-}$ mice are seemingly resistant to cell death evoked by chloroacetaldehyde (CAA) whereas cerebellar neurons from $XPA^{-/-}$ mice have increased vulnerability to cell death elicited by CAA (Kisby et al. 2004) and UV radiation (Enokido et al. 1997), as compared with neurons from wild-type animals. This suggests that XPA may specifically influence neuronal survival following DNA damage in mature cells.

Neurons are capable of nucleotide excision repair (Brooks 1998; Nouspikel and Hanawalt 2002); however, the induction of nucleotide excision repair factors following injury in the brain has been relatively unexamined. ERCC6 mRNA expression is induced in both neurons and astrocytes in the rodent brain following ischemia (Sun et al. 2002), suggesting that some members of this pathway may respond to injury.

Mismatch Repair

In mismatch repair, DNA glycosylases that recognize mismatched bases or insertions or deletions introduced by the DNA polymerase excise the damaged region. This is followed by helicase-mediated DNA unwinding, endonucleolytic cleavage of the damage site, exonucleolytic processing of the DNA ends, and repair completion by DNA polymerases and ligases. Human mismatch repair recognition genes include hMSH2, hMSH3 and hMSH6, which are believed to recruit hMLH1 and hPMS2. DNA repair synthesis is then facilitated by PCNA, RPA, and DNA polymerase δ/ε (Karran and Bignami 1999).

Mismatch repair is functional in mature neurons (Brooks et al. 1996; Marietta et al. 1998). The impact of deficiencies in mismatch repair in neuronal populations in the brain are unclear, but humans with inherited mutations in mismatch repair genes appear to be vulnerable to developing certain types of brain tumors (de Vos et al. 2005; Hegde et al. 2005; Menko et al. 2004). Mismatch repair protein induction following injury has not been extensively examined in the brain, but the MSH2 protein has been reported to increase following seizures (Belloni et al. 1999) and mice deficient in mismatch repair show increased vulnerability to seizures and seizure-evoked hippocampal cell loss (Francisconi et al. 2006). This suggests that like base excision and nucleotide excision repair pathways, mismatch repair may be induced following injury in the brain.

Double-Strand Break Repair

As previously mentioned, our major focus is DSB induction and repair, given the lethal nature of these lesions and the need for more investigation in this area with regard to neuronal injury and survival mechanisms. Thus, in the next section, we will cover what is currently known about DSB induction and repair in the brain and discuss some of our most recent findings that may shed some light on how DSBs and their repair may influence neuronal fate following seizures.

Early Events

DSBs are considered to be the most lethal of all DNA lesions, and, thus, DSB repair may be particularly important to neuronal vulnerability following neurological insults. Surprisingly, while being potentially a very important contributor to vulnerability to neuronal injury, our knowledge of DSB formation, detection, and repair comes almost exclusively from nonneural cells. DSBs are repaired by several pathways, including homologous recombination, single-strand annealing, and nonhomologous-end-joining (NHEJ) (Singleton and Jeggo 1999). There is tremendous overlap in the initial detection and signaling events occurring before each of these repair pathways. One of the earliest events to happen after a DSB is the phosphorylation of histone H2A.X (termed γ-H2AX) over megabase domains near the lesion site (Fig. 16.2a). Thousands of H2A.X molecules are phosphorylated near each DSB, occurring within minutes of ionizing radiation. It has been suggested that ATM is primarily responsible for this phosphorylation event, but other phosphatidylinositol 3-kinase (PI_3K) family members, such as ATR and DNA-PK, have all been implicated in γ-H2AX formation following various types of injury (Friesner et al. 2005; Hickson et al. 2004; Park et al. 2003; Stiff et al. 2004). Although several groups have used γ-H2AX as a marker of DSBs in neurons (Kim et al. 2004; Nowak et al. 2006), our laboratory was the first to demonstrate that γ-H2AX nuclear foci are rapidly formed following activation of glutamate receptors, *in vitro*, in primary cortical neuronal cultures (Crowe et al. 2006). In this

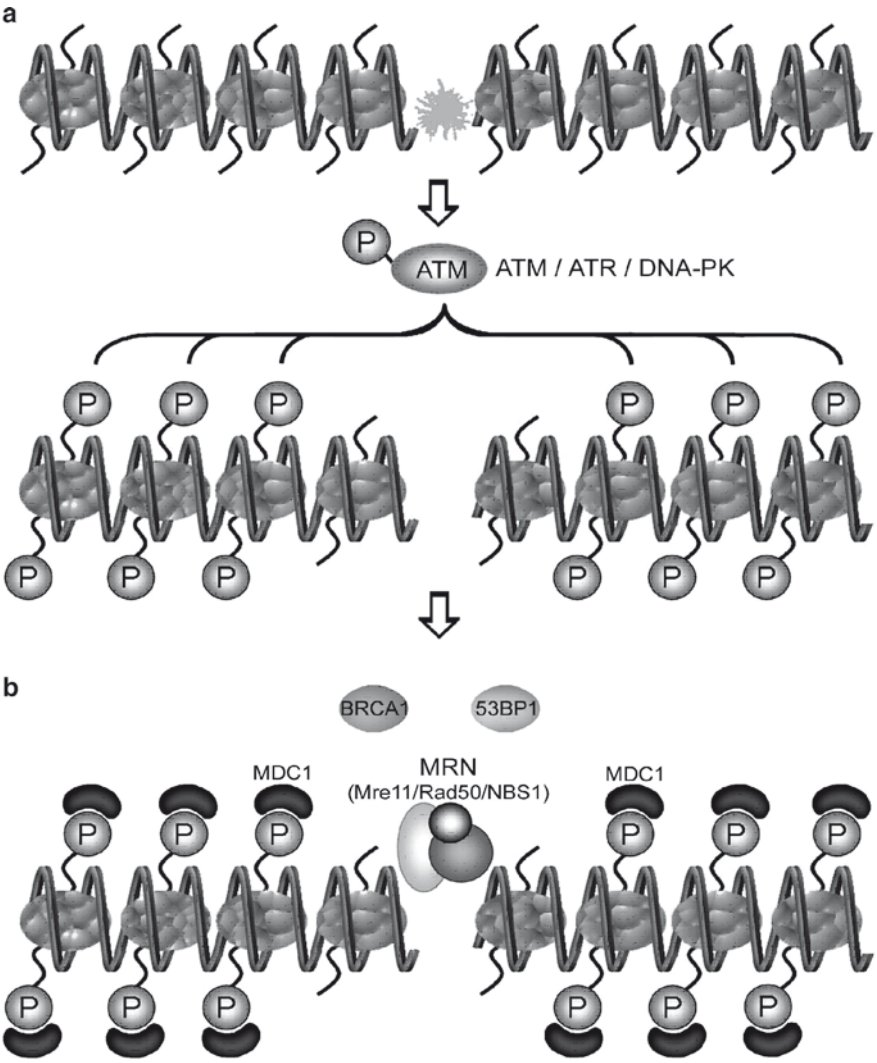

Fig. 16.2 Early events in DSB detection- and repair-related signaling. (**a**) Following a DSB, histone H2A.X is phosphorylated on C-terminal serine residues over megabase domains by ATM and/or other PI_3K family members, such as ATR or DNA-PK, near the lesion site. (**b**) DSB signaling and repair factors are recruited to the lesions site, including the MRN (Mre11/NBS1/Rad50) complex, BRCA1, 53BP1, and MDC1

study, we found that the extent and temporal profile of γ-H2AX formation following glutamate receptor activation corresponded to that observed following low doses of ionizing radiation (IR), a DNA damaging agent that consistently and dose-dependently produces DSBs (IR experiments were performed to create a baseline

for subsequent studies involving glutamate receptor activation). We observed γ-H2AX formation in a majority of neurons following *nonlethal* IR doses or concentrations of glutamate receptor agonists, NMDA or AMPA *resulting in minimal cell death*, suggesting that insults not necessarily resulting in cell death may be sufficient to induce DNA damage and DSB-related repair pathways in neurons. We also characterized several distinct types of γ-H2AX foci observed in neurons and described their quantification methods and exclusion criteria. Gradual disappearance of γ-H2AX foci in surviving neurons suggested ongoing repair, although the significance of disappearing foci is yet unclear (Bouquet et al. 2006; Crowe et al. 2006). This suggestion was further supported by our observation of frequent colocalization of this chromatin modification with the DNA repair factor Mre11 (Crowe et al. 2006) (see below).

As previously discussed, experimental models of continuous seizures, such as those caused by the systemic administration of kainic acid, result in specific patterns of excitotoxic neuronal loss in endangered neuronal populations (particularly in the CA1 and CA3 of the hippocampus; the amygdala; and the entorhinal, perirhinal, and piriform cortices) (Kondratyev and Gale 2001; Kondratyev et al. 2001; Schwob et al. 1980; Sperk et al. 1983) following injurious seizure durations. Furthermore, the degree of this seizure-evoked injury increases with seizure duration (Kondratyev and Gale 2001; Tuunanen et al. 1999). Conversely, relatively short durations of seizure activity, such as continuous seizures lasting less than 30 min in duration and brief, repeated minimal ECS do not trigger neuronal loss in endangered cells. Moreover, pretreatment with noninjurious seizures (either brief, repeated minimal ECS or continuous seizures lasting <30 min in duration) can result in either protection from or tolerance to subsequent seizure-evoked injury (Kondratyev and Gale 2001; Kondratyev et al. 2001; Najm et al. 1998; Plamondon et al. 1999; Zhang et al. 2002). This raises several questions when considering the role of DSBs and their repair in neuronal injury and resilience following seizures. First, are DSBs induced following continuous seizures, and, if so, does this induction occur in all endangered populations or only those that are susceptible to seizure-evoked cell death? Second, are DSB detection and repair machinery recruited following seizures, or does seizure-evoked DNA damage persist over time? Third, do noninjurious seizures evoke any degree of DSBs, and is there a distinction between noninjurious continuous seizures and brief, repeated minimal ECS? Finally, do neuroprotective strategies such as seizure preconditioning attenuate subsequent seizure-evoked DNA damage and/or facilitate repair? Although these questions remain to be answered, there is some indication that DSBs and associated repair processes may influence the transition from cell endangerment to cell death.

Given that DNA DSBs are the most lethal form of DNA lesion, and our *in vitro* results showing rapid γ-H2AX response following glutamate receptor activation, we hypothesized that DSB detection and repair machinery may be a determinant of vulnerability to seizure-induced cell death. *To test this hypothesis, we performed a series of in vivo studies investigating* H2A.X phosphorylation following injurious and noninjurious seizures. We found that following continuous seizures that are either associated with subsequent massive cell death (2 h of kainate-induced status

epilepticus) or not causing significant neurodegeneration (≤30 min of kainate-induced status epilepticus), γ-H2AX increased in neurons in numerous brain areas, including hippocampal CA3 (Fig. 16.3), CA1, dentate gyrus, as well as entorhinal cortex, amygdala, and thalamic nuclei (not shown). These increases were observed immediately following termination of the seizures with diazepam, suggesting that γ-H2AX is an early marker of DNA damage in neurons following seizures. In the majority of brain areas studied, the number of γ-H2AX foci increased as a function of status epilepticus duration (i.e., the longer the duration the greater the number of foci). Moreover, increases in γ-H2AX immunoreactivity were seen in vulnerable brain regions following much shorter durations of seizure activity (i.e., 5 min of status epilepticus or 30 min of intermittent seizure activity preceding the onset of status epilepticus). It is important to note that durations of seizure activity this short are usually not associated with any signs of injury. This further underscores that γ-H2AX foci formation is potentially an especially sensitive marker of seizure-induced neuronal endangerment. Interestingly, pre-exposure to noninjurious ECS did not result in significant γ-H2AX foci formation in any of the brain regions examined (not shown). This supports the notion that *minimal* ECS does not result in neuronal damage or injury. Furthermore, our preliminary data indicate that ECS preconditioning attenuated the γ-H2AX increases caused by subsequent injurious

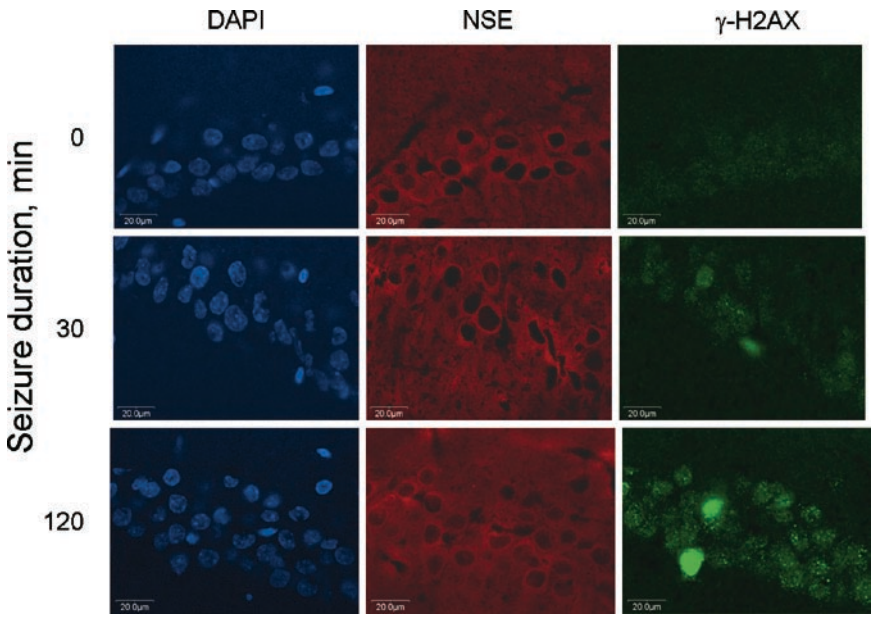

Fig. 16.3 Formation of γ-H2AX foci *in vivo* after kainic acid-induced continuous seizures lasting for 0 (control), 30, or 120 min in the CA3 region of the hippocampus. Seizures were induced as described previously (Kondratyev et al. 2001). DAPI staining reveals nuclear morphology, NSE (Neuron-Specific Enolase) is a neuronal marker, γ-H2AX shows phosphorylated histone H2A.X. Scale bar, 20 μm

seizures (i.e., 2 h of status epilepticus) as compared to the foci formation following 2 h of status epilepticus in nonpreconditioned animals. These data may provide some insight into the mechanism subserving the neuroprotective effects of preexposure to minimal ECS.

Recruitment of DSB detection- and repair-related factors is another early event following DSB induction (Fig. 16.2b). The MRN (Mre11/NBS1/Rad50) complex, BRCA1 and BRCA2, 53BP1 (Anderson et al. 2001), Rad51, and MDC1 (Stewart et al. 2003; Stucki et al. 2005; Stucki and Jackson 2006) all form ionizing radiation-induced foci that colocalize with γ-H2AX. Although there is evidence suggesting that γ-H2AX recruits these factors to the lesion site (Fernandez-Capetillo et al. 2004), the exact temporal relationship of these factors accruing around the DNA site is still an area of intense investigation. It currently appears that although γ-H2AX may not be required for the initial recruitment of DNA repair machinery (Celeste et al. 2003), it is necessary for retaining these factors (Celeste et al. 2003) and possibly for maintaining chromatin structure in an accessible state (Kruhlak et al. 2006). Notably, deficiencies in NBS1 results in neural death during development, and a lack of ATM causes progressive neurodegeneration. This suggests that early events in DSB recognition and signaling may influence neuronal fate following injury, although there are few studies that have examined this issue. Using confocal laser microscopy, we have shown that the majority of γ-H2AX foci optically colocalized with Mre11 following glutamate receptor activation in cortical neurons *in vitro* (Crowe et al. 2006). The patterns of co-localization were similar to those observed after IR, a known source of DSBs, suggesting mobilization of DSB repair pathways following excitotoxic injury. To confirm the true nature of colocalization of γ-H2AX with Mre11, we performed fluorescence resonance energy transfer (FRET) analysis of colocalization following IR or glutamate receptor activation in primary neuronal cultures (unpublished observation, Fig. 16.4). Consistent with our previously published results, IR increased γ-H2AX formation in a dose-dependent manner, and it also increased the amount of γ-H2AX colocalization with Mre11, as demonstrated by FRET analysis (Fig. 16.4, yellow color corresponds to a "true" co-localization, as revealed by FRET technique). Treatment with NMDA (15 μm) for 10 min also resulted in FRET positivity, indicating recruitment of the DNA repair machinery following glutamate receptor activation (Fig. 16.4). As expected, no FRET was detected in control neurons (not shown). Moreover, to the best of our knowledge, this is the first ever demonstration of colocalization of γ-H2AX with Mre11 using FRET in neurons and only the second demonstration of such colocalization using FRET ever documented (Rapp and Greulich 2004).

In summary, these results provide key evidence that DSB DNA damage/repair mechanisms can be implicated in vulnerability and/or resistance to glutamate-mediated toxicity. It remains to be determined whether γ-H2AX colocalizes with DSB repair factors following seizure activity *in vivo*. However, similarities of the γ-H2AX response in neuronal cultures and *in vivo*, following seizures, suggest that DSB repair mechanisms may be important contributors to vulnerability and/or resistance to seizure-evoked injury *in vivo*.

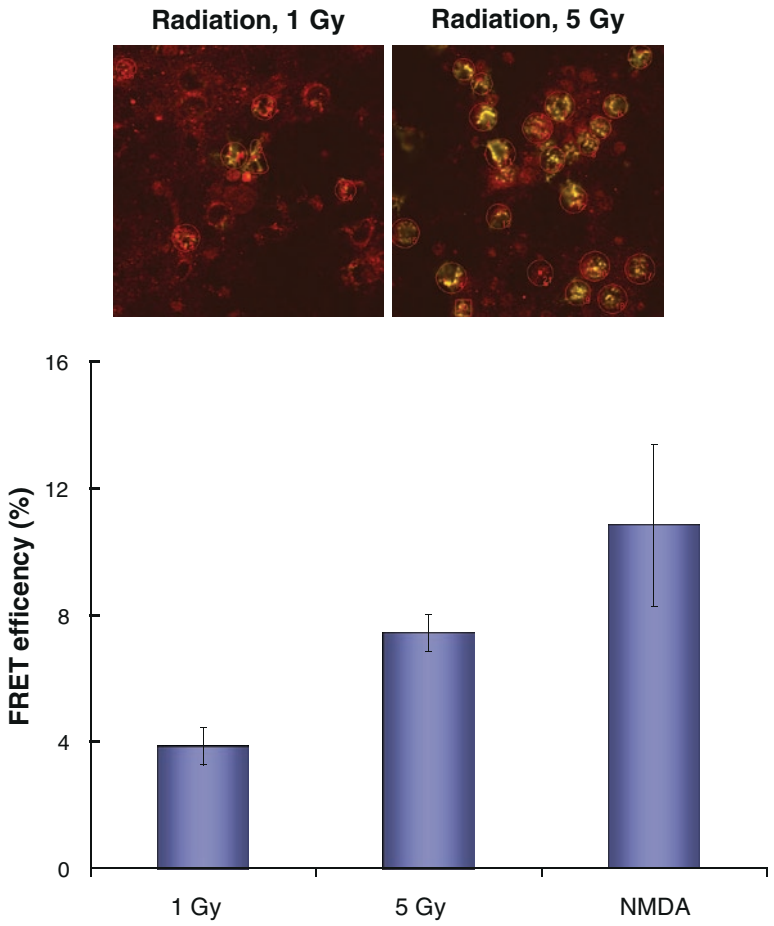

Fig. 16.4 Colocalization of γ-H2AX with Mre-11 as determined by using the FRET technique. Fourteen days-in-vitro primary cortical neurons were treated either with NMDA (15 μM) or ionizing radiation (1 Gy or 5 Gy), as described in (Crowe et al. 2006). Control cultures received either vehicle or sham-irradiation. Collection and analysis of FRET by the sensitized emission method was based on technique described previously (Gordon et al. 1998; Xia and Liu 2001). Photographs are representative images of co-localization following ionizing radiation (the extent of co-localization as visualized by FRET technique is seen in *yellow*). Data represent means ± SD

Delayed Events

Following the early events after DSB induction, DSB repair proceeds mainly through NHEJ in mammalian cells (Friedberg et al. 1995; Jackson 2002). Although the precise components of NHEJ are unknown, six major factors of the NHEJ repair pathway have been identified to date: DNA-PK [composed of the autoantigens Ku70 and Ku 80 and the DNA-dependent protein kinase catalytic subunit (DNA-

PKcs)], Artemis, XRCC4, ligase IV, and the recently identified Cernunnos–XLF protein (Sekiguchi and Ferguson 2006). It is unclear how many other factors are required for successful DSB repair, but additional factors, such as 53BP1 (Nakamura et al. 2006), WRN helicase (Cheng et al. 2004; Li and Comai 2000), the Mre11/Rad50/NBS-1 (MRN) complex (Huang and Dynan 2002; Trujillo et al. 1998), and the polypeptide complex composed of polypyrimidine tract-binding protein-associated splicing factor (PSF) and a 54-kDa nuclear RNA-binding protein (p54(nrb)) (Bladen et al. 2005) have all been suggested to participate in this repair pathway.

After cellular detection of a DNA strand break, Ku70 and Ku80 form a heterodimer and bind to the free DNA ends. Following DNA binding, the Ku70/Ku80 complex is believed to then recruit DNA-PKcs. Although DNA-PKcs and the Ku heterodimer can bind to DNA independently of each other (Hammarsten and Chu 1998; Yaneva et al. 1997), Ku increases the affinity of DNA-PKcs for DNA ends (West et al. 1998) and is thought to facilitate efficient DSB repair (Dynan and Yoo 2002). Moreover, recent data suggest Ku may also act to recruit the ligase complex (Chen et al. 2000; Nick McElhinny et al. 2000) and pol mu and pol lambda (Ma et al. 2004), underscoring its important role in this repair process.

DNA-PK is activated upon the binding and phosphorylation of DNA-PKcs. The DNA-PK complex is then joined by Artemis, which in turn is phosphorylated by DNA-PK and possibly other PI_3 kinases such as ATM and ATR. Following its activation, Artemis is thought to be responsible for the nucleolytic processing of the DNA ends prior to joining (Jeggo and O'Neill 2002; Ma et al. 2005; Sekiguchi and Ferguson 2006). Once the DNA ends are ready for ligation, the XRCC4/ligase multimer together with Cernunnos–XLF mediates DNA end-joining (Ahnesorg et al. 2006; Buck et al. 2006; Sekiguchi and Ferguson 2006).

Mature neurons are capable of repairing DNA DSBs *in vitro* (Gobbel et al. 1998; Merlo et al. 2005; Vyjayanti and Rao 2006). Furthermore, cellular extracts from the rodent brain possess DNA end-joining activity (Ren and de Ortiz 2002). This suggests that mature neurons have the ability to repair DSBs.

NHEJ factors have been implicated in neuronal vulnerability to injury (Chechlacz et al. 2001; Culmsee et al. 2001; Neema et al. 2005; Shackelford et al. 1999; Vemuri et al. 2001) and in neurodegenerative pathologies (Davydov et al. 2003; Shackelford 2006; Vyjayanti and Rao 2006). Furthermore, this pathway may be induced by both injury and by neuroprotective preconditioning paradigms (i.e., ischemic preconditioning). Both Ku 80 DNA binding activity and protein expression were increased following ischemic preconditioning and/or brief ischemic events (Shackelford et al. 1999; Sugawara et al. 2001). Although these data suggest that DSB repair influences neuronal viability following injury, the regulation of DSB repair machinery following neuronal insults in general and in particular, after seizures remains relatively unexamined. Our own studies described below have demonstrated that seizures result in similar upregulation of Ku70 protein. We hypothesized that continuous seizures will result in the upregulation of DNA repair machinery (i.e., Ku70) in vulnerable brain regions. Furthermore, we predicted that the regulation of DNA repair machinery will depend on seizure

duration, and thus, the degree of seizure-evoked injury. Moreover, we predicted that early induction of DSB repair mechanisms may be a compensatory process following noninjurious seizures, and this harnessing of cellular self-defense systems may underlie the neuroprotection afforded by preconditioning the brain with noninjurious seizures.

Ku70 Protein Levels Increase Following Continuous Seizures

A rapid and transient increase in the expression of nuclear Ku70 protein could be detected in the rhinal cortex (defined in the legend to Fig. 16.5) following 30 min of continuous seizures [Fig. 16.5a (top) and b (light bars)]. This increase could be detected as early as 8.5 h after the start of continuous seizures. By 20 h after the onset of continuous seizures, Ku70 protein expression returned to that observed in vehicle (saline)-injected control animals. There was a trend towards a downregulation in Ku70 protein 20 h after the start of continuous seizures; this decrease, however, did not reach statistical significance.

A similar pattern of seizure-evoked Ku70 protein expression was observed in the rhinal cortex following 120 min of continuous seizures [Fig. 16.5a (bottom) and b (dark bars)]. Following 120 min of continuous seizures, a significant increase in Ku70 protein expression could be detected 6 h following the start of continuous seizures. This increase in Ku70 protein expression occurred slightly earlier than the increase induced by continuous seizures lasting 30 min in duration. As observed following 30 min of seizures, Ku70 protein levels returned to those of vehicle-injected control animals by 10 h after the start of continuous seizures, and there was a trend towards a downregulation by 20 h after seizure onset that did not reach significance. We did not detect a change in Ku70 protein levels following either seizure duration in the hippocampus or in the olfactory bulb (not shown).

Intriguingly, we also observed an increase in Ku70 protein following both seizure durations in the cytosolic fraction of the rhinal cortex (Fig. 16.6a, b) but not in the hippocampus or the olfactory bulb (not shown).

Ku70 Protein Levels Are Not Affected by ECS Treatment

Chronic ECS treatment (7 days) (Fig. 16.7a) or acute treatments (1 or 3 days, not shown) did not appear to affect nuclear or cytosolic Ku70 protein levels in the rhinal cortex at either 8 or 24 h after the last ECS session. Similarly, neither chronic nor acute ECS altered Ku70 levels in the nucleus or cytosol in the hippocampus.

Preexposure to ECS Prevents Seizure-Induced Upregulation of Ku70 Protein

Pretreatment with chronic ECS (7 days), prevented the nuclear Ku70 protein increases observed 6 h after the start of 120 min of continuous seizures (Fig. 16.7b). It does not appear that ECS delayed the induction of Ku70 protein expression in response to continuous seizures as Ku70 protein expression levels were unaltered

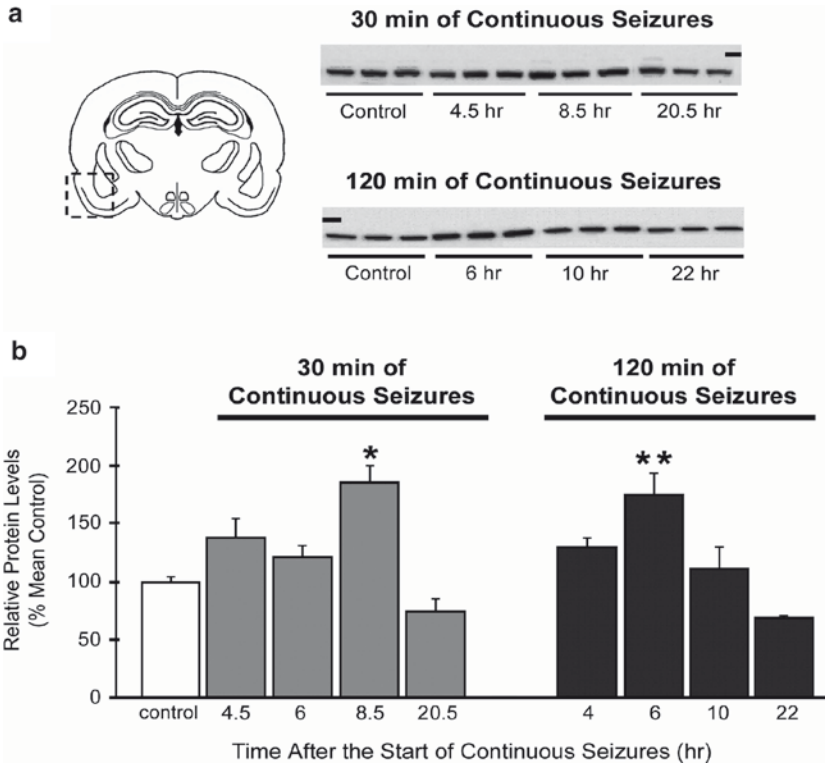

Fig. 16.5 Nuclear Ku70 protein levels increase in the rhinal cortex following 30 or 120 min of continuous seizures. Seizures were induced by systemic administration of kainic acid, as described previously (Kondratyev and Gale 2001; Kondratyev et al. 2001). (**a**) and (**b**), Ku70 protein expression detected by Western blotting. (**a**) Representative Western blots of Ku70 protein expression as a function of time after the start of 30 min (*top*) or 120 min (*bottom*) of continuous seizures. Protein from the nuclear extracts of rhinal cortices (see box drawn on schematic of coronal rat brain section; Bregma-3.6. The sample referred to here as "rhinal cortex" corresponded to a single piece of tissue containing the entorhinal and perirhinal cortex as well as the intervening lateral periamygdaloid cortex and the posterior piriform cortex) from kainic acid treated rats or from saline-injected controls were separated on 12.5% SDS–PAGE gels, transferred onto PVDF membranes, and probed with rabbit antihuman Ku70 antibody (Chemicon; 1:8,000). Chemiluminescent signal was detected by exposure to Kodak Biomax film. *Black bar* indicates the location of bovine serum albumin protein (82.6 kDa) present in the prestained standard. (**b**) Quantification of nuclear Ku70 protein amounts following 30 min (*light bars*) or 120 min (*dark bars*) of continuous seizures. The optical densities of the bands were measured and protein levels were calculated relative to the standard. Data are expressed as the percent of the mean value for vehicle-treated control animals (% mean control ± SEM) ($N=4$ for all groups, $^{*}p<0.05$ and $^{**}p<0.01$ as compared to vehicle-injected controls; ANOVA followed by post hoc Tukey)

as compared with those in rhinal cortices from saline-injected rats (controls) at 10.5 h following the start of continuous seizures.

These results provide evidence that Ku70 protein expression increases in the rhinal cortex in response to injurious and noninjurious kainic acid-induced seizures.

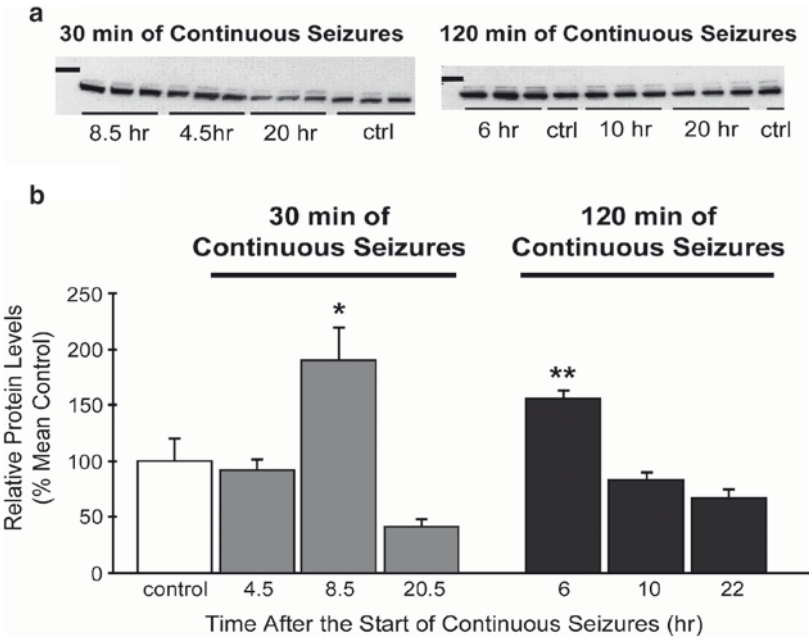

Fig. 16.6 Cytosolic Ku70 protein levels increase in the rhinal cortex following 30 or 120 min of kainic acid-evoked continuous seizures. (**a**) and (**b**), Ku70 protein expression detected by Western blotting. (**a**) Representative Western blots of cytosolic Ku70 protein expression as a function of time after the start of 30 min (*left*) or 120 min (*right*) of continuous seizures. Western blots of Ku70 from cytosolic protein extracts were obtained as described in the legend for Fig. 5. *Black bar* indicates the location of bovine serum albumin protein (82.6 kDa) present in the prestained standard. (**b**) Quantification of cytosolic Ku70 protein amounts following 30 min (*light bars*) or 120 min (*dark bars*) of continuous seizures. The optical densities of the bands were measured and protein levels were calculated relative to the standard. Data are expressed as the percent of the mean value for vehicle-treated control animals (% mean control ± SEM) ($N=4$ for all groups, $^{*}p<0.05$ and $^{**}p<0.01$ as compared to vehicle-injected controls; ANOVA followed by post hoc Tukey)

Furthermore, we show that pretreatment with brief, repeated minimal ECS prevents the Ku70 protein induction observed following 120 min of continuous seizures.

Most studies in the brain investigating injury-evoked changes in DNA repair factors have focused on the effects of injurious insults (i.e., ischemia), which result in cell death (Chen et al. 2003; Fujimura et al. 1999a, c, 2000; Hermann et al. 2001; Kim et al. 2001; Lewen et al. 2001; Morita-Fujimura et al. 1999). In these studies, DNA repair protein or gene expression was evaluated at time points immediately preceding and/or occurring concomitantly with the appearance of cell death markers. Thus, the injury-evoked changes in DNA repair machinery observed in these studies are likely related to cell death processes as opposed to an initial response to injury-evoked DNA damage. Notably, *early* after injury, prior to the initiation of cell death processes, increases in DNA repair factors have been observed following

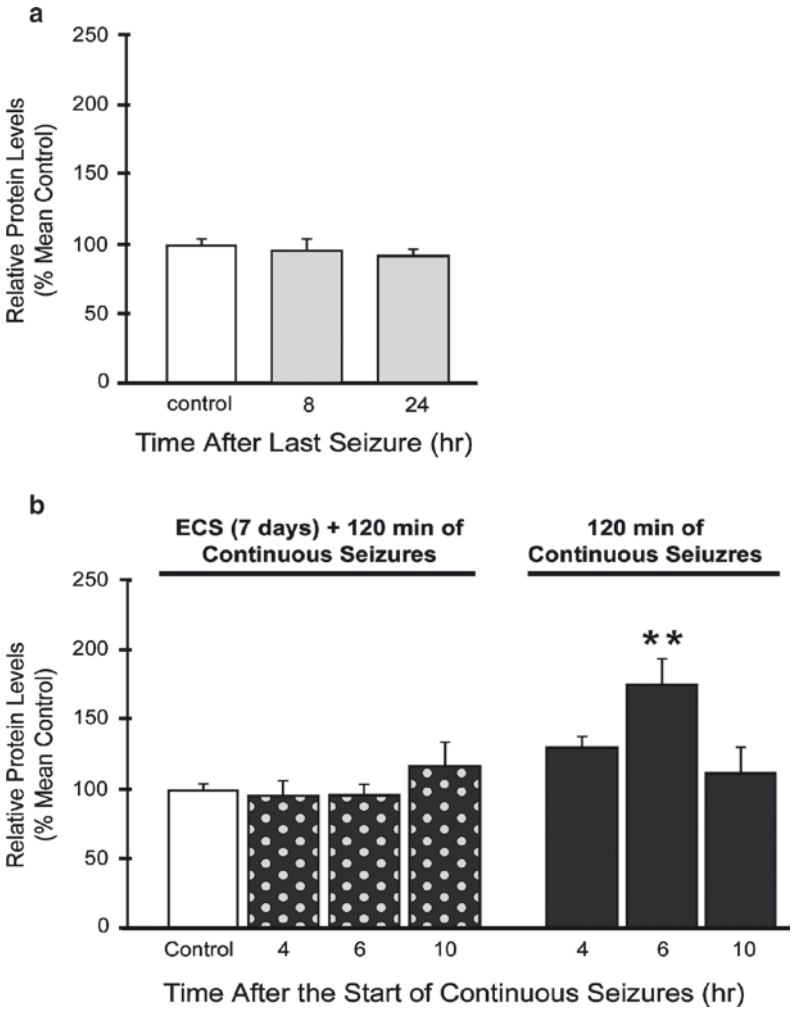

Fig. 16.7 Pretreatment with 7 days of minimal electroconvulsive seizures (ECS) prevents the increase in nuclear Ku70 protein levels evoked by 120 min of kainic acid-induced continuous seizures. Status epilepticus and ECS were induced as described previously (Kondratyev et al. 2001). (**a**) and (**b**), Quantification of relative nuclear Ku70 protein levels detected by Western blotting. The optical densities of the bands were measured and protein levels were calculated relative to the standard. Data are expressed as the percent of the mean value for vehicle-treated control animals (% mean control ± SEM). (**a**) ECS alone (7 days) did not alter nuclear Ku70 protein levels in the rhinal cortex ($N = 5$ for all groups). (**b**) Pretreatment with ECS (*spotted bars*) prevented the increase in Ku70 protein levels observed following 120 min of continuous seizures (*black bars*; reproduced from Fig. 6b for reference comparison) ($N = 5$ for ECS + 120 min of seizures, $N = 4$ for 120 min of seizures, $^{**}p < 0.01$ as compared to vehicle-injected controls; ANOVA followed by post hoc Tukey)

ischemic events (Chen et al. 2003; Hermann et al. 2001; Lin et al. 2000; Ling et al. 1999) and kainic acid-induced seizures (Belloni et al. 1999; Quach et al. 2005). This pattern of early DNA repair factor induction and subsequent decrease occurring during the cell death phase after the injury is similar to the induction of Ku70 we observed between 6 and 8 h following the initiation of continuous seizures and the trend towards a downregulation in Ku70 expression occurring 20 h later. Although we did not find a significant decrease in Ku70 protein expression 20 h after the start of continuous seizures, prior studies in our lab did reveal a significant downregulation of Ku70 protein at 48 and 72 h following 120 min of continuous seizures (unpublished data).

The overall modest increase in Ku70 protein levels observed following kainic acid-induced seizures is similar to that observed by others following brief ischemia and may be explained by the high levels of constitutive Ku70 protein expression in the normal brain, especially in glia (Sugawara et al. 2001). In our hands, preliminary immunohistochemical studies of Ku70 protein expression in the rhinal cortex and hippocampus in untreated-control animals have revealed high Ku70 protein expression in the nuclei of small-bodied cells throughout the brain and in the cytoplasm of larger cells located in the pyramidal cell layers of the hippocampal CA subfields and in the entorhinal cortex that are presumably neurons given their location and morphological features (data not shown). Thus, a high baseline level of Ku70 protein in particular cellular compartments may make injury-induced changes difficult to detect using gross measures such as Western blotting. Notably, although particular hippocampal subdomains are highly vulnerable to seizure-induced injury (i.e., neurons in the CA1 and CA3 subfields and the dentate hilar region), we did not observe an increase in Ku70 protein in this brain region after even injurious seizures. The cellular heterogeneity (and injury resistant CA2 subfield and dentate granule cells) may make it difficult to detect Ku70 protein induction in specific hippocampal subpopulations when measuring overall protein changes using immunoblotting. Unfortunately, however, at the time of these studies, the available Ku70 antibodies did not yield clear, consistent staining in fixed brain tissue. A detailed examination of seizure-evoked Ku70 protein expression at the subcellular level, thus, could not be performed, and it remains to be determined whether the increases in Ku70 protein expression following seizures occurred in neurons, glia, or both cell types.

As mentioned earlier, pretreatment with particular seizure paradigms including kindling (Kelly and McIntyre 1994) and short durations of kainic acid-induced seizures (Najm et al. 1998; Plamondon et al. 1999; Zhang et al. 2002) result in a "tolerance" to subsequent seizure episodes. This is similar to ischemic tolerance, where exposure to a brief, ischemic event protects the brain from subsequent ischemia-induced injury. These "tolerance" paradigms are suggestive of inducible mechanisms influencing vulnerability to excitotoxicity (a form of cellular self-defense). The induction of Ku70 protein we observed following 30 min of KA-evoked seizures may thus be similar to the induction of Ku70 protein (Shackelford et al. 1999; Sugawara et al. 2001) and DNA binding activity (Shackelford et al. 1999) observed after ischemic preconditioning, reflecting a compensatory mechanism that contributes

to the "tolerance" phenomenon. Multiple DNA repair factors have been reported to increase after ischemic preconditioning (Chen et al. 1998, 2003; Nagayama et al. 2000b), raising the question as to whether or not inducible DNA repair contributes to the neuroprotection afforded by preconditioning. This is possible given that we observed γ-H2AX foci formation after very short durations of continuous seizures (≤30 min), which would suggest the induction of some degree of DNA damage and recruitment of repair-related machinery.

Repeated, minimal ECS is also neuroprotective, and, thus, is another paradigm of seizure "preconditioning" (Kondratyev et al. 2001; Masco et al. 1995). It has been suggested, however, that ECS is not a form of "tolerance" as is ischemic preconditioning or preexposure to KA-evoked seizures (Kondratyev et al. 2001), and that minimal ECS affords neuroprotection via different mechanisms than those responsible for tolerance phenomena. Intriguingly, following ECS there is an *increase* in antioxidant activity and decrease in oxidative damage in the hippocampus, an area susceptible to seizure-evoked injury (Barichello et al. 2004a, b). Furthermore, there is no evidence for the induction of DNA single-strand breaks, presumably a precursor for the induction of DSBs following oxidative damage, after repeated ECS (Khan et al. 1995), and similarly, in our previously-mentioned studies, we did not observe detectable induction of γ-H2AX following brief, repeated minimal ECS. Taken together, these data suggest that repeated, minimal ECS are not only noninjurious in terms of frank cell death but may be noninjurious at the subcellular level. This may explain why we did not observe a change in Ku70 protein levels following ECS alone. Although the data presented in this chapter suggest that ECS may decrease the extent of seizure-evoked injury upstream of DNA damage, further studies investigating the effects of ECS on DNA damage and repair following subsequent seizures are necessary to draw any definitive conclusions.

Interestingly, we also observed an increase in the rhinal cortex in cytosolic Ku70 protein levels following non-injurious (30 min) and injurious (120) durations of continuous seizures. It is unknown why Ku70 would increase in the cytoplasm following insults, but it is likely that this protein has a function apart from DNA repair. It has been suggested that Ku70 acts to prevent apoptosis by binding to the cell-death promoting protein, Bax, and preventing its translocation to the mitochondria (Sawada et al. 2003). Although the precise role of Bax in seizure-induced neuronal death is unclear, it is attractive to speculate that the cytosolic increases in Ku70 protein expression reflect a second, DNA repair-*independent* role for this protein following seizures.

16.3 Concluding Remarks

Taken together with data previously published by others, our data suggest that DSB detection and repair machinery are induced in response to seizure-induced injury. Furthermore, both early (phosphorylation of H2A.X) and late (regulation of Ku70 protein) processes in DSB detection and repair appear to occur in neurons following

continuous seizures. Moreover, rapid formation of nuclear foci associated with DNA DSBs in neuronal populations vulnerable to seizure-induced damage following very brief durations of seizure activity suggest that elements of DSB detection pathway (i.e., γ-H2AX formation and its co-localization with the repair factor, Mre11) may serve as especially sensitive markers of seizure-evoked damage as well as of efficacy of neuroprotective intervention. It remains to be elucidated whether or not early detection and recruitment of DSB repair machinery contributes to neuroprotection evoked by seizure preconditioning. An especially promising avenue for future research is to examine whether or not molecular manipulations to enhance DSB repair in neurons will be effective in preventing seizure-induced cell death.

The role of DNA damage and repair in mediating the transition from cell endangerment to cell death in the aftermath of seizures is an important factor to consider, especially when trying to determine why some endangered neuronal populations are "resilient" following injurious seizures. Although these cells do not die, the consequence of seizure-induced DNA damage is unknown. The extent to which this damage is repaired (and the fidelity of such repair) has yet to be determined, and it is possible the resilient cells may contribute to the pathological changes occurring after injurious seizures. Furthermore, although not discussed in this chapter, seizure-evoked changes in histone proteins, such as those we observed for histone H2A.X, may influence neuronal fate and function following neural insults. The contribution of such posttranslational histone modifications to neuronal injury and to neuroprotection remains a compelling avenue for future studies.

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References

Aguirre N, Beal MF, Matson WR, Bogdanov MB (2005) Increased oxidative damage to DNA in an animal model of amyotrophic lateral sclerosis. Free Radic Res 39:383–388

Ahnesorg P, Smith P, Jackson SP (2006) XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. Cell 124:301–313

Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD, Jenner P, Halliwell B (1997) Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. J Neurochem 69:1196–1203

An SJ, Kang TC, Park SK, Hwang IK, Cho SS, Chung MH, Won MH (2002) Oxidative DNA damage and alteration of glutamate transporter expressions in the hippocampal Ca1 area immediately after ischemic insult. Mol Cells 13:476–480

Anderson L, Henderson C, Adachi Y (2001) Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. Mol Cell Biol 21:1719–1729

Araneda S, Mermet N, Verjat T, Angulo JF, Radicella JP (2001) Expression of Kin17 and 8-OxoG DNA glycosylase in cells of rodent and quail central nervous system. Brain Res Bull 56:139–146

Arnett SD, Osbourn DM, Moore KD, Vandaveer SS, Lunte CE (2005) Determination of 8-oxoguanine and 8-hydroxy-2′-deoxyguanosine in the rat cerebral cortex using microdialysis sampling and capillary electrophoresis with electrochemical detection. J Chromatogr B Analyt Technol Biomed Life Sci 827:16–25

Barichello T, Bonatto F, Agostinho FR, Reinke A, Moreira JC, Dal-Pizzol F, Izquierdo I, Quevedo J (2004a) Structure-related oxidative damage in rat brain after acute and chronic electroshock. Neurochem Res 29:1749–1753

Barichello T, Bonatto F, Feier G, Martins MR, Moreira JC, Dal-Pizzol F, Izquierdo I, Quevedo J (2004b) No evidence for oxidative damage in the hippocampus after acute and chronic electroshock in rats. Brain Res 1014:177–183

Barnham KJ, Masters CL, Bush AI (2004) Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov 3:205–214

Bazan NG, Birkle DL, Tang W, Reddy TS (1986) The accumulation of free arachidonic acid, diacylglycerols, prostaglandins, and lipoxygenase reaction products in the brain during experimental epilepsy. Adv Neurol 44:879–902

Bellissimo MI, Amado D, Abdalla DS, Ferreira EC, Cavalheiro EA, Naffah-Mazzacoratti MG (2001) Superoxide dismutase, glutathione peroxidase activities and the hydroperoxide concentration are modified in the hippocampus of epileptic rats. Epilepsy Res 46:121–128

Belloni M, Uberti D, Rizzini C, Ferrari-Toninelli G, Rizzonelli P, Jiricny J, Spano P, Memo M (1999) Distribution and kainate-mediated induction of the DNA mismatch repair protein MSH2 in rat brain. Neuroscience 94:1323–1331

Ben-Ari Y, Repressa A, Tremblay E, Nitecka L (1986) Selective and non-selective seizure related brain damage produced by kainic acid. Adv Exp Med Biol 203:647–657

Bishnoi M, Patil CS, Kumar A, Kulkarni SK (2007) Co-administration of acetyl-11-keto-beta-boswellic acid, a specific 5-lipoxygenase inhibitor, potentiates the protective effect of COX-2 inhibitors in kainic acid-induced neurotoxicity in mice. Pharmacology 79(1):34–41

Bladen CL, Udayakumar D, Takeda Y, Dynan WS (2005) Identification of the polypyrimidine tract binding protein-associated splicing factor.p54(nrb) complex as a candidate DNA double-strand break rejoining factor. J Biol Chem 280:5205–5210

Bogdanov M, Brown RH, Matson W, Smart R, Hayden D, O'Donnell H, Flint BM, Cudkowicz M (2000) Increased oxidative damage to DNA in ALS patients. Free Radic Biol Med 29:652–658

Bouquet F, Muller C, Salles B (2006) The loss of gammaH2AX signal is a marker of DNA double strand breaks repair only at low levels of DNA damage. Cell Cycle 5:1116–1122

Brooks PJ (1998) Detection of excision nuclease in cell-free extracts from the adult mammalian brain. Mutat Res 408:37–46

Brooks PJ (2002) DNA repair in neural cells: basic science and clinical implications. Mutat Res 509:93–108

Brooks PJ, Marietta C, Goldman D (1996) DNA mismatch repair and DNA methylation in adult brain neurons. J Neurosci 16:939–945

Bruce AJ, Baudry M (1995) Oxygen free radicals in rat limbic structures after kainate-induced seizures. Free Radic Biol Med 18:993–1002

Buck D, Malivert L, de Chasseval R, Barraud A, Fondaneche MC, Sanal O, Plebani A, Stephan JL, Hufnagel M, le Deist F, Fischer A, Durandy A, de Villartay JP, Revy P (2006) Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. Cell 124:287–299

Cardozo-Pelaez F, Song S, Parthasarathy A, Hazzi C, Naidu K, Sanchez-Ramos J (1999) Oxidative DNA damage in the aging mouse brain. Mov Disord 14:972–980

Celeste A, Fernandez-Capetillo O, Kruhlak MJ, Pilch DR, Staudt DW, Lee A, Bonner RF, Bonner WM, Nussenzweig A (2003) Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol 5:675–679

Chechlacz M, Vemuri MC, Naegele JR (2001) Role of DNA-dependent protein kinase in neuronal survival. J Neurochem 78:141–154

Chen J, Uchimura K, Stetler RA, Zhu RL, Nakayama M, Jin K, Graham SH, Simon RP (1998) Transient global ischemia triggers expression of the DNA damage-inducible gene GADD45 in the rat brain. J Cereb Blood Flow Metab 18:646–657

Chen L, Trujillo K, Sung P, Tomkinson AE (2000) Interactions of the DNA ligase IV-XRCC4 complex with DNA ends and the DNA-dependent protein kinase. J Biol Chem 275:26196–26205

Chen D, Minami M, Henshall DC, Meller R, Kisby G, Simon RP (2003) Upregulation of mitochondrial base-excision repair capability within rat brain after brief ischemia. J Cereb Blood Flow Metab 23:88–98

Cheng WH, von Kobbe C, Opresko PL, Arthur LM, Komatsu K, Seidman MM, Carney JP, Bohr VA (2004) Linkage between Werner syndrome protein and the Mre11 complex via Nbs1. J Biol Chem 279:21169–21176

Chinopoulos C, Adam-Vizi V (2006) Calcium, mitochondria and oxidative stress in neuronal pathology. Novel aspects of an enduring theme. FEBS J 273:433–450

Chuang YC, Chang AY, Lin JW, Hsu SP, Chan SH (2004) Mitochondrial dysfunction and ultrastructural damage in the hippocampus during kainic acid-induced status epilepticus in the rat. Epilepsia 45:1202–1209

Clarke DD, Sokoloff L (1999) Circulation and energy metabolism of the brain. In: Sigel GJ, Agranoff BW, Albers RW, Fisher SK, Uhler MD (eds) Basic neurochemistry: molecular, cellular, and medical aspects. Lippincott-Raven, Philadelphia, pp 637–669

Cock HR, Tong X, Hargreaves IP, Heales SJ, Clark JB, Patsalos PN, Thom M, Groves M, Schapira AH, Shorvon SD, Walker MC (2002) Mitochondrial dysfunction associated with neuronal death following status epilepticus in rat. Epilepsy Res 48:157–168

Coyle JT, Puttfarcken P (1993) Oxidative stress, glutamate, and neurodegenerative disorders. Science 262:689–695

Crowe SL, Movsesyan VA, Jorgensen TJ, Kondratyev A (2006) Rapid phosphorylation of histone H2A.X following ionotropic glutamate receptor activation. Eur J Neurosci 23:2351–2361

Cui J, Holmes EH, Liu PK (1999) Oxidative damage to the c-fos gene and reduction of its transcription after focal cerebral ischemia. J Neurochem 73:1164–1174

Cui J, Holmes EH, Greene TG, Liu PK (2000) Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain. FASEB J 14:955–967

Culmsee C, Bondada S, Mattson MP (2001) Hippocampal neurons of mice deficient in DNA-dependent protein kinase exhibit increased vulnerability to DNA damage, oxidative stress and excitotoxicity. Brain Res Mol Brain Res 87:257–262

Dal Pizzol F, Quevedo J, Streck E, Walz R, Moreira JC (2007) Changes in lipid composition in hippocampus early and late after status epilepticus induced by kainic acid in wistar rats. Metab Brain Dis 22:25–29

Davydov V, Hansen LA, Shackelford DA (2003) Is DNA repair compromised in Alzheimer's disease? Neurobiol Aging 24:953–968

de Vos M, Hayward B, Bonthron DT, Sheridan E (2005) Phenotype associated with recessively inherited mutations in DNA mismatch repair (MMR) genes. Biochem Soc Trans 33: 718–720

Dringen R, Hirrlinger J (2003) Glutathione pathways in the brain. Biol Chem 384:505–516

Dringen R, Kussmaul L, Hamprecht B (1998) Detoxification of exogenous hydrogen peroxide and organic hydroperoxides by cultured astroglial cells assessed by microtiter plate assay. Brain Res Brain Res Protoc 2:223–228

Dringen R, Pawlowski PG, Hirrlinger J (2005) Peroxide detoxification by brain cells. J Neurosci Res 79:157–165

Du F, Eid T, Lothman EW, Kohler C, Schwarcz R (1995) Preferential neuronal loss in layer III of the medial entorhinal cortex in rat models of temporal lobe epilepsy. J Neurosci 15:6301–6313

Dynan WS, Yoo S (2002) Interaction of Ku protein and DNA-dependent protein kinase catalytic subunit with nucleic acids. Nucleic Acids Res 26(7):1551–1559

Edwards M, Rassin DK, Izumi T, Mitra S, Perez-Polo JR (1998) APE/Ref-1 responses to oxidative stress in aged rats. J Neurosci Res 54:635–638

Endres M, Biniszkiewicz D, Sobol RW, Harms C, Ahmadi M, Lipski A, Katchanov J, Mergenthaler P, Dirnagl U, Wilson SH, Meisel A, Jaenisch R (2004) Increased postischemic brain injury in mice deficient in uracil-DNA glycosylase. J Clin Invest 113: 1711–1721

Englander EW, Ma H (2006) Differential modulation of base excision repair activities during brain ontogeny: implications for repair of transcribed DNA. Mech Ageing Dev 127:64–69

Englander EW, Greeley GH Jr, Wang G, Perez-Polo JR, Lee HM (1999) Hypoxia-induced mitochondrial and nuclear DNA damage in the rat brain. J Neurosci Res 58:262–269

Enokido Y, Inamura N, Araki T, Satoh T, Nakane H, Yoshino M, Nakatsu Y, Tanaka K, Hatanaka H (1997) Loss of the xeroderma pigmentosum group A gene (XPA) enhances apoptosis of cultured cerebellar neurons induced by UV but not by low-K+ medium. J Neurochem 69:246–251

Fernandez-Capetillo O, Lee A, Nussenzweig M, Nussenzweig A (2004) H2AX: the histone guardian of the genome. DNA Repair (Amst) 3:959–967

Ferrante RJ, Browne SE, Shinobu LA, Bowling AC, Baik MJ, MacGarvey U, Kowall NW, Brown RH Jr, Beal MF (1997) Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. J Neurochem 69:2064–2074

Francisconi S, Codenotti M, Ferrari TG, Uberti D, Memo M (2006) Mitochondrial dysfunction and increased sensitivity to excitotoxicity in mice deficient in DNA mismatch repair. J Neurochem 98:223–233

Friedberg EC, Walker GC, Siede W (1995) DNA repair and mutagenesis. ASM Press, Washington, DC

Friesner JD, Liu B, Culligan K, Britt AB (2005) Ionizing radiation-dependent gamma-H2AX focus formation requires ataxia telangiectasia mutated and ataxia telangiectasia mutated and Rad3-related. Mol Biol Cell 16:2566–2576

Fujikawa DG (1996) The temporal evolution of neuronal damage from pilocarpine-induced status epilepticus. Brain Res 725:11–22

Fujikawa DG, Shinmei SS, Cai B (2000a) Kainic acid-induced seizures produce necrotic, not apoptotic, neurons with internucleosomal DNA cleavage: implications for programmed cell death mechanisms. Neuroscience 98:41–53

Fujikawa DG, Shinmei SS, Cai B (2000b) Seizure-induced neuronal necrosis: implications for programmed cell death mechanisms. Epilepsia 41(Suppl 6):S9–S13

Fujimura M, Morita-Fujimura Y, Kawase M, Chan PH (1999a) Early decrease of apurinic/apyrimidinic endonuclease expression after transient focal cerebral ischemia in mice. J Cereb Blood Flow Metab 19:495–501

Fujimura M, Morita-Fujimura Y, Narasimhan P, Copin JC, Kawase M, Chan PH (1999b) Copper-zinc superoxide dismutase prevents the early decrease of apurinic/apyrimidinic endonuclease and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. Stroke 30:2408–2415

Fujimura M, Morita-Fujimura Y, Sugawara T, Chan PH (1999c) Early decrease of XRCC1, a DNA base excision repair protein, may contribute to DNA fragmentation after transient focal cerebral ischemia in mice. Stroke 30:2456–2462

Fujimura M, Morita-Fujimura Y, Noshita N, Yoshimoto T, Chan PH (2000) Reduction of the DNA base excision repair protein, XRCC1, may contribute to DNA fragmentation after cold injury-induced brain trauma in mice. Brain Res 869:105–111

Gabbita SP, Lovell MA, Markesbery WR (1998) Increased nuclear DNA oxidation in the brain in Alzheimer's disease. J Neurochem 71:2034–2040

Gobbel GT, Bellinzona M, Vogt AR, Gupta N, Fike JR, Chan PH (1998) Response of postmitotic neurons to X-irradiation: implications for the role of DNA damage in neuronal apoptosis. J Neurosci 18:147–155

Gordon GW, Berry G, Liang XH, Levine B, Herman B (1998) Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. Biophys J 74:2702–2713

Gupta YK, Briyal S (2006) Protective effect of vineatrol against kainic acid induced seizures, oxidative stress and on the expression of heat shock proteins in rats. Eur Neuropsychopharmacol 16:85–91

Gupta YK, Gupta M, Kohli K (2003) Neuroprotective role of melatonin in oxidative stress vulnerable brain. Indian J Physiol Pharmacol 47:373–386
Hamilton ML, Van Remmen H, Drake JA, Yang H, Guo ZM, Kewitt K, Walter CA, Richardson A (2001) Does oxidative damage to DNA increase with age? Proc Natl Acad Sci USA 98:10469–10474
Hammarsten O, Chu G (1998) DNA-dependent protein kinase: DNA binding and activation in the absence of Ku. Proc Natl Acad Sci USA 95:525–530
Hayashi T, Sakurai M, Itoyama Y, Abe K (1999) Oxidative damage and breakage of DNA in rat brain after transient MCA occlusion. Brain Res 832:159–163
Hayashi M, Araki S, Kohyama J, Shioda K, Fukatsu R, Tamagawa K (2004) Brainstem and basal ganglia lesions in xeroderma pigmentosum group A. J Neuropathol Exp Neurol 63:1048–1057
Hegde MR, Chong B, Blazo ME, Chin LH, Ward PA, Chintagumpala MM, Kim JY, Plon SE, Richards CS (2005) A homozygous mutation in MSH6 causes Turcot syndrome. Clin Cancer Res 11:4689–4693
Hegde ML, Gupta VB, Anitha M, Harikrishna T, Shankar SK, Muthane U, Subba RK, Jagannatha Rao KS (2006) Studies on genomic DNA topology and stability in brain regions of Parkinson's disease. Arch Biochem Biophys 449:143–156
Henshall DC, Sinclair J, Simon RP (1999) Relationship between seizure-induced transcription of the DNA damage-inducible gene GADD45, DNA fragmentation, and neuronal death in focally evoked limbic epilepsy. J Neurochem 73:1573–1583
Henshall DC, Sinclair J, Simon RP (2000) Spatio-temporal profile of DNA fragmentation and its relationship to patterns of epileptiform activity following focally evoked limbic seizures. Brain Res 858:290–302
Hermann DM, Kuroiwa T, Hata R, Gillardon F, Ito U, Mies G (2001) Expression of redox factor-1, p53-activated gene 608 and caspase-3 messenger RNAs following repeated unilateral common carotid artery occlusion in gerbils – relationship to delayed cell injury and secondary failure of energy state. Neuroscience 102:779–787
Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NM, Orr AI, Reaper PM, Jackson SP, Curtin NJ, Smith GC (2004) Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. Cancer Res 64:9152–9159
Huang J, Dynan WS (2002) Reconstitution of the mammalian DNA double-strand break end-joining reaction reveals a requirement for an Mre11/Rad50/NBS1-containing fraction. Nucleic Acids Res 30:667–674
Intano GW, Cho EJ, McMahan CA, Walter CA (2003) Age-related base excision repair activity in mouse brain and liver nuclear extracts. J Gerontol A Biol Sci Med Sci 58:205–211
Itoh M, Hayashi M, Shioda K, Minagawa M, Isa F, Tamagawa K, Morimatsu Y, Oda M (1999) Neurodegeneration in hereditary nucleotide repair disorders. Brain Dev 21:326–333
Jackson SP (2002) Sensing and repairing DNA double-strand breaks. Carcinogenesis 23(5): 687–696
Jeggo P, O'Neill P (2002) The Greek Goddess, Artemis, reveals the secrets of her cleavage. DNA Repair (Amst) 1:771–777
Kajitani K, Yamaguchi H, Dan Y, Furuichi M, Kang D, Nakabeppu Y (2006) MTH1, an oxidized purine nucleoside triphosphatase, suppresses the accumulation of oxidative damage of nucleic acids in the hippocampal microglia during kainate-induced excitotoxicity. J Neurosci 26: 1688–1698
Karahalil B, Hogue BA, de Souza-Pinto NC, Bohr VA (2002) Base excision repair capacity in mitochondria and nuclei: tissue-specific variations. FASEB J 16:1895–1902
Karran P, Bignami M (1999) Mismatch repair and cancer. In: Smith PJ, Jones CJ (eds) DNA recombination and repair. Oxford, Oxford University Press, pp 66–98
Kato H, Liu Y, Araki T, Kogure K (1991) Temporal profile of the effects of pretreatment with brief cerebral ischemia on the neuronal damage following secondary ischemic insult in the gerbil: cumulative damage and protection effects. Brain Res 553:238–242
Kawaguchi K, Hickey RW, Rose ME, Zhu L, Chen J, Graham SH (2005) Cyclooxygenase-2 expression is induced in rat brain after kainate-induced seizures and promotes neuronal death in CA3 hippocampus. Brain Res 1050:130–137

Kawase M, Fujimura M, Morita-Fujimura Y, Chan PH (1999) Reduction of apurinic/apyrimidinic endonuclease expression after transient global cerebral ischemia in rats: implication of the failure of DNA repair in neuronal apoptosis. Stroke 30:441–448

Kelly ME, McIntyre DC (1994) Hippocampal kindling protects several structures from the neuronal damage resulting from kainic acid-induced status epilepticus. Brain Res 634: 245–256

Khan A, Lai H, Nishimura Y, Mirolo MH, Singh NP (1995) Effects of ECS on DNA single-strand breaks in rat brain cells. Convuls Ther 11:114–121

Kim H, Bing G, Jhoo W, Ko KH, Kim WK, Suh JH, Kim SJ, Kato K, Hong JS (2000) Changes of hippocampal Cu/Zn-superoxide dismutase after kainate treatment in the rat. Brain Res. 853:215–226

Kim GW, Noshita N, Sugawara T, Chan PH (2001) Early decrease in DNA repair proteins, Ku 70 and Ku86, and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. Stroke 32:1401–1407

Kim SJ, Kim JE, Moon IS (2004) Paraquat induces apoptosis of cultured rat cortical cells. Mol Cells 17:102–107

Kisby GE, Milne J, Sweatt C (1997) Evidence of reduced DNA repair in amyotrophic lateral sclerosis brain tissue. Neuroreport 8:1337–1340

Kisby GE, Lesselroth H, Olivas A, Samson L, Gold B, Tanaka K, Turker MS (2004) Role of nucleotide- and base-excision repair in genotoxin-induced neuronal cell death. DNA Repair (Amst) 3:617–627

Kohji T, Hayashi M, Shioda K, Minagawa M, Morimatsu Y, Tamagawa K, Oda M (1998) Cerebellar neurodegeneration in human hereditary DNA repair disorders. Neurosci Lett 243:133–136

Kondratyev A, Gale K (2001) Temporal and spatial patterns of DNA fragmentation following focally or systemically-evoked status epilepticus in rats. Neurosci Lett 310:13–16

Kondratyev A, Sahibzada N, Gale K (2001) Electroconvulsive shock exposure prevents neuronal apoptosis after kainic acid-evoked status epilepticus. Brain Res Mol Brain Res 91:1–13

Krishna TH, Mahipal S, Sudhakar A, Sugimoto H, Kalluri R, Rao KS (2005) Reduced DNA gap repair in aging rat neuronal extracts and its restoration by DNA polymerase beta and DNA-ligase. J Neurochem 92:818–823

Krokan HE, Nilsen H, Skorpen F, Otterlei M, Slupphaug G (2000) Base excision repair of DNA in mammalian cells. FEBS Lett 476:73–77

Kruhlak MJ, Celeste A, Dellaire G, Fernandez-Capetillo O, Muller WG, McNally JG, Bazett-Jones DP, Nussenzweig A (2006) Changes in chromatin structure and mobility in living cells at sites of DNA double-strand breaks. J Cell Biol 172:823–834

Kruman II, Schwartz E, Kruman Y, Cutler RG, Zhu X, Greig NH, Mattson MP (2004) Suppression of uracil-DNA glycosylase induces neuronal apoptosis. J Biol Chem 279:43952–43960

Kubota Y, Nash RA, Klungland A, Schar P, Barnes DE, Lindahl T (1996) Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase beta and the XRCC1 protein. EMBO J 15:6662–6670

Lan J, Henshall DC, Simon RP, Chen J (2000) Formation of the base modification 8-hydroxyl-2′-deoxyguanosine and DNA fragmentation following seizures induced by systemic kainic acid in the rat. J Neurochem 74:302–309

Lan J, Li W, Zhang F, Sun FY, Nagayama T, O'Horo C, Chen J (2003) Inducible repair of oxidative DNA lesions in the rat brain after transient focal ischemia and reperfusion. J Cereb Blood Flow Metab 23:1324–1339

Laposa RR, Cleaver JE (2001) DNA repair on the brain. Proc Natl Acad Sci USA 98:12860–12862

Lee HM, Wang C, Hu Z, Greeley GH, Makalowski W, Hellmich HL, Englander EW (2002) Hypoxia induces mitochondrial DNA damage and stimulates expression of a DNA repair enzyme, the Escherichia coli MutY DNA glycosylase homolog (MYH), in vivo, in the rat brain. J Neurochem 80:928–937

Lewen A, Sugawara T, Gasche Y, Fujimura M, Chan PH (2001) Oxidative cellular damage and the reduction of APE/Ref-1 expression after experimental traumatic brain injury. Neurobiol Dis 8:380–390

Li B, Comai L (2000) Functional interaction between Ku and the Werner syndrome protein in DNA end processing. J Biol Chem 275:39800
Li S, Zheng J, Carmichael ST (2005) Increased oxidative protein and DNA damage but decreased stress response in the aged brain following experimental stroke. Neurobiol Dis 18:432–440
Li W, Luo Y, Zhang F, Signore AP, Gobbel GT, Simon RP, Chen J (2006) Ischemic preconditioning in the rat brain enhances the repair of endogenous oxidative DNA damage by activating the base-excision repair pathway. J Cereb Blood Flow Metab 26:181–198
Liang LP, Ho YS, Patel M (2000) Mitochondrial superoxide production in kainate-induced hippocampal damage. Neuroscience 101:563–570
Lin LH, Cao S, Yu L, Cui J, Hamilton WJ, Liu PK (2000) Up-regulation of base excision repair activity for 8-hydroxy-2′-deoxyguanosine in the mouse brain after forebrain ischemia-reperfusion. J Neurochem 74:1098–1105
Ling X, Zhang LM, Huang YL, Bao WL, Sun FY (1999) Neuronal ERCC6 mRNA expression in rat brain induced by a transient focal cerebral ischemia. Zhongguo Yao Li Xue Bao 20:15–20
Liu PK, Hsu CY, Dizdaroglu M, Floyd RA, Kow YW, Karakaya A, Rabow LE, Cui JK (1996) Damage, repair, and mutagenesis in nuclear genes after mouse forebrain ischemia-reperfusion. J Neurosci 16:6795–6806
Lovell MA, Gabbita SP, Markesbery WR (1999) Increased DNA oxidation and decreased levels of repair products in Alzheimer's disease ventricular CSF. J Neurochem 72:771–776
Lovell MA, Xie C, Markesbery WR (2000) Decreased base excision repair and increased helicase activity in Alzheimer's disease brain. Brain Res 855:116–123
Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J, Yankner BA (2004) Gene regulation and DNA damage in the ageing human brain. Nature 429:883–891
Ma Y, Lu H, Tippin B, Goodman MF, Shimazaki N, Koiwai O, Hsieh CL, Schwarz K, Lieber MR (2004) A biochemically defined system for mammalian nonhomologous DNA end joining. Mol Cell 16:701–713
Ma Y, Schwarz K, Lieber MR (2005) The Artemis:DNA-PKcs endonuclease cleaves DNA loops, flaps, and gaps. DNA Repair (Amst) 4:845–851
MacGregor DG, Higgins MJ, Jones PA, Maxwell WL, Watson MW, Graham DI, Stone TW (1996) Ascorbate attenuates the systemic kainate-induced neurotoxicity in the rat hippocampus. Brain Res 727:133–144
Marietta C, Palombo F, Gallinari P, Jiricny J, Brooks PJ (1998) Expression of long-patch and short-patch DNA mismatch repair proteins in the embryonic and adult mammalian brain. Brain Res Mol Brain Res 53:317–320
Masco D, Sahibzada N, Gale K (1995) Electroconvulsive shock prevents apoptotic and excitotoxic cell death. Proc Soc Neurosci 21:326.12
McMurray CT (2005) To die or not to die: DNA repair in neurons. Mutat Res 577:260–274
Meira LB, Devaraj S, Kisby GE, Burns DK, Daniel RL, Hammer RE, Grundy S, Jialal I, Friedberg EC (2001) Heterozygosity for the mouse Apex gene results in phenotypes associated with oxidative stress. Cancer Res 61:5552–5557
Mendez DR, Cherian L, Moore N, Arora T, Liu PK, Robertson CS (2004) Oxidative DNA lesions in a rodent model of traumatic brain injury. J Trauma 56:1235–1240
Menko FH, Kaspers GL, Meijer GA, Claes K, van Hagen JM, Gille JJ (2004) A homozygous MSH6 mutation in a child with cafe-au-lait spots, oligodendroglioma and rectal cancer. Fam Cancer 3:123–127
Merlo D, Di Stasi AMM, Bonini P, Mollinari C, Cardinale A, Cozzolino F, Wisden W, Garaci E (2005) DNA repair in post-mitotic neurons: a gene-trapping strategy. Cell Death Differ 12:307–309
Mishima K, Tanaka T, Pu F, Egashira N, Iwasaki K, Hidaka R, Matsunaga K, Takata J, Karube Y, Fujiwara M (2003) Vitamin E isoforms alpha-tocotrienol and gamma-tocopherol prevent cerebral infarction in mice. Neurosci Lett 337:56–60
Morita-Fujimura Y, Fujimura M, Kawase M, Chan PH (1999) Early decrease in apurinic/apyrimidinic endonuclease is followed by DNA fragmentation after cold injury-induced brain trauma in mice. Neuroscience 93:1465–1473

Motte J, Fernandes MJ, Baram TZ, Nehlig A (1998) Spatial and temporal evolution of neuronal activation, stress and injury in lithium-pilocarpine seizures in adult rats. Brain Res 793: 61–72

Nagayama T, Lan J, Henshall DC, Chen D, O'Horo C, Simon RP, Chen J (2000a) Induction of oxidative DNA damage in the peri-infarct region after permanent focal cerebral ischemia. J Neurochem 75:1716–1728

Nagayama T, Simon RP, Chen D, Henshall DC, Pei W, Stetler RA, Chen J (2000b) Activation of poly(ADP-ribose) polymerase in the rat hippocampus may contribute to cellular recovery following sublethal transient global ischemia. J Neurochem 74:1636–1645

Najm IM, Hadam J, Ckakraverty D, Mikuni N, Penrod C, Sopa C, Markarian G, Luders HO, Babb T, Baudry M (1998) A short episode of seizure activity protects from status epilepticus-induced neuronal damage in rat brain. Brain Res 10(1–2):72–75

Nakamura K, Sakai W, Kawamoto T, Bree RT, Lowndes NF, Takeda S, Taniguchi Y (2006) Genetic dissection of vertebrate 53BP1: a major role in non-homologous end joining of DNA double strand breaks. DNA Repair (Amst) 5(6):741–749

Neema M, Navarro-Quiroga I, Chechlacz M, Gilliams-Francis K, Liu J, Lamonica K, Lin SL, Naegele JR (2005) DNA damage and nonhomologous end joining in excitotoxicity: neuroprotective role of DNA-PKcs in kainic acid-induced seizures. Hippocampus 15:1057–1071

Nick McElhinny SA, Snowden CM, McCarville J, Ramsden DA (2000) Ku recruits the XRCC4-ligase IV complex to DNA ends. Mol Cell Biol 20:2996–3003

Nilsen H, Rosewell I, Robins P, Skjelbred CF, Andersen S, Slupphaug G, Daly G, Krokan HE, Lindahl T, Barnes DE (2000) Uracil-DNA glycosylase (UNG)-deficient mice reveal a primary role of the enzyme during DNA replication. Mol Cell 5:1059–1065

Norbury CJ, Zhivotovsky B (2004) DNA damage-induced apoptosis. Oncogene 23:2797–2808

Nouspikel T, Hanawalt PC (2000) Terminally differentiated human neurons repair transcribed genes but display attenuated global DNA repair and modulation of repair gene expression. Mol Cell Biol 20:1562–1570

Nouspikel T, Hanawalt PC (2002) DNA repair in terminally differentiated cells. DNA Repair (Amst) 1:59–75

Nowak E, Etienne O, Millet P, Lages CS, Mathieu C, Mouthon MA, Boussin FD (2006) Radiation-induced H2AX phosphorylation and neural precursor apoptosis in the developing brain of mice. Radiat Res 165:155–164

Onem G, Aral E, Enli Y, Oguz EO, Coskun E, Aybek H, Ozcan AV, Sacar M, Bir LS, Baltalarli A, Baycu C (2006) Neuroprotective effects of L-carnitine and vitamin E alone or in combination against ischemia-reperfusion injury in rats. J Surg Res 131:124–130

Park EJ, Chan DW, Park JH, Oettinger MA, Kwon J (2003) DNA-PK is activated by nucleosomes and phosphorylates H2AX within the nucleosomes in an acetylation-dependent manner. Nucleic Acids Res 31:6819–6827

Patel MN (2002) Oxidative stress, mitochondrial dysfunction, and epilepsy. Free Radic Res 36:1139–1146

Patel M (2004) Mitochondrial dysfunction and oxidative stress: cause and consequence of epileptic seizures. Free Radic Biol Med 37:1951–1962

Patel M, Li QY (2003) Age dependence of seizure-induced oxidative stress. Neuroscience 118:431–437

Patel M, Li QY, Chang LY, Crapo J, Liang LP (2005) Activation of NADPH oxidase and extracellular superoxide production in seizure-induced hippocampal damage. J Neurochem 92:123–131

Phillis JW, Horrocks LA, Farooqui AA (2006) Cyclooxygenases, lipoxygenases, and epoxygenases in CNS: their role and involvement in neurological disorders. Brain Res Brain Res Rev 52(2):201–243

Plamondon H, Blondeau N, Heurteaux C, Lazdunski M (1999) Mutually protective actions of kainic acid epileptic preconditioning and sublethal global ischemia on hippocampal neuronal death: involvement of adenosine A1 Receptors and KATP Channels. J Cereb Blood Flow Metab 19(12):1296–1308

Quach N, Chan T, Lu TA, Schreiber SS, Tan Z (2005) Induction of DNA repair proteins, Ref-1 and XRCC1, in adult rat brain following kainic acid-induced seizures. Brain Res 1042:236–240

Raji NS, Krishna TH, Rao KS (2002) DNA-polymerase alpha, beta, delta and epsilon activities in isolated neuronal and astroglial cell fractions from developing and aging rat cerebral cortex. Int J Dev Neurosci 20:491–496

Rao KS (2007) DNA repair in aging rat neurons. Neuroscience 145:1330–1340

Rao KS, Annapurna VV, Raji NS (2001) DNA polymerase-beta may be the main player for defective DNA repair in aging rat neurons. Ann N Y Acad Sci 928:113–120

Rapp A, Greulich KO (2004) After double-strand break induction by UV-A, homologous recombination and nonhomologous end joining cooperate at the same DSB if both systems are available. J Cell Sci 117:4935–4945

Rass U, Ahel I, West SC (2007) Defective DNA repair and neurodegenerative disease. Cell 130:991–1004

Ren K, de Ortiz SP (2002) Non-homologous DNA end joining in the mature rat brain. J Neurochem 80:949–959

Rolig RL, McKinnon PJ (2000) Linking DNA damage and neurodegeneration. Trends Neurosci 23:417–424

Rutten BP, Korr H, Steinbusch HW, Schmitz C (2003) The aging brain: less neurons could be better. Mech Ageing Dev 124:349–355

Sasahira M, Lowry T, Simon RP, Greenberg DA (1995) Epileptic tolerance: prior seizures protect against seizure-induced neuronal injury. Neurosci Lett 185:95–98

Satrustegui J, Richter C (1984) The role of hydroperoxides as calcium release agents in rat brain mitochondria. Arch Biochem Biophys 233:736–740

Sawada M, Sun W, Hayes P, Leskov K, Boothman DA, Matsuyama S (2003) Ku 70 suppresses the apoptotic translocation of Bax to mitochondria. Nat Cell Biol 5:320–329

Schwob JE, Fuller T, Price JL, Olney JW (1980) Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. Neuroscience 5:991–1014

Sekiguchi JM, Ferguson DO (2006) DNA double-strand break repair: a relentless hunt uncovers new prey. Cell 124:260–262

Shackelford DA (2006) DNA end joining activity is reduced in Alzheimer's disease. Neurobiol Aging 27:596–605

Shackelford DA, Tobaru T, Zhang S, Zivin JA (1999) Changes in expression of the DNA repair protein complex DNA-dependent protein kinase after ischemia and reperfusion. J Neurosci 19:4727–4738

Sharma S (2007) Age-related nonhomologous end joining activity in rat neurons. Brain Res Bull 73:48–54

Shin HJ, Lee JY, Son E, Lee DH, Kim HJ, Kang SS, Cho GJ, Choi WS, Roh GS (2007) Curcumin attenuates the kainic acid-induced hippocampal cell death in the mice. Neurosci Lett 416:49–54

Simmet T, Seregi A, Hertting G (1987) Formation of sulphidopeptide-leukotrienes in brain tissue of spontaneously convulsing gerbils. Neuropharmacology 26:107–110

Simonian NA, Coyle JT (1996) Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol 36:83–106

Singleton BK, Jeggo PA (1999) Double-strand break repair and V(D)J recombination. In: Smith PJ, Christopher JJ (eds) DNA recombination and repair. Oxford University Press, Oxford, pp 16–37

Sloviter RS, Dean E, Sollas AL, Goodman JH (1996) Apoptosis and necrosis induced in different hippocampal neuron populations by repetitive perforant path stimulation in the rat. J Comp Neurol 366:516–533

Slupphaug G, Kavli B, Krokan HE (2003) The interacting pathways for prevention and repair of oxidative DNA damage. Mutat. Res. 531:231–251

Sperk G, Lassmann H, Baran H, Kish SJ, Seitelberger F, Hornykiewicz O (1983) Kainic acid induced seizures: neurochemical and histopathological changes. Neuroscience 10:1301–1315

Stewart GS, Wang B, Bignell CR, Taylor AM, Elledge SJ (2003) MDC1 is a mediator of the mammalian DNA damage checkpoint. Nature 421:961–966

Stiff T, O'Driscoll M, Rief N, Iwabuchi K, Lobrich M, Jeggo PA (2004) ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. Cancer Res 64:2390–2396

Stucki M, Jackson SP (2006) gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. DNA Repair (Amst) 5:534–543

Stucki M, Clapperton JA, Mohammad D, Yaffe MB, Smerdon SJ, Jackson SP (2005) MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell 123:1213–1226

Sugawara T, Noshita N, Lewen A, Kim GW, Chan PH (2001) Neuronal expression of the DNA repair protein Ku 70 after ischemic preconditioning corresponds to tolerance to global cerebral ischemia. Stroke 32:2388–2393

Sumanont Y, Murakami Y, Tohda M, Vajragupta O, Watanabe H, Matsumoto K (2006) Prevention of kainic acid-induced changes in nitric oxide level and neuronal cell damage in the rat hippocampus by manganese complexes of curcumin and diacetylcurcumin. Life Sci 78:1884–1891

Sun FY, Lin X, Mao LZ, Ge WH, Zhang LM, Huang YL, Gu J (2002) Neuroprotection by melatonin against ischemic neuronal injury associated with modulation of DNA damage and repair in the rat following a transient cerebral ischemia. J Pineal Res 33:48–56

Tijsterman M, Verhage RA, Brouwer J (1999) Transcription-coupled and global genome repair in yeast and humans. In: Smith PJ, Jones CJ (eds) DNA recombination and repair. Oxford University Press, Oxford, pp 139–165

Tretter L, dam-Vizi V (2005) Alpha-ketoglutarate dehydrogenase: a target and generator of oxidative stress. Philos Trans R Soc Lond B Biol Sci 360:2335–2345

Trujillo KM, Yuan SS, Lee EY, Sung P (1998) Nuclease activities in a complex of human recombination and DNA repair factors Rad50, Mre11, and p95. J Biol Chem 273:21447–21450

Tuunanen J, Lukasiuk K, Halonen T, Pitkanen A (1999) Status epilepticus-induced neuronal damage in the rat amygdaloid complex: distribution, time-course and mechanisms. Neuroscience 94:473–495

Ueda Y, Yokoyama H, Niwa R, Konaka R, Ohya-Nishiguchi H, Kamada H (1997) Generation of lipid radicals in the hippocampal extracellular space during kainic acid-induced seizures in rats. Epilepsy Res 26:329–333

Vemuri MC, Schiller E, Naegele JR (2001) Elevated DNA double strand breaks and apoptosis in the CNS of scid mutant mice. Cell Death Differ 8:245–255

Vogel R, Wiesinger H, Hamprecht B, Dringen R (1999) The regeneration of reduced glutathione in rat forebrain mitochondria identifies metabolic pathways providing the NADPH required. Neurosci Lett 275:97–100

Vyjayanti VN, Rao KS (2006) DNA double strand break repair in brain: reduced NHEJ activity in aging rat neurons. Neurosci Lett 393:18–22

Wang J, Markesbery WR, Lovell MA (2006) Increased oxidative damage in nuclear and mitochondrial DNA in mild cognitive impairment. J Neurochem 96:825–832

West RB, Yaneva M, Lieber MR (1998) Productive and nonproductive complexes of Ku and DNA-dependent protein kinase at DNA termini. Mol Cell Biol 18:5908–5920

Xia Z, Liu Y (2001) Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. Biophys J 81:2395–2402

Yaneva M, Kowalewski T, Lieber MR (1997) Interaction of DNA-dependent protein kinase with DNA and with Ku: biochemical and atomic-force microscopy studies. EMBO J 16:5098–5112

Zaleska MM, Wilson DF (1989) Lipid hydroperoxides inhibit reacylation of phospholipids in neuronal membranes. J Neurochem 52:255–260

Zhang WR, Hayashi T, Sasaki C, Sato K, Nagano I, Manabe Y, Abe K (2001) Attenuation of oxidative DNA damage with a novel antioxidant EPC-K1 in rat brain neuronal cells after transient middle cerebral artery occlusion. Neurol Res 23:676–680

Zhang X, Cui SS, Wallace AE, Hannesson DK, Schmued LC, Saucier DM, Honer WG, Corcoran ME (2002) Relations between brain pathology and temporal lobe epilepsy. J Neurosci 22:6052–6061

Zhang B, Tanaka J, Yang L, Yang L, Sakanaka M, Hata R, Maeda N, Mitsuda N (2004) Protective effect of vitamin E against focal brain ischemia and neuronal death through induction of target genes of hypoxia-inducible factor-1. Neuroscience 126:433–440

Zoccarato F, Cavallini L, Alexandre A (2004) Respiration-dependent removal of exogenous H2O2 in brain mitochondria: inhibition by Ca2+. J Biol Chem 279:4166–4174

Chapter 17
Activation of Caspase-Independent Programmed Pathways in Seizure-Induced Neuronal Necrosis

Denson G. Fujikawa

17.1 Excitotoxicity and Seizure-Induced Neuronal Death

Our current concept of how prolonged epileptic seizures (status epilepticus, or SE) kill neurons originated in the 1970s from the pioneering work done by John Olney and associates. Olney reported in 1969 that monosodium glutamate killed neurons in the hypothalamic arcuate nucleus, a region that lacks a blood–brain barrier (Olney 1969). Subsequently, Olney and associates found that administration of glutamate (GLU), the most abundant excitatory neurotransmitter in the brain, killed hypothalamic neurons in the infant mouse (Olney 1971), and that systemic administration of a GLU analogue, kainic acid (KA) to the adult rodent resulted in SE and neuronal death (Olney et al. 1974). In 1985, Olney put forth his excitotoxic hypothesis as it applies to SE (Olney 1985). This hypothesis, which states that excessive presynaptic GLU release results in the death of postsynaptic neurons, has proved to be remarkably robust, and is applicable to a wide variety of acute neuronal insults, as mentioned in the Introduction.

17.1.1 Verification of the Excitotoxic Hypothesis with Respect to Seizures

Microdialysis studies of the hippocampus and piriform cortex, in which microdialysis probes are placed to measure extracellular GLU (GLU_o) concentrations before and during SE, have produced mixed results (Lehmann et al. 1985; Wade et al. 1987; Lallement et al. 1991; Bruhn et al. 1992; Millan et al. 1993; Tanaka et al. 1996; Smolders et al. 1997). At least part of the reason for finding a lack of

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increased GLU_o could be that these probes are relatively large, typically 2 mm in diameter, so that only overall GLU_o is measured, rather than synaptic concentrations. GLU_o activates the *N*-methyl-D-aspartate (NMDA) subtype of GLU receptor. Despite the uncertain results of the microdialysis studies, experiments in which NMDA-receptor antagonists were given systemically have shown that these agents are remarkably neuroprotective, despite ongoing electrographic seizure discharges (Fariello et al. 1989; Clifford et al. 1990; Fujikawa et al. 1994; Fujikawa 1995).

17.2 The Morphology of Cell Death

A developmental classification of cell death morphology (Clarke 1990) has been adopted by investigators studying acute neuronal injury. In this classification, type I cell death is apoptotic, type II is autophagic, and type III is divided into IIIa (nonlysosomal) and IIIb (cytoplasmic, corresponding to our current understanding of necrotic cell death). However, this classification has been misused by investigators who do not pay detailed attention to morphology. Using surrogate markers is insufficient because all of the nonultrastructural surrogate markers studied to date are not specific (for example, TUNEL staining, DNA laddering, and annexin V externalization). In addition, biochemical markers have been used to identify the type of cell death. But these markers should not be used to define morphology, because morphology all too often is not sufficiently defined, and mistakes are too often made.

Morphological neuronal apoptosis in vivo occurs in fetal or postnatal brain (Ikonomidou et al. 1999, 2000; Ishimaru et al. 1999). By postnatal day 21 (P21), neuronal apoptosis becomes undetectable if naturally occurring and very low if induced an NMDA-receptor antagonist (Ikonomidou et al. 1999). Almost all in vitro cell culture experiments have used fetal neurons, and all have used low magnification fluorescence photomicrographs to label neurons "apoptotic" if they showed nuclear pyknosis (shrinkage). Ironically, this feature makes their neurons conform to necrotic rather than apoptotic morphology (Fujikawa et al. 1999, 2000a, 2002, 2007; Fujikawa 2000, 2002, 2005).

Contrary to in vitro cell culture experiments, in which cells exposed to an overwhelming stimulus show cell swelling and then lyse, presumbably thereafter to disappear, it was shown in the 1970s and 1980s (Brown 1977; Griffiths et al. 1983, 1984; Evans et al. 1984; Auer et al. 1985a, b), and was recently rediscovered that cerebral ischemia and SE in vivo produce electron-dense, shrunken neurons with pyknotic (shrunken) nuclei, plasma membrane disruption, and cytoplasmic vacuoles, many of which are irreversibly damaged mitochondria and all of which are markers of cellular necrosis (Colbourne et al. 1999; Fujikawa et al. 1999, 2000a, b, 2002). One should be cautious in using the results from in vitro experiments of fetal or neonatal cells dispersed in cell culture to explain what happens in the adult brain in vivo.

17.2.1 The Morphology of Seizure-Induced Neuronal Death

In the 1980s, Meldrum and colleagues showed mitochondrial calcium accumulation in electron-dense, shrunken neurons using the oxalate-pyroantimonate technique in ultrastructural brain sections (Griffiths et al. 1983, 1984; Evans et al. 1984). The shrunken, electron-dense neurons showed nuclear pyknosis and cytoplasmic vacuoles, corresponding to what we now know as neuronal necrosis (Colbourne et al. 1999; Fujikawa et al. 1999, 2000a, b, 2002) (Fig. 17.1). In addition, seizure-induced necrotic neurons show internucleosomal DNA cleavage, or DNA laddering, a programmed process requiring endonuclease activation (Fujikawa et al. 1999, 2000a, b, 2002) (Fig. 17.1). The calcium accumulation in swollen mitochondria predated our understanding of how calcium entered neurons destined to die. Nevertheless, the ultrastructural description of seizure-induced neuronal necrosis was correct.

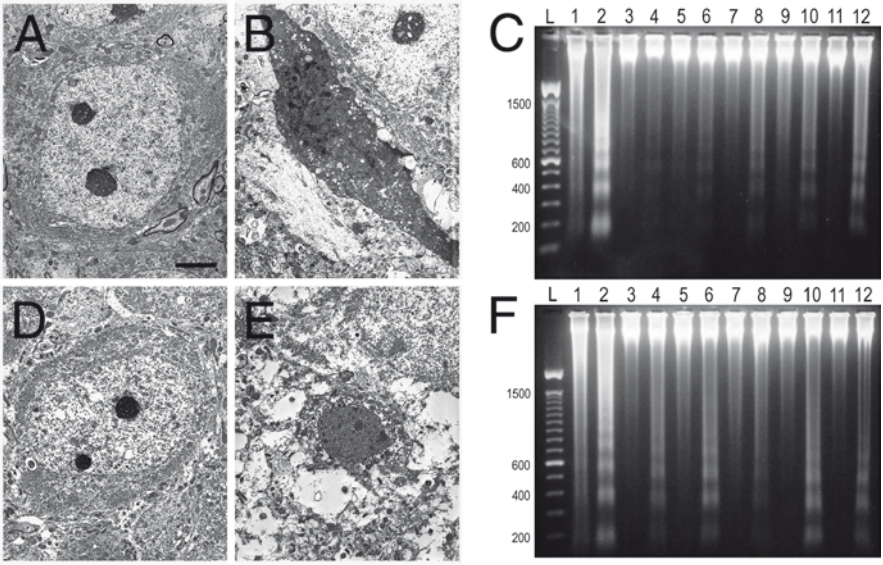

Fig. 17.1 Seizure-induced necrotic neurons show internucleosomal DNA cleavage (DNA laddering). (**A**) and (**D**) are electron photomicrographs of ventral hippocampal CA1 neurons in control rats given lithium chloride and normal saline instead of pilocarpine 24 (**A**) and 72 h (**D**) later. (**B**) and (**E**) are electron photomicrographs of necrotic neurons 24 (**B**) and 72 h (**D**) after 3-h lithium-pilocarpine-induced status epilepticus (LPCSE). The electron-dense neuronal shrinkage, nuclear pyknosis and cytoplasmic disruption are apparent, as well as the astrocytic end feet swelling surrounding the necrotic neurons. (**C**) and (**F**) show DNA agarose gel electrophoresis results 24 and 72 h after normal saline or 3-h LPCSE. Lanes 1 and 2 in (**C**) and (**F**) show control and apoptotic thymocytic tissue, controls for DNA laddering. The odd-numbered lanes are control tissue and the even-numbered lanes seizure tissue from dorsal hippocampus (3 and 4), ventral hippocampus (5 and 6), neocortex (7 and 8), amygdala and piriform cortex (9 and 10) and entorhinal cortex (11 and 12). The 180-base-pair DNA laddering can be seen in the seizure lanes at both 24 and 72 h time points. From Fujikawa (2005), with permission from MIT Press

Our current understanding of the morphology of seizure-induced neuronal death in the adult brain is in remarkable agreement with the ultrastructural morphology of cerebral ischemic and hypoglycemic neuronal death, both of which are excitotoxic in origin, and both of which show unmistakable earmarks of neuronal necrosis in vivo (Auer et al. 1985a, b; Colbourne et al. 1999). This suggests that a common mechanism, presynaptic glutamate release from depolarization of neurons, as well as reversal of glutamate uptake by astrocytes, underlies these pathological conditions. After traumatic CNS injury in the adult rodent, ultrastructural photomicrographs (1) have been misinterpreted as showing apoptotic neurons (Colicos and Dash 1996); their Fig. 3B and 3C show microglia, Fig. 4B shows a microglial cell and Fig. 4B and 4C shows a necrotic neuron), (2) have suggested both necrotic and apoptotic morphology, predominantly the former, but with DNA laddering (Rink et al. 1995), which we have shown occurs in necrotic neurons, or (3) have suggested neuronal necrosis (Whalen et al. 2008), based on early propidium iodide labeling (their Figs. 2,3,5 and 6) and ultrastructure (their Fig. 9B and 9C).

17.3 The Time Course of the Appearance of Necrotic Neurons Following Seizure Onset

How soon after the onset of prolonged seizures does neuronal death begin to appear? A study of the evolution of neuronal death from seizures has shown that after 40 min of seizures, acidophilic neurons by H & E stain, the light-microscopic equivalent of ultrastructural neuronal necrosis (Fujikawa et al. 1999, 2000a, 2002, 2007) appeared in 14 brain regions, with more appearing in more brain regions as seizure duration and recovery periods increased (Fujikawa 1996). The maximum amount of seizure-induced neuronal necrosis occurred 24 h after 3-h SE. For example, in the piriform cortex 24 h after 3-h SE, neuronal death occurred in greater than 75% of the neurons (Fujikawa 1996). Thus, investigating recovery periods longer than 24 h in generalized seizures will not contribute to an understanding of the programmed mechanisms responsible for killing the neurons, because neuronal necrosis will already have occurred in the vast majority of neurons.

17.4 Apoptosis and the Caspase-Dependent Pathways of Cell Death

In general, caspase activation, via either the extrinsic, death-receptor pathway involving activation of caspase-8, or the intrinsic, mitochondrial pathway involving activation of caspase-9, with subsequent downstream activation of caspase-3 by either pathway, is associated with cellular apoptosis. It is also true that in general, caspase activation occurs in immature cells in vitro or in vivo and in immortalized cell lines. Despite this, there are in vivo studies that describe caspase activation in SE in the adult rodent brain (Henshall et al. 2000; Kondratyev and Gale 2000).

Kondratyev and colleagues showed neuroprotection with a caspase inhibitor in hippocampus, which did not show caspase-3 activation (Kondratyev and Gale 2000). The reason is that the caspase inhibitor, z-DEVD-fmk, is not specific for caspases (Rozman-Pungerčar et al. 2003; Knoblach et al. 2004; Bizat et al. 2005). With respect to the other study, the reason for the discrepancy is not clear. Although the caspase-3 zymogen and not the active fragment was used for immunofluorescence microscopy, a DEVD cleavage assay for caspase-3-like activity showed an eightfold increase 8 h after 45-min focal seizures (they used 40-min seizures in subsequent studies) (Henshall et al. 2000). The same group has shown upstream caspase activation in both the extrinsic and intrinsic pathways (Henshall et al. 2001a, b). The model that they used, 40 min of focal SE, could possibly explain the difference in results. In this respect, their results were confirmed by another group using their model but with 60 min of SE (Li et al. 2006).

However, there is evidence that caspase activation is age-dependent, and that it occurs in hypoxia-ischemia in the neonatal and not the adult brain (Hu et al. 2000; Liu et al. 2004). Moreover, it has been shown that neither the extrinsic caspase-8 death-receptor pathway nor the intrinsic mitochondrial caspase-9 death receptor pathway is activated in neurons following generalized SE in the adult rat (Fujikawa et al. 2007), and caspase-3, the central downstream effector caspase, is also not activated in neurons after generalized SE in the adult brain (Fujikawa et al. 2002; Narkilahti et al. 2003; Takano et al. 2005; Araújo et al. 2008).

17.5 Caspase-Independent Programmed Mechanisms in Necrotic Cell Death

Earlier chapters by Rieckher and Tavernarakis, Krantic and Susin, Kang et al. and Liu et al. have pointed out that in pathologically induced cell death that programmed mechanisms other than caspase activation, which occurs most often in morphologically apoptotic cells, are activated. In the nematode *C. elegans* hyperactive mutations of ion channels result in cellular necrosis, which is similar to excitotoxic neuronal death in rodents (see chapter by Rieckher and Tavernarakis). In *C. elegans* lysosomal involvement in cellular necrosis has been shown in mutants defective in lysosomal function (Artal-Sanz et al. 2006). In wild-type nematodes, cathepsins are released and because of an acidic intracellular pH, brought about by the vacuolar H^+-ATPase in lysosomal membranes, the result is cellular necrosis (Syntichaki et al. 2005). As in transient global ischemia in primates (see chapter by Tonchev and Yamashima), calpain I (μ-calpain), the calcium-dependent cytoplasmic cysteine protease, is involved because downregulation of calpains and cathepsins by RNA interference (RNAi) reduces cell death (Syntichaki et al. 2002). In addition, AIF translocation to nuclei is dependent on calpain I activation in hippocampus in TGI (Cao et al. 2007); (also see chapter by Liu et al.).

In mouse embryonic fibroblasts in cell culture, Moubarak and colleagues showed, using a large panel of gene knockout cells, that DNA damage by an

alkylating agent or inhibition of a nuclear enzyme (topoisomerase II) brought about cellular necrosis by an orderly sequence of events: poly(ADP-ribose) polymerase-1 (PARP-1) activation, calpain I activation, Bax translocation from cytosol to mitochondria, followed by apoptosis-inducing factor (AIF) release from mitochondria and translocation to nuclei, producing necrotic cell death (Moubarak et al. 2007); (also see chapter by Krantic and Susin).

Finally, NMDA application to cortical neurons in culture produces an excitotoxic death characterized by PARP-1 activation, which forms poly(ADP-ribose) (PAR) polymers that translocate to mitochondria, releasing AIF, which translocates to nuclei, resulting in neuronal death (Yu et al. 2002, 2006; Andrabi et al. 2006) (also see chapter by Kang et al.). The authors call this cell death "parthanatos," from "PAR" and "thanatos," or death. Morphologically, this neuronal death, with shrunken, pyknotic nuclei by fluorescence microscopy, corresponds to neuronal necrosis (Fujikawa et al. 1999, 2000a, b, 2002, 2007). Olney's group does not recognize the word "necrosis," saying that it is a word for "death" and is therefore meaningless in terms of cell death classification. However, many investigators world-wide use the word to refer to a particular type of cell death that corresponds to what we have characterized as "neuronal necrosis" and to what the Olney group calls "excitotoxic neuronal death." They are one and the same.

17.5.1 Caspase-Independent Programmed Mechanisms in Seizure-Induced Neuronal Necrosis

The controversy regarding the activation and importance of caspases in SE-induced neuronal necrosis has already been addressed. There is no controversy as to the importance of caspase-independent programmed mechanisms in SE-induced neuronal necrosis. The recent study by Moubarak and colleagues (Moubarak et al. 2007) showed the sequential activation of PARP-1 and calpain I, then Bax translocation to mitochondrial membranes, followed by AIF release from mitochondria and translocation to cellular nuclei, a process that they called "programmed necrosis." They showed this in mouse embryonic fibroblasts in cell culture, in which DNA damage was created by exposure to a DNA alkylating agent or by inhibition of the nuclear enzyme topoisomerase II. These results provide a unifying hypothesis that could apply to excitotoxic neuronal death in general and SE-induced neuronal necrosis in particular.

PARP-1, AIF, Calpain I, Cytochrome c and Endonuclease G

In generalized SE both PARP-1 (Wang et al. 2007) and calpain I activation (Araújo et al. 2008; Wang et al. 2008) have been shown to contribute to hippocampal neuronal death (Figs. 17.2–17.5), and in the studies by Wang et al. (2007, 2008), there was associated translocation of AIF to nuclei, with reduction of AIF translocation with

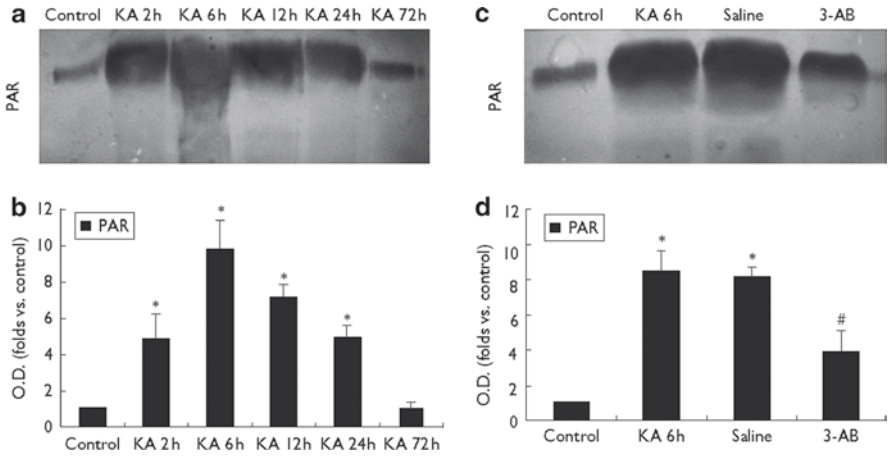

Fig. 17.2 Effects of PARP-1 inhibition on poly(ADP-ribose) (PAR) polymer formation in the rat hippocampus following 60-min SE. (**a**) shows the time course of PAR formation in rat hippocampus from 2 to 72 h after 60-min SE, and (**c**) shows the effect of 3-aminobenzamide (3-AB), a PARP-1 inhibitor, given i.p. 15 min before intracerebroventricular (i.c.v.) injection of kainic acid, 6 h after SE. Increases in PAR occurred 2, 6, 12 and 24 h after SE, with the maximal increase 6 h after SE (**b**). When compared to SE hippocampus, 6 h after SE, 3-AB inhibited PAR formation by more than 50% (**d**). From Wang et al. (2007), with permission from Lippincott Williams & Wilkins

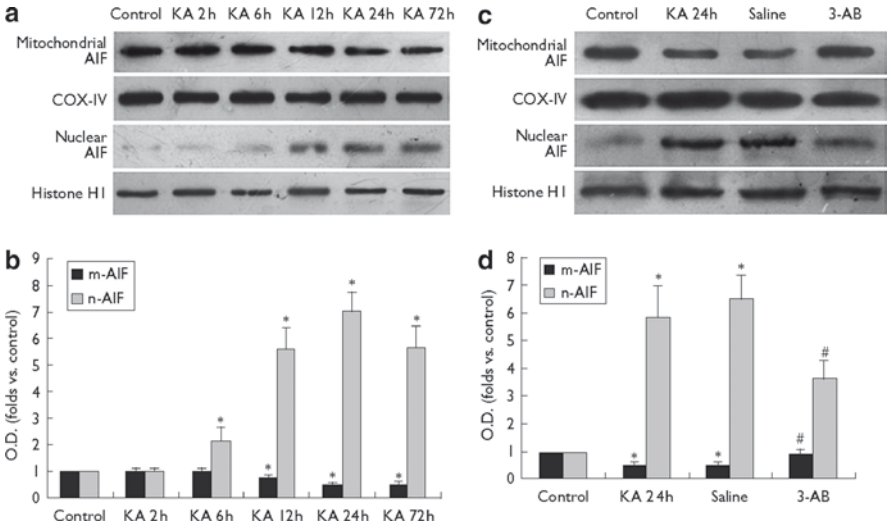

Fig. 17.3 Effects of the PARP-1 inhibitor 3-aminobenzamide (3-AB) on nuclear translocation of AIF following 60-min SE. (**a**) shows the time course of AIF translocation to hippocampal nuclei from 2 to 72 h after SE, and (**c**) shows the effect of 3-AB, given i.p. 15 min before i.c.v. kainic acid injection, 24 h after SE. Increases in nuclear AIF occurred 6, 12, 24 and 72 h after SE, with the maximal increase 24 h after SE (**b**). 3-AB reduced nuclear translocation of AIF by 50% (**d**). COX-IV is a marker of the mitochondrial fraction and Histone H1 is a marker of the nuclear fraction. From Wang et al. (2007), with permission from Lippincott Williams & Wilkins

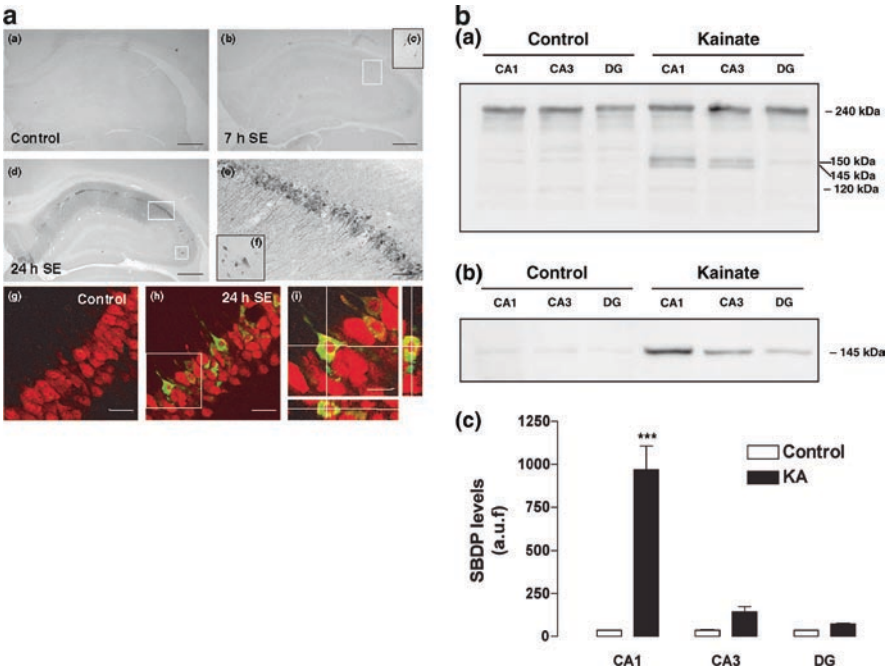

Fig. 17.4 Calpain activation 24 h after the onset of kainic acid-induced SE, assessed by expression of the calpain I-cleaved fragment of αII-spectrin (spectrin breakdown product, or SBDP). In **a**, SBDP expression occurred in dorsal hippocampal CA1 neurons 24 h after seizures began, but not in controls and only minimally 7 h after seizures began (**a**–**f**). (**c**) is the inset of the *white-boxed area* in (**b**), (**e**) is the *larger white-boxed area* in (**d**), and (**f**) is a higher magnification photomicrograph of pyramidal neurons in the *smaller white boxed area* in (**d**). Scale bars 400 μm in (**a**, **b**) and (**d**) and 50 μm in (**e**). (**g**–**i**) are confocal photomicrographs of hippocampal pyramidal neurons expressing the neuronal marker NeuN (*red*) and SBDP (*green*). SBDP appeared 24 h after seizure onset (**h**) and (**i**) but not in a saline-injected control (**g**). Scale bars 20 μm in (**g**) and (**h**) and 8 μm in (**i**). **b** shows western blots of lysates from hippocampal CA1, CA3 and dentate gyrus (DG), demonstrating in (**a**) full-length αII-spectrin (240 kDa) and SBDPs 150, 145 and 120 kDa, with the appearance of the calpain-cleaved 150 and 145 kDa SBDPs but not the caspase-3-cleaved 120 kDa SBDP 24 h after SE. (**b**) shows results using an antibody that recognizes only the 145 kDa calpain SBDP, and (**c**) shows quantitation of (**b**), with a markedly increased 145 kDa SBDP in CA1. From Araújo et al. (2008), with permission from Blackwell Publishing

Fig. 17.5 (continued) after SE, with maximal expression 3 days after SE. Cleaved (activated) caspase-3 (20 kD) appeared 1–7 days after SE, with maximal expression 7 days after SE. (Narkilahti et al. (2003) showed that late activation of caspase-3 occurs in astrocytes, not neurons). In **b** (**C**) and (**D**) and (**C′**) and (**D′**) show that the calpain I inhibitor MDL 28170 reduced significantly the formation of calpain-cleaved αSpII and truncated (15 kDa) Bid, AIF translocation from mitochondria to nuclei and Cyt C translocation from mitochondria to cytosol. B-actin is a loading control, COX-IV is a marker of the mitochondrial fraction and "histone" is a marker of the nuclear fraction. From Wang et al. (2008), with permission from Elsevier Inc

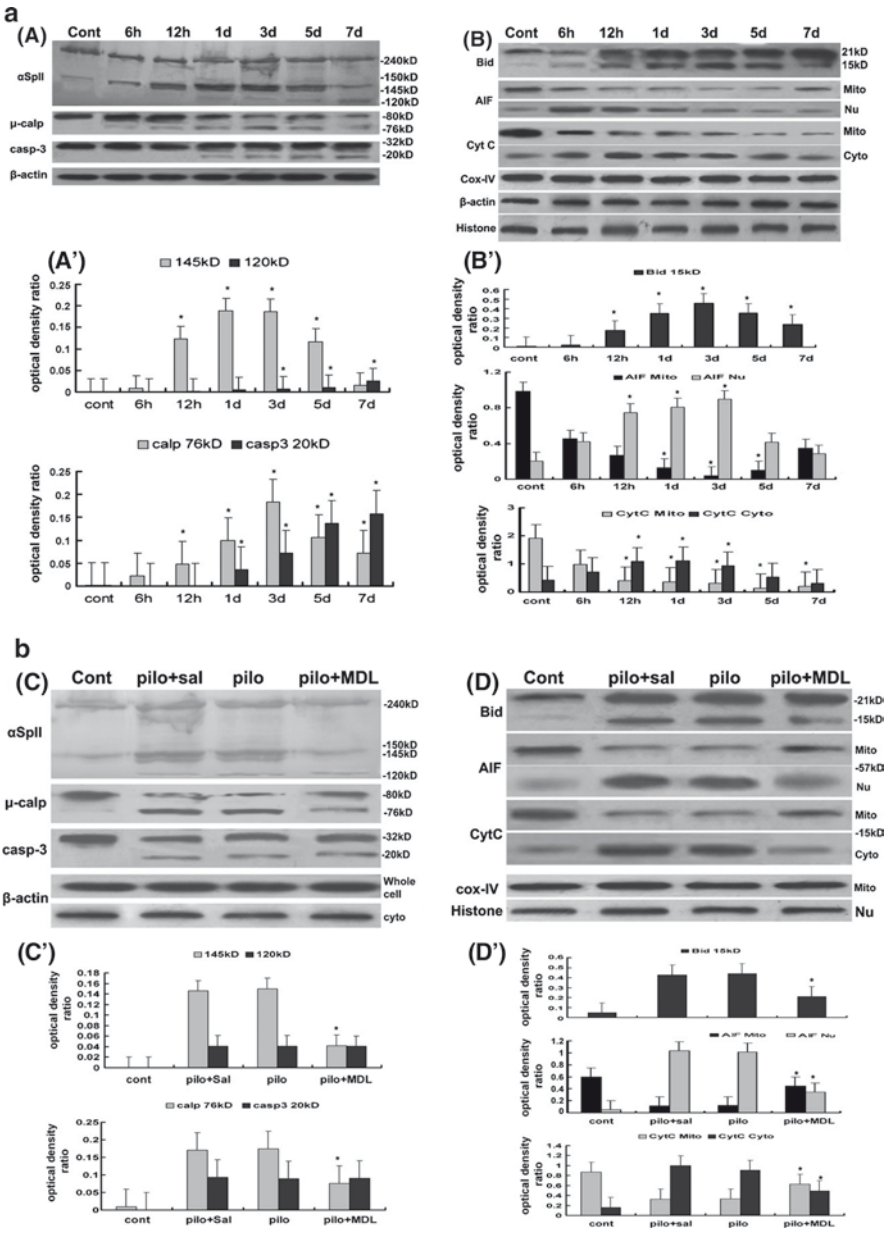

Fig. 17.5 In **a** (**A**) and (**B**) show western blots of αII-spectrin (αSpII), calpain I (μ-calp), caspase-3 (casp-3), Bid, AIF and cytochrome *c* (Cyt C) from 6 h to 7 days after 2-h lithium-pilocarpine-induced SE (LPCSE). (**A′**) and (**B′**) show quantitation of (**A**) and (**B**). The calpain-I cleaved 145 kDa αSpII fragment increased significantly 12 h and 1, 3 and 5 days after SE, cleaved Bid (15 kDa) was significantly elevated 12 h and 1–7 days after SE, AIF translocated from mitochondria to nuclei 12 h and 1 and 3 days after SE, and Cyt C translocated from mitochondria to cytosol 12 h and 1 and 3 days after SE. Cleaved (activated) calpain I (76 kD) appeared 12 h and 1–7 days

PARP-1 and calpain I inhibitors (Figs. 17.2, 3, and 5). However, in the studies by Wang et al. (2007, 2008) they did not confirm translocation of AIF to neuronal nuclei, as only western blots were done. In this regard, Narkilahti and colleagues showed that late activation of caspase-3 following SE occurs almost exclusively in astrocytes and not neurons (Narkilahti et al. 2003). As mentioned previously, Moubarak and colleagues have shown that PARP-1 activation leads to calpain I activation and AIF release from mitochondria and translocation to nuclei, with subsequent cell death (Moubarak et al. 2007).

In addition to the previously cited studies, AIF has been shown to be necessary for SE-induced hippocampal neuronal death in AIF-deficient Harlequin mice (Cheung et al. 2005) and in minocycline-treated mice (Heo et al. 2006). Finally, mitochondrial endonuclease G (endoG) has been shown to translocate to neuronal nuclei 5 days following KASE (Wu et al. 2004). AIF is associated with large-scale (50 kilobase) DNA cleavage (Susin et al. 1999) and endoG produces internucleosomal (180 base-pair) DNA cleavage (Li et al. 2001; Parrish et al. 2001).

There is also a study in which wild-type and calpastatin-deficient mice were subjected to KASE by focal injection of KA into hippocampus (Takano et al. 2005). Calpastatin is an endogenous inhibitor of calpain I. The authors found that AIF and endoG translocation to hippocampal nuclei occurred in the calpastatin-deficient mice, and that caspase-3, the principal effector caspase, was not activated. As was noted in section 17.4, "Apoptosis and the Caspase-Dependent Pathways of Cell Death," other studies have also noted a lack of caspase-3 activation following generalized SE (Fujikawa et al. 2002; Narkilahti et al. 2003; Araújo et al. 2008), and neither caspase-9 nor caspase-8, initiator caspases in the intrinsic (mitochondrial) and extrinsic pathways, respectively, are activated following lithium-pilocarpine-induced SE (LPCSE) (Fujikawa et al. 2007).

We have found that the mitochondrial proteins cytochrome *c* (cyt *c*) and AIF and the enzyme endoG translocate to neuronal nuclei in the piriform cortex within the first 60 min of generalized SE, persisting and becoming more widespread as neurons become shrunken and necrotic 6 and 24 h after 3-h lithium-pilocarpine-induced SE (LPCSE) (Zhao et al. Submitted for publication). In the LPCSE seizure model, maximal neuronal necrosis occurs 24 h following 3-h SE (Fujikawa 1996; Fujikawa et al. 1999, 2000a, b, 2002, 2007). Cytochrome *c* (in vivo) has not so far been shown to translocate to cellular nuclei, and so its presence there quite early with persistence thereafter raises interesting questions, which we will attempt to answer in future studies.

Translocation of cytochrome *c* to the cytoplasm is known to activate caspase-9, through formation of an "apoptosome" with Apaf-1 and dATP (Li et al. 1997). Cytochrome *c* translocation to cytoplasm has been reported in generalized SE, without evidence of caspase activation (Heo et al. 2006) or with late caspase-3 activation, days after calpain I activation (Wang et al. 2008). Narkilahti et al. (2003) showed that in generalized SE that activated caspase-3 appeared primarily in astrocytes in hippocampus 2 and 7 days after SE. The increasing amount of activated caspase-3 from 1 to 7 days after SE found by Wang et al. (2008) by western blotting could also have occurred in astrocytes, as immunohistochemistry was not done.

A unique in vitro study found that DNA damage to HeLa cells or cerebellar granule cells in culture produced nuclear translocation of cyt *c* within 60 min (Nur-E-Kamal et al. 2004). This was associated with translocation of acetylated histone H2A from the nucleus to the cytoplasm and with chromatin condensation. Our in vivo results confirm nuclear translocation of cyt *c* within the first 60 min of generalized SE (Zhao et al. Submitted for publication). Nuclear translocation of cyt *c* points to a heretofore unexplored pathological mechanism for this mitochondrial respiratory chain protein.

Lysosomal Cathepsins, DNase II, Calpain I, and Reactive Oxygen Species

Calpain I has been shown to bind to lysosomal membranes, with subsequent release of the cysteinyl protease cathepsin B to the cytoplasm and DNase II to the nucleus of hippocampal neurons following transient global ischemia in primates (Yamashima et al. 1996, 1998; Tsukada et al. 2001). Recently, it was shown in rat hippocampal slices subjected to oxygen-glucose deprivation that NMDA-receptor activation activates calpain I, which causes lysosomal membrane permeabilization (LMP) and the release of cathepsins B and D to cytosol (Windelborn and Lipton 2008). In this model, superoxide ($^{\bullet}O_2^-$) levels were increased, and reducing reactive oxygen species (ROS) levels blocked LMP. In addition, NMDA-receptor blockade, as well as inhibition of pathways producing arachidonic acid, blocked LMP and ROS production (Windelborn and Lipton 2008).

We have also found that in addition to the mitochondrial proteins AIF and cyt *c* and the enzyme endoG, the lysosomal enzymes cathepsins B and D and DNase II translocate to neuronal nuclei in the piriform cortex within the first 60 min of generalized SE, persisting and becoming more widespread as neurons become shrunken and necrotic 6 and 24 h after 3-h lithium-pilocarpine-induced SE (LPCSE) (Zhao et al. Submitted for publication). As mentioned previously, maximal neuronal necrosis occurs 24 h following 3-h LPCSE (Fujikawa 1996; Fujikawa et al. 1999, 2000a, b, 2002, 2007). Like cyt *c* (in vivo), neither cathepsin B or D has been shown to translocate to cellular nuclei in vitro or in vivo. As with cyt *c*, their presence there quite early, with persistence thereafter, raises interesting questions which we will also investigate in future studies.

p53 in Status Epilepticus

p53, a tumor-suppressor gene, is activated by SE, and inhibition of protein synthesis prevents p53 mRNA expression and neuronal death (Schreiber et al. 1993; Sakhi et al. 1994) (also see chapter by Tan and Schreiber). p53 has also been shown to regulate the Bax-dependent, mitochondrial caspase cell death pathway (Chipuk et al. 2004). As we have shown that KASE produces morphologically necrotic neurons, these findings suggest another programmed pathway activated by programmed necrosis.

Autophagy in Status Epilepticus

Recently, there has been an increasing amount of attention to type II programmed cell death (Clarke 1990), or macroautophagy, most often referred to simply as "autophagy." Classically, autophagic mechanisms are triggered by starvation, in which internal digestion of cellular components within autophagosomes is an attempt to keep cells alive. Controversy has centered on whether in pathologic insults autophagy is an attempt to protect cells or whether it actively contributes to cell death (Edinger and Thompson 2004; Shintani and Klionsky 2004; Codogno and Meijer 2005; Tsujimoto and Shimizu 2005). A recent report has shown that KASE induces transient autophagic stress in mouse hippocampus, manifested by an increase of LC3-II, a specific marker of autophagic vacuoles, 4–6 h after systemic KA injection (Shacka et al. 2007). This does not, of course, establish whether autophagy contributes to SE-induced neuronal necrosis or whether it is simply a bystander effect. Further studies are warranted.

17.6 Translating an Understanding of Mechanisms to a Neuroprotective Strategy in Refractory Human Status Epilepticus

The most direct and theoretically most effective neuroprotective approach in excitotoxic neuronal death in general, and SE in particular, would be to administer an NMDA-receptor antagonist. By blocking postsynaptic NMDA receptors, excessive calcium influx would be prevented, thereby preventing activation of downstream biochemical pathways that produce neuronal necrosis. However, there are two caveats to this simplistic approach. The first is that NMDA-receptor antagonists have been shown to produce delayed neuronal necrosis in adult rats (Fix et al. 1993) and neuronal apoptosis in neonatal rats (Ikonomidou et al. 1999). However, in the adult brain, preventing or ameliorating the widespread, severe neuronal necrosis produced by SE should take precedence over any lesser and less widespread adverse effects of NMDA-receptor antagonists.

We advocated the use of NMDA-receptor antagonists following SE more than 10 years ago, based upon their remarkable neuroprotective effects (Fujikawa et al. 1994; Fujikawa 1995). We and others showed years ago that NMDA-receptor antagonists are remarkably neuroprotective in the face of ongoing seizure discharges, which are not eliminated by NMDA-receptor antagonists, including ketamine (Fariello et al. 1989; Clifford et al. 1990; Fujikawa et al. 1994; Fujikawa 1995). The conclusion of these studies is that ketamine and other NMDA-receptor antagonists provide neuroprotection that is independent of an antiepileptic effect. Despite this, ketamine has finally been advocated for use in refractory SE in humans, not because of its neuroprotective properties, but because of a delayed antiepileptic effect (Borris et al. 2000; Bleck 2005). Borris et al. (2000) showed that given after 60 min of spontaneous SE from electrical hippocampal stimulation in the rat, ketamine abolished seizure discharges,

and that it was ineffective in doing so after 15 min of SE. This is consistent with our data, but as we have shown, after 60 min of SE, neuronal necrosis will already have begun to occur in many brain regions (Fujikawa 1996). Immediate use of ketamine should be considered because of its neuroprotective properties, with the added benefit of a potential delayed antiepileptic effect.

The second caveat is that once NMDA-receptor-mediated calcium influx has begun, receptor blockade will have diminishing efficacy the longer after seizure onset it is instituted. The influx of calcium activates enzymes such as calpain I and neuronal nitric oxide synthase (nNOS), the latter of which produces nitric oxide (NO), which in turn forms peroxynitrite ($ONOO^-$), a free radical that can damage DNA, activating PARP-1. Activation of calpain I and PARP-1 results in the release of death-promoting proteins and enzymes from mitochondria and lysosomes that cause the degradation of cytoplasmic proteins and nuclear chromatin. Thus, administration of a cocktail of inhibitors of, for example, NMDA receptors, calpain I, nNOS and PARP-1, to name but a few currently known essential targets, would seem to have the best chance of salvaging still-viable neurons, but with diminishing returns the longer the SE continues.

References

Andrabi SA, Kim S-W, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD, Poirier GG, Dawson VL, Dawson TM (2006) Poly(ADP-ribose) (PAR) polymer is a death signal. Proc Natl Acad Sci USA 103:18308–18313

Araújo IM, Gil JM, Carreira BP, Mohapel P, Petersen A, Pinheiro PS, Soulet D, Bahr BA, Brundin P, Carvalho CM (2008) Calpain activation is involved in early caspase-independent neurodegeneration in the hippocampus following status epilepticus. J Neurochem 105:666–676

Artal-Sanz M, Samara C, Syntichaki P, Tavernarakis N (2006) Lysosomal biogenesis and function is critical for necrotic cell death in Caenorhabditis elegans. J Cell Biol 173:231–239

Auer RN, Kalimo H, Olsson Y, Siesjo BK (1985a) The temporal evolution of hypoglycemic brain damage. I. Light- and electron-microscopic findings in the rat cerebral cortex. Acta Neuropathol (Berl) 67:13–24

Auer RN, Kalimo H, Olsson Y, Siesjo BK (1985b) The temporal evolution of hypoglycemic brain damage. II. Light- and electron-microscopic findings in the hippocampal gyrus and subiculum of the rat. Acta Neuropathol (Berl) 67:25–36

Bizat N, Galas MC, Jacquard C, Boyer F, Hermel JM, Schiffmann SN, Hantraye P, Blum D, Brouillet E (2005) Neuroprotective effect of zVAD against the neurotoxin 3-nitropropionic acid involves inhibition of calpain. Neuropharmacology 49:695–702

Bleck TP (2005) Refractory status epilepticus. Curr Opin Crit Care 1:117–120

Borris DJ, Bertram EH, Kaipur J (2000) Ketamine controls prolonged status epilepticus. Epilepsy Res 42:117–122

Brown AW (1977) Structural abnormalities in neurones. J Clin Pathol 30(Suppl 11):155–169

Bruhn T, Cobo M, Berg M, Diemer NH (1992) Limbic seizure-induced changes in extracellular amino acid levels in the hippocampal formation: a microdialysis study of freely moving rats. Acta Neurol Scand 86:455–461

Cao G, Xing J, Xiao X, Liou AK, Gao Y, Yin XM, Clark RS, Graham SH, Chen J (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. J Neurosci 27:9278–9293

Cheung EC, Melanson-Drapeau L, Cregan SP, Vanderluit JL, Ferguson KL, McIntosh WC, Park DS, Bennett SA, Slack RS (2005) Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms. J Neurosci 25:1324–1334

Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M, Green DR (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303:1010–1014

Clarke PGH (1990) Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol 181:195–213

Clifford DB, Olney JW, Benz AM, Fuller TA, Zorumski CF (1990) Ketamine, phencyclidine, and MK-801 protect against kainic-acid-induced seizure-related brain damage. Epilepsia 31:382–390

Codogno P, Meijer AJ (2005) Autophagy and signaling: their role in cell survival and cell death. Cell Death Differ 12(Suppl 2):1509–1518

Colbourne F, Sutherland GR, Auer RN (1999) Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J Neurosci 19:4200–4210

Colicos MA, Dash PK (1996) Apoptotic morphology of dentate granule cells following experimental cortical impact injury in rats: possible role in spatial memory deficits. Brain Res 739:120–131

Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16:663–669

Evans MC, Griffiths T, Meldrum BS (1984) Kainic-acid seizures and the reversibility of calcium loading in vulnerable neurons in the hippocampus. Neuropathol Appl Neurobiol 10:285–302

Fariello RG, Golden GT, Smith GG, Reyes PF (1989) Potentiation of kainic acid epileptogenicity and sparing from neuronal damage by an NMDA receptor antagonist. Epilepsy Res 3: 206–213

Fix AS, Horn JW, Wightman KA et al (1993) Neuronal vacuolization and necrosis induced by the noncompetitive N-methyl-D-aspartate (NMDA) antagonist MK(+)801 (dizocilpine maleate): a light and electron microscopic evaluation of the rat retrosplenial cortex. Exp Neurol 123:204–215

Fujikawa DG (1995) The neuroprotective effect of ketamine administered after status epilepticus onset. Epilepsia 36:186–195

Fujikawa DG (1996) The temporal evolution of neuronal damage from pilocarpine-induced status epilepticus. Brain Res 725:11–22

Fujikawa DG (2000) Confusion between neuronal apoptosis and activation of programmed cell death mechanisms in acute necrotic insults. Trends Neurosci 23:410–411

Fujikawa DG (2002) Apoptosis: ignoring morphology and focusing on biochemical mechanisms will not eliminate confusion. Trends Pharmacol Sci 23:309–310

Fujikawa DG (2005) Prolonged seizures and cellular injury: understanding the connection. Epilepsia 7:S3–S11

Fujikawa DG, Daniels AH, Kim JS (1994) The competitive NMDA-receptor antagonist CGP 40116 protects against status epilepticus-induced neuronal damage. Epilepsy Res 17:207–219

Fujikawa DG, Shinmei SS, Cai B (1999) Lithium-pilocarpine-induced status epilepticus produces necrotic neurons with internucleosomal DNA fragmentation in adult rats. Eur J Neurosci 11:1605–1614

Fujikawa DG, Shinmei SS, Cai B (2000a) Kainic acid-induced seizures produce necrotic, not apoptotic, neurons with internucleosomal DNA cleavage: implications for programmed cell death mechanisms. Neuroscience 98:41–53

Fujikawa DG, Shinmei SS, Cai B (2000b) Seizure-induced neuronal necrosis: implications for programmed cell death mechanisms. Epilepsia 41(Suppl 6):S9–S13

Fujikawa DG, Ke X, Trinidad RB, Shinmei SS, Wu A (2002) Caspase-3 is not activated in seizure-induced neuronal necrosis with internucleosomal DNA cleavage. J Neurochem 83:229–240

Fujikawa DG, Shinmei SS, Zhao S, Aviles ER Jr (2007) Caspase-dependent programmed cell death pathways are not activated in generalized seizure-induced neuronal death. Brain Res 1135:206–218

Griffiths T, Evans M, Meldrum BS (1983) Intracellular calcium accumulation in rat hippocampus during seizures induced by bicuculline or L-allylglycine. Neuroscience 10:385–395

Griffiths T, Evans MC, Meldrum BS (1984) Status epilepticus: the reversibility of calcium loading and acute neuronal pathological changes in the rat hippocampus. Neuroscience 12:557–567

Henshall DC, Chen J, Simon RP (2000) Involvement of caspase-3-like protease in the mechanism of cell death following focally evoked limbic seizures. J Neurochem 74:1215–1223

Henshall DC, Bonislawski DP, Skradski SL, Araki T, Lan J-Q, Schindler CK, Meller R, Simon RP (2001a) Formation of the Apaf-1/cytochrome c complex precedes activation of caspase-9 during seizure-induced neuronal death. Cell Death Diff 8:1169–1181

Henshall DC, Bonislawski DP, Skradski SL, Lan J-Q, Meller R, Simon RP (2001b) Cleavage of Bid may amplify caspase-8-induced neuronal death following focally evoked limbic seizures. Neurobiol Dis 8:568–580

Heo K, Cho Y-J, Cho K-J, Kim H-W, Kim H-J, Shin HY, Lee BI, Kim GW (2006) Minocycline inhibits caspase-dependent and -independent cell death pathways and is neuroprotective against hippocampal damage after treatment with kainic acid in mice. Neurosci Lett 398:195–200

Hu BR, Liu CL, Ouyang Y, Blomgren K, Siejö BK (2000) Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. J Cereb Blood Flow Metab 20:1294–1300

Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler V, Dikranian K, Tenkova TI, Stefovska V, Turksi L, Olney JW (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283:70–74

Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Horster F, Tenkova T, Dikranian K, Olney JW (2000) Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287:1056–1060

Ishimaru MJ, Ikonomidou C, Tenkova TI, Der TC, Dikranian K, Sesma MA, Olney JW (1999) Distinguishing excitotoxic from apoptotic neurodegeneration in the developing rat brain. J Comp Neurol 408:461–476

Knoblach SM, Alroy DA, Nikolaeva M, Cernak I, Stoica BA, Faden AI (2004) Caspase inhibitor z-DEVD-fmk attenuates calpain and necrotic cell death in vitro and after traumatic brain injury. J Cereb Blood Flow Metab 24:1119–1132

Kondratyev A, Gale K (2000) Intracerebral injection of caspase-3 inhibitor prevents neuronal apoptosis after kainic acid-evoked status epilepticus. Mol Brain Res 75:216–224

Lallement G, Carpentier P, Collet A, Pernot-Marino I, Baubichon D, Blanchet G (1991) Effects of soman-induced seizures on different extracellular amino acid levels and on glutamate uptake in rat hippocampus. Brain Res 563:234–240

Lehmann A, Hagberg H, Jacobson I, Hamberger A (1985) Effects of status epilepticus on extracellular amino acids in the hippocampus. Brain Res 359:147–151

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–489

Li L, Luo X, Wang X (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412:95–99

Li T, Lu C, Xia Z, Xiao B, Luo Y (2006) Inhibition of caspase-8 attenuates neuronal death induced by limbic seizures in a cytochrome c-dependent and Smac/DIABLO-independent way. Brain Res 1098:204–211

Liu CL, Siesjö BK, Hu BR (2004) Pathogenesis of hippocampal neuronal death after hypoxia-ischemia changes during brain development. Neuroscience 127:113–123

Millan MH, Chapman AG, Meldrum BS (1993) Extracellular amino acid levels in hippocampus during pilocarpine-induced seizures. Epilepsy Res 14:139–148

Moubarak RS, Yuste VJ, Artus C, Bouharrour A, Greer PA, Menissier-de Murcia J, Susin SA (2007) Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. Mol Cell Biol 27:4844–4862

Narkilahti S, Pirtillä TJ, Lukasiuk K, Tuunanen J, Pitkänen A (2003) Expression and activation of caspase 3 following status epilepticus. Eur J Neurosci 18:1486–1496

Nur-E-Kamal A, Gross SR, Pan Z, Balklava Z, Ma J, Liu LF (2004) Nuclear translocation of cytochrome c during apoptosis. J Biol Chem 279:24911–24914

Olney JW (1969) Brain lesions, obesity and other disturbances in mice treated with monosodium glutamate. Science 164:719–721

Olney JW (1971) Glutamate-induced neuronal necrosis in the infant mouse hypothalamus. An electron microscopic study. J Neuropathol Exp Neurol 30:75–90

Olney JW (1985) Excitatory transmitters and epilepsy-related brain damage. In: Smythies JR, Bradley RJ (eds) International review of neurobiology, vol 27. Academic, Orlando, pp 337–362

Olney JW, Rhee V, Ho OL (1974) Kainic acid: a powerful neurotoxic analogue of glutamate. Brain Res 77:507–512

Parrish J, Li L, Klotz K, Ledwich D, Wang X, Xue D (2001) Mitochondrial endonuclease G is important for apoptosis in *C elegans*. Nature 412:90–94

Rink A, Fung KM, Trojanowski JQ, Lee VM-Y, Neugebauer E, McIntosh TK (1995) Evidence of apoptotic cell death after experimental traumatic brain injury in the rat. Am J Pathol 147:1575–1583

Rozman-Pungerčar J, Kopitar-Jerala N, Bogyo M, Turk D, Vasiljeva O, Štefe I, Vandenabeele P, Brőmme D, Pulzdar V, Fonović M, Trstenjak-Prebanda M, Dolenc I, Turk V, Turk B (2003) Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity. Cell Death Diff 10:881–888

Sakhi S, Bruce A, Sun N, Tocco G, Baudry M, Schreiber SS (1994) p53 induction is associated with neuronal damage in the central nervous system. Proc Natl Acad Sci USA 91:7525–7529

Schreiber SS, Tocco G, Najm I, Thompson RF, Baudry M (1993) Cycloheximide prevents kainate-induced neuronal death and c-fos expression in adult rat brain. J Mol Neurosci 4:149–159

Shacka JJ, Lu J, Xie ZL, Uchiyama Y, Roth KA, Zhang J (2007) Kainic acid induces early and transient autophagic stress in mouse hippocampus. Neurosci Lett 414:57–60

Shintani T, Klionsky DJ (2004) Autophagy in health and disease: a double-edged sword. Science 306:990–995

Smolders I, Van Belle K, Ebinger G, Michotte Y (1997) Hippocampal and cerebellar extracellular amino acids during pilocarpine-induced seizures in freely moving rats. Eur J Pharmacol 319:21–29

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446

Syntichaki P, Xu K, Driscoll M, Tavernarakis N (2002) Specific aspartyl and calpain proteases are required for neurodegeneration in *C. elegans*. Nature 419:939–944

Syntichaki P, Samara C, Tavernarakis N (2005) The vacuolar H+-ATPase mediates intracellular acidification required for neurodegeneration in C. elegans. Curr Biol 15:1249–1254

Takano J, Tomioka M, Tsubuki S, Higuchi M, Nobuhisa Iwata N, Itohara S, Maki M, Saido TC (2005) Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. J Biol Chem 280:16175–16184

Tanaka K, Graham SH, Simon RP (1996) The role of excitatory neurotransmitters in seizure-induced neuronal injury in rats. Brain Res 737:59–63

Tsujimoto Y, Shimizu S (2005) Another way to die: autophagic programmed cell death. Cell Death Differ 12(Suppl 2):1528–1534

Tsukada T, Watanabe M, Yamashima T (2001) Implications of CAD and DNase II in ischemic neuronal necrosis specific for the primate hippocampus. J Neurochem 79:1196–1206

Wade JV, Samson FE, Nelson SR, Pazdernik TL (1987) Changes in extracellular amino acids during soman- and kainic acid-induced seizures. J Neurochem 49:645–650

Wang SJ, Wang SH, Song ZF, Liu XW, Wang R, Chi ZF (2007) Poly(ADP-ribose) polymerase inhibitor is neuroprotective in epileptic rat via apoptosis-inducing factor and Akt signaling. Neuroreport 18:1285–1289

Wang S, Wang S, Shan P, Song Z, Dai T, Wang R, Chi Z (2008) mu-Calpain mediates hippocampal neuron death in rats after lithium-pilocarpine-induced status epilepticus. Brain Res Bull 76:90–96

Whalen MJ, Dalkara T, You Z, Qiu J, Bermpohl D, Mehta N, Suter B, Bhide PG, Lo EH, Ericsson M, Moskowitz MA (2008) Acute plasmalemmal permeability and protracted clearance of injured cells after controlled cortical impact in mice. J Cereb Blood Flow Metab 28:490–505

Windelborn JA, Lipton P (2008) Lysosomal release of cathepsins causes ischemic damage in the rat hippocampal slice and depends on NMDA-mediated calcium influx, arachidonic acid metabolism, and free radical production. J Neurochem 106:56–69

Wu Y, Dong M, Toepfer NJ, Fan Y, Xu M, Zhang J (2004) Role of endonuclease G in neuronal excitotoxicity in mice. Neurosci Lett 264:203–207

Yamashima T, Saido TC, Takita M, Miyazawa A, Yamano J, Miyakawa A, Nishiyo H, Yamashima J, Kawashima S, Ono T, Yoshioka T (1996) Transient brain ischemia provokes Ca2+, PIP2 and calpain responses prior to delayed neuronal death in monkeys. Eur J Neurosci 8:1932–1944

Yamashima T, Kohda Y, Tsuchiya K, Ueno T, Yamashita J, Yoshioka T, Kominami E (1998) Inhibition of ischaemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074: a novel strategy for neuroprotection based on 'calpain-cathepsin hypothesis'. Eur J Neurosci 10:1723–1733

Yu S-W, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Yu S-W, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Proc Natl Acad Sci USA 103:18314–18319

Zhao S, Aviles ER Jr, Fujikawa DG (Submitted for publication) Nuclear translocation of mitochondrial cytochrome *c* and lysosomal cathepsins B and D within the first 60 minutes of generalized seizures

Concluding Remarks

Denson G. Fujikawa

An underlying theme of this book is that acute pathological neurological processes, despite their apparent widely varying presentations, have at their core the central underlying triggering mechanism – excitotoxicity. That is because excessive neuronal depolarization, with excessive glutamate (GLU) release, both presynaptically and from reversal of astrocytic GLU uptake, occurs in each condition – cerebral ischemia, traumatic central nervous system (CNS) injury, hypoglycemia, and status epilepticus (SE). In a way, SE is the "purest" form of excitotoxicity, because the other processes have superimposed upon excessive neuronal depolarization an additional and modifying condition, be it reduced or absent cerebral blood flow (CBF) for a period of time, with or without reperfusion, traumatic impact to brain or spinal cord, or a low glucose concentration for a period of time, with or without resumption of a normal concentration. The excessive release of GLU activates an excessive number of ionotropic postsynaptic *N*-methyl-D-aspartate (NMDA) receptors, allowing excessive Ca^{2+} influx into these neurons, thereby triggering the biochemical cascades that produce irreversible damage and cell death.

The cysteine proteases that cleave proteins at aspartate (Asp) residues (caspases) have been studied extensively, both in vitro and, to a lesser extent, in vivo. Two primary caspase cascades are triggered within cells destined for a morphologically apoptotic death – the intrinsic (mitochondrial) caspase-9 pathway, and the extrinsic death receptor Fas caspase-8 pathway. Both initiator caspases activate the principal effector caspase, caspase-3, which when cleaved translocates to cell nuclei. Active caspase-3 can activate caspase-activated DNase (CAD, also known as DNA fragmentation factor 40, or DFF 40) by cleaving its heterodimer, inhibitor of CAD (ICAD, or DFF 45), which dissociates from CAD (Liu et al. 1997, 1998; Enari et al. 1998; Sakahira et al. 1998).

However, in recent years caspase-independent mechanisms of programmed cell death have been described (Kitanaka and Kuchino 1999; Denecker et al. 2001; Proskuryakov et al. 2003; Syntichaki and Tavernarakis 2003). These mechanisms are evolutionarily conserved within the CNS from nematodes (see Riekher and Tavernarakis, Chap. 2) to mouse cortical neurons in cell culture (see Kang et al., Chap. 5) to hippocampal neurons in vivo in primates subjected to transient global ischemia (see Tonchev and Yamashima, Chap. 10). This book has focused on the

caspase-independent programmed mechanisms triggered by excessive Ca^{2+} influx into postsynaptic neurons, in vitro and in vivo.

Mitochondrial mechanisms have been studied most extensively, especially those involving the Ca^{2+}-activated cysteine protease calpain I (μ-calpain), apoptosis-inducing factor (AIF), and poly(ADP-ribose) polymerase-1 (PARP-1). There is less information regarding lysosomal mechanisms, but it is apparent that calpain-mediated lysosomal cathepsin release is important for neuronal necrosis in the rat hippocampal slice subjected to oxygen-glucose deprivation (Windelborn and Lipton 2008) and in transient global ischemia in the primate (Yamashima et al. 1998; Yamashima 2000; Yamashima et al. 2003; Yamashima 2004). We have found that in generalized (also referred to as "limbic") SE both mitochondrial and lysosomal death-promoting proteins and enzymes translocate to neuronal nuclei as early as 60 min after seizure onset (Zhao et al. submitted for publication). Nuclear mechanisms, which are important and to date have been focused primarily on brief descriptions of large-scale (50 kb) and internucleosomal (180 base-pair) DNA cleavage, chromatin condensation and nuclear pyknosis (shrinkage), and autophagy, are very likely to be the areas of increasing interest in the future. Not discussed in this book are mechanisms involving the endoplasmic reticulum and unfolded protein response, and those involving fragmentation of the Golgi apparatus (Mukherjee et al. 2007; Nakagomi et al. 2008; Nogueira et al. 2008), both of which are important and should be considered in any comprehensive assessment of programmed cell death mechanisms.

Because of the wide variety of intracellular events that are triggered by an excitotoxic stimulus, it would seem that all should be taken in consideration in describing what happens to neurons after the inciting stimulus and in formulating a strategy of neuroprotection. Of course, blocking NMDA receptors can prevent activation of the downstream intracellular events that are the subject of this book. Delayed NMDA-receptor blockade can prevent further Ca^{2+} influx, but cannot undo activation of calpain I, PARP-1, nNOS, and reactive oxygen species (ROS) production. So it is remarkable that inhibition of a single enzyme, such as calpain I and PARP-1, or trapping free radicals with a single agent, or inhibiting mitochondrial membrane permeability with a single drug, such as cyclosporin A or minocycline, has proven to be so successful in rescuing neurons. A possible explanation is sequential activation of, for example, activated PARP-1 causing activation of calpain I, as shown by Moubarak et al. (2007), so that inhibition of either would have the same effect. However, mitochondrial Ca^{2+} uptake, with subsequent production of ROS (see Davis and Sullivan, Chap. 11), which disrupts plasma and intracellular membranes, would still occur, and the converse, reducing ROS alone, would not affect PARP-1 and calpain I activation. So administering a cocktail of these inhibitors and drugs simultaneously might provide greater benefit. If not, would it mean that our conceptual framework is faulty and that we should rethink our conceptual approach to these intracellular events? Whatever the answers to these questions, they will undoubtedly point future research toward new and exciting directions.

Personal Blurb for Springer Book

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References

Denecker G, Vercammen D, Declerq W, Vandermeele P (2001) Apoptotic and necrotic cell death induced by death domain receptors. Cell Mol Life Sci 58:356–370

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391:43–50

Kitanaka C, Kuchino Y (1999) Caspase-independent programmed cell death with necrotic morphology. Cell Death Diff 6:508–515

Liu X, Peng L, Widlak P, Zou H, Luo X, Garrard WT, Wang X (1998) The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc Natl Acad Sci USA 95:8461–8466

Liu X, Zou H, Slaughter C, Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89:175–184

Moubarak RS, Yuste VJ, Artus C, Bouharrour A, Greer PA, Menissier-de Murcia J, Susin SA (2007) Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. Mol Cell Biol 27:4844–4862

Mukherjee S, Chiu R, Leung SM, Shields D (2007) Fragmentation of the Golgi apparatus: an early apoptotic event independent of the cytoskeleton. Traffic 8:369–378

Nakagomi S, Barsoum MJ, Bossy-Wetzel E, Sutterlin C, Malhotra V, Lipton SA (2008) A Golgi fragmentation pathway in neurodegeneration. Neurobiol Dis 29:221–231

Nogueira E, Fidalgo M, Molnar A, Kyriakis J, Force T, Zalvide J, Pombo CM (2008) SOK1 translocates from the Golgi to the nucleus upon chemical anoxia and induces apoptotic cell death. J Biol Chem 283:16248–16258

Proskuryakov SY, Knoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? Exp Cell Res 283:1–16

Sakahira H, Enari M, Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96–99

Syntichaki P, Tavernarakis N (2003) The biochemistry of neuronal necrosis: rogue biology? Nat Rev Neurosci 4:672–684

Windelborn JA, Lipton P (2008) Lysosomal release of cathepsins causes ischemic damage in the rat hippocampal slice and depends on NMDA-mediated calcium influx, arachidonic acid metabolism, and free radical production. J Neurochem 106(1):56–69

Yamashima T (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. Prog Neurobiol 62:273–295

Yamashima T (2004) Ca^{2+}-dependent proteases in ischemic neuronal death: a conserved 'calpain-cathepsin cascade' from nematodes to primates. Cell Calcium 36:285–293

Yamashima T, Kohda Y, Tsuchiya K, Ueno T, Yamashita J, Yoshioka T, Kominami E (1998) Inhibition of ischaemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074: a novel strategy for neuroprotection based on 'calpain-cathepsin hypothesis'. Eur J Neurosci 10:1723–1733

Yamashima T, Tonchev AB, Tsukada T, Saido TC, Imajoh-Ohmi S, Momoi T, Kominami E (2003) Sustained calpain activation associated with lysosomal rupture executes necrosis of the postischemic CA1 neurons in primates. Hippocampus 13:791–800

Zhao S, Aviles ER, Jr., Fujikawa DG Nuclear translocation of mitochondrial cytochrome *c* and lysosomal cathepsins B and within the first 60 minutes of generalized seizures (submitted for publication)

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