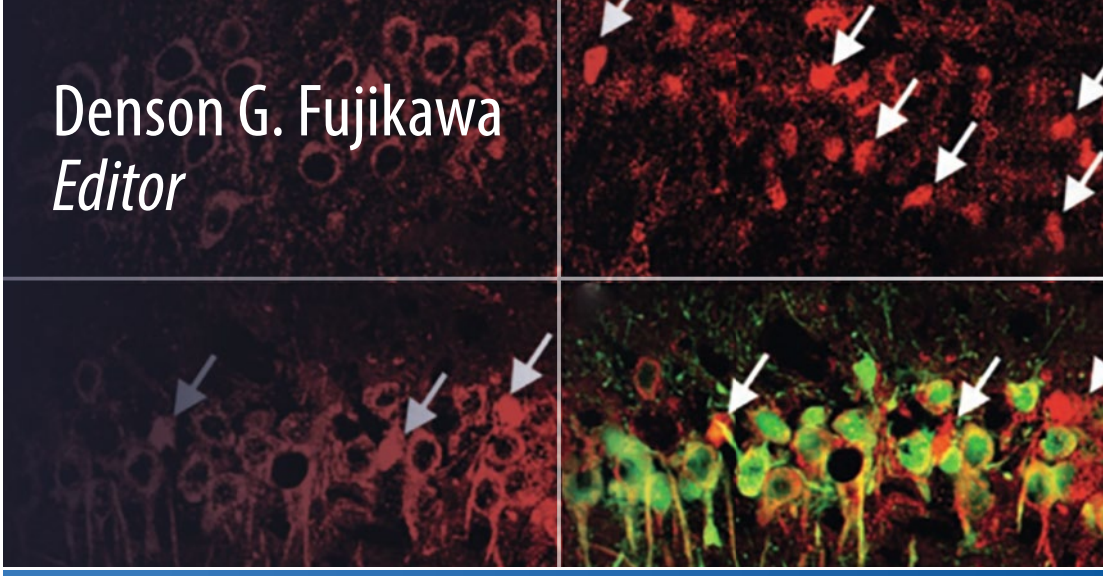

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Editor
Denson G. Fujikawa
Department of Neurology
VA Greater Los Angeles Healthcare System
North Hills, CA, USA

Department of Neurology and Brain Research Institute
David Geffen School of Medicine
University of California at Los Angeles
Los Angeles, CA, USA

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Cover Caption: AIF translocation in vivo following global ischemia is prevented by overexpression of calpastatin. Representative immunofluorescence of apoptosis-inducing factor (AIF, red) from non-ischemic CA1 (a) or 72 h after global ischemia (b– d). AAV–calpastatin, a calpain inhibitory protein (c, d), or the empty vector (b) was infused 14 d before ischemia, and brain sections were double-label immunostained for AIF (red) and calpastatin overexpression (green, d). Note that the 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)

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This book is dedicated to the memory of John W. Olney, M.D. (1932–2015), the father of excitotoxicity.

Introduction

In the Introduction to the first edition of this book, the history behind the concept of excitotoxicity was described, from John Olney's initial descriptions of the phenomenon (Olney 1969, 1971; Olney et al. 1974) through subsequent studies identifying the mechanisms by which excessive glutamate release presynaptically and by reversal of astrocytic glutamate uptake results in postsynaptic neuronal death. A synaptic mechanism was identified in 1983 (Rothman 1983), followed by identification of calcium entry via the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor (MacDermott et al. 1986) and excessive calcium entry through the NMDA receptor-operated cation channel as the mechanism by which neurons died (Choi 1987; Choi et al. 1987). Early electron-microscopic studies of neuronal death from 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) showed electron-dense, shrunken neurons with pyknotic nuclei containing irregular chromatin clumps and dilated mitochondria, which the authors called "dark-cell degeneration." These were morphologically identical to the neurons that Olney found following exposure to glutamate or its analogues. We now call these neurons "necrotic," to differentiate them from "apoptotic" neurons (Fujikawa 2000, 2002), both of which die from different programmed mechanisms.

As was emphasized in the First Edition, excitotoxicity underlies all acute neuronal injuries, from cerebral ischemia, traumatic CNS injury, status epilepticus and hypoglycemia (Fujikawa 2010). Excessive intracellular calcium activates the cytosolic calcium-dependent enzymes calpain I and neuronal nitric oxide synthase (nNOS). Among other actions, calpain I is responsible for mitochondrial release of cytochrome *c*, apoptosis-inducing factor (AIF) and endonuclease G (endoG), and lysosomal release of cathepsins B and D and DNase II, all of which translocate to the neuronal nucleus and participate in its destruction. nNOS uses L-arginine as a substrate to form nitric oxide (NO), which reacts with superoxide (O_2-) to form the toxic free radical peroxynitrite ($ONOO-$). Peroxynitrite, with other free radicals generated by mitochondria exposed to the high intracellular calcium concentration (Beal 1996), damages the plasma membrane, mitochondrial and lysosomal membranes and causes double-stranded nuclear DNA cleavage.

The nuclear DNA repair enzyme poly(ADP-ribose) polymerase-1 (PARP-1) produces poly(ADP-ribose) (PAR) polymers to repair DNA double-strand breaks, and excess PAR polymers exit nuclei and translocate to mitochondria membranes, where they, in addition to calpain I, trigger the exit of AIF from mitochondrial membranes to neuronal nuclei (Andrabi et al. 2006; Yu et al. 2006), where it recruits migration inhibitory factor (MIF), a PARP-1-dependent, AIF-associated nuclease (PAAN) to the nucleus, where MIF cleaves single-stranded DNA into large-scale DNA fragments (Wang et al. 2016). Ted and Valina Dawson in their chapter provide details of the PARP-1 pathway.

Two new areas are covered in the current edition: the role of extra-synaptic NMDA receptors in excitotoxic necrosis and a separate necrotic pathway uncovered by inhibition of caspase-8 *in vitro*: necroptosis. The first topic was first described 14 years ago (Hardingham and Bading 2002) but was not covered in the first edition of the book. Evidence was put forth that it is extra-synaptic NMDA receptors that are responsible for excitotoxicity by inhibiting cAMP-response element binding protein (CREB) activity and brain-derived neurotrophic factor (BDNF) gene expression, whereas synaptic NMDA receptors did the opposite and actually provides a neuroprotective effect that is overwhelmed by extra-synaptic NMDA-receptor activation (Hardingham and Bading 2002). Dr. Michel Baudry in his chapter reinforces this concept and gives evidence that calpain I (also known as μ-calpain) is activated by synaptic NMDA receptors, whereas calpain II (also known as m-calpain) is activated by extra-synaptic NMDA receptors. On the other hand, Dr. Jun Chen's group in their chapter of the First Edition provided evidence that calpain I activation activates AIF by cleaving it in the mitochondrial membrane, which results in its exit from the mitochondrial membrane and translocation to neuronal nuclei; they have updated their chapter for this edition.

In recent years another necrotic pathway has been described, which has been dubbed "necroptosis" (Degterev et al. 2005). In cell culture, after inhibition of caspases with a broad-spectrum caspase inhibitor (z-VAD.fmk), investigators have found that cells subjected to a lethal insult had a necrotic morphology, and that the pathway involved three key proteins: receptor-interacting protein 1 and 3 (RIP1 and RIP3; also known as RIP1 kinase and RIP3 kinase) and mixed lineage kinase domain-like protein (MLKL) (Degterev et al. 2008; Sun et al. 2012). This pathway has been shown to occur *in vivo* in cerebral ischemia (Yin et al. 2015; Miao et al. 2015; Xu et al. 2016; Vieira et al. 2014) and traumatic brain injury (Liu et al. 2016). Drs. Vieira and Carvalho in their chapter provide evidence that oxygen-glucose deprivation (OGD) of hippocampal neurons *in vitro* and transient global cerebral ischemia (TGCI) *in vivo* up-regulate RIP3 and induce necroptotic neuronal necrosis. Overexpression of RIP3 worsened and knock-down of RIP3 reduced necroptosis in OGD. Dr. Tao in his chapter shows that the necroptotic pathway is activated following traumatic brain injury and that Necrostatin-1 (Nec-1), a RIP1 inhibitor, is neuroprotective.

If both the excitotoxic and necroptotic pathways are activated following acute neuronal injury, do each contribute separately to neuronal necrosis, producing an additive effect, or are there interactions between the two, and if so, what are they

and what is the outcome? The non-competitive NMDA-receptor antagonist MK-801 and the nNOS inhibitor 7-nitroindazole both reduced RIP3 nitrosylation and neuronal necrosis in the hippocampal CA1 region following TGCI (Miao et al. 2015). On the other hand, cathepsin B release from lysosomes, which occurs in excitotoxicity, was reduced by Nec-1 following TGCI (Yin et al. 2015). So there appears to be cross-talk between the two programmed necrotic pathways. Further interactions between the two pathways and their consequences will undoubtedly be elucidated in future research.

Department of Neurology
VA Greater Los Angeles Healthcare System
North Hills, CA, USA

Denson G. Fujikawa

Department of Neurology and Brain Research Institute
David Geffen School of Medicine,
University of California at Los Angeles
Los Angeles, CA, USA

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Contents

Part I
General Considerations

Chapter 1
Excitotoxic Programmed Cell Death Involves Caspase-Independent Mechanisms

Ted M. Dawson and Valina L. Dawson

Abstract Excitotoxicity is a common pathological process in many neurologic and 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 followed by nuclear accumulation of macrophage migration inhibitory factor (MIF). MIF's nuclease activity serves as the final executioner in excitotoxicity by shredding genomic DNA. Interfering with PARP activation, PAR signaling or MIF nuclease activity offers therapeutic interventions that could protect against a variety of neuronal injury due to a variety of insults involving glutamate excitotoxicity.

Keywords Poly (ADP-ribose) polymerase-1 (PARP-1) · Parthanatos · Poly (ADP-ribose) (PAR) · Apoptosis inducing factor (AIF) · Macrophage migration inhibitory factor (MIF) · Glutamate

1.1 Introduction

In conditions like brain trauma, ischemia/reperfusion, stroke, neurodegenerative diseases, brain injury, and cellular stress, neurons, glia and the vasculature succumb to cell death processes. Cell death, in general, is a complex process involving multiple pathways (Galluzzi et al. 2012, 2015). Apoptosis and necrosis are the two traditional described pathways of cell death based on morphology. Apoptosis involves the formation of apoptotic bodies that are cleared through mechanisms that avoid

T. M. Dawson (✉) · V. L. Dawson
Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, USA
e-mail: vdawson@jhmi.edu; tdawson@jhmi.edu

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eliciting inflammatory responses. Necrosis, on the other hand, involves massive cell swelling, inflammation and rupture of cellular structures. The morphologic classification of cell death has now been replaced by biochemical definitions (Galluzzi et al. 2012) in which there are different forms of regulated and programmed cell death (Galluzzi et al. 2015). Accidental cell death has been coined as the form of cell death that involved rupture of cellular structures. During the development of living organisms or homeostatic control of cell number in mature organisms, cell death is a programmed physiological process that is executed by the activation of caspases; and has an apoptotic morphology. Regulated necrosis is defined by biochemical assessments that are quantifiable and these pathways share many common biochemical events as well as morphologic features of both necrosis and apoptosis in cell death execution (Dawson and Dawson 2017). This chapter reviews the underlying mechanisms accounting for glutamate excitotoxicity that primarily involves regulated necrosis and in particular, parthanatos (Fatokun et al. 2014).

1.2 Excitotoxicity

Excitotoxicity is a process of neuronal injury mediated by excitatory amino acids (Arundine and Tymianski 2004; Lai et al. 2014; Mehta et al. 2013; 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 (Lai et al. 2014; Mehta et al. 2013; Meldrum 1992). α-Amino-3-hydroxy-5-methyl-4- isoxalone propionic acid (AMPA) and kainate receptors are activated by AMPA and by kainate, respectively (Nakanishi 1992). These non-NMDA-type receptors are also involved in excessive intracellular calcium influx and excitotoxicity (Arundine and Tymianski 2003, 2004; Dawson and Dawson 2017). 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, phospholipases and other Ca^{2+}-dependent enzymes (Dawson and Dawson 2017). The activation of nNOS occurs through postsynaptic density-95 (PSD-95) dependent mechanisms since PSD-95 inhibitors prevent the downstream consequences of glutamate excitotoxicity (Aarts et al. 2002; Sattler et al. 1999;

Soriano et al. 2008; Sun et al. 2008). nNOS activation leads to the overproduction of NO through the conversion of L-arginine to L-citrulline. NO can exert many roles as a signaling molecule in neurons (Dawson et al. 1992). Generation of excess NO can be neurotoxic (Dawson et al. 1991, 1993; Samdani et al. 1997). NO can combine with $O_2^{\bullet-}$ in the mitochondria to generate more toxic peroxynitrite ($ONOO^-$), which can cause oxidative or nitrosative injury to cellular proteins, lipids and DNA (Beckman and Koppenol 1996; Szabo and Dawson 1998; Xia et al. 1996). Injury to DNA causes a massive activation of poly (ADP-ribose) polymerase-1, which ultimately triggers cell death via the process of parthanatos (Dawson and Dawson 2017; Fatokun et al. 2014) (Fig. 1.1). PARP-1 can also be activated through mechanisms that do not involve DNA damage, in that aminoacyl-tRNA synthetase complex interacting multifunctional protein-2 (AIMP2) directly activates PARP-1 initiating parthanatos (see Fig. 1.3) (Lee et al. 2013).

1.3 Role of PARP-1 and PAR Polymer in Excitotoxicity

Poly (ADP-ribose) polymerases (PARPs) are known to play key roles in DNA repair (de Murcia and Menissier de Murcia 1994; de Murcia et al. 1994). PARP-1 is the founding member of the PARP family, which includes 18 different isoforms based on protein sequence homology to the PARP-1 catalytic domain (Hottiger et al. 2010). 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) (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 (Chatterjee et al. 1999). 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 (Beck et al. 2014) (Fig. 1.2). 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, diabetes and many other disorders (Fatokun et al. 2014; Virag and Szabo 2002). In the nervous system, PARP-1 activation is triggered by excitotoxic stimuli (Eliasson et al. 1997; Mandir et al. 2000; Zhang et al. 1994).

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^+ (Fatokun et al. 2014; 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).

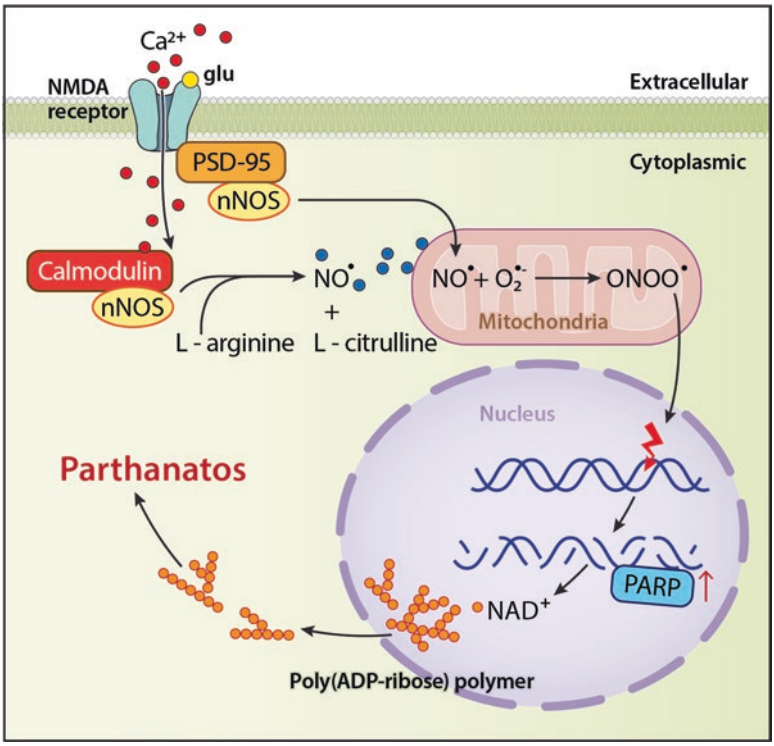

Fig. 1.1 PARP-1 and PAR mediated cell death in excitotoxicity. Over-stimulation of NMDA receptors by glutamate (yellow circle) results in the influx of Ca^{2+} (red circles), which binds calmodulin and activates neuronal nitric oxide (NO) synthase (nNOS), to convert L-arginine to NO and L-citrulline. nNOS is tethered to the NMDA receptor via postsynaptic density-95 (PSD-95) protein. 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 ($O_2^{\bullet-}$). Peroxynitrite damages DNA, which results in over activation of PARP (PARP↑), depletion of NAD+, and generation of PAR polymer, leading to parthanatos

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; Goto et al. 2002). 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^+ (Dawson and Dawson 2017; 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

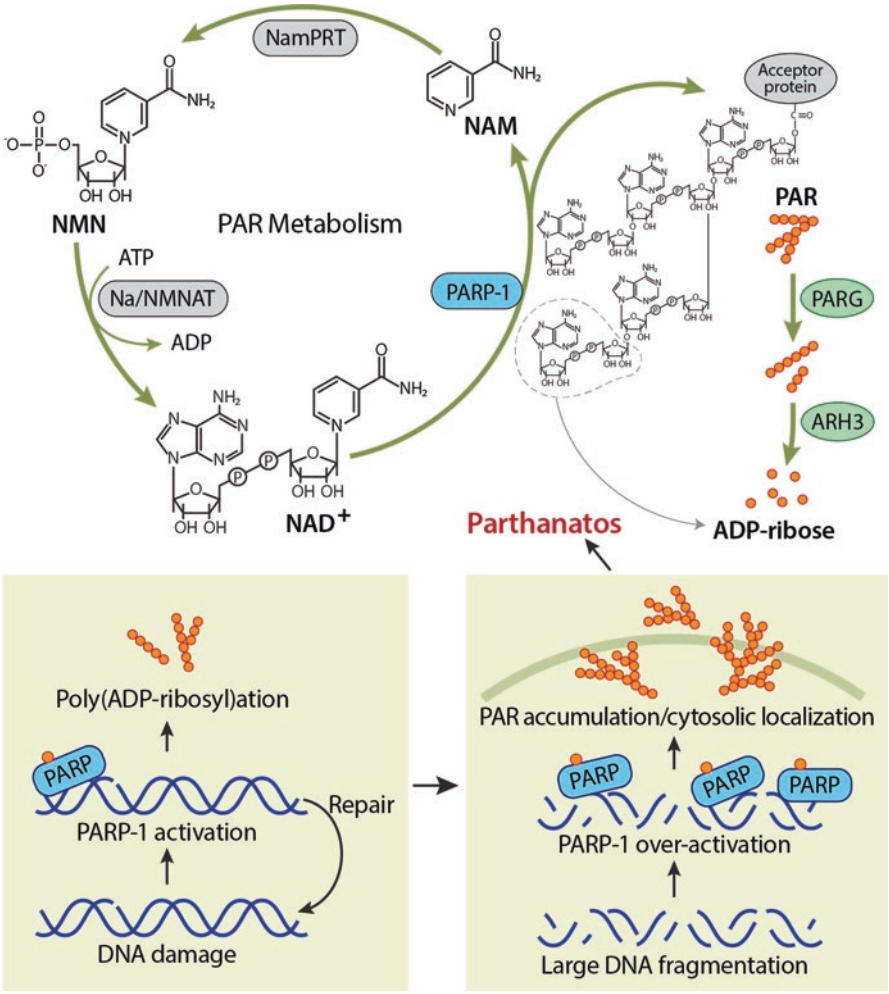

Fig. 1.2 PAR metabolism. PARP-1 utilizes NAD+ as a substrate for synthesis of PAR polymers. In the process of PAR formation, nicotinamide (NAM), a product of NAD+ hydrolysis, is first converted into nicotinamide mononucleotide (NMN) and then into NAD+ by nicotinamide phosphoribosyl transferase (NamPRT) and nicotinamide mononucleotide adenylyl transferases (Na/NMNAT-1, -2, and -3), respectively. Mild DNA damage or breaks activate the activation of 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. Poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribose-(arginine) protein hydrolase-3 (ARH3) degrade PAR polymers

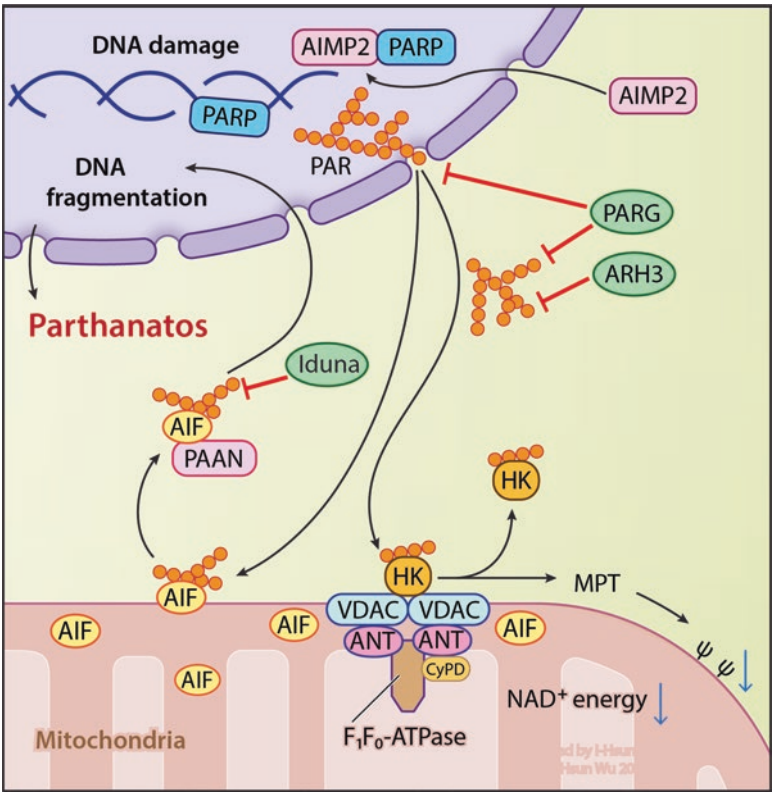

Fig. 1.3 Parthanatos. In parthanatos, a caspase-independent cell death, apoptosis inducing factor (AIF) acts as the mitochondrial factor to mediate cell death. DNA damage or aminoacyl tRNA synthetase complex interacting multifunctional protein (AIMP2) activates poly (ADP-ribose) (PAR) polymerase 1 (PARP-1). PAR generated by activation of PARP-1 translocates from the nucleus to the mitochondria, where it binds AIF, inducing AIF release from the mitochondria. AIF then binds the parthanatos AIF associated nuclease (PAAN) where they translocate to the nucleus and PAAN shreds genomic DNA acting as the final executioner in parthanatos. PAR polymer also binds and inactivates hexokinase (HK), which accounts for the energy depletion due to activation of PARP-1. PARG or ARH3 degrade PAR polymer, preventing parthanatos. Iduna, a PAR- dependent ubiquitin E3 ligase, is an inhibitor of parthanatos. Abbreviations: *ANT* adenine nucleotide translocase, *CyPD* cyclophilin D, *F_1F_0-ATPase* ATP synthase, *MPT* mitochondrial membrane permeability transition, *VDAC* voltage-dependent anion channel, *Ψ* mitochondrial membrane potential. Figure adapted from Dawson and Dawson (2017)

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 due to a toxic accumulation of PAR. PAR, generated by PARP-1 in the nucleus, travels to the cytosol to induce cell death (Fig. 1.1). 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 several 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 roles of individual PARP isoforms in 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 for neuronal excitotoxicity (Dawson and Dawson 2017; Fatokun et al. 2014). 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 (Yu et al. 2002). Caspase activation, which is a hallmark of apoptotic cell death, does not play a primary role in parthanatos, as broad-spectrum caspase inhibitors, knockout of Apoptotic protease activating factor-1 (APAF-1) or BAX are unable to protect cells (Cregan et al. 2004; Yu et al. 2002). 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.

1.4 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 (Fig. 1.2). Many genes encoding different PARPs have been identified, whereas only a single gene encoding 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). 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 (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 shows that a 39-kDa ADP-ribose-(arginine) protein hydrolase (ARH3) has PARG-like activity (Oka et al. 2006). ARH3 seems to play a neuroprotective role as well by reducing PAR levels in the cytoplasm that are generated by PARG, preventing the release of AIF by PAR polymer (Mashimo et al. 2013).

1.5 Mitochondria in PAR-Induced Cell Death: Role of AIF and Hexokinase

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 (Cyt C), AIF, Smac/Diablo, and Omi/HtrA2 (Green and Kroemer 2004; Kroemer et al. 2007; Newmeyer and Ferguson-Miller 2003; Tait and Green 2010). 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 the cytoplasm ensures that the brake that IAPs provide on caspase activation is removed. Release of Cyt-C into the cytosol leads to APAF-1 binding and initiates cell death through assembly of the apoptosome complex (Li et al. 1997; Liu et al. 1996; Zou et al. 1997). Besides Cyt C and APAF-1, the apoptosome requires pro-caspase 9 as the initiator caspase and dATP. In this complex, caspase 9 is cleaved and activated, which in turn activates downstream caspases that include the effector caspase, caspase 3 (Green and Kroemer 2004; Kroemer et al. 2007; Newmeyer and Ferguson-Miller 2003; Tait and Green 2010). Active caspase 3 has many cellular substrates, including α-foldrin, PARP-1, plasma membrane Ca^{2+} pump (PMCA) and inhibitor of caspase- activated DNase (ICAD). Caspase-activated DNase (CAD) 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). Although there is evidence that caspase activation occurs during excitotoxic cell death and in a variety of injuries to the nervous system, it seems to play a secondary role in excitotoxic cell death (Dawson and Dawson 2017; Lipton 1999).

AIF, on the other hand, directly translocates to the nucleus to initiate large scale chromatin condensation and caspase-independent cell death during glutamate excitotoxicity (Fig. 1.3) (Cregan et al. 2004; Wang et al. 2004, 2011, 2016; Yu et al. 2002).

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; Wang et al. 2004; Yu et al. 2002, 2006). Moreover, PARP-1 activation is required for AIF release from the mitochondria since knockout of PARP-1 completely prevents AIF translocation from the mitochondria to the nucleus (Wang et al. 2004; Yu et al. 2002). PAR generated from PARP-1 is necessary and sufficient to induce the release of AIF from the mitochondria where PAR directly binds to mitochondrial AIF (Andrabi et al. 2006; Wang et al. 2011; Yu et al. 2006). Mutating the PAR binding domain of AIF prevents the release of AIF from the mitochondria and is protective against glutamate excitotoxicity (Wang et al. 2011). In addition to PARG, Iduna (RNF146) prevents glutamate excitotoxicity by preventing AIF translocation from the mitochondria to the nucleus by interfering with PAR signaling (Andrabi et al. 2011). Iduna is a PAR-dependent ubiquitin E3 ligase that targets PARylated or PAR binding proteins for proteasomal degradation (Callow et al. 2011; Kang et al. 2011; Zhang et al. 2011). Future experiments are required to identify Iduna substrates that play a role in parthanatos. How PAR leaves the nucleus and translocates to the mitochondria also remains an unresolved issue, but there are likely PAR binding proteins or PARylated proteins that translocate from the nucleus to the mitochondria after PARP-1 activation (Dawson and Dawson 2017). Some of these may be Iduna substrates.

PAR also binds to hexokinase (HK) during parthanatos, where PAR inhibits HK activity (Andrabi et al. 2014; Fouquerel et al. 2014). This leads to inhibition of glycolysis, which accounts for the reduction in NAD^+ and ATP that has been observed following PARP-1 activation (Alano et al. 2010; Ha and Snyder 1999). Thus, the energy collapse following PARP-1 activation is not directly due the consumption of NAD^+ by PARP-1 activity, but instead is due to PAR- dependent inhibition of HK (Fig. 1.3). The mitochondrial substrates glutamine and pyruvate can rescue the PAR-HK dependent defects in glycolysis, supporting the notion that the reduction in NAD^+ is due to PAR inhibition of HK (Andrabi et al. 2014; Dawson and Dawson 2017; Fouquerel et al. 2014).

1.6 Parthanatos Associated AIF Nuclease (PAAN)

Once AIF enters the nucleus it causes the nucleus to undergo nuclear condensation and genomic DNA is cleaved into large (20–50 Kb) fragments. Although AIF binds to DNA, it does not have any nuclease activity, and it was assumed that AIF binds to and activates a DNA nuclease. The identity of this nuclease has remained a mystery for almost two decades. Recently our group definitively identified the first parthanatos- associated AIF nuclease (PAAN) as macrophage migration inhibitory

factor (MIF) (Fig. 1.3) (Wang et al. 2016). MIF is a Mg^{2+}/Ca^{2+} dependent nuclease. Following PARP-1 activation AIF binds to MIF and carries it into the nuclease where MIF cleaves genomic DNA into large fragments. Neuronal culture containing MIF mutants that lack nuclease activity or fail to bind to AIF are resistant to glutamate excitotoxicity and stimuli that induce parthanatos. Moreover, mice with MIF mutants that lack nuclease activity or fail to bind to AIF are resistant to stroke (Wang et al. 2016). The development of MIF nuclease inhibitors as neuroprotective agents holds particular promise as they would not interfere with DNA repair, like PARP inhibitors (Dawson and Dawson 2017).

1.7 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 pro-cell death factors. In the case of parthanatos, cell death is initiated by nuclear translocation and mitochondrial release of AIF. PAR polymer induces the structural change of a number of cellular proteins by either the process of PARylation by PARP or through non-covalent interactions. Recent studies indicate that human neurons predominantly die via parthanatos in response to glutamate excitotoxicity indicating that this form of cell death is particularly important to human neurologic injury (Xu et al. 2016). Inhibition of PARP-1 and 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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Chapter 2
To Survive or to Die: How Neurons Deal with it

Yubin Wang, Xiaoning Bi, and Michel Baudry

Abstract Unlike the majority of cells in the organism, neurons have only two options during their entire existence, to survive or to die. As a result, they have evolved elaborate mechanisms to determine which path they will follow in response to a multitude of internal and external signals, and to the wear-and-tear associated with the aging process. Until recently, activation of the calcium-dependent protease, calpain, had been traditionally associated with neurodegeneration. This chapter will review recent findings that indicate that two of the major calpain isoforms present in the brain, calpain-1 and calpain-2, play opposite functions in neuronal survival/death. Thus, calpain-1 activation, downstream of synaptic NMDA receptors, is part of a neuronal survival pathway through the truncation of PHLPP1 and the stimulation of the Akt pathway. In contrast, calpain-2 activation is downstream of extrasynaptic NMDA receptors and is neurodegenerative through the truncation of the phosphatase, STEP, and the activation of the p38 protein kinase. These findings have major significance for our understanding of neurological conditions associated with neurodegeneration and for the development of new therapeutic approaches to prevent neuronal death in these disorders.

Keywords Calpain-1 · Calpain-2 · Neuronal death · Neuronal survival · NMDA receptors · Akt · STEP

Y. Wang · M. Baudry (✉)
Graduate College of Biomedical Sciences, Western University of Health Sciences, Pomona, CA, USA
e-mail: mbaudry@westernu.edu

X. Bi
College of Osteopathic Medicine of the Pacific, Western University of Health Sciences, Pomona, CA, USA

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2.1 Introduction

Neurons have to perform several basic functions, including growing (from the time of differentiation), migrating, responding and adapting to external and internal stimuli, and surviving or dying, as a result of continuous challenges and the deleterious effects of the aging process. Numerous reviews have discussed the role of calpain in neurodegeneration in general (Vosler et al. 2008; Yildiz-Unal et al. 2015), and in stroke (Anagli et al. 2009; Koumura et al. 2008) and in traumatic brain injury (TBI) (Kobeissy et al. 2015; Liu et al. 2014). Likewise, numerous studies have attempted to use calpain inhibitors to reduce neurodegeneration in both stroke and TBI (Anagli et al. 2009; Bartus et al. 1994a, b; Cagmat et al. 2015; Hong et al. 1994; Li et al. 1998; Markgraf et al. 1998; Siklos et al. 2015; Tsubokawa et al. 2006). While some studies have reported some positive effects of calpain inhibitors in TBI (Thompson et al. 2010), other studies have not confirmed these results. In particular, overexpression of the endogenous calpain inhibitor, calpastatin, was reported to reduce the formation of the Spectrin Breakdown Product (SBDP), resulting from calpain-mediated truncation of spectrin, a widely used biomarker of calpain activation and potentially neurodegeneration (Yan and Jeromin 2012), but had no effect on neurodegeneration (Schoch et al. 2012). Another recent study concluded that even a blood-brain barrier- and cell-permeable calpain inhibitor, SNJ-1945, did not have a sufficient efficacy and a practical therapeutic window in a model of controlled cortical impact (Bains et al. 2013).

Several reasons could account for the failure to develop clinical applications of such inhibitors, including their lack of specificity/potency/selectivity (Donkor 2011), and the incomplete knowledge regarding the functions of the major calpain isoforms in the brain, calpain-1 and calpain-2 (aka μ- and m-calpain). Work from our laboratory over the last 5 years has revealed new features of these two enzymes, which significantly changed our understanding of their functions in the brain. Specifically, we found that calpain-1 and calpain-2 play opposite functions in both synaptic plasticity and neuroprotection/neurodegeneration (Baudry and Bi 2016). Thus, calpain-1 activation is required for theta burst stimulation-induced long-term potentiation (LTP) and for certain types of learning and memory, and is neuroprotective (Wang et al. 2013, 2014). Calpain-1 is neuroprotective due to the degradation of the PH domain and Leucine rich repeat Protein Phosphatase 1 (PHLPP1β) and the resulting activation of the Akt survival pathway. On the other hand, calpain-2 activation limits the magnitude of LTP and restricts learning, and is neurodegenerative due to the cleavage of STEP and the stimulation of death pathways (Wang et al. 2013, 2014). In addition, we found that ischemia-induced damage to retinal ganglion cells was exacerbated in calpain-1 knock-out mice, indicating that calpain-1 inhibition is likely to counteract the potential beneficial effects of calpain-2 inhibition if non-selective calpain inhibitors are used (Wang et al. 2016b). These findings could account for the failure of the previous studies to convincingly demonstrate the role of calpain in neurodegeneration, and for the lack of clear efficacy of the previously tested calpain inhibitors, which did not discriminate between calpain-1 and

calpain-2. It is also important to stress that calpain activation has also been implicated in diffuse axonal injury (Wang et al. 2012a), which has been proposed to represent an important component of the pathophysiology of TBI (Xiong et al. 2013), although at this point, there is no information regarding which calpain isoform is involved.

In this chapter, we will first discuss how calpain-1 and calpain-2 activation appear to be closely related to the stimulation of synaptic and extra-synaptic NMDA receptors, respectively. We will then review the mechanisms underlying the neuroprotective effects of calpain-1 activation, which will be followed by a discussion of the mechanisms involved in calpain-2-mediated neurodegeneration. These two aspects will be illustrated by studies using intra-ocular NMDA injection to produce acute neurodegeneration of retinal ganglion cells. Finally, we will discuss the potential clinical implications of these findings and our current efforts to develop selective calpain-2 inhibitors as a new approach for neuroprotection in conditions associated with acute neurodegeneration.

2.2 Calpains and NMDA Receptors

NMDARs play critical roles in both physiological and pathological conditions, and several studies have shown that NMDA receptor localization is responsible for opposite consequences of NMDA receptor stimulation for neuronal survival or death; thus, synaptic NMDAR activation provides neuroprotection, while extrasynaptic NMDARs are linked to pro-death pathways (Hardingham and Bading 2010). The Akt and MAP kinase/extracellular signal-regulated kinase (ERK1/2) pathways are two key pro-survival pathways downstream of synaptic NMDARs (Hardingham et al. 2001; Papadia et al. 2005; Wang et al. 2012b). Akt phosphorylates and inhibits various pro-apoptotic substrates, such as glycogen synthase kinase-3 (GSK3), forkhead box O (FOXO) (Soriano et al. 2006), apoptosis signal-regulating kinase 1 (ASK1) (Kim et al. 2001), p53 (Yamaguchi et al. 2001), and Bcl2-associated death promoter (BAD) (Downward 1999). On the other hand, ERK1/2 activates the survival nuclear transcription factor, cyclic-AMP response element binding protein (CREB) (Hardingham et al. 2001). Although some protein kinases linking NMDARs to Akt and ERK have been found (Krapivinsky et al. 2003; Perkinton et al. 2002), how Akt and ERK1/2 were activated by synaptic but not extrasynaptic NMDARs was not clearly understood until recently.

PH domain and Leucine rich repeat Protein Phosphatase 1 (PHLPP1) exhibits two splice variants, PHLPP1α and PHLPP1β, which share amino acid sequence similarity but have different sizes (140 kDa and 190 kDa, respectively). PHLPP1α dephosphorylates Akt at Ser473 in cancer cells (Gao et al. 2005) and neurons (Jackson et al. 2010), and its down-regulation is related to cell survival in CNS (Chen et al. 2013; Liu et al. 2009; Saavedra et al. 2010). PHLPP1β inhibits ERK1/2

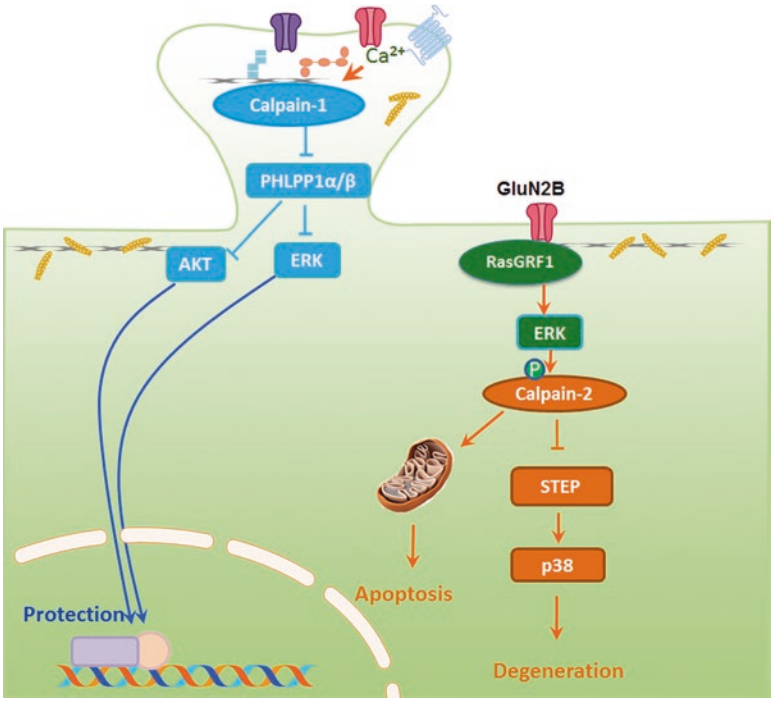

Fig. 2.1 Schematic representation of the links between synaptic and extrasynaptic NMDARs and calpain-1 and calpain-2. Calpain-1 is rapidly stimulated by the calcium influx generated by synaptic NMDA receptor activation, resulting in PHLPP1α/β degradation. This produces the activation of Akt and ERK, which triggers the stimulation of neuroprotective cascades. On the other hand, extrasynaptic NMDA receptors containing NR2B subunits trigger ERK activation, calpain-2 phosphorylation/activation and the activation of STEP and p38, leading to neurodegeneration. Moreover, calpain-2 activation has been linked to apoptosis through the truncation of anti-apoptotic factors

by binding and trapping its activator Ras in the inactive form (Shimizu et al. 2003). PHLPP1β was previously shown to be degraded by calpain in hippocampus, and its degradation contributes to novel object recognition memory (Shimizu et al. 2007). Thus, PHLPP1 was a good candidate to link NMDA receptor stimulation to Akt and ERK regulation.

Using primary neuronal cultures, we showed that calpain-1 and calpain-2 are activated by different NMDAR populations (synaptic vs. extrasynaptic NMDARs) and regulate different substrates (PHLPP1 and STEP) to produce opposite effects on neuronal fate (neuroprotection and neurodegeneration) (Fig. 2.1). Interestingly, calpain-induced cleavage of PHLPP1β and the resulting ERK activation were previously shown to regulate synaptic plasticity (Shimizu et al. 2007). We showed that calpain-1-mediated PHLPP1β degradation was specifically triggered by synaptic but not extra-synaptic NMDAR activation and contributed to the neuroprotective effects of synaptic NMDAR activation. In addition, PHLPP1α, which

dephosphorylates and inhibits Akt, was also cleaved by calpain-1 following synaptic NMDAR activation. Calpain cleavage of PHLPP1 1α and β was necessary and sufficient for synaptic NMDAR-induced activation of the Akt and ERK pathways, since calpain inhibition blocked, while PHLPP1 knockdown mimicked, the effects of synaptic NMDAR activation on Akt and ERK pathways. PHLPP1 suppressed Akt and ERK pathways under basal conditions; following synaptic NMDAR activation, calpain cleaves PHLPP1α and β, thus releasing the inhibition of these two major pro-survival signaling cascades in neurons. Consistently, calpain-1-mediated cleavage of PHLPP1 was required for the neuroprotective effects of synaptic NMDARs, as calpain inhibition blocked the neuroprotection elicited by synaptic NMDAR activation. We further confirmed these results using PHLPP1 knockdown, as down-regulation of PHLPP1 not only suppressed the blockade of neuroprotection caused by calpain inhibition but also induced neuroprotection without synaptic NMDAR activation. Consistent with our results, a recent study reported that PHLPP1 knockout mice are more resistant to ischemic brain injury (Chen et al. 2013). Thus, PHLPP1 should be considered as a novel potential target for the treatment of neurodegenerative diseases.

As previously reported (Xu et al. 2009), we found that calpain activated by extrasynaptic NMDAR stimulation cleaved STEP and caused neuronal death (Wang et al. 2013). It had previously been proposed that prolonged or excessive activation of calpain was responsible for calpain-mediated neurotoxicity, whereas brief and limited calpain activation could be involved in the regulation of synaptic plasticity. However, prolonged activation of synaptic NMDARs (by Bic and 4-AP treatment) for as long as 3 days did not result in STEP cleavage, nor in neuronal damage, but produced neuroprotection against starvation and oxidative stress. On the other hand, activation of extrasynaptic NMDARs did not affect PHLPP1 or its downstream pathways, strongly suggesting that there are two separate pools of calpain downstream of synaptic and extrasynaptic NMDARs, which regulate different substrates and therefore exert separate functions.

The possibility that calpain-1 and calpain-2 could exert different roles in CNS had not been extensively discussed. However, the discovery that calpain-2 could be activated by phosphorylation (Zadran et al. 2010), coupled with the identification of PTEN as a specific calpain-2 substrate (Briz et al. 2013), raised the possibility that calpain-1 and calpain-2 could play distinct functions. Interestingly, synaptic NMDAR activation did not result in the degradation of PTEN, a specific calpain-2 substrate, further supporting the idea that synaptic NMDAR activation does not activate calpain-2. The use of calpain-1 and calpain-2 specific inhibitors also confirmed this idea, as a calpain-2 specific inhibitor did not affect synaptic NMDAR-dependent PHLPP1 cleavage and neuroprotection but blocked extrasynaptic NMDAR-dependent STEP cleavage and neurotoxicity. In contrast, a calpain-1 specific inhibitor blocked synaptic NMDAR-mediated effects but not extrasynaptic NMDAR-mediated neurotoxicity. Down-regulation of calpain-1 and calpain-2 by specific siRNAs in cultured neurons also indicated that only calpain-1 knockdown blocked synaptic NMDAR-mediated neuroprotective pathways. In addition, knockdown of calpain-2 but not calpain-1, by AAV-shRNA transfection increased survival

of primary hippocampal neurons following NMDA treatment (Bevers et al. 2009). Results obtained in cultured neurons were further confirmed using a model of NMDA-induced neurotoxicity in acute hippocampal slices from young mice, which had previously indicated that NMDA treatment of acute hippocampal slices caused neurotoxicity in young but not adult rats (Zhou and Baudry 2006), probably because young rats have more NR2B-containing NMDARs, which are preferentially localized extrasynaptically (Tovar and Westbrook 1999). In hippocampal slices prepared from young calpain-1 knock-out mice, NMDA induced the degradation of STEP but not PHLPP1, and exacerbated neurotoxicity, as compared to slices prepared from wild-type mice. On the other hand, calpain-2 specific inhibition by applying either a selective calpain-2 inhibitor in slices from wild-type mice or a non-selective calpain inhibitor in slices from calpain-1 knock-out mice blocked NMDA-induced degradation of STEP and suppressed neurotoxicity (Wang et al. 2013).

Together, these results demonstrate that calpain-1 is preferentially activated by synaptic NMDAR stimulation, whereas calpain-2 is preferentially activated by extrasynaptic NMDAR stimulation. Calpain-1 was shown to be localized in synaptic compartments (Perlmutter et al. 1988), where it could regulate synaptic function through its action on synaptic elements such as cytoskeletal and scaffolding proteins, as well as glutamate receptors (Liu et al. 2008). Little is known regarding the ultrastructural localization of calpain-2 in neurons. One of the newly discovered physiological roles of calpain-2 is to regulate activity-dependent local protein synthesis (Briz et al. 2013; Wang and Huang 2012), which takes place not in synapses but in nearby extrasynaptic areas (Frey and Morris 1998; Steward and Wallace 1995). In addition, calpain-2 has been reported to control synaptogenesis in dendritic shafts through constitutive proteolysis of the cytoskeletal protein, cortactin (Mingorance-Le Meur and O'Connor 2009). These findings would suggest that calpain-2 is localized, at least in part, in extrasynaptic domains (Fig. 2.1).

The existence of separate signaling pathways for calpain-1 and calpain-2 suggested that these two calpain isoforms belong to different protein scaffolds, which could segregate them in different neuronal compartments. PHLPP1 could be cleaved by both purified calpain-1 and calpain-2 in membrane fractions, yet it was cleaved only by calpain-1 following synaptic NMDAR activation in hippocampal slices, suggesting that substrate specificity for calpains depends not only on amino acid sequences within substrates, but also on localization and scaffolding of both substrates and calpains in neurons. Co-immunoprecipitation experiments confirmed that NR2A-containing NMDARs, PSD95, calpain-1 and PHLPP1, form a complex in neurons. Furthermore, synaptic NMDAR activity recruited calpain-1 to this NMDAR multi-protein complex; such recruitment could facilitate the proteolysis of PHLPP1 and possibly other calpain-1 substrates in the complex. In contrast, calpain-2 was not present in this complex under basal conditions nor was it recruited by activity, consistent with the absence of calpain-2 activation following synaptic NMDAR activation. It is likely that a calpain-2-containing multi-protein complex is associated with extrasynaptic NMDARs. How could activation of extrasynaptic NMDARs results in calpain-2 activation? It has been repeatedly shown that NR2B subunits are enriched in extrasynaptic NMDARs (Papouin and Oliet 2014), and that

their activation is critical for excitotoxicity (Chazot 2004). Interestingly, NR2B directly binds RasGRF1, which provides a link between NMDAR activation and ERK activation (Krapivinsky et al. 2003). As we have shown that ERK activation directly phosphorylates and activates calpain-2 (Zadran et al. 2010), this pathway is likely responsible for the prolonged activation of calpain-2 following stimulation of extrasynaptic NMDA receptors (Fig. 2.1). In addition, we discussed elsewhere the existence of different PDZ binding domains in the C-terminal of calpain-1 and calpain-2, which could account for their differential subcellular distribution (Baudry and Bi 2016).

2.3 Calpain-1 Activation and Neuroprotection

As discussed above, calpain-1 is downstream of synaptic NMDARs and as such, we postulated that it has a neuroprotective function. This notion was supported by results obtained in cultured neurons, where we demonstrated that calpain-1 activation following stimulation of synaptic NMDARs was neuroprotective against starvation- and oxidative stress-mediated neurotoxicity (Wang et al. 2013). Previous studies have shown that normal stimulation of synaptic NMDA receptors is required to limit the extent of apoptotic neuronal death during the postnatal period, as blockade of these receptors during this period increases the extent of apoptotic neuronal death (Monti and Contestabile 2000). Calpain activity is higher in cerebellum than in cortex or hippocampus across different mammalian species (Baudry et al. 1986). An immunohistochemical study revealed that the major calpain isoform expressed in cerebellar neurons is calpain-1 (Hamakubo et al. 1986). Calpain-1 activity in cerebellum during prenatal and early postnatal period is high, as compared to that in adulthood (Simonson et al. 1985), suggesting a potential role for calpain-1 in cerebellar development. Interestingly, a *CAPN1* missense mutation in the Parson Russell Terrier dog breed has been associated with spinocerebellar ataxia (Forman et al. 2013).

Loss of cerebellar granule cells (CGCs) induced by different mechanisms results in ataxia (Hashimoto et al. 1999; Kim et al. 2009; Pennacchio et al. 1998; Shmerling et al. 1998). NMDAR activity is essential for CGC survival during the critical stage of cerebellar development (Monti and Contestabile 2000; Balazs et al. 1988; Monti et al. 2002; Moran and Patel 1989), although the underlying mechanism has remained elusive. NMDAR-induced activation of the nuclear factor CREB is required (Monti et al. 2002), and CREB is a target of the pro-survival kinase Akt (Du and Montminy 1998).

As discussed above, synaptic NMDAR-mediated calpain-1 activation results in the degradation of PHLPP1. PHLPP1 dephosphorylates and inhibits Akt, and is involved in tumorigenesis (Chen et al. 2011), circadian clock (Masubuchi et al. 2010), learning and memory process (Wang et al. 2014; Shimizu et al. 2007), and autophagy (Arias et al. 2015). Calpain-1-mediated degradation of PHLPP1 activates Akt and promotes neuronal survival (Wang et al. 2013), and we postulated that

calpain-1 mediated regulation of PHLPP1 and Akt could be involved in NMDAR-dependent CGC survival during postnatal development.

We analyzed apoptosis in the brain during the postnatal period in wild-type and calpain-1 KO mice (Wang et al. 2016a). Calpain-1 KO mice exhibited abnormal cerebellar development, including enhanced apoptosis of CGCs during the early postnatal period, and reduced granule cell density and impaired synaptic transmission from parallel fiber to Purkinje cells in adulthood, resulting in an ataxia phenotype. All these defects are due to deficits in the calpain-1/PHLPP1/Akt pro-survival pathway in developing granule cells, since treatment with an Akt activator during the postnatal period or crossing calpain-1 KO mice with PHLPP1 KO mice restored most of the observed alterations in cerebellar structure and function in calpain-1 KO mice (Wang et al. 2016a). To reverse reduced pAkt levels in cerebellum of calpain-1 KO mice during the early postnatal period, we treated them from PND1 to PND7 with a PTEN inhibitor, bisperoxovanadium (bpV) (0.5 mg/kg, i.p., twice daily), which has been shown to activate Akt (Boda et al. 2014; Li et al. 2009; Mao et al. 2013). BpV injection significantly increased pAkt levels in cerebellum of developing KO mice, and completely prevented the enhanced apoptosis in cerebellum and cerebrum of calpain-1 KO mice at PND7 (Fig. 2.2).

Thus the NMDAR/calpain-1/PHLLP1/Akt pro-survival pathway is active in developing CGCs, where it limits the extent of CGC apoptosis. Increased PHLPP1 and decreased pAkt levels were found in cerebellar homogenates of calpain-1 KO mice, indicating that calpain-1 activity normally reduces PHLPP1 levels and maintains Akt activated during the postnatal period in cerebellum. The density of pAkt-positive puncta was reduced in cerebellar granular layer but not in Purkinje or molecular layer of calpain-1 KO mice, suggesting that calpain-1-dependent regulation of Akt only takes place in CGCs but not in other cerebellar cell types. Down-regulation of PHLPP1 restored normal levels of pAkt in developing cerebellum of calpain-1 KO mice, indicating that PHLPP1 is downstream of calpain-1 and that its level is important for Akt regulation. Finally, reduced Akt activity was associated with enhanced CGC apoptosis in calpain-1 KO mice, while increased Akt activity was associated with reduced CGC apoptosis in bpV-injected WT and in mice lacking both calpain-1 and PHLPP1.

NMDAR- and calpain-1-mediated neuronal survival during brain development was not limited to CGCs, as enhanced apoptosis was present in other brain regions such as cortex, striatum and hippocampus in developing calpain-1 KO mice (Fig. 2.2). Importantly, calpain-1-mediated neuroprotection is also present in human brain, as calpain-1 mutations resulting in lack of function are associated with cerebellar ataxia (Wang et al. 2016a; Gan-Or et al. 2016). Furthermore, the important roles of calpain-1 in hippocampal neuronal survival during development and in synaptic plasticity in the adult (Wang et al. 2014; Zhu et al. 2015) may contribute to the cognitive decline found in ataxia patients with *CAPN1* mutations.

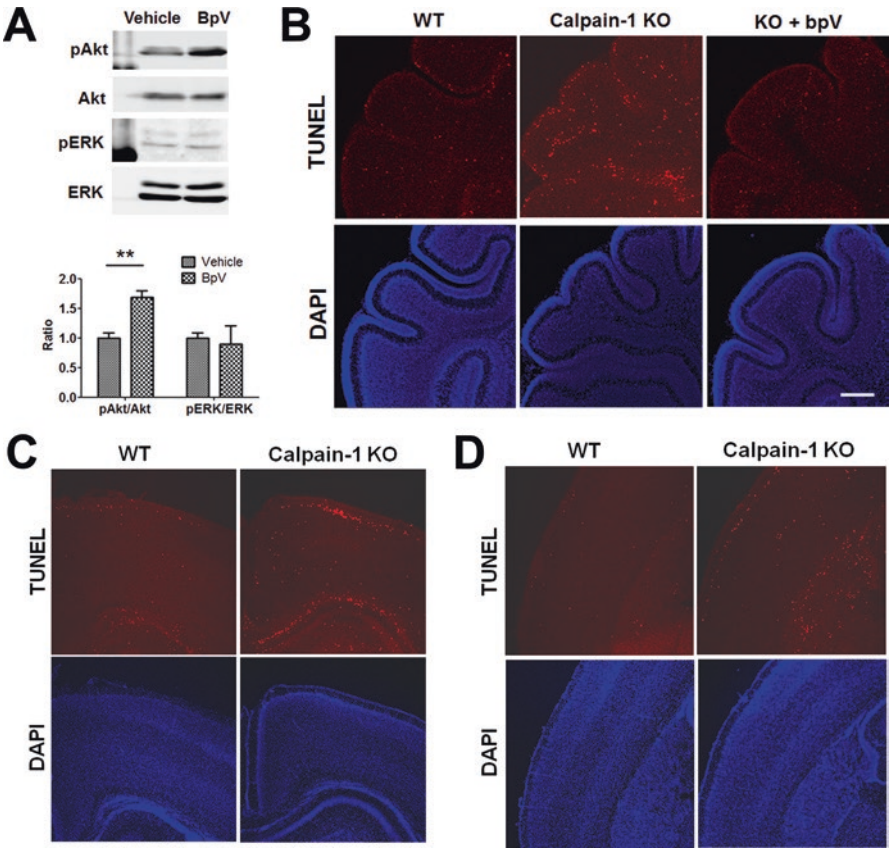

Fig. 2.2 Effects of bpV on apoptosis and Akt in telencephalon of calpain-1 (CAPN1) KO mice during the postnatal period. (**a**–**c**) TUNEL and DAPI staining of coronal sections at various anterior-posterior levels of PND7 calpain-1 KO mice injected from PND1 to PND7 with vehicle or a PTEN inhibitor, bisperoxovanadium (bpV) (0.5 mg/kg, i.p., twice daily). Note the clear decrease in TUNEL staining in bpv-injected calapin-1 KO mice. (**d**, **e**) Levels of Akt and p-Akt and ERK and p-ERK in cortex of PND7 calpain-1 KO mice injected from PND1 to PND7 with vehicle or bpv (0.5 mg/kg, i.p., twice daily). Results are expressed as means ± SEM of four experiments. **$p < 0.05$, Student's t-test

2.4 Calpain-2 and Neurodegeneration

As mentioned above, there is abundant literature linking calpain activation with neurodegeneration. However, very few studies have explored the specific contributions of calpain-1 and calpain-2 in neurodegeneration. Our *in vitro* studies clearly indicated that calpain-2 activation, but not calpain-1 activation was responsible for NMDA-induced excitotoxicity through the activation of STEP. A similar study indicated that down-regulation of calpain-2 but not calpain-1 also increased neuronal survival following NMDA treatment of cultured hippocampal neurons (Bevers et al.

2009). In order to further analyze the role of calpain-2 in neurodegeneration in vivo, we used a model consisting of direct intraocular NMDA injection in mice. Calpain activation had been previously involved in retinal cell death induced by NMDAR activation (Chiu et al. 2005; Shimazawa et al. 2010). To test the specific roles of calpain-1 and calpain-2 in this process, wild-type (WT) mice were injected systemically with a calpain-2 selective inhibitor (C2I), Z-Leu-Abu-CONH-CH_2-C_6H_3 (3, 5-$(OMe)_2$) (Wang et al. 2013, 2014), 30 min before NMDA intravitreal injection. Levels of SBDP and of PHLPP1, were determined in retinal extracts 6 h after NMDA injection (Fig. 2.3a–c). Akt levels were also measured as a loading control. Levels of SBDP were significantly increased and those of PHLPP1 decreased after NMDA injection, as compared to control (PBS intravitreal injection), suggesting that calpain was activated after NMDA injection. Systemic (intraperitoneal; i.p.) injection of C2I significantly suppressed NMDA-induced changes in SBDP but not in PHLPP1, suggesting that C2I systemic injection selectively inhibited calpain-2 but not calpain-1 activation in retina after intravitreal NMDA injection.

Six days after intravitreal injection of NMDA or PBS to WT mice, frozen retinal sections were prepared and H&E staining was performed to evaluate cell numbers in the ganglion cell layer (GCL) and the thickness of the Inner Plexiform Layer (IPL), which contains RGC dendrites. NMDA injection (NMDA plus Vehicle) significantly reduced cell numbers in the GCL and IPL thickness, while PBS injection (PBS plus Vehicle) had no effect on these parameters (Fig. 2.3d–f). Systemic injection of C2I 30 min before and 6 h after NMDA injection significantly suppressed the reduction in GCL cell numbers and IPL thickness (Fig. 2.3d–f), suggesting that calpain-2 activation contributes to NMDA-induced cell death in GCL.

In calpain-1 KO mice, GCL cell number and IPL thickness were not affected by vehicle injection. However, the effects of NMDA injection on GCL cell number and IPL thickness were larger than in WT mice (Fig. 2.3g–i). GCL cell death in calpain-1 KO mice after NMDA injection was significantly more severe than that in WT mice (Fig. 2.3j), suggesting that calpain-1 supports cell survival in GCL after NMDA injection. Systemic injection of C2I to calpain-1 KO mice partially but significantly reversed NMDA-induced decrease in GCL cell number and IPL thickness

Fig. 2.3 (continued) (2 μl of 2.5 mM). Mice were injected i.p. with vehicle (10% DMSO) or C2I (0.3 mg/kg) 30 min before intravitreal injection. Quantification of the ratios of SBDP/Akt (**b**) and PHLPP1/Akt (**c**). n = 4. *p < 0.05, ***p < 0.001. One-way ANOVA followed by Bonferroni test. (**d**) H&E staining of naive, PBS- (control) or NMDA- (2 μl of 2.5 mM) treated retina from WT mice injected i.p. with vehicle (10% DMSO) or C2I (0.3 mg/kg) 30 min before and 6 h after NMDA injection. H&E staining was performed 7 days after injection. Scale bar = 30 μm. Quantification of cell numbers in GCL (**e**) and thickness of IPL (**f**). Six sections in each eye were analyzed. n = 4–8 (eyes). *p < 0.05, **p < 0.01, One-way ANOVA followed by Bonferroni test. (**g**) H&E staining of PBS- (control) and NMDA- (2 μl of 2.5 mM) treated retina from calpain-1 KO mice injected i.p. with vehicle or C2I (0.3 mg/kg) 30 min before and 6 h after NMDA injection. H&E stain was done 7 days after injection. Scale bar = 30 μm. Quantification of cell number in GCL (**h**) and thickness of IPL (**i**). n = 6. *p < 0.05, **p < 0.01, ***p < 0.001, One-way ANOVA followed by Bonferroni test. (**j**) GCL cell numbers in NMDA-treated WT and KO mice without and with C2I treatment. n = 6. **p < 0.01. Two-tailed t-test

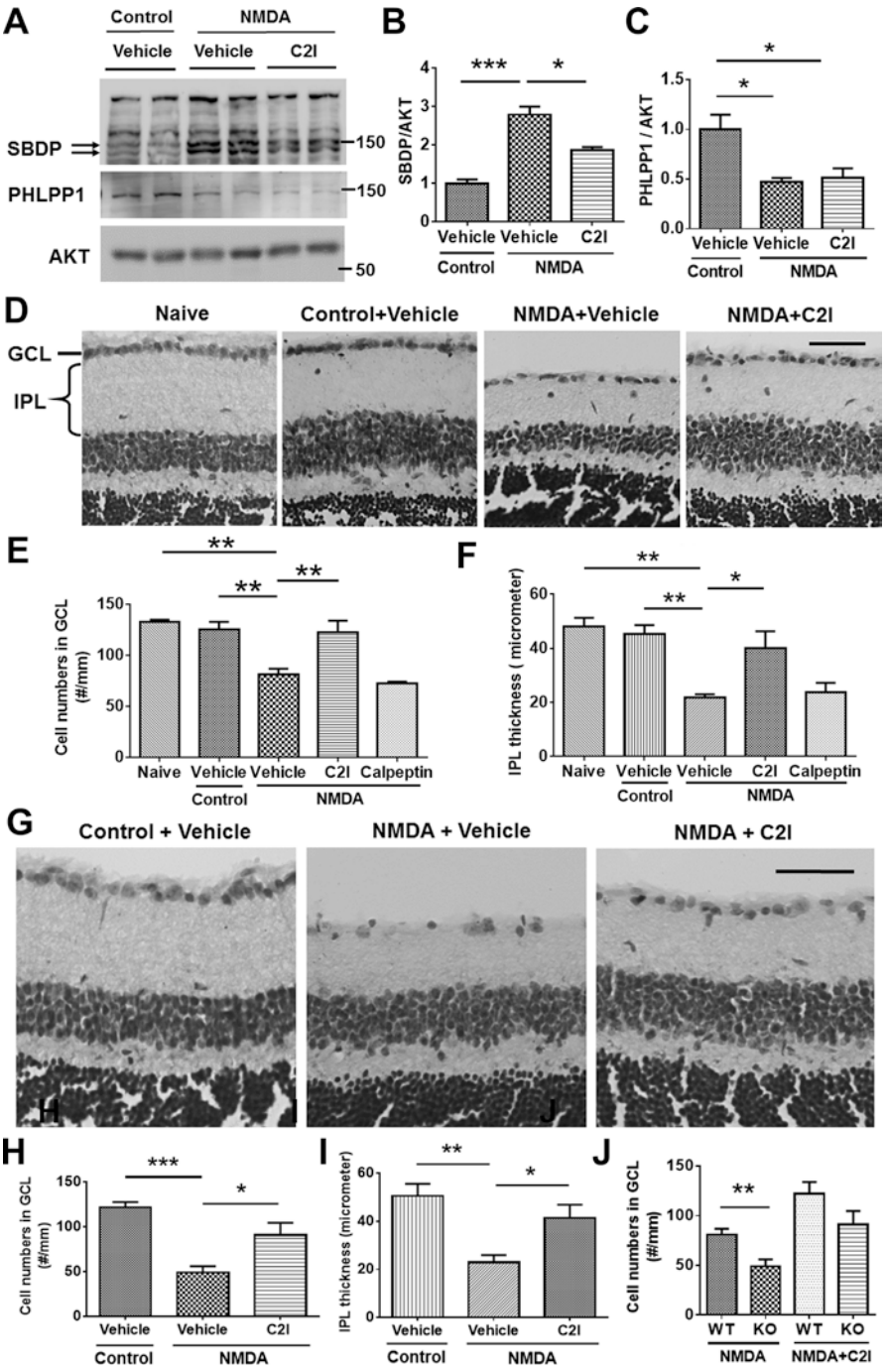

Fig. 2.3 Calpain-2 inhibition reduces, while calpain-1 knockout exacerbates cell death in ganglion cell layer induced by NMDA intravitreal injection. (**a**) Representative immunoblot of indicated proteins in mouse retinal extracts 6 h after intravitreal injection of PBS (control) or NMDA

(Fig. 2.3g–i). A very similar pattern of results was obtained in a different model of acute glaucoma, consisting in a brief period of increased intraocular pressure (Wang et al. 2016b). Furthermore, recent studies in a mouse model of TBI also support the notion that calpain-2 activation is prolonged and responsible for neuronal death, while calpain-1 activation is neuroprotective (Wang et al 2017).

2.5 Clinical Implications of Specific Calpain-2 Inhibition and Calpain-1 Activation

Our results clearly demonstrate that calpain-1 and calpain-2 have opposite functions in both synaptic plasticity and neuronal survival/death after acute insults. Thus, calpain-1 activation is required for LTP induction and for hippocampus-dependent learning and is neuroprotective both during the postnatal developmental period and in adulthood following acute insults. On the other hand, calpain-2 activation limits the extent of hippocampus-dependent learning and is neurodegenerative following acute insults, and in particular excitotoxicity. Our results have important implications for the development of new approaches for treating diseases associated with excitotoxicity, such as epilepsy, stroke, Alzheimer's and Parkinson's disease, Huntington disease and ischemia. In all these cases, it has been suggested that extrasynaptic NMDAR activation and STEP degradation are involved in neurodegeneration. Our results would, therefore, suggest that specific inhibition of calpain-2 but not calpain-1 would have neuroprotective effects under these conditions. Conversely, overexpression or activation of calpain-1, by cleaving PHLPP1 and stimulating pro-survival cascades, could also have beneficial effects. In addition, calpain-2 activation is involved in regulating the magnitude of long-term potentiation (LTP) in hippocampus, due to the existence of a molecular brake consisting in calpain-2-mediated PTEN degradation and stimulation of m-TOR dependent PHLPP1β synthesis (Wang et al. 2014). We also showed that low doses of a selective calpain-2 inhibitor facilitate learning in normal mice, while higher doses, which inhibit calpain-1, impair learning. Thus, a selective calpain-2 inhibitor could be extremely beneficial for preventing neurodegeneration, while facilitating certain forms of learning and memory. As discussed above, a selective calpain-2 inhibitor prevented death of retinal ganglion cells and maintained vision in a mouse model of acute glaucoma (Wang et al. 2016b). Calpain inhibitors have previously been proposed to represent potential treatments for a variety of eye disorders, including glaucoma and macular degeneration (Azuma and Shearer 2008; Paquet-Durand et al. 2007), and further studies are needed to assess the potential use of selective calpain-2 inhibitors for these disorders. Calpain inhibition has been proposed to represent a therapeutic approach for stroke and TBI, although this notion has not been supported by a variety of experiments. We postulate that the use of selective calpain-2 inhibitors might overcome the problems associated with that of non-selective calpain inhibitors. Our results in a mouse model of TBI supports this notion, as we have found that

post-treatment with a selective calpain-2 inhibitor provides a highly significant degree of neuroprotection and facilitates behavioral recovery (Wang et al 2017). The potential use of selective calpain-2 inhibitors for chronic neurodegenerative disorders needs to be further evaluated. It is important to note that calpain has been proposed to participate in neurodegeneration associated with Parkinson's disease as well as Alzheimer's disease, and it is tempting to speculate that selective calpain-2 inhibitors might also be beneficial in these disorders.

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Part II
Traumatic Brain Injury

Chapter 3
Oxidative Damage Mechanisms in Traumatic Brain Injury and Antioxidant Neuroprotective Approaches

Edward D. Hall, Indrapal N. Singh, and John E. Cebak

Abstract This chapter reviews our current knowledge of the role of oxidative damage mechanisms and pharmacological antioxidant neuroprotective strategies for inhibiting reactive oxygen species (ROS) and reactive nitrogen species (RNS)-mediated secondary injury following traumatic brain injury (TBI). First of all, the chemistry of the main forms of oxidative damage: lipid peroxidation, carbonylation and nitration are presented as well as the interactions of oxidative damage with other secondary injury mechanisms including glutamate-mediated excitotoxicity, intracellular calcium overload and mitochondrial dysfunction. Secondly, the general mechanistic approaches to interrupting oxidative damage are presented: decreasing ROS/RNS formation or scavenging ROS and RNS-derived radicals, inhibition of lipid peroxidation propagation, chelation of iron, which is a potent catalyst of lipid peroxidation reactions, scavenging of neurotoxic aldehydic lipid peroxidation products ('carbonyls'), and enhancement of the expression of the pleiotopic Nrf2-antioxidant response element (ARE) pathway that controls the synthesis of several endogenous antioxidant enzymes and chemical antioxidants. Pharmacological examples of compounds that effectively inhibit oxidative damage and produce neuroprotective effects in animal TBI models by each of these various approaches are presented. Finally, the results of large phase III clinical trials with the either the radical scavenger polyethylene glycol-coupled superoxide dismutase (PEG-SOD) or the 21-aminosteroid lipid peroxidation inhibitor tirilazad are revisited in which the latter compound was found to selectively improve survival after moderate and severe TBI, particularly in male patients, suggesting that successful clinical translation of neuroprotective antioxidant compounds, or combinations of mechanistically complimentary antioxidants, should be possible.

E. D. Hall (✉) · I. N. Singh
Spinal Cord & Brain Injury Research Center (SCoBIRC) and Department of Neuroscience, University of Kentucky Medical Center, Lexington, KY, USA
e-mail: edhall@uky.edu; ising2@uky.edu

J. E. Cebak
Lincoln Memorial University DeBusk College of Osteopathic Medicine, Harrogate, TN, USA

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3.1 Introduction

At present, there are no FDA-approved pharmacological therapies for acute treatment of traumatic brain injury (TBI) patients that are conclusively proven to mitigate the often devastating neurological effects of their injuries. However, the possibility of discovering and developing an effective 'neuroprotective' treatment that will limit posttraumatic brain damage and improve neurological recovery is based upon the fact that even though some of the neural injury is due to the primary mechanical injury to the parenchymal neurons, glia and vascular elements, the majority of post-traumatic neurodegeneration is due to a pathophysiological secondary injury cascade triggered initially by massive release of glutamate and its excitotoxic effects that occur during the first minutes, hours and days following the injury, which exacerbates the damaging effects of the primary injury. One of the most validated "secondary injury" mechanisms, as revealed in experimental TBI studies, that contributes to glutamate-mediated excitotoxic neurodegeneration, involves the downstream increase in reactive oxygen species (ROS) that cause oxygen radical-induced oxidative damage to brain cell lipids and proteins. This chapter outlines the key sources of reactive oxygen species (ROS), including highly reactive (i.e. rapidly oxidizing) free radicals, the pathophysiological mechanisms associated with oxidative neural damage and pharmacological antioxidants that have been shown to produce neuroprotective effects that limit excitotoxic neurodegeneration in preclinical TBI models, one of which has revealed some evidence of neuroprotective efficacy in a major pathological subset of TBI patients in a large phase III clinical trial.

3.2 Reactive Oxygen Species and Reactive Nitrogen Species

The term reactive oxygen species (ROS) includes oxygen-derived radicals such as the modestly reactive superoxide radical ($O_2^{\bullet -}$) and the highly reactive hydroxyl ($OH^{\bullet}$) radical as well as non-radicals such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), the latter often referred to as a reactive nitrogen species (RNS). The cascade of posttraumatic oxygen radical reactions begins in response to the primary mechanical injury triggering neuronal depolarization, due to the voltage-dependent opening of sodium (Na^+) and calcium (Ca^{2+}) channels, which causes a massive increase in intracellular Ca^{2+} that stimulates rapid elevations in extracellular glutamate levels that excessively stimulates N-methyl-aspartate (NMDA) glutamate receptors, causing a further exacerbation of the injury-induced increase in intracellular Ca^{2+}. This voltage-dependent and glutamate receptor-mediated intracellular Ca^{2+} overload initiates multiple downstream neurodegenerative processes,

one of which is the increased generation of oxygen free radicals that initiate oxidative damage to brain cell phospholipid membranes and proteins. The primordial oxygen free radical that comes from several pathophysiological sources involves the single electron (e^-) reduction of an oxygen molecule (O_2) to produce superoxide ($O_2^{\bullet-}$). Superoxide can be generated from several sources; one of the main ones is $O_2^{\bullet-}$ leakage from complex I of the mitochondrial electron transport chain in Ca^{2+}-overloaded brain mitochondria. However, $O_2^{\bullet-}$ is considered by many free radical chemists and biologists to be a modestly reactive radical that can potentially react with other molecules to give rise to much more reactive, and thus more potentially damaging, radical species. The reason that $O_2^{\bullet-}$ is only modestly reactive is that it can act as either an oxidant by stealing an electron from another oxidizable molecule or it can act as a reductant by which it donates its unpaired electron to another radical species, thus acting as an antioxidant. However, if $O_2^{\bullet-}$ reacts with a proton (H^+) to form a hydroperoxyl radical ($HO\bullet_2$) this results in a superoxide form that is much more likely to cause oxidation (i.e. act as an electron stealer).

One of the most important endogenous antioxidants is the enzyme superoxide dismutase (SOD) which rapidly catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 and oxygen. At low pH, $O_2^{\bullet-}$ can dismutate spontaneously. The formation of highly reactive oxygen radicals, which have unpaired electron(s) in their outer molecular orbitals, and the propagation of chain reactions are fueled by non-radical ROS, which do not have unpaired electron(s), but are chemically reactive. For example, $OH^{\bullet}$ radicals are generated in the iron-catalyzed Fenton reaction, where ferrous iron (Fe^{2+}) is oxidized to form $OH^{\bullet}$ in the presence of H_2O_2 ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$). Superoxide, acting as a reducing agent (i.e. an electron donor), can actually donate its unpaired electron to ferric iron (Fe^{3+}), cycling it back to the ferrous state in the Haber-Weiss reaction ($O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$), thus driving subsequent Fenton reactions and increased production of $OH^{\bullet}$. Under physiological conditions, iron is tightly regulated by its transport protein, transferrin and storage protein, ferritin, both of which bind the ferric (Fe^{3+}) form. This reversible bond of transferrin and ferritin with iron decreases with declining pH (below pH 7). Indeed, tissue acidosis is known to occur in the traumatized CNS that will cause the release of iron and initiation of iron-dependent oxygen radical production. A second source of iron comes from hemoglobin released into the blood during injury-induced hemorrhage.

Although $O_2^{\bullet-}$ is much less reactive than $OH^{\bullet}$ radical, its reaction with nitric oxide ($NO^{\bullet}$) radical forms the highly reactive oxidizing agent, peroxynitrite (PN: $ONOO^-$).This reaction ($O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$) occurs at a very high rate constant that out competes SOD's ability to convert $O_2^{\bullet-}$ into H_2O_2. Subsequently, at physiological pH, $ONOO^-$ will largely undergo protonation to form peroxynitrous acid (ONOOH) or it can react with carbon dioxide (CO_2) to form nitrosoperoxycarbonate ($ONOOCO_2^-$). The ONOOH can break down to form highly reactive nitrogen dioxide ($NO\bullet_2$) and $OH^{\bullet}$ ($ONOOH \rightarrow NO^{\bullet}_2 + OH^{\bullet}$). Alternatively, the $ONOOCO_2^-$ can decompose into $NO^{\bullet}_2$ and carbonate radical ($CO_3^{\bullet-}$) ($ONOOCO_2^- \rightarrow NO\bullet_2 + CO_3^{\bullet-}$).

3.3 Lipid Peroxidation

Increased production of reactive free radicals (i.e. "oxidative stress") in the injured brain has been shown to cause "oxidative damage" to cellular lipids and proteins, leading to functional compromise and cell death in both the microvascular and brain parenchymal compartments. Extensive study shows that a major form of radical-induced oxidative damage involves oxidative attack on cell membrane polyunsaturated fatty acids, triggering the process of lipid peroxidation (LP) that is characterized by three distinct steps: initiation, propagation and termination (Gutteridge 1995), which are shown in Fig. 3.1 in the context of radical-induced LP of arachidonic acid. **Initiation:** LP is initiated when highly reactive oxygen radicals (e.g. OH•, NO_2•, $CO_3^{\bullet -}$) react with polyunsaturated fatty acids such as arachidonic acid (AA), linoleic acid (LA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), resulting in disruptions in cellular and subcellular membrane integrity. Initiation of LP begins when ROS-induced hydrogen atom (H^+) and its one associated electron is abstracted from an allylic carbon. The basis for the susceptibility of the allylic carbon of the polyunsaturated fatty acid having one of its electrons stolen by a highly electrophilic free radical is that the carbon is surrounded by two relatively electronegative double bonds which tend to pull one of the electrons from the carbon. Consequently, a reactive free radical has an easy time pulling the hydrogen electron off of the carbon because the commitment of the carbon electron to staying

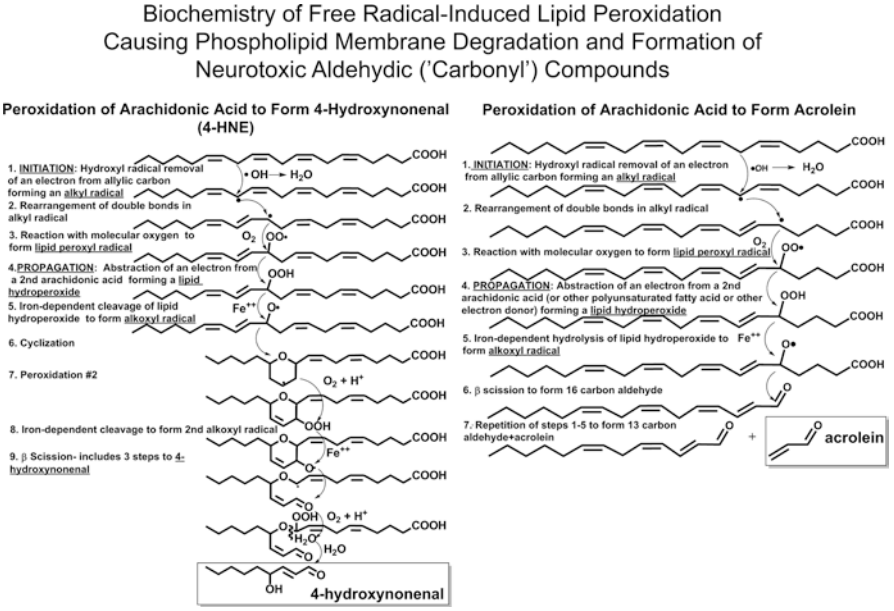

Fig. 3.1 Biochemistry involved in the initiation, propagation and termination reactions of arachidonic acid during lipid peroxidation, with the resulting formation of the aldehydic end-products 4-hydroxynonenal (4-HNE) and acrolein

paired with it has been weakened by the surrounding electronegative double bonds. This results in the original radical being quenched while the polyunsaturated fatty acid (L) becomes a lipid radical (L•) due to its having lost an electron. **Propagation:** Subsequently, in the propagation step, the unstable L• reacts with O_2 to form a lipid peroxyl radical (LOO•). The LOO• in turn extracts a hydrogen atom from an adjacent polyunsaturated fatty acid, yielding a lipid hydroperoxide (LOOH) and a second L•, which sets off a series of propagation "chain" reactions.

Termination: These propagation reactions are terminated in the third step, when the substrate becomes depleted and a lipid radical reacts with another radical to yield potentially neurotoxic non-radical aldehydic end products. One of those end-products that is often used to measure LP is the three carbon-containing malondialdehyde (MDA) which is mainly a stable non-toxic compound that when measured represents a LP 'tombstone'. In contrast, two highly neurotoxic aldehydic products of LP (commonly referred to as 'carbonyls') are 4-hydroxynonenal (4-HNE) or 2-propenal (acrolein), both of which have been well characterized in CNS injury experimental models (Bains and Hall 2012; Hall et al. 2010; Hamann and Shi 2009). These latter two aldehydic LP end products covalently bind to proteins and amino acids (lysine, histidine, arginine) by either Schiff base or Michael addition reactions altering their structure and functional properties. Immunohistochemical and immunoblotting (western, slot, dot) techniques are commonly used to measure 4-HNE or acrolein-modified proteins (i.e. 'protein carbonyls') in the injured brain (Hall and Bosken 2009).

3.4 Free Radical-Induced Protein Carbonylation and Nitration

Free radicals can cause various forms of oxidative protein damage. Firstly, a major mechanism involves carbonylation by reaction of various free radicals with susceptible amino acids such as arginine, lysine and histidine. The protein carbonyls thus formed are measurable through immunoblotting after derivatization of the carbonyl groups with diphenylhydrazine (DNPH). Indeed, the measurement of protein carbonyls by the so-called DNPH assay has long been used to measure free radical-induced protein oxidation. However, the carbonyl assay also picks up protein carbonyls that are present due to covalent binding of LP-derived 4-HNE and acrolein to cysteine residues, in addition to those resulting from direct free radical-induced amino acid oxidation. Thus, as a result, the carbonyl assay is as much an indirect index of LP as it is of direct radical-induced protein oxidation.

Secondly, $NO•_2$ can nitrate the three position of aromatic amino acids tyrosine or phenylalanine in proteins; 3-NT is a specific footprint of PN-induced cellular damage. Similarly, lipid peroxyl radicals (LOO•) can promote nitration of aromatic amino acids by producing initial oxidation (i.e. loss of an electron) which would enhance the ability of $NO•_2$ to nitrate the phenyl ring**.** Multiple commercially

available polyclonal and monoclonal antibodies are available for immunoblot or immunohistochemical measurement of proteins that have been nitrated by PN.

3.5 Interaction of Oxidative Damage with Other Secondary Injury Mechanisms

The impact of ROS/RNS production is heightened when oxygen radicals feed back and amplify other secondary injury pathways creating a continuous cycle of ion imbalance, Ca^{2+} buffering impairment, mitochondrial dysfunction, glutamate-induced excitotoxicity and microvascular disruption. One example of ROS-induced ionic disruption arises from LP-induced damage to the plasma membrane ATP-driven Ca^{2+} pump (Ca^{2+}-ATPase) and Na^+ pump (Na^+/K^+-ATPase), which contributes to increases in intracellular Ca^{2+} concentrations, mitochondrial dysfunction and additional ROS production. Both Ca^{2+}-ATPase and Na^+/K^+-ATPase disruptions result in further increases in intracellular Ca^{2+} and Na^+ accumulation respectively (Bains and Hall 2012), the latter causing reversal of the Na^+/Ca^{2+} exchanger which further exacerbates intracellular Ca^{2+} (Rohn et al. 1993, 1996). As already noted above, PN formed from mitochondrial Ca^{2+} overload also contributes to mitochondrial dysfunction. Specifically, nitric oxide (NO•), formed from mitochondrial NOS, which in turn reacts with $O_2^{\bullet -}$ to produce the highly toxic PN, which impairs respiratory and Ca^{2+} buffering capacity via its derived free radicals (Bringold et al. 2000). Indeed increased PN-derived 3NT and 4HNE has been detected during the time of mitochondrial dysfunction and correlates with respiratory and Ca^{2+} buffering impairment (Sullivan et al. 2007). Increased synaptosomal 4-HNE content is associated with impaired synaptosomal glutamate and amino acid uptake (Carrico et al. 2009; Zhang et al. 1996). Glutamate and NMDA- induced damage in neuronal cultures is attenuated with LP inhibition, confirming LP and oxidative damage as promoters of glutamate excitotoxicity (Monyer et al. 1990; Pellegrini-Giampietro et al. 1990).

3.6 Mechanisms for Pharmacological Inhibition of Oxidative Damage

Based upon the discussion above concerning oxidative stress (increased ROS/RNS) and oxidative damage (LP, protein oxidation and nitration), a number of potential mechanisms for its inhibition are apparent which fall into five categories. The first category includes compounds that inhibit the initiation of LP and other forms of oxidative damage by **attenuating the formation of ROS or RNS species**. For instance, nitric oxide synthase (NOS) inhibitors exert an indirect antioxidant effect by limiting $NO^{\bullet}$ production and thus PN formation. However, they also have the

potential to interfere with the physiological roles that NO• is responsible for, including antioxidant effects which are due to its important role as a scavenger of lipid peroxyl radicals (e.g. LOO• + NO• → LOONO) (Hummel et al. 2006). Another approach to blocking posttraumatic radical formation is the inhibition of the enzymatic (e.g. cyclooxygenase, 5-lipoxygenases) arachidonic acid (AA) cascade during which $O_2^{\bullet -}$ is produced as a by-product of prostanoid and leukotriene synthesis. Kontos and colleagues (Kontos 1989; Kontos and Wei 1986) and Hall (1986) have shown that cyclooxygenase-inhibiting non-steroidal anti-inflammatory agents (e.g. indomethacin, ibuprofen) are vaso- and neuro-protective in TBI models.

Another example of an indirect approach for reducing the formation of ROS/RNS in the injured brain is via the inhibition of brain mitochondrial functional failure with the drug cyclosporine A which has been shown to reduce mitochondrial permeability transition pore (mPTP) formation by blocking cyclophilin D interaction with other components of the pathological pore which has been shown to lessen mitochondrial free radical formation and consequently attenuate LP and nitrative mitochondrial protein oxidative damage (Mbye et al. 2008; Sullivan et al. 1999).

A second indirect LP inhibitory approach involves **chemically scavenging the radical species** (e.g. $O_2^{\bullet -}$, OH•, NO•$_2$, $CO_3^{\bullet -}$) before they have a chance to steal an electron from a polyunsaturated fatty acid and thus initiate LP. The use of pharmacologically-administered SOD represents an example of this strategy. Another example concerns the use of the nitroxide antioxidant tempol which has been shown to catalytically scavenge the PN-derived free radicals NO•$_2$ and $CO_3^{\bullet -}$ (Carroll et al. 2000). In either case, a general limitation to these first two approaches and antioxidant agents that work by this mechanism is that they would be expected to have a short therapeutic window and would have to be administered rapidly in order to have a chance to interfere with the initial posttraumatic "burst" of free radical production that has been documented in TBI models (Kontos and Wei 1986; Hall et al. 1993). While it is believed that ROS, including PN production, persists several hours after injury, the major portion is an early event that peaks in the first 60 min after injury, making it clinically impractical to pharmacologically inhibit, unless the antioxidant compound is already "on board" when the TBI occurs (Fig. 3.2).

In contrast to the above indirect-acting antioxidant mechanisms, the third category involves stopping the "chain reaction" propagation of LP once it has begun. The most demonstrated way to accomplish this is by **scavenging of lipid peroxyl (LOO•) radicals**. The prototype scavenger of these lipid radicals is alpha tocopherol or vitamin E (Vit E) which can donate an electron from its phenolic hydroxyl moiety to quench a LOO•. However, the scavenging process is stoichiometric (1 Vit E can only quench 1 LOO•) and in the process vitamin E loses its antioxidant efficacy and becomes Vitamin E radical (LOO• + Vit E → LOOH + Vit E•). Although Vit E• is relatively unreactive (i.e. harmless), it also cannot scavenge another LOO• until it is reduced back to its active form by receiving an electron from other endogenous antioxidant reducing agents such as ascorbic acid (Vitamin C) or glutathione (GSH). While this tripartite LOO• antioxidant defense system (Vit E, Vit C, GSH) works fairly effectively in the absence of a major oxidative stress, numerous studies have shown that each of these antioxidants are rapidly consumed during the early

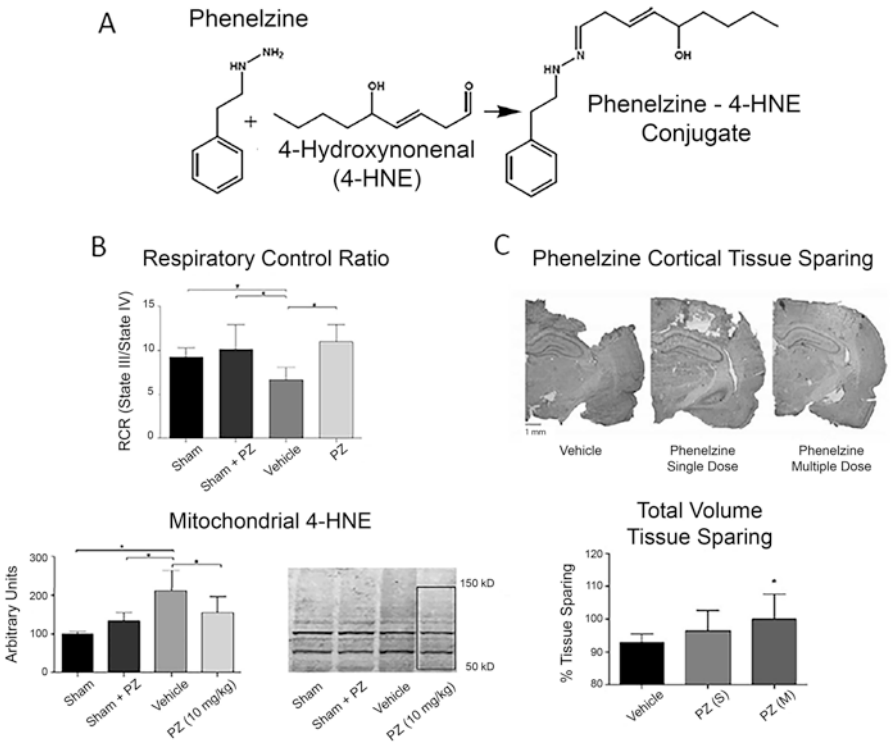

Fig. 3.2 (**a**) Chemical scavenging mechanism involved in the reactivity of the hydrazine-containing compound phenelzine with 4-HNE. (**b**) (Top): Effects of repeated phenelzine (PZ: 10 mg/kg s.c. 15 min after injury followed by maintenance dosing (5 mg/kg s.c.) every 12 h) on cortical mitochondrial bioenergetics 72 h following severe controlled cortical impact TBI. Mitochondrial respiration was measured with a Clark-type electrode expressed as respiratory control ratio (RCR). The RCR is rate of oxygen consumption during State III divided by State IV respiration. Animals received PZ rats were euthanized at 72 h. Sham and Sham + PZ groups were significantly different compared to Vehicle groups. RCR of PZ treatment was significantly increased compared to vehicle and not significantly different from either sham control variant (Sham or Sham + PZ). One-way ANOVA ($F = 7.7$, $df = 3, 24$, $P < 0.009$) followed by Student Newman-Keuls post-hoc test. $*p < 0.05$. Error bars represent ±SD; n = 8–9 rats per group except sham where n = 5 rats per group. (**b**) (Bottom): Repeated PZ reduces 4-hydroxynonenal (4-HNE) accumulation in mitochondria 72 h after TBI. As revealed by quantitative western blot (see sample blot and bar graph), 4-HNE-modified proteins were significantly elevated in the Vehicle group compared to both Sham groups. PZ treatment group exhibited significantly reduced oxidative damage compared to Vehicle group, but did not return to Sham levels. ANOVA ($F = 9.9$, $df = 3, 24$, $p < 0.0002$) followed by Student Newman-Keuls post-hoc test. $*p < 0.05$. (**c**) Repeated PZ reduces cortical neurodegeneration 72 h after TBI: Coronal sections of ipsilateral rat brains rat taken at 1.2× magnification. **Left**: Vehicle (0.9% saline) treated rat brain injected 15 min after TBI; **Center**: Phenelzine (PZs) single 10 mg/kg s.c. dose treated animal; **Right**: Rat brain of PZ-treated with a multiple dosing paradigm (PZm): single subcutaneous injection of PZ 15 min after injury, followed by maintenance dosing of 5 mg/kg every 12 h thereafter. All groups (Vehicle, PZ(S), PZ(M)) were euthanized 72 h after first injection. Black bar under the photomicrographs represents 1 mm. The graph below the photos shows percent of cortical tissue sparing followed by either Vehicle (saline), PZ(S), or PZ(M) treatment. Rats were euthanized in all treatment paradigms at 72 h after first injection. PZs did not exhibit a statistically significant amount of cortical tissue sparing when compared to Vehicle. However, PZm significantly increased the total volume of spared cortical tissue. One-way ANOVA followed by Dunnett's post-hoc test. $*p < 0.05$ compared to Vehicle. Error bars represent mean ± SD; n = 6 rats for vehicle group; n = 8 rats per group for drug-treated rats. These data are reproduced with permission from Cebak et al. (2017)

minutes and hours after CNS injury (Hall et al. 1989, 1992). Thus, it has long been recognized that more effective brain penetrable pharmacological $LOO^{\bullet}$ scavengers are needed. Furthermore, compared to antioxidants that are scavengers of the initial post-TBI oxygen radical burst, it is reasonable to theorize that antioxidants that interrupt the LP process after it has begun would be able to exert a more clinically practical neuroprotective effect (i.e. possess a longer antioxidant therapeutic window).

An additional approach to inhibiting the propagation of LP reactions is to **chelate free iron**, either ferrous (Fe^{2+}) or ferric (Fe^{3+}), which potently catalyzes the breakdown of lipid hydroperoxides (LOOH), an essential event in the continuation of LP chain reactions in cellular membranes. The prototypical iron-chelating drug which chelates Fe^{3+}, is the tri-hydroxamic acid compound deferoxamine.

The fourth antioxidant category that has begun to be explored for neuroprotection following TBI concerns **pharmacological scavenging of LP-derived aldehydic (carbonyl-containing) breakdown products 4-HNE and acrolein**. As introduced earlier, these highly neurotoxic compounds have high affinity for covalently binding to basic amino acid residues including histidine, lysine, arginine and cysteine. These modifications have been shown to inhibit the activities of a variety of enzymatic proteins (Halliwell and Gutteridge 2008). Also, 4-HNE and acrolein, formed by LP oxidative damage, are also associated with stimulating additional free radical generation (i.e. oxidative stress) in injured CNS tissue (Hamann and Shi 2009). Several compounds have been identified that are able to antagonize this "carbonyl stress" by covalently binding to reactive LP-derived aldehydes. Two commercially available FDA-approved drugs that have been tested in TBI models are D-penicillamine and phenelzine, whose neuroprotective effects will be briefly discussed in the next section of this chapter.

A fifth antioxidant category that is theoretically an attractive broad spectrum mechanistic approach for achieving neuroprotection in TBI involves **pharmacologically activating the body's endogenous pleiotropic antioxidant defense system** that is largely regulated by nuclear factor E2-related factor 2/antioxidant response element (Nrf2/ARE) signaling at the transcriptional level (Kensler et al. 2007). As will be discussed below, Nrf2 activation and the up-regulation of antioxidant and anti-inflammatory genes, which has been previously described in experimental models of stroke and neurodegenerative disease (Shih et al. 2003), appears to be particularly promising in TBI models. Indeed, it has been documented that in the mouse controlled cortical impact TBI paradigm the injury itself upregulates Nrf2 and antioxidant gene expression. However, the time course of that antioxidant response occurs simultaneously with the time course of posttraumatic LP in brain tissue (Miller et al. 2014). Thus, what is needed is a compound that speeds up and increases the magnitude of the post-TBI Nrf2/ARE activation in the injured brain so that is has a chance to attenuate the peak of posttraumatic oxidative neural damage. Two such naturally occurring compounds that have been shown to be protective in TBI models are sulforaphane, found in high concentrations in broccoli, and carnosic acid, found in the herb rosemary.

Finally, a sixth strategy for achieving antioxidant neuroprotection in injured brain tissue involves **protecting the neural mitochondrion** which is essential for maintaining ATP production via the multi-complex electron transport chain as well as for its role in excessive post-TBI cytoplasmic Ca^{2+} accumulation. While mitochondrial Ca^{2+} buffering is one of the major functions of cellular mitochondria, as the intra-mitochondrial Ca^{2+} concentration increases, this leads to increases in mitochondrial $O_2^{\bullet-}$ as well as activation of a mitochondrial NOS that produces $NO^{\bullet}$. These two radicals rapidly combine to generate peroxynitrite and its derived highly reactive radicals ($OH^{\bullet}$, $NO\bullet_2$, $CO_3^{\bullet-}$) which cause damage NO• leakage, causing oxidative damage to the electron transport chain (Bains and Hall 2012). Ultimately, mitochondrial dysfunction triggers the formation of the multi-component mitochondrial permeability transition pore (mPTP), which when it opens triggers mitochondrial permeability transition (mPT) loss of ionic gradients and leakage of important mitochondrial proteins (e.g. cytochrome C). Cyclosporine A (CsA), in addition to its immunosuppressive properties caused by inhibition of calcineurin, also has the ability to prevent mPTP formation by binding to one of the mPTP components, cyclophilin D, preventing the latter from joining mPTP complex, which is required in order for mPT to take place. Consequently, CsA acts to rescue the mitochondrion, preserves membrane potential and lessens additional ROS generation and oxidative damage. This has repeatedly been demonstrated in TBI models (Mbye et al. 2008; Sullivan et al. 1999). Because CsA is not an electron-donor or radical scavenger, its antioxidant action is consequently indirect. In other words, by preventing mPT from occurring it decreases mitochondrial ROS generation and thus indirectly limits oxidative damage. That this protective effect of CsA has little or nothing to do with its inhibition of calcineurin is due to the demonstration that the non-immunosuppressive CsA analog NIM811 does not inhibit calcineurin, but does bind to cyclophilin D, is just as protective as CsA in terms of mitochondrial function in the injured brain and able to reduce lesion volume (Mbye et al. 2008, 2009; Readnower et al. 2011)

3.7 Neuroprotective Effects of Pharmacological Antioxidants in TBI Patients and Models

TBI Clinical Trial Results with PEG-SOD and Tirilazad: During the past 30 years, there has been an intense effort to discover and develop pharmacological agents for acute treatment of TBI. This has included two compounds that possess free radical scavenging/antioxidant properties, including polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) and the LP inhibitor tirilazad, that were tested in phase III clinical trials in a pathologically heterogeneous population of TBI patients (Langham et al. 2000; Marshall et al. 1998; Narayan et al. 2002). However, each of these trials was a therapeutic failure in that no overall benefit was documented. These failures have been hypothesized to be due to several factors (Narayan et al. 2002).

PEG-SOD: As mentioned earlier, the initial studies of free radical scavenging compounds in TBI models were carried out with Cu/Zn SOD based upon the work of Kontos and colleagues, who showed that post-traumatic microvascular dysfunction was initiated by $O_2^{\bullet-}$ generated as a by-product of the arachidonic acid cascade, which is massively activated during the first minutes and hours after TBI (Kontos 1989; Kontos and Wei 1986; Kontos and Povlishock 1986). Their work showed that administration of SOD prevented the post-traumatic microvascular dysfunction. This led to clinical trials in which the more metabolically stable polyethylene glycol (PEG)-conjugated SOD was examined in moderate and severe TBI patients when administered within the first 8 h after injury. Although an initial small phase II study showed a positive trend, subsequent multi-center phase III studies failed to show a significant benefit in terms of increased survival or improved neurological outcomes (Muizelaar et al. 1995). One theoretical reason may be that a large protein like SOD is unlikely to have much brain penetrability and therefore its radical scavenging effects may be limited to the microvasculature. A second reason may be that attempting to scavenge the short-lived inorganic radical $O_2^{\bullet-}$ may be associated with a very short therapeutic window, as suggested above. As pointed out earlier, the time course of measurable post-traumatic $OH^{\bullet}$ formation in the injured rodent brain has been shown to largely run its course by the end of the first hour after TBI (Hall et al. 1993; Smith et al. 1994). A more rational strategy would be to inhibit the LP that is triggered by the initial burst of inorganic radicals. A comparison of the time course of LP with that of post-traumatic $OH^{\bullet}$ shows that LP reactions continue to build beyond the first post-traumatic hours (Smith et al. 1994) and may continue for 3–4 days (Du et al. 2004; Miller et al. 2014; Hall et al. 2012). Despite the failure of PEG-SOD in human TBI, experimental studies have shown that transgenic mice that over-express Cu/Zn SOD are significantly protected against post-TBI pathophysiology and neurodegeneration (Chan et al. 1995; Gladstone et al. 2002; Lewen et al. 2000; Mikawa et al. 1996; Xiong et al. 2005). This fully supports the importance of post-traumatic $O_2^{\bullet-}$ in post-traumatic secondary injury, despite the fact that targeting this primordial radical is probably not the best antioxidant strategy for acute TBI compared to trying to stop the downstream LP process that is initiated by the early increases in $OH^{\bullet}$, $NO\bullet_2$ and $CO_3^{\bullet-}$.

Tirilazad: Consistent with that rationale, the 21-aminosteroid LP inhibitor tirilazad was discovered, which inhibits free radical-induced LP by a combination of $LOO^{\bullet}$ scavenging and a membrane-stabilizing action that limits the propagation of LP reactions between an $LOO^{\bullet}$ and an adjacent polyunsaturated fatty acid (Hall et al. 1994). The protective efficacy of tirilazad has been demonstrated in multiple animal models of acute TBI in mice (Hall et al. 1988), rats (McIntosh et al. 1992) and cats (Dimlich et al. 1990). While the compound is largely localized in the microvascular endothelium, the post-traumatic disruption of the BBB is known to allow the successful penetration of tirilazad into the brain parenchyma as noted earlier (Hall et al. 1992). Other mechanistic data derived from the rat controlled cortical impact and the mouse diffuse concussive head injury models have definitively shown that a major effect of tirilazad is to lessen post-traumatic microvascular damage including BBB opening (Hall et al. 1992; Smith et al. 1994).

Tirilazad was taken into clinical development in the early 1990s, and following a small phase II dose-escalation study that demonstrated the drug's safety in TBI patients, it was evaluated in two phase III multi-center clinical trials for its ability to improve neurological recovery in moderately and severely injured closed TBI patients. One trial was conducted in North America and the other in Europe. In both trials, TBI patients were treated within 4 h after injury with either vehicle or tirilazad (10 mg/kg i.v. q6h for 5 days). The North American trial was never published, due to a major confounding imbalance in the randomization of the patients to placebo or tirilazad in regards to injury severity and pre-treatment neurological status. In contrast, the European trial that 1120 enrolled had much better randomization balance and was published (Marshall et al. 1998). As observed for PEG-SOD, tirilazad failed to show a significant beneficial effect of tirilazad in either moderate (GCS = 9–12) or severe (GCS = 4–8) patient categories. However, a post hoc analysis showed that moderately-injured male TBI patients with traumatic subarachnoid hemorrhage (tSAH) had a significantly lower incidence of 6 month mortality after treatment with tirilazad (6%) compared to placebo (24%, $p < 0.042$). In severely injured males with tSAH, tirilazad also lessened mortality from 43% in placebo-treated to 34% ($p < 0.026$). This result is consistent with the fact that tirilazad is also highly effective in reducing SAH-induced brain edema and vasospasm in animal models of SAH (Hall et al. 1994). Nevertheless, additional trials would have been required in order to establish the neuroprotective utility of tirilazad in tSAH patients in order to gain FDA approval. However, the sponsoring company Pharmacia & Upjohn opted not to continue the compound's development for TBI although tirilazad was successfully approved and marketed for use in aneurysmal SAH (aSAH) in several western European countries, Australia, New Zealand and South Africa, based upon its demonstrated efficacy in phase III aSAH trials conducted in those countries (Kassell et al. 1996; Lanzino and Kassell 1999). Therefore, the apparent post hoc-identified benefit in tSAH patients is consistent with tirilazad's prospectively demonstrated efficacy in aSAH patients, also mainly observed in males.

Effects of Other Direct and Indirect-Acting Lipid Peroxidation Inhibitors: In addition to tirilazad, several other LP inhibitors have been reported to be effective neuroprotectants in TBI models. These include the lipid peroxyl radical ($LOO^{\bullet}$) scavenging 2-methylaminochromans U-78517F and U-83836E (Hall et al. 1991; Mustafa et al. 2010, 2011), the pyrrolopyrimidine U-101033E (Hall et al. 1997; Xiong et al. 1997, 1998), OPC-14117 (Mori et al. 1998) and the naturally-occurring $LOO^{\bullet}$ scavengers curcumin (Sharma et al. 2009; Wu et al. 2006) and resveratrol (Ates et al. 2007; Sonmez et al. 2007), the indoleamine melatonin (Beni et al. 2004; Cirak et al. 1999; Mesenge et al. 1998; Ozdemir et al. 2005a, b) and lastly, the endogenous antioxidant lipoic acid (Toklu et al. 2009). In the case of curcumin and resveratrol, these are potent $LOO^{\bullet}$ scavengers due to their possession of multiple phenolic hydroxyl groups that can donate electrons to $LOO^{\bullet}$ radicals. Melatonin also has $LOO^{\bullet}$ scavenging capability (Longoni et al. 1998), but in addition appears to react with PN (Zhang et al. 1999). Lipoic acid may also have $LOO^{\bullet}$ scavenging effects, but these are more likely to be indirect via the regeneration (i.e. re-reduction)

of other endogenous electron-donating antioxidants, including vitamin E, glutathione and vitamin C.

Among these LP inhibitors, arguably the most potent and effective $LOO^{\bullet}$ scavenging LP inhibitor yet discovered is the 2-methylaminochroman compound U-83836E which combines the $LOO^{\bullet}$ scavenging antioxidant chroman ring structure of vitamin E with the bis-pyrrolopyrimidine moiety of tirilazad. The phenolic chroman antioxidant moiety, after it sacrifices it phenolic electron to scavenge an $LOO^{\bullet}$, can be re-reduced by endogenous ascorbic acid (vitamin C) or glutathione (GSH) making it able to quench a second and then a third $LOO^{\bullet}$, etc. The bis-pyrrolopyrimidine moiety, on the other hand, can also scavenge multiple moles of $LOO^{\bullet}$ by a true catalytic mechanism (Hall et al. 1991; Hall et al. 1995). Thus, U-83836E, is a dual functionality $LOO^{\bullet}$ scavenger that is understandably more effective than either vitamin E, tirilazad (Hall et al. 1991) and possibly the other naturally-occurring $LOO^{\bullet}$ scavengers such as curcumin, resveratrol, melatonin and lipoic acid. Furthermore, U-83836E possesses a high degree of lipophilicity endowing it with a high affinity for membrane phospholipids where LP takes place. Studies from the authors' laboratory in the mouse CCI-TBI model have shown that U-83836E is able to reduce post-traumatic LP and protein nitration and preserve mitochondrial respiratory function, and lessen calpain-mediated neuronal cytoskeletal degradation and decrease injured tissue (Mustafa et al. 2010, 2011).

Nitroxide Antioxidants and Peroxynitrite Scavengers: In addition to the lipid peroxyl ($LOO^{\bullet}$) radical scavengers, the neuroprotective effects of a family of nitroxide-containing antioxidants have also been examined in experimental TBI models. These are sometimes referred to as "spin-trapping agents" and include α-phenyl-tert-butyl nitrone (PBN) and its thiol analog NXY-059 and tempol. Both PBN and tempol have been shown to be protective in rodent TBI paradigms (Awasthi et al. 1997; Marklund et al. 2001). As mentioned earlier, tempol has been shown by the author and colleagues to catalytically scavenge PN-derived $NO\bullet_2$ and $CO_3^{\bullet-}$ (Carroll et al. 2000; Bonini et al. 2002), and to reduce post-traumatic oxidative damage (both LP and protein nitration), preserve mitochondrial function, decrease calcium-activated, calpain-mediated cytoskeletal damage and reduce neurodegeneration in mice subjected to a severe controlled cortical impact-induced focal TBI (Deng-Bryant et al. 2008). Earlier, another laboratory reported that tempol can reduce post-traumatic brain edema and improve neurological recovery in a rat contusion injury model (Beit-Yannai et al. 1996; Zhang et al. 1998). However, the neuroprotective effect of tempol, administered alone, is associated with a therapeutic window of an hour or less in the mouse controlled cortical impact TBI (CCI-TBI) model. Moreover, tempol is not effective at directly inhibiting LP in the latter model (Deng-Bryant et al. 2008).

Effects of the Iron Chelator Deferoxamine: The prototype iron chelator deferoxamine, which binds ferric (Fe^{3+}) iron and thereby would lessen the catalytic effects of iron on LP, has also been reported to have beneficial actions in preclinical TBI or TBI-related models (Gu et al. 2009; Long et al. 1996). However, deferoxamine is hindered by its limited brain penetration and rapid plasma elimination rate. To counter

the latter limitation, a dextran-coupled deferoxamine has been synthesized that has been reported to significantly improve early neurological recovery in a mouse diffuse TBI model (Panter et al. 1992). Much of this activity, however, is probably due to microvascular antioxidant protection because of limited brain penetrability. Another caveat to the iron-chelation antioxidant neuroprotective approach that is at least relevant to the ferric iron chelators such as deferoxamine is that at they can cause a pro-oxidant effect in that their binding of Fe^{3+} can actually drive the oxidation of ferrous to ferric iron which can increase superoxide radical formation in the process ($Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}$).

Effects of Carbonyl Scavengers: We have previously demonstrated that D-penicillamine is able to scavenge PN (Althaus et al. 1994) and to protect brain mitochondria from PN-induced respiratory dysfunction in isolated rat brain mitochondria (Singh et al. 2007). D-Penicillamine has also been documented to form an irreversible bond to primary aldehydes, enabling it to scavenge neurotoxic LP-derived carbonyl compounds such as 4-HNE and acrolein (Wood et al. 2008). Consistent with that mechanism of action, D-penicillamine was shown to attenuate the levels 4-HNE-modified brain mitochondrial proteins after exposure of isolated mitochondria to 4-HNE (Singh et al. 2007). The PN scavenging action of D-penicillamine along with its carbonyl scavenging capability may jointly explain our previous findings that acutely administered penicillamine can improve early neurological recovery of mice subjected to moderately severe concussive TBI (Hall et al. 1999).

More recently, it has been demonstrated that a variety of FDA-approved hydrazine ($–NH–NH_2$)-containing compounds including the anti-hypertensive agent hydralazine and the anti-depressant phenelzine can react with the carbonyl moieties of 4-HNE or acrolein, which prevents the latter from binding to susceptible amino acids in proteins (Galvani et al. 2008). Most impressive is the fact that the application of hydrazines can rescue cultured cells from 4-HNE toxicity even when administered after the 4-HNE has already covalently bound to cellular proteins (Galvani et al. 2008). Consistent with this effect being neuroprotective, others have shown that hydralazine inhibits either compression or acrolein-mediated injuries to ex vivo spinal cord (Hamann et al. 2008). However, hydralazine, which is a potent vasodilator, would be difficult to administer in vivo after either spinal cord injury or TBI in which hypotension is already a common pathophysiological problem. In contrast, another FDA-approved hydrazine-containing drug phenelzine, used for certain depressive patients, should not compromise blood pressure as readily as hydralazine. Accordingly, a recently published paper has shown that phenelzine administration to rats subjected to acute contusion SCI mitigated post-SCI neuropathic pain, reduces motor deficits and improves spinal cord tissue sparing (Chen et al. 2016). Earlier studies have demonstrated neuroprotective efficacy in a rodent ischemia-reperfusion stroke model that were attributed to reducing ‘aldehyde load’ in the stroke-injured brain (Wood et al. 2006).

In vitro studies in our laboratory have documented the ability of phenelzine to protect isolated rat brain mitochondria from the respiratory depressant effects of 4-HNE, together with a concentration-related attenuation of the accumulation of

4-HNE modified mitochondrial proteins. More recently, we have observed that phenelzine is able to protect isolated mitochondria from respiratory functional depression and modification of mitochondrial proteins following application of the more highly reactive aldehyde acrolein (Cebak et al. 2017). Subsequent in vivo studies in the rat controlled cortical impact TBI model have found that a single 10 mg/kg s.c. dose of phenelzine can also reduce early (3 h) posttraumatic mitochondrial respiratory failure as well as reducing cortical lesion volume at 14 days post-injury (Singh et al. 2013). To better define the optimal neuroprotective use of phenelzine, additional in vivo TBI studies have demonstrated that repeated dosing with phenelzine over a 60 h post-TBI period is capable of protecting delayed mitochondrial failure at its peak at 72 h in the same TBI model along with a reduction in cortical lesion volume that is greater than that seen with a single early dose. This makes sense in that the adequate carbonyl-scavenging drug levels logically need to be maintained during the 72 h long time course of posttraumatic generation of LP-derived neurotoxic aldehydes (Cebak et al. 2017).

Effects of Nrf2/ARE Signaling Activators: The body's endogenous antioxidant defense system is largely regulated by nuclear factor E2-related factor 2/antioxidant response element (Nrf2/ARE) signaling at the transcriptional level (Zhang 2006; Kensler et al. 2007). Nrf2 activation and the up-regulation of antioxidant and anti-inflammatory genes represents a valid neurotherapeutic intervention in CNS injury and has been previously described in various experimental models of stroke and neurodegenerative diseases (Shih et al. 2003). More recently, the role of Nrf2/ARE activation in SCI has been explored as a targeted neuroprotective strategy for both TBI and SCI. Indeed, studies in Nrf2 (−/−) mice demonstrated increased spinal cord edema and expression of inflammatory cytokines compared to wild-type Nrf2 mice following SCI (Mao et al. 2010; Mao et al. 2011). In mild rat thoracic SCI, it has been reported that Nrf2 levels increase as early as 30 min post-injury and remain elevated through 3 days. In the same study, application of the natural product sulforaphrane, a Nrf2/ARE signaling activator, significantly reduced contusion volume and increased post-SCI coordination. These positive outcomes were a result of sulforaphrane-induced increases in Nrf2, glutamine and decreases in inflammatory cytokines, IL-1β and TBFα (Wang et al. 2012).

The mRNA levels of Nrf2-regulated antioxidant enzymes, heme oxygenase (HO-1) and NADPH:quinone oxidoreductase-1 (NQO1), are up-regulated 24 h post TBI (Yan et al. 2008). In addition, Nrf2-knockout mice are susceptible to increased oxidative stress and neurologic deficits following TBI compared to their wild-type counterparts (Hong et al. 1994). Administration of sulforaphane is also neuroprotective in various animal models of TBI, specifically reducing cerebral edema and oxidative stress and improving BBB function and cognitive deficits (Dash et al. 2009). Studies by Chen et al. (2011) demonstrated increased expression of Nrf2 and HO-1 in the cortex of the rat subarachnoid hemorrhage model. Treatment with sulforaphane further increased the expression of Nrf2, HO-1, NQ01 and glutathione S-transferase-α1 (GST-α1), resulting in the reduction of brain edema, cortical neuronal death and motor deficits (Chen et al. 2011). Tert-butylhydroquinone, another

activator of Nrf2, protects against TBI-induced inflammation and damage via reduction in NF-KB activation and TNFα and IL-1β production following injury in the mouse closed head injury model (Jin et al. 2011). Collectively these studies demonstrate a significant neuroprotective role of Nrf2 signaling through the activation of antioxidant enzymes and reduction of oxidative secondary injury responses following CNS injury. Thus, Nrf2 activation may be a prime candidate for the attenuation of oxidative stress and subsequent neurotoxicity in TBI via the development of small-molecule activators of the Nrf2/ARE pathway.

Recent work in our laboratory has revealed that following controlled cortical impact TBI in mice, there is indeed a progressive activation of the Nrf2-ARE system in the traumatically-injured brain as evidenced by an increase in HO-1 mRNA and protein that peaks at 72 h after TBI. However, this effect does not precede, but rather it is coincident with the post-injury increase in LP-related 4-HNE (Miller et al. 2014). Therefore, it is apparent that this endogenous neuroprotective antioxidant response needs to be pharmacologically enhanced and/or sped up if it is to be capable of exerting acute post-TBI neuroprotection. Our laboratory is currently studying another Nrf2-ARE activator, natural product carnosic acid, that has been shown by others to more effectively induce this antioxidant defense system than the prototype sulforaphane (Satoh et al. 2008). We have shown that administration of carnosic acid to non-TBI mice is able to significantly increase the resistance of cortical mitochondria harvested 48 h later to the respiratory depressant effects of the in vitro applied 4-HNE together with in decrease in 4-HNE modification of mitochondrial proteins (Miller et al. 2013). Subsequently, we have administered a single 1 mg/kg i.p. dose of carnosic acid to mice at 15 min after controlled cortical impact TBI and observed that the compound is able to significantly preserve respiratory function along with a reduction in the level of LP-mediated damage in mitochondria harvested from the injured cortex at 24 h after TBI (Miller et al. 2015). Furthermore, carnosic acid's antioxidant effects were still apparent at 48 h post-injury in terms of an attenuation of 4-HNE and 3-NT in the injured cortical tissue together with a decrease in Ca^{2+}-activated, calpain-mediated neuronal cytoskeletal degradation. In regards to the latter neuronal protective effect, a decrease in 48 h cytoskeletal degradation was also shown to occur even with a post-TBI treatment delay of 8 h. Ongoing studies are evaluating the behavioral recovery and tissue protective effects of carnosic acid whether these are achievable with a clinically practical therapeutic window.

3.8 Combination Antioxidant Treatment of Traumatic Brain Injury

Antioxidant neuroprotective therapeutic discovery directed at acute TBI has consistently been focused upon attempting to inhibit the secondary injury cascade by pharmacological targeting of a single oxidative damage mechanism. As presented above, these efforts have included either enzymatic scavenging of superoxide radicals with SOD (Muizelaar et al. 1995) or inhibition of LP with tirilazad

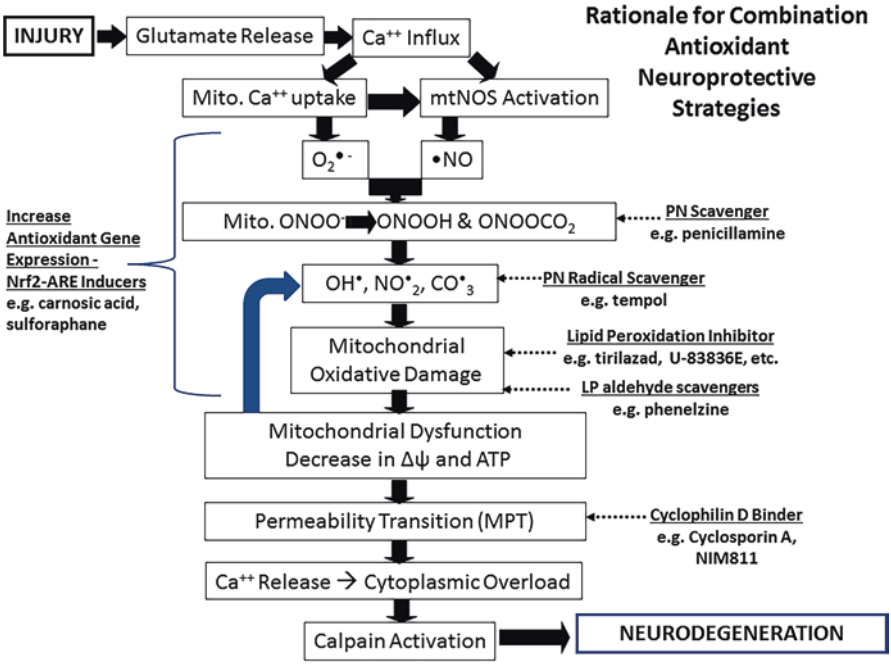

Fig. 3.3 Rationale for the combination of two or more antioxidant strategies to achieve a more effective and consistent (i.e. less variable) neuroprotective effect in the injured brain

(Marshall et al. 1998). While each of these strategies alone has shown protective efficacy in animal models of TBI, phase III clinical trials with either compound failed to demonstrate a statistically significant positive effect although post hoc subgroup analysis suggests that the microvascularly localized tirilazad may have efficacy in moderate and severe TBI patients with tSAH (Marshall et al. 1998). While many reasons have been identified as possible contributors to the failure, one logical explanation has to do with the possible need to interfere at multiple points in the oxidative damage portion of the secondary injury cascade either simultaneously or in a phased manner in order to achieve a clinically demonstrable level of neuroprotection. To begin to address this hypothesis, we are currently exploring the possibility that reducing posttraumatic oxidative damage more completely and less variably might be achievable by combined treatment with two or more mechanistically complimentary antioxidant compounds. Figure 3.3 summarizes the overall rationale for a multi-mechanistic antioxidant therapy for TBI. It is anticipated that the combination of two or three antioxidant mechanistic strategies may improve the extent of neuroprotective efficacy, lessen the variability of the effect and possibly provide a longer therapeutic window of opportunity compared to the window for the individual strategies. Figure 3.4 shows preliminary, not yet published data, suggesting that combination treatment of the PN radical scavenger tempol with the LP inhibitor U-83836E in mice subjected to controlled cortical impact TBI was more effective in

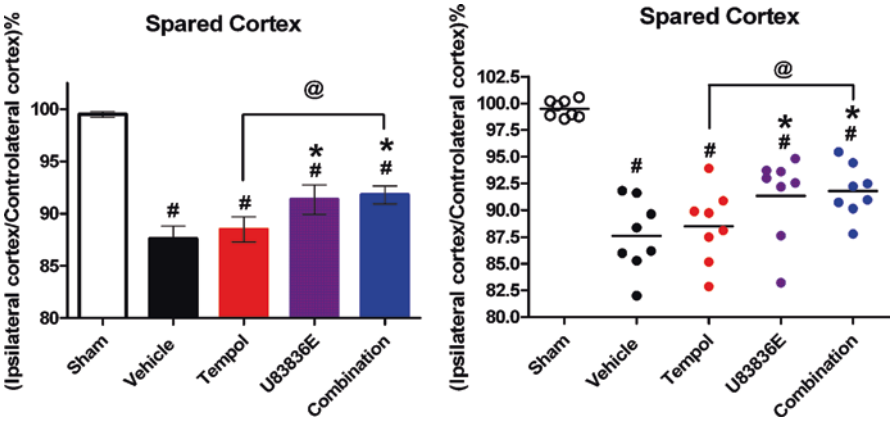

Fig. 3.4 Preliminary data on the neuroprotective effects of 15 min post-injury administration of tempol, U83836E or the combination on cortical tissue sparing. U83836E and the combination both significantly improved tissue sparing whereas tempol in this experiment did not. However, only the combination significantly out-performed tempol. As in scatter plot on the right, the variability in the combination group was considerably lower than in the single treatment groups. All values = mean ± SEM for N = 8/group; #$p < 0.05$ vs sham; *$p < 0.05$ vs. vehicle, @$p < 0.05$ tempol alone vs. combination

reducing 7 day post-TBI cortical tissue damage as well as resulting in a reduction in the variability of the data to half of that seen in the parallel groups treated with the either of the two drugs alone.

In other published studies, we have observed that combined treatment with an LP inhibitor with an inhibitor of excitotoxic glutamate release increases the neuroprotective therapeutic window. Using an infant rat model of shaken baby-induced brain damage model, we have documented that treatment with riluzole, an inhibitor of excitotoxic glutamate release, attenuated cortical neurodegeneration measured at 14 days post-TBI, but the therapeutic window for this neuroprotective effect was limited to the initial riluzole dose having to be administered during the first hour after TBI. However, when the infant rats received the LP inhibitor tirilazad at 30 min after TBI, it increased the neuroprotective therapeutic window for riluzole to 4 h after TBI (Smith and Hall 1998). Thus, combination treatments may extend the neuroprotective efficacy window significantly. Accordingly, combination neuroprotective therapy might be able to improve efficacy, reduce variability and improve the therapeutic window for achievement of clinically measurable neuroprotection in TBI patients, although additional work remains to be conducted to determine whether that neuroprotective hypothesis is correct.

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Chapter 4
Mitochondrial Damage in Traumatic CNS Injury

W. Brad Hubbard, Laurie M. Davis, and Patrick G. Sullivan

Abstract Traumatic brain injury (TBI) is ever-present in societal issues and puts a tremendous economic burden on U.S. healthcare and affected individuals. Symptomology after TBI is sometimes subtle without outward signs of injury and neurological impairments can be long-lasting. Unfortunately, the underlying cellular mechanisms that occur in response to TBI have not fully been deduced and as such there is no FDA-approved treatment for the resulting neurological pathology. In this chapter, we will review the essential role of mitochondria in the CNS while highlighting numerous functions of this crucial organelle under basal conditions. Furthermore, we review the bevy of literature showing the dysfunction of mitochondria after TBI. After TBI, these normal mitochondrial functions become dysregulated in response to elevated Ca^{2+} mitochondrial buffering and further cause downstream damage. The loss of adequate membrane potential can lead to lower ATP levels and necrotic cell death. Ca^{2+} overload also leads to the formation of the mitochondrial permeability transition pore and cytochrome c-mediated apoptosis. Aberrant levels of reactive oxygen species resultant from mitochondrial dysfunction cause increased lipid peroxidation and presence of 4-hydroxynonenal. All of these secondary cascades involved in mitochondrial dysfunction present unique therapeutic targets that investigators throughout the years have evaluated. The development of new pharmacological tools, with translatable potential, targeting mitochondrial pathways will further validate the critical role of mitochondria in outcomes after TBI and hopefully advance research to find an effective treatment.

Keywords Mitochondria · Traumatic brain injury · Electron transport chain · Cell death · Reactive oxygen species

W. B. Hubbard · L. M. Davis · P. G. Sullivan (✉)
Department of Neuroscience & The Spinal Cord & Brain Injury Research Center (SCoBIRC), The University of Kentucky Chandler College of Medicine, Lexington, KY, USA
e-mail: patsullivan@uky.edu

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4.1 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 the underlying neural damage can present without any outward sign of physical damage and the victims are usually cognitively impaired, it has become a largely 'silent epidemic' (Jennett 1998; Thurman et al. 1999; Jager et al. 2000; Langlois et al. 2006; CDC 2015). 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 and often develop prolonged and chronic neurological symptoms (CDC 2015). The growing injured population has presented our society with an enormous economic and social burden, as these patients are commonly unable to properly reintegrate into their previous professional and social networks. An estimated 43% of hospitalized TBI patients sustain long-term disability, highlighting the need for rehabilitative care and support for these patients throughout their lives (Selassie et al. 2008). There is a substantial economic impact with initial health care costs and long term support for TBI totaling tens of billions dollars per year nationally (Langlois et al. 2006). There are some clinical options designed to allow people to survive their injuries, which include assuaging acute brain edema, decreasing intracranial pressure, and preventing of peripheral complications; however there is a void of treatment to mitigate neuropathology or to attenuate and 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 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. Actual brain injuries that were labeled under the umbrella term of SS were potentially the result of combat conducted in "trenches," where explosives fragments as well as reflected blast overpressure could impact soldiers (Mott 1916). Symptoms of SS ranged from headaches to depression with lack of standard symptom scoring. It had become 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 effect 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 (Langlois et al. 2006; Rapoport et al. 2006; CDC 2015). 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. 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; Losoi et al. 2016).

Figure 4.1 shows the "iceberg" effect of TBI incidence according to the CDC. TBI accounts for over 50,000 deaths per year, though this is only the tip of the iceberg in terms of those whose lives are impacted by TBI and have to receive medical care. The alarming statistic is that the number of individuals who sustain TBIs that are not treated or diagnosed is inestimable. Persons in this category can often lead lives with complications that go unresolved. Although great strides have been made in TBI education and diagnosis (ED visits for TBI-related injures doubled from 2003 to 2010), these efforts need to continue to provide the ability for diagnosis for all TBIs (CDC 2015). This can range from informing the general public about brain injury as well as clinical head trauma management. As advances in medicine continue, patients are able to survive injuries that would be fatal if treated several decades ago in the battlefield and clinic (Okie 2005; Warden 2006). For multiple organ injury sustained from motor vehicle accidents or severe falls, necessary steps must be taken to observe brain damage early on, despite other high priority injuries to the patient (Leong et al. 2013; Sharma et al. 2014). It has also been shown that multi/polytrauma coupled with TBI can change the inflammatory dynamics in the prognosis of TBI, so interaction with other injuries should be accounted for in treatment (McDonald et al. 2016).

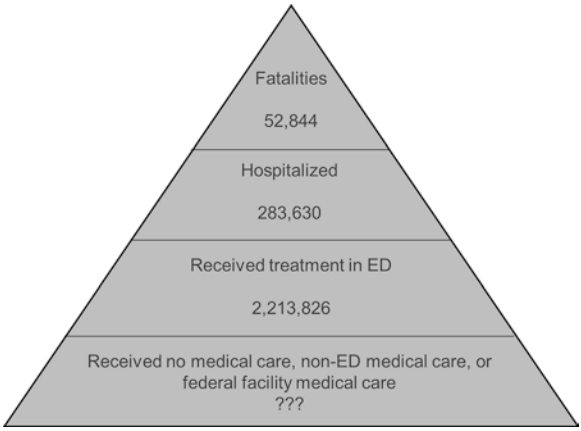

Fig. 4.1 The iceberg effect of TBI. Although there are over 50,000 fatalities yearly in the US, this is only a small fraction of the people who are affected each year by TBIs. With respect to TBI-related fatalities, over five times more people are hospitalized and over 40 times more people are treated in an Emergency Department (ED). These numbers greatly underestimate the total amount of people who sustain a TBI

In recent years, mild TBI has become a major concern of military medicine, as over 80% of TBI are classified as mild by the Department of Defense. Since 2000, the number of TBIs diagnosed in U.S. service members steadily increased until 2011, where the peak reached around 34,000 diagnoses (DoD 2014). Blast has caused the majority of injuries and TBIs in recent war zones, and has been labeled as a signature injury of recent U.S. involvement in Middle Eastern conflicts, such as Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) (Warden 2006; Hoge et al. 2008; Snell and Halter 2010). 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, this injury population also has an increased predisposition to the development of post-traumatic stress disorder (PTSD). Several studies have found that Veterans from recent military conflicts (OIF/OEF) predominantly present with a combination of conditions, namely chronic pain, PTSD, and persistent postconcussive symptoms (PPCS) (Clark et al. 2007a, 2009; Lew et al. 2009; Sayer et al. 2009). For clinical reports, TBI refers to the initial insult sustained and its pathology, whereas PPCS relates to the expression of symptoms following TBI (Marshall et al. 2015). 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). Mild TBI is a growing concern in the US civilian population as the

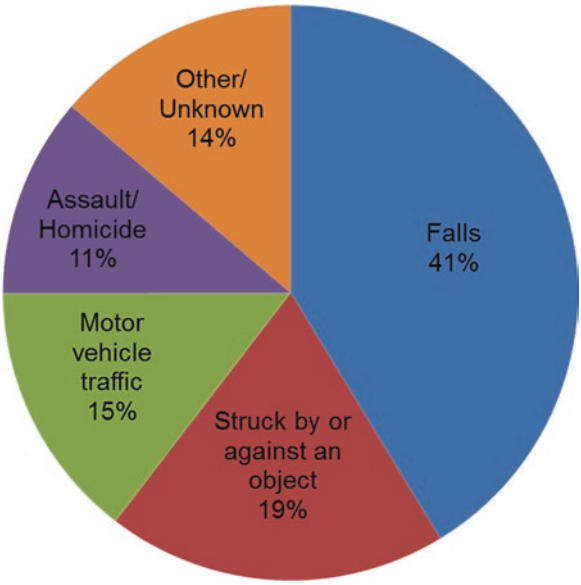

Fig. 4.2 Mechanism of TBI for Emergency Department (ED) visits. Falls and being struck by or against an object are the two leading causes of ED visits for TBI-related incidents. These type of insults often results in "mild" TBIs that do not result in fatalities

leading causes of emergency department (ED) visits due to TBI (Fig. 4.2) are falls and "struck by/against" incidents, which often are non-severe impacts (CDC 2015). 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 (Yang et al. 2008; CDC 2015). As knowledge is gained, it has become apparent that special precautions are needed after pediatric head trauma and return to play/return to classroom initiatives and protocols garner more attention. However, implementation of these novel protocols is limited due to the unique challenges they present (Halstead et al. 2013; Dettmer et al. 2014). 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). Unique considerations are required for these populations; children and adolescents can face setbacks in cognitive and functional development after sustained TBI, while older adults have a higher mortality risk and slower rates of functional recovery (Cifu et al. 1996; Susman et al. 2002; Anderson et al. 2006; Gerrard-Morris et al. 2010). Over 50,000 deaths are attributed to TBI each year, as well as 283,000 hospitalizations and 2,200,000 ED visits (Fig. 4.1). 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 U.S. can reach $50 billion dollars per year. This figure increases to $60 billion when lost societal 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). Despite life satisfaction for some individuals after sustaining

TBI, numbers are still low, reportedly at 30% 2 years after injury, for the general population, stressing a clear need for the development of neuroprotective therapies and effective protocols for the treatment of TBI (Corrigan et al. 2001).

4.2 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). This dependence on oxygen supply for mitochondria is further exaggerated in neural tissue where mitochondria play an indispensable role in supplying energy for CNS function. For example, the brain makes up ~2% of body mass but the brain consumes ~20% of oxygen taken into the body. 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).

Mitochondria have been studied since the end of the nineteenth century, and these organelles have proven to be one of the most important discoveries in the history of cellular research. Mitochondrial function has been the root of many discoveries ever since Kolliker, Altman, and Benda described their presence in cells in the later part of the nineteenth century. 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. In 1997, the Nobel Prize in Chemistry was awarded to Boyer and Walker for their discovery of the enzymatic mechanism underlying the synthesis of ATP. The role mitochondria play in intercellular cascades continues to expand for normal and disease states of cell function (Pagliarini and Rutter 2013). Although we have learned much over the past century about mitochondrial bioenergetics, there remains a great deal to be discovered.

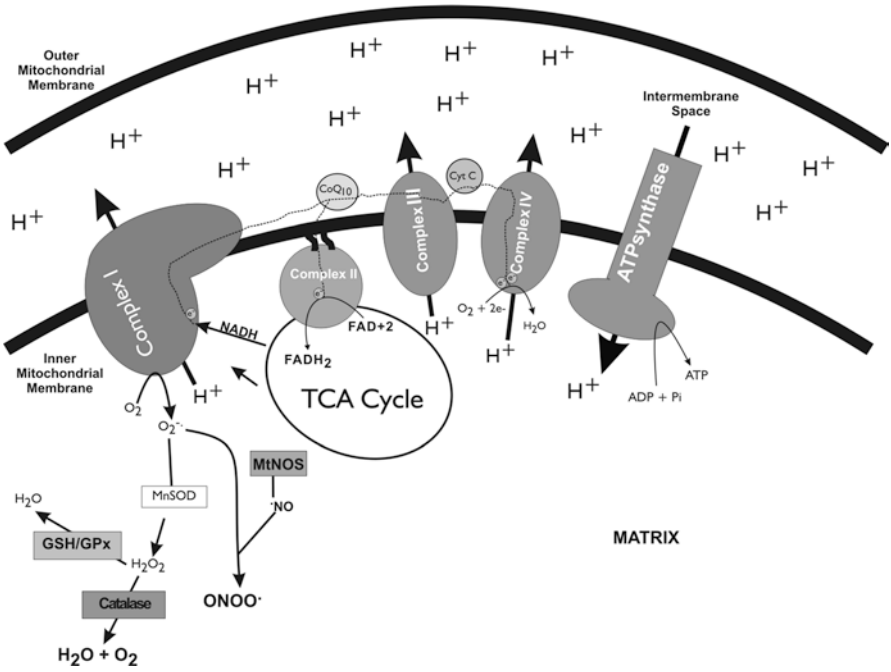

Fig. 4.3 Normal mitochondrial function, utilizing five protein complexes which make up the electron transport chain (ETC). The proton gradient established by the ETC is what drives Complex V to create ATP, the energy source for cellular processes

In order to discuss mitochondrial dysfunction we must first discuss normal mitochondrial function (Fig. 4.3). 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. 4.3). 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. 4.3). 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 this electron and transports it to Complex IV (Cytochrome-C Oxidase) which displaces another proton into the IMS via complex IV (Fig. 4.3). 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. 4.3). 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. 4.3).

4.3 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 EAAs 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+} into the cell, leading to a loss of 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 $[Ca^{2+}]_i$ lead to cell death. Excessive $[Ca^{2+}]_i$ can damage the structure of nucleic acids and proteins, interfere with kinase activity, and activate proteases or phospholipases, 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.

4.4 Free Radicals, ΔΨ, 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 ($\bullet O_2^-$) when kinetic factors favor one-electron reduction (Murphy 2009). 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. With normal mitochondria function, H_2O_2 is rapidly converted to H_2O via catalase and glutathione peroxidase, but extensive H_2O_2 efflux can occur when there is a high NADH/NAD^+ in the matrix or a highly reduced coenzyme Q (CoQ) pool accompanied by high proton motive force in isolated mitochondria (Murphy 2009). H_2O_2 has the potential to be converted to the highly reactive hydroxyl radical (•OH) via the Fenton reaction, underlying ROS neurotoxicity (Thomas et al. 2009). •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 (Fig. 4.4). Such oxidative injury results in significant alterations in cellular function. Free radicals can also damage DNA, leading to activation of DNA repair enzymes, such as poly-ADP ribose polymerase (PARP) (Prins et al. 2013). PARP activation has been shown to deplete NAD^+ pools in the cytoplasm and lead to eventual cell death (Cipriani et al. 2005; Alano et al. 2010). 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 ΔΨ such that hyperpolarization (high ΔΨ) 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 ΔΨ prevents oxidation of cytochrome b1 in the Q cycle). In other words, at a high ΔΨ, protons can no longer be pumped out of the matrix (against the electrochemical proton gradient) by the chain resulting in electron transport stalling. This leads to a sustained low level of intermediates, which increases the chance that electrons escape from these intermediates, thereby reducing oxygen and increasing ROS production. Since the magnitude of ROS production is largely dependent on- and correlates with—ΔΨ, even a modest reduction via increased proton conductance (decreases ΔΨ, the electrochemical proton gradient) across the mitochondrial inner membrane (uncoupling) reduces ROS formation (Sullivan et al. 2004b; Pandya et al. 2007).

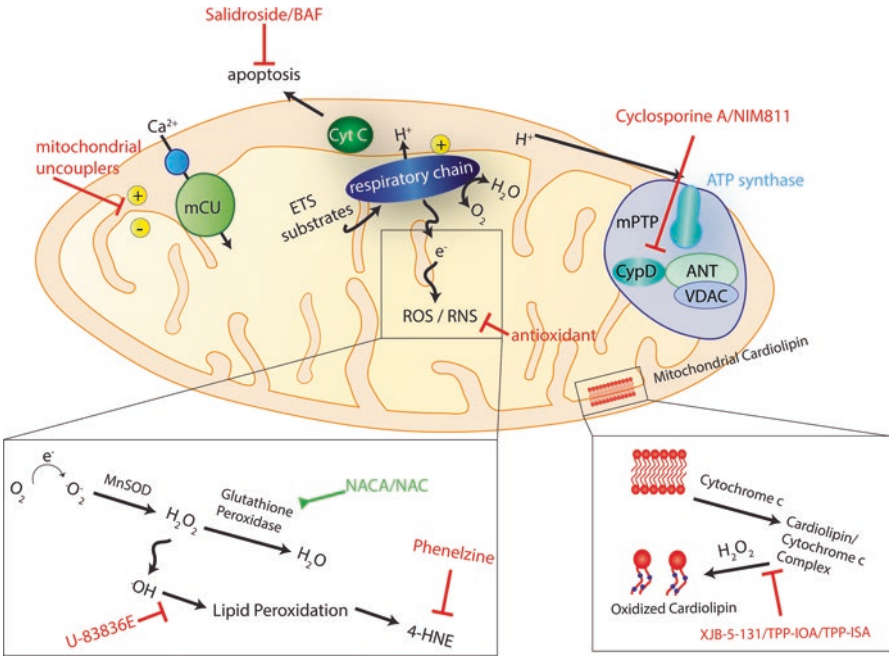

Fig. 4.4 Mitochondrial-targeted therapeutic approaches to combat eventual ROS overload and cell death. *BAF* boc-aspartyl(OMe)-fluoromethylketone, *Cyt C* cytochrome c; *mCU* mitochondrial calcium uniporter, *ETS* electron transport chain, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *NIM811* N-methyl-4-isoleucine cyclosporin, *mPTP* mitochondrial permeability transition pore, *CypD* cyclophilin D, *ANT* adenine nucleotide translocase, *VDAC* voltage-dependent anion channel, *MnSOD* manganese superoxide dismutase, *NACA* N-acetylcysteineamide, *NAC* N-acetylcysteine, *4-HNE* 4-hydroxynonenal, *TPP-IOA* triphenylphosphonium conjugated imidazole oleic acid, *TPP-ISA* triphenylphosphonium conjugated imidazole stearic acid. Adapted from Yonutas et al. (2016)

4.5 Mitochondria and 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, 2002, 2005; Nicholls and Budd 2000; Hatton 2001; Pellock et al. 2001; Schurr 2002; Tieu et al. 2003; Lifshitz et al. 2004; Sullivan 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. 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^+ 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, 2005). Calcium seems to affect primarily complex-driven respiration; damage to this major site of electron acceptance 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).

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, 2009). Membrane uncouplers, such as 2,4-dinitrophenol (2,4-DNP) and carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP) ("mitochondrial uncouplers" in Fig. 4.4), have been shown to decrease Ca^{2+} burden and lower oxidative damage, but optimal dosage is extremely important (Pandya et al. 2007, 2009).

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. 2000b, 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 comprised of 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. Recent data has also implicated a role for the ATP synthase as either a component or modulator of the mPTP, but it is still controversial (Jonas et al. 2015; Richardson and Halestrap 2016). The mPTP allows nonspecific conductance of matrix and intermembrane space components to the cytosol, where they can activate detrimental signaling cascades leading to cell death. It has been repeatedly shown that cyclosporine A (CsA) (and CsA analogs) inhibits opening of the mPTP (Fig. 4.4), leading to stable mitochondrial membrane potential and calcium homeostasis

(Sullivan et al. 1999, 2000a, b, 2011; Mbye et al. 2008; Readnower et al. 2011). Specifically, NIM811, a non-immunosuppressive CsA analog, at 10 mg/kg improves tissue sparing, mitochondrial functionality, and reduces 4-HNE levels (Readnower et al. 2011). Recently, it has also been shown that CsA improves respiration by targeting the more damaged synaptic mitochondria, relative to non-synaptic mitochondria (Kulbe et al. 2016).

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 endonuclease G (Endo G), both of which are responsible for nuclear DNA degradation. Cytochrome c translocation and subsequent DNA fragmentation has been reported 1 h and 4 h, respectively, after controlled cortical impact (CCI); cytochrome c cytosolic fractions were higher after injury in animals lacking MnSOD, suggesting an oxidative-dependent mechanism (Lewen et al. 2001). It has been show that cytochrome c translocation induces apoptotic pathways (Fig. 4.4), potentially even necrosis (Lewen et al. 2001; Sullivan et al. 2002).

As described previously, a common byproduct of normal mitochondrial function is the production of ROS. However, TBI-induced mitochondrial damage leads to Ca^{2+} loading which 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).

ROS accumulation can also activate PARP, which leads to cell death (Duan et al. 2007). PARP1 is elevated 8 h after CCI and inhibition of PARP activity has been shown to decrease lesion size, maintain NAD^+ balance and improve functional performance in mice (LaPlaca et al. 2001; Satchell et al. 2003). N-Acetylcysteineamide (NACA), as a pre-cursor, can increase levels of glutathione in order to reduce ROS accumulation. NACA, which has more CNS bioavailability compared to N-acetylcysteine (NAC), administration, has been shown to improve cortical tissue

sparing and reduce oxidative damage (HNE levels) in the brain, as well as restoring glutathione content (Fig. 4.4) (Pandya et al. 2014).

Pharmacological studies have been performed to target lipid peroxidation (Fig. 4.4) (Mustafa et al. 2011; Ji et al. 2012; Singh et al. 2013). Phenelzine, a scavenger of lipid peroxidation-derived reactive aldehyde 4-hydroxynonenal (HNE), given 15 min after CCI, prevented uncoupling after energy in contused brain tissue and also increased spared tissue (Singh et al. 2013). Another lipid peroxidation inhibitor, U-83836E, has been reported to attenuate oxidative damage, preserve Ca^{2+} buffering capacity, and reduce calpain-mediated cytoskeletal damage after CCI (Mustafa et al. 2010; Mustafa et al. 2011). Ji et al. has demonstrated that mitochondria-targeted small molecule inhibitors are needed to target cardiolipin oxidation, which is central to neuronal death pathways (Ji et al. 2012). Cardiolipin oxidation is a major pathway in lipid peroxidation and provides a target for therapeutic inhibitors of mitochondrial-related pathology after TBI (Anthonymuthu et al. 2016). Among these inhibitors are XJB-5-131, which prevents $\bullet O_2^-$ and H_2O_2 formation, and triphenylphosphonium conjugated imidazole oleic acid (TPP-IOA) and stearic acid (TPP-ISA), which prevent cytochrome c/cardiolipin complex peroxidase activity (Fig. 4.4) (Atkinson et al. 2011; Ji et al. 2012, 2015).

Finally, antiapoptotic approaches have been explored to preserve mitochondria after TBI (Fig. 4.4) (Clark et al. 2007b; Chen et al. 2012). Salidroside sparks an antioxidant mechanisms and increases survival signaling via PI3K/Akt pathway, aiding mitochondrial protection (Chen et al. 2012). Boc-aspartyl(OMe)-fluoromethylketone (BAF) is a caspase inhibitor that has been reported to lower cytochrome c release and brain tissue loss (Clark et al. 2007b).

Many different models of TBI have been employed in rodents to study the effects of mitochondrial dysfunction, such as controlled cortical impact (CCI) and non-impact rotational head injury. Mitochondrial dysfunction is established and easily observed in these models of moderate to severe TBI, though it is unknown whether aberrant mitochondrial function is present after mild TBI. Fluid percussion injury (FPI) has been employed to study mitochondrial function after a "mild" insult. mPTP opening, deficits in Ca^{2+} uptake in surviving mitochondria, and ETC alterations have been reported in various groups after FPI (Xing et al. 2013; Murugan et al. 2016; Sun and Jacobs 2016). While these groups label low-level FPI as "mild" TBI, it is not representative of mild closed head injury. Few studies on mitochondrial-related decreased metabolic parameters (NAA and ATP-ADP ratio) after closed head injuries (weight drop model) have been reported (Vagnozzi et al. 2005, 2007; Di Pietro et al. 2014). These models also show ROS accumulation after mild TBI (Tavazzi et al. 2007). Recently, mild TBI has risen to prominence in the preclinical research community due to its prevalence and inconspicuous nature. While there have been limited mitochondrial dysfunction studies performed in mild TBI, findings of ROS accumulation and cell death suggest that mitochondrial play a central role in mild injury as well.

4.6 Closing Remarks

Strategies that target specific mitochondrial mechanisms have proven beneficial in preclinical studies as therapeutic interventions following central nervous system (CNS) injury (Yonutas et al. 2016). The combination of all experimental data presented demonstrates 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. CsA and its derivatives) of the mitochondrial permeability transition (mPT) in vivo to test this hypothesis. In addition, targeting of ROS accumulation, cardiolipin oxidation, lipid peroxidation and apoptosis has proven successful in preclinical studies. The development of new pharmacological tools, with translatable potential, that specifically target the mPT and other mitochondrial pathological pathways will provide further support for the vital role of mitochondrial regulation in CNS injury and may prove beneficial as possible treatments for this and other neurodegenerative conditions.

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Chapter 5
Neuroprotective Agents Target Molecular Mechanisms of Programmed Cell Death After Traumatic Brain Injury

Lu-Yang Tao

Abstract The review is to update the current state of knowledge in post-TBI pathophysiological mechanisms, including programmed cell death mechanisms and mechanism-based preclinical pharmacological intervention used in animal models. Their effects on cell death, inflammatory events, and prolonged motor and cognitive deficits will be summarized, and their potential success for clinical application will be evaluated. Many of the above-mentioned mechanisms may be important targets for limiting the consequences of TBI.

Keywords Traumatic brain injury · Cell death · Neural protection · Plasmalemma

5.1 Introduction

Traumatic brain injury (TBI), a major cause of morbidity and mortality (Jin et al. 2015), represents the quintessential neuropsychiatric paradigm with a combination of effects in cognition, personality, and the risk for psychiatric disorders (Santopietro et al. 2015). However, the hope for an effective treatment is derived from the fact that much of the post- traumatic damage to the injured brain is caused by a secondary injury cascade of pathochemical and pathophysiological events that exacerbates the primary mechanical TBI (Mustafa et al. 2010). Therefore, TBI is a significant clinical problem with few therapeutic interventions successfully translated to the clinic.

The goal of this chapter is to update the current state of knowledge in post-TBI pathophysiological mechanisms, including programmed cell death mechanisms and mechanism-based preclinical pharmacological intervention used in animal models. Their effects on cell death, inflammatory events, prolonged motor and cognitive deficits will be summarized, and their potential success for clinical application will be evaluated. Many of the above mentioned mechanisms may be important targets for limiting the consequences of TBI.

L.-Y. Tao (✉)
Department of Forensic Medicine, Soochow University, Suzhou, China
e-mail: taoluyang@suda.edu.cn

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5.2 Programmed Cell Death

Cell death is broadly classified into three types: necrosis, apoptosis (type 1 programmed cell death [PCD]) and autophagy (type 2 PCD) (Edinger and Thompson 2004). It has been identified that necrosis is the majority and apoptosis is the minority (Krysko et al. 2008), and necrosis can also be programmed, which has been called necroptosis (Degterev et al. 2005).

Traumatic brain injury (TBI) results in neuronal apoptosis, autophagic cell death and necroptosis (Wang et al. 2012). A greater understanding of the time course of cell death following traumatic brain injury (TBI) is important for clinical treatment. Our *in vivo* study evaluated the time course of TBI-induced cell death through dUTP nick-end labeling (TUNEL), Fluoro-Jade B, and propidium iodide (PI) staining using a fluorescent staining double-labeling method. Our results indicated PI labeling is more sensitive and reliable than TUNEL and Fluoro-Jade B staining for detecting cell death following traumatic brain injury. Moreover, PI labeling can function as a reliable marker to estimate the entire time course of cell death (Luo et al. 2010a, b). TBI elicited a significant increase in the number of PI-positive cells from 10 min to 21 days, and the count peaked in the 24 h and 48 h group (Luo et al. 2010a, b, 2011).

5.2.1 Apoptosis

Apoptosis has been attributed to programmed cell death in TBI (Tehranian et al. 2008). In mammals, two major molecular pathways can initiate the apoptotic cascade, the cell death-receptor pathway and the mitochondrial pathway (Hengartner 2000). The two pathways can both lead to caspase-3 activation and apoptotic cell death. The former is triggered by members of the death receptor superfamily such as Fas (CD95) or TNF-R1 (Siegel et al. 2000), whereas the latter is activated by stimuli involving mitochondrial membrane permeabilization (MMP) and then release of proapoptotic mitochondrial proteins (cytochrome c, etc.) into the cytosol, leading to activation of caspases and cell death (Halestrap et al. 2000; Jiang and Wang 2000; Lin et al. 2009).

Apoptosis is a caspase-dependent cell death modality. The proteolytic activation of caspases in apoptotic cells drives cell rounding, retraction of pseudopodes, reduction of cellular volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), and plasma membrane blebbing (Vanden Berghe et al. 2015).

Caspase activation by the extrinsic pathway involves the binding of extracellular death ligands (such as FasL or tumor necrosis factor-a (TNFa)) to transmembrane death receptors. Attachment of death receptors to their cognate ligands provokes the recruitment of adaptor proteins, such as the Fas-associated death domain protein (FADD), which in turn recruits and aggregates several molecules of caspase-8,

thereby promoting its autoprocessing and activation (Song et al. 2016). Active caspase-8 then proteolytically processes and activates caspase-3 and caspase-7, provoking further caspase activation events that culminate in substrate proteolysis and cell death. In some situations, extrinsic death signals can cross talk with the intrinsic pathway through caspase-8-mediated proteolysis of the BH3-only protein BID (BH3-interacting domain death agonist). Truncated BID (tBID) can promote release of mitochondrial cytochrome c and assembly of the apoptosome (comprising ~7 molecules of apoptotic protease-activating factor-1 (APAF1) and the same number of caspase-9 homodimers). In the intrinsic pathway, diverse stimuli that provoke cell stress or damage typically activate one or more members of the BH3-only protein family. BH3-only proteins act as pathway-specific sensors for various stimuli and are regulated in distinct ways. BH3-only protein activation above a crucial threshold overcomes the inhibitory effect of the anti-apoptotic B cell lymphoma-2 (BCL-2) family members and promotes the assembly of BAK–BAX oligomers within mitochondrial outer membranes. These oligomers permit the efflux of intermembrane space proteins, such as cytochrome c, into the cytosol. On release from mitochondria, cytochrome c can seed apoptosome assembly (Song et al. 2016). Active caspase-9 then propagates a proteolytic cascade of caspase activation events. The granzyme B-dependent caspase activation involves the delivery of this protease into the target cell through specialized granules that are released from cytotoxic T lymphocytes (CTL) or natural killer (NK) cells. CTL and NK granules contain numerous granzymes as well as a pore-forming protein, perforin, which oligomerizes in the membranes of target cells to permit entry of the granzymes. Granzyme B, similar to the caspases, also cleaves its substrates after Asp residues and can process BID as well as caspase-3 and caspase-7 to initiate apoptosis (Taylor et al. 2008; Song et al. 2016).

Similar to the above research, our study also showed that TBI induced a marked increase in number of TUNEL positive cells, the release of cytochrome c from mitochondria to cytosol, activation of caspase-3, up-regulation of Bax, and down-regulation of Bcl-2, indicating TBI results in apoptosis (Luo et al. 2010a, b, 2011).

5.2.2 Autophagy

Autophagy is an evolutionarily conserved pathway that leads to degradation of proteins and entire organelles in cells undergoing stress (Pozuelo-Rubio 2011). There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (Klionsky 2005). Microautophagy involves the direct engulfment of the cargo by the lysosomal membrane. Chaperone-mediated autophagy is characterized by transfer of cytosolic proteins with a KFERQ motif to the lysosome by chaperone proteins, followed by their direct import via the lysosomal-associated membrane protein type 2A (LAMP2A) translocation complex. Finally, macroautophagy (hereafter simply called autophagy), is the most studied type of autophagy

and involves the formation around the cargo of a double-membrane vesicle named the autophagosome that subsequently fuses with the lysosome to form autolysosomes (Wesselborg and Stork 2015).

Currently, p62 is identified as one of the specific substrates that are degraded through the autophagy pathway (Ichimura et al. 2008; Komatsu and Ichimura 2010). This degradation is mediated by interaction with LC3, a mammalian homologue of Atg8, which is recruited to the phagophore/isolation membrane and remains associated with the completed autophagosome (Pankiv et al. 2007; Zheng et al. 2009). In this autophagy pathway, 3-methyladenine (3-MA) is a relatively selective inhibitor of the Class III phoshatidylinositol-3-kinase (PI3K); the latter can interact with Beclin-1 to participate in the formation of autophagosomes (Petiot et al. 2000). Moreover, bafliomycin A1 (BFA) inhibits autophagy through inhibiting vacuolar H + -ATPase (Boya et al. 2005).

Recent studies have shown that autophagy is increased after TBI (Lai et al. 2008; Zhang et al. 2008). The increased LC3 immunostaining is found mainly in neurons at 24 h post-TBI (Liu et al. 2008). However, few experimental studies have addressed the role of autophagy in traumatic damage and neurologic outcome. By using autophagy inhibitors 3-MA and bafliomycin A1 (BFA), our study suggests that the autophagy pathway is involved in the pathophysiologic responses after TBI, and inhibition of this pathway may help attenuate traumatic damage and functional outcome deficits (Luo et al. 2011).

Autophagic cell death has been identified as a mechanism of programmed cell death. However, whether this is death due to autophagy or not remains controversial (Kroemer and Levine 2008). During conditions of nutrient limitation, autophagy is used to generate amino acids and energy to maintain cell viability through the bulk degradation of cytoplasmic material (Messer 2016). Accordingly, the presence of autophagy in dying cells has been proposed to be a stress response mechanism to prolong cell viability. Nevertheless, recent studies strongly support autophagy as a process that can promote programmed cell death (Lin and Baehrecke 2015).

Many of the original studies describing autophagy-induced death relied on the observation of autophagy in dying cells and did not examine autophagic flux (Messer 2016). In autophagic flux studies, while increased autophagic flux may be protective after TBI, after more severe trauma inhibition of autophagic flux may contribute to neuronal cell death, indicating disruption of autophagy as a part of the secondary injury mechanism (Sarkar et al. 2014; Lipinski et al. 2015). The concept of autophagic cell death is based on observations of increased morphological features (e.g., accumulation of autophagic vesicles) in dying cells (Galluzzi et al. 2012). Recently, a novel form of autophagy-dependent cell death has been described, autosis, which not only meets the criteria in claim (i.e., blocked by autophagy inhibition, independent of apoptosis or necrosis), but also demonstrates unique morphological features and a unique ability to be suppressed by pharmacological or genetic inhibition of Na^+/K^+-ATPase (Liu et al. 2013).

5.2.3 Necroptosis

In recent years, the traditional concept of necrosis has been seriously challenged. Tumor necrosis factor (TNF) receptor (TNFR) superfamily are strong regulators of apoptosis (Chan et al. 2003). However, TNFR-1, Fas and TNF-related apoptosis-inducing ligand receptor (TRAILR) can also trigger an alternative form of cell death as "programmed necrosis", namely TNF-induced programmed necrosis was facilitated by TNFR-2 signaling and caspase inhibition. It is conceivable that when the apoptotic caspases fail to be activated, then cells undergo necroptosis as an alternative death pathway (Han et al. 2011).

Necroptosis is a newly discovered caspases-independent programmed necrosis pathway which can be triggered by activation of death receptor. Necroptosis is currently the best-characterized form of regulated necrosis and is mediated by the action of receptor interacting protein kinase 1 (RIPK1) and RIPK3, and mixed lineage kinase domain-like (MLKL) in response to death receptors, Toll- and NOD-like receptors, T-cell receptor, genotoxic stress, and viruses (Linkermann and Green 2014; Zhou and Yuan 2014). As a caspase-independent cell death mode, necroptosis exists as an alternative form of programmed cell death when caspase dependent apoptosis is blocked (Christofferson and Yuan 2010). However, definite interactions between necroptosis and apoptosis are far from obvious.

Previous studies showed that TBI initiates physiopathologic cascades of cell death signals and induces multiple cell death modes (Luo et al. 2010a, b, 2011; Werner and Engelhard 2007). Beyond the classical programmed cell death pathways, apoptosis (type1) and autophagy (type2), necroptosis is a newly discovered caspase-independent programmed necrotic mode. It has been demonstrated that the occurrence of these cell death modes participate in neural injury in TBI and ischemia models (Luo et al. 2010a, b, 2011; Meloni et al. 2011; Xu et al. 2010a, b; You et al. 2008).

Being a specific inhibitor of RIPK1, necrostatin-1 (NEC-1) was found to potently depress necroptotic cell death (Degterev et al. 2005). Previous work showed that NEC-1, a specific necroptosis inhibitor, could reduce tissue damage and functional impairment through inhibiting of necroptosis following TBI (You et al. 2008). However, several lines of evidence indicate that necroptosis has indeterminate relationships with the other two types of programmed cell death (Zhang et al. 2011). We have shown that multiple cell death pathways participate in the development of TBI, and NEC-1 inhibits apoptosis and autophagy simultaneously. These interactions may further explain how NEC-1 reduces TBI-induced tissue damage and functional deficits and reflect the interrelationship among necrosis, apoptosis and autophagy (Wang et al. 2012).

5.2.4 *Interactions Between Autophagy, Apoptosis and Necroptosis*

A breakthough has been made in the understanding of the role of Bcl-2 in controlling autophagy via its interaction with Beclin1 (Erlich et al. 2006; Sadasivan et al. 2008). Autophagy is activated, and can coexist or occur sequentially with apoptosis (Uchiyama et al. 2008). The process of autophagy acts to both increase and decrease apoptosis. Autophagy limits apoptosis through degradation of pro-apoptotic stimuli such as damaged mitochondria or cytotoxic protein aggregates (Xue et al. 2001).

As a caspase-independent cell death mode, necroptosis exists as an alternative form of programmed cell death when caspase-dependent apoptosis is blocked (Christofferson and Yuan 2010). However, definite interactions between necroptosis and apoptosis are far from obvious. Caspase-3, defined as an 'executioner' caspase, can be activated via amplification of extrinsic or intrinsic apoptotic signals (Cheema et al. 1999; Zou et al. 1997). On the contrary, Bcl-2 is an anti-apoptotic member of B-cell lymphoma-2 (Bcl-2) family of proteins and plays an important role in regulation of both caspase-dependent and caspase-independent apoptosis (Graham et al. 2000). To date, the effects of NEC-1 on apoptosis are still controversial. NEC-1 could not change the activation of caspase-3 or number of TUNEL-positive cell in the ischemic brain (Degterev et al. 2005). Moreover, NEC-1 reversed shikonin-induced necroptosis to apoptosis (Han et al. 2009). On the other hand, activation of caspase-3 induced by 11′-deoxyverticillin-A in human colon carcinoma cell death was also partially inhibited by NEC-1 (Zhang et al. 2011). The causes of these differences are probably associated with the different cell types or different strategies in particular protocols. Here, in our TBI protocol, we validated an anti-apoptotic role of NEC-1 through inhibiting Bcl-2 decline and caspase-3 activation after TBI.

Autophagy is a probable downstream consequence of necroptosis rather than a contributing factor to necroptosis and is activated as a clean-up mechanism for cell death (Degterev et al. 2005; Bell et al. 2008). Treatment with autophagy inhibitors also inhibits Z-VAD-FMK induced cell death, and knockdown of autophagy related genes such as beclin-1 and atg7 were shown to inhibit necroptosis in L929 cells (Yu et al. 2004). These results suggest an interaction between necroptosis and autophagy.

5.3 Mechanism-Based Preclinical Pharmacological Intervention

The mechanisms underlying TBI are very complex, including membrane integrity, apoptosis, autophagy, necroptosis, mitochondrial dysfunction, inflammation, oxidative stress, and excitotoxicity. Drug treatments have been classified according to which categories of pathogenic mechanisms they target. Currently, there are no Food and Drug Administration (FDA)-approved pharmacologic agents for the treatment of those with TBI (Kulbe et al. 2016).

5.3.1 Membrane-Resealing Agents

Plasmalemma permeability plays an important role in the secondary neuronal death induced by TBI (Cullen et al. 2011; Serbest et al. 2005). Poloxamer 188 (P188) is an amphiphilic copolymer of polyoxyethylene and polyoxypropylene and is used as a pharmaceutical excipient. P188 can restore plasma membrane integrity, help to seal damaged cell membranes, and can have a cytoprotective action in many types of cells (Serbest et al. 2005; Greenebaum et al. 2004). We have found that plasmalemma permeability contributes to TBI-induced blood-brain barrier (BBB) disruption, brain edema and neural cell apoptosis. Maintaining plasmalemma integrity is important for TBI, and P188 may be a potential drug in clinical applications (Bao et al. 2012).

Our recent study investigated the effects of plasmalemmal resealing by P188 on neuronal autophagy in TBI. The results revealed that plasma membranes were resealed after TBI, in which P188 aggravated autophagy in vivo (Bao et al. 2016).

In addition, acute membrane damage due to traumatic brain injury (TBI) is a critical precipitating event (Lenz et al. 2007). However, the subsequent effects of the mechanical trauma, including mitochondrial and lysosomal membrane permeability, remain elusive. We have shown that injured neurons have undergone mitochondrial and lysosomal membrane permeability damage, and the mechanism can be exploited with pharmacological interventions. P188's neuroprotection appears to involve a relationship between cathepsin B and tBid-mediated mitochondrial initiation of cell death (Luo et al. 2013a, b).

5.3.2 Anti-Necroptosis Agents

Necroptosis is a newly discovered caspase-independent programmed necrosis pathway which can be triggered by activation of death receptor. NEC-1, identified as a specific inhibitor of RIPK1, effectively inhibits necroptosis (Degterev et al. 2005, 2008).

Recently, studies found that NEC-1 could change the activities of other types of cell death- associated factors. For example, (1) NEC-1 prevented glutamate-induced nuclear translocation of apoptosis inducing factor (AIF), production of reactive oxygen species (ROS) and poly ADP-ribose polymerase (PARP) activation (Xu et al. 2007, 2010a, b); (2) NEC-1 efficiently reduced arachidonic acid (AA)-induced cell death via blocking reactive oxygen species (ROS) production and c-Jun N-terminal kinases (JNKs) activation (Kim et al. 2010); and (3) NEC-1 inhibited Z-VAD-FMK- induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in a mouse model of Huntington's disease (Zhu et al. 2011). These findings showed that NEC-1 could alter other cell death modes besides necroptosis, at least in certain conditions. These accessory effects of NEC-1 coincide with the potential role of RIPK-1, a multifunctional protein, which is involved in different pathways of cell death and survival (Degterev et al. 2008; Festjens et al. 2007).

Besides reducing the amount of cell injury and tissue damage, NEC-1 improves functional outcome after TBI (Degterev et al. 2005). We found that multiple cell death pathways participate in the development of TBI, and NEC-1 inhibits apoptosis and autophagy simultaneously. These coactions may further explain how NEC-1 can reduce TBI-induced tissue damage and functional deficits and reflect the interrelationship among necrosis, apoptosis and autophagy (Wang et al. 2012).

5.3.3 Anti-Inflammatory Agents

Mechanical disruption of brain triggers a cascade of events leading to BBB breakdown, brain edema and inflammation after TBI (Lenzlinger et al. 2001; Uryu et al. 2002). Activation of pro-inflammatory cytokines is an important neuroinflammatory response. Among proinflammatory cytokines, TNF-a, IL-1b and IL-6 appear to play a determinant role in disrupting blood–brain barrier, and accelerating the formation of cerebral edema (Wang et al. 2007). Elevated TNF-a, IL-1b and IL-6 have been detected in the brain parenchyma within the early hours after brain injury in both humans and rodents (Chao et al. 1995; Winter et al. 2002). Furthermore, previous studies have shown that inhibiting TNF-a, IL-1b and IL-6 induced by TBI is neuroprotective (Jones et al. 2005; Shohami et al. 1997). Our study indicates that TBI activates pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), the MAPK pathways together with lipoxin A4 (LXA4) receptor in astrocytes, and these mechanisms may be inhibited by pharmacological interventions (Luo et al. 2013a, b).

In addition, NF-κB upregulation has been demonstrated in neurons and glial cells in response to experimental injury and neuropathological disorders, where it has been related to both neurodegenerative and neuroprotective activities (Sanz et al. 2002). It has been generally recognized that NF-κB plays important roles in the regulation of apoptosis and inflammation as well as innate and adaptive immunity. We sought first to investigate the effect of the NF-κB inhibitor SN50, which inhibits NF-κB nuclear translocation, cell death, inflammatory pathways and behavioral deficits in our mouse TBI models. Our results imply that through NF-κB/TNF-α/cathepsin networks SN50 may contribute to regulation of TBI-induced extrinsic and intrinsic apoptosis, and inflammatory pathways, which partly determined the fate of injured cells in our TBI model. The protective effects of SN50 preconditioning may be partly related to suppression of apoptosis as well as reduction of inflammatory cytokines like TNF-α. However, further study is needed to completely elucidate the protective mechanism of SN50 on TBI-induced cell death and to establish its clinical utility in the treatment of TBI.

5.3.4 Mitochondrial Protective Agents

Mitochondria, the primary energy-generating system in most eukaryotic cells, have been shown to be a crucial participant in TBI pathophysiology (Gajavelli et al. 2015), undergoing constant changes in size and shape. As central mediators of the secondary injury cascade, mitochondria are promising therapeutic targets for prevention of cellular death and dysfunction after TBI. One of the most promising and extensively studied mitochondrial targeted TBI therapies is inhibition of the mitochondrial permeability transition pore (mPTP) by the FDA-approved drug, cyclosporine A (CsA) (Kulbe et al. 2016). Kulbe et al. report that synaptic mitochondria sustain more damage than non-synaptic mitochondria 24 h after severe controlled cortical impact injury (CCI), and that intraperitoneal administration of CsA (20 mg/kg) 15 min after injury improves synaptic and non-synaptic respiration, with a significant improvement being seen in the more severely impaired synaptic population (Kulbe et al. 2016).

Furthermore, mitochondrial morphology is orchestrated by a well- conserved cellular machinery, comprised of dynamin-related GTPases, dynamin-related protein 1 (Drp1) for fission and mitofusions (Mfn1 and Mfn2) and optic atrophy-1 (OPA1) for fusion (Purnell and Fox 2013; Wang et al. 2013a, b). Drp1, which is targeted to the outer mitochondrial membrane, is primarily found in the cytosol, and it localizes to discrete spots on mitochondrial surfaces to initiate fission by interaction with Fis1 (Chan 2006; Qi et al. 2013; Sharp et al. 2015). Mdivi-1, a mitochondrial division inhibitor, is a highly efficacious small molecule acting as a selective inhibitor of Drp1 (Zhang et al. 2013a, b, c).

Disturbed regulation of mitochondrial dynamics, the balance of mitochondrial fusion and fission, has been implicated in neurodegenerative diseases, such as Parkinson′s disease and cerebral ischemia/reperfusion (Knott and Bossy-Wetzel 2008; Lackner and Nunnari 2009; Ong et al. 2010). However, the role of mitochondrial dynamics in traumatic brain injury has not been illuminated. Our study was to investigate the role of Mdivi-1, a small molecule inhibitor of a key mitochondrial fission protein Drp1, in TBI-induced cell death and functional outcome. Protein expression of Drp1 was first investigated. Our data imply that inhibition of Drp1 may help attenuate TBI-induced functional outcome and cell death through maintaining normal mitochondrial morphology and inhibiting activation of apoptosis (Wu et al. 2016).

5.3.5 Hydrogen Sulfide (H_2S)

Hydrogen sulfide (H_2S) has been known as a toxic gas characterized by its offensive odor, described as the smell of rotten eggs, and its being an environment pollutant (Abe and Kimura 1996; Martelli et al. 2012). It had long been assumed that H_2S

exists in animal tissues at very low concentrations because of its toxicity, although it could be produced endogenously (Kimura 2010). H_2S is a lipid-soluble, endogenously produced gaseous messenger molecule collectively known as gasotransmitters (Kimura 2010). Over the last several decades, gasotransmitters have emerged as potent cytoprotective mediators in various models of tissue and cellular injury. Current evidence suggests that endogenous H_2S in the brain is produced from L-cysteine by the pyridoxal 50-phosphate-dependent enzyme, cystathionine-beta-synthase (CBS) (Enokido et al. 2005).

In our study, we investigated changes of H_2S and its possible role in the pathogenesis after TBI. We found that: (1) down-regulation of endogenous H_2S pathway after brain injury and the level of H_2S was in parallel with the expression of CBS in the cortex and hippocampus (Zhang et al. 2013a, b, c); (2) CBS may be implicated in neuronal death and the pathophysiology of brain after TBI (Zhang et al. 2013a, b, c); (3) a protective effect and therapeutic potential of H_2S in the treatment of brain injury and the protective effect against TBI may be associated with regulating apoptosis and autophagy (Zhang et al. 2014). Our data may provide a novel pathway to learn the underlying molecular and cellular mechanisms of CNS after TBI and a novel strategy for the treatment of CNS trauma. Future studies attempting to characterize the functional consequences of H_2S on cellular apoptosis, inflammation and the identification of their substrates and downstream signaling targets are now possible.

3-mercaptopyruvate sulfurtransferase (3-MST) is a novel hydrogen sulfide (H_2S)-synthesizing enzyme that may be involved in cyanide degradation and in thiosulfate biosynthesis (Frasdorf et al. 2014). In recent years, considerable attention has been focused on the biochemistry and molecular biology of H_2S-synthesizing enzymes. In contrast, there have been few concerted attempts to investigate the changes in the expression of the H_2S-synthesizing enzymes with disease states (Shibuya et al. 2009). We found that 3-MST is mainly located in living neurons and may be implicated in the autophagy of neurons and in the pathophysiology of brain after TBI (Zhang et al. 2016).

5.3.6 Others: Cathepsin B Inhibitor (CBI), and Humanin

Cathepsins B is one of the major lysosomal cysteine proteases that might be important in the intracellular protein catabolism in neurons and also plays an important role in apoptotic and necrotic programmed cell death (Guicciardi et al. 2004; Chwieralski et al. 2006; Krysko et al. 2008). Ellis et al. (2005) reported that in areas adjacent to the injury epicenter following spinal cord injury in the rat, cathepsin B enzymatic activity was significantly increased, and cathepsin B immunoreactivity appeared to be elevated in neurons. A lysosomal-mitochondrial axis theory of cell death has also been proposed (Kim et al. 2006). Specific inhibitors of cathepsin B, such as cystatin A and CBI (a selective cathepsin B inhibitor), can protect cells against ischemic hippocampal neuronal death (Yamashima et al. 1998; Tsuchiya

et al. 1999) and excitotoxic striatal cell death (Wang et al. 2008). We found that CBI inhibits TBI-induced cell death through the programmed cell necrosis and mitochondria-mediated apoptotic pathways (Luo et al. 2010a, b).

Humanin (HN) is as an endogenous peptide that inhibits Alzheimer disease (AD)-relevant neuronal cell death (Hashimoto et al. 2001). HNG, a variant of HN in which the 14th amino acid serine was replaced with glycine, can reduce infarct volume and improve neurological deficits after ischemia/reperfusion injury (Xu et al. 2008; Zhao et al. 2011). We have found that HNG treatment improves morphological and functional outcomes after TBI in mice, and the protective effect of HNG against TBI may be associated with down-regulating apoptosis and autophagy (Wang et al. 2013a, b).

5.4 Conclusions and Perspective

Traumatic brain injury (TBI), a common cause of disability and death worldwide, causes cell death and behavioral deficits (Al Nimer et al. 2015; Jin et al. 2015). TBI-induced programmed cell death includes apoptosis, autophagic cell death and necroptosis (Wang et al. 2012). There have been limited advances in the therapeutic strategies to counter brain injury. Except for conservative management, neuroprotection and neural recovery are still the main therapeutic strategies.

Our review updates the current state of knowledge in post-TBI pathophysiological mechanisms, mainly including programmed cell death mechanisms. Mechanism-based preclinical pharmacological intervention is summarized, including membrane-resealing agents, anti-autophagy agents, anti-necroptosis agents, anti-inflammatory agents, mitochondrial protective agents and others.

Novel therapeutic agents may even have differential effects at different points in the progression of TBI. There may be numerous pathologically and/or genetically distinct forms of TBI, which might be best targeted with different therapies. Furthermore, biomarker discovery may help us to recognize the earliest symptoms of TBI, ultimately improving prognosis and contributing to clinical trials with TBI patients.

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Part III
Focal Cerebral Ischemia

Chapter 6
Involvement of Apoptosis-Inducing Factor (AIF) in Neuronal Cell Death Following Cerebral Ischemia

Nikolaus Plesnila and Carsten Culmsee

Abstract Delayed neuronal death is a hallmark of infarct development and sustained functional impairment in rodent models of focal cerebral ischemia, an experimental paradigm resembling ischemic stroke in humans. The exact molecular pathophysiology of this still enigmatic event is not only of academic interest but may hold the key for novel therapeutic strategies for human stroke. There is general understanding that acute lack of perfusion leads to rapid necrotic-oncotic cell death in the core of the ischemic infarct. In contrast, conditions associated with severely reduced, but not immediately lethal reductions of cerebral blood flow in the ischemic penumbra likely result in delayed and more programmed type of neuronal cell death. Based on results first obtained from non-neuronal cells, cysteine aspartate proteases (caspases) were described as key modulators of this process. More recently, however, it became clear that also caspase-independent mechanisms play a significant role for ischemia-induced delayed neuronal cell loss. In this chapter, we review the role of one of the first described caspase-independent cell death proteins, apoptosis-inducing factor (AIF), for post-ischemic brain damage. Our conclusion is that there is compelling evidence for a causal role of AIF in neuronal cell death following experimental stroke and other neurological disorders associated with cerebral ischemia. Hence, AIF and other, more recently described subtypes of caspase-independent cell death may provide promising targets for therapeutic interventions in cerebrovascular disease.

Keywords Stroke · Focal cerebral ischemia · Apoptosis inducing factor · AIF · Programmed cell death

N. Plesnila (✉)
Institute for Stroke and Dementia Research (ISD), University of Munich Medical Center, Munich, Germany
e-mail: nikolaus.plesnila@med.uni-muenchen.de

C. Culmsee
Clinical Pharmacy—Pharmacology and Toxicology, Faculty of Pharmacy, Philipps-University of Marburg, Marburg, Germany
e-mail: culmsee@uni-marburg.de

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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. 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 options for stroke patients are recanalization of large brain supplying arteries by local or systemic administration of recombinant tissue plasminogen activator (rtPA) and/or by mechanical removal of clots/emboli with stent retrievers (Hussain et al. 2016). A major limitation of these early therapeutic approaches, however, is that both require brain CT and MRI imaging in order to exclude hemorrhagic stroke and to localize the occluded vessel. Accordingly, rtPA lysis and mechanical recanalization can only be initiated after affected patients are admitted to a specialized center. By the time diagnostic procedures have been completed the therapeutic window for both procedures, i.e. 4.5 h after the onset of ischemia, has shortened significantly or even closed. As a result less than 10% of all stroke patients are subjected to recanalization therapy (Adams et al. 2007). The remaining 90% may only hope for spontaneous reperfusion, which in most cases, however, occurs too late to prevent penumbral cell death, the main mechanism underlying infarct outgrowth and the subsequent loss of neurological function (Molina et al. 2001). Hence, novel treatment strategies are warranted which are able to prolong neuronal survival in the ischemic penumbra, i.e., under compromised cerebral blood flow conditions.

It is well accepted that even after reperfusion cell death signaling pathways triggered 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 of transient 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 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, features not present in classical necrotic cell death which is associated with cell lysis and nuclear decomposition.

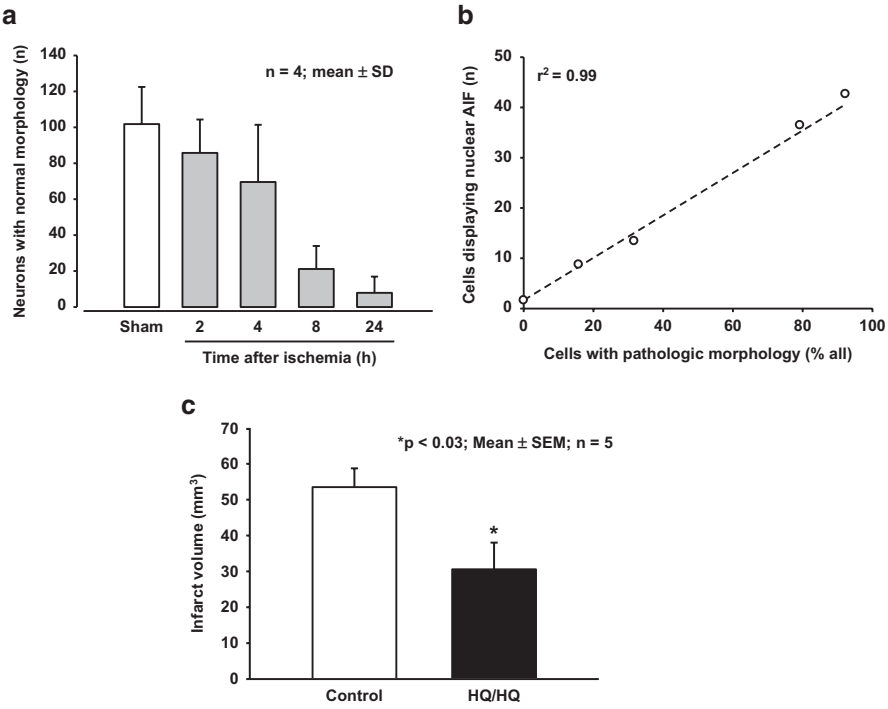

Fig. 6.1 Delayed neuronal cell death in the ischemic penumbra and correlation with nuclear AIF following transient focal cerebral ischemia in mice (**a**) Following 60 min of middle cerebral artery occlusion (MCAo) the majority of neurons (~70%) in the ischemic penumbra, i.e. the cerebral cortex, stay alive for at least 4 h. Despite sufficient blood flow 24 h after MCAo over 90% of neurons which 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). (**c**) In Harlequin mutant mice (HQ) which have a reduced expression of AIF protein due to a proviral insertion in the *aif* gene, 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)

Cell shrinkage and nuclear condensation following cerebral ischemia 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 irrespective if 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 various forms of programmed cell death, such as apoptosis, necroptosis, or parthanatos (Andrabi et al. 2011; Galluzzi et al. 2014; Vanden Berghe et al. 2014). More than 20 years ago Linnik et al. and Charriaut-Merlangue et al. were the first to demonstrate that nuclear condensation following cerebral ischemia was the result of DNA damage and endonuclease activation (Charriaut-Marlangue et al. 1996;

Linnik et al. 1995). These findings 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 was derived in the same year from experiments from the same laboratory using pan-caspase and caspase-3 specific 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 Gao 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 focused 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 has not yet been 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 in 2001 it was demonstrated that the BH3-only Bcl-2 family 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. This was supported by recent experiments on Bax- deficient mice, which showed a similar level of neuroprotection as Bid- deficient animals (D'Orsi et al. 2015). 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 for-

mation (Plesnila 2004; Plesnila et al. 2001; Yin et al. 2002). 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, (Cao et al. 2002; D'Orsi et al. 2015; Kilic et al. 2002; Martinou et al. 1994; Plesnila et al. 2001; Wiessner et al. 1999), i.e., mechanisms upstream of caspase-3 activation such as Bid and Bax 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 neurotoxicity, 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 (Becattini et al. 2006; Cao et al. 2002; Cregan et al. 2002; Culmsee et al. 2005).

AIF is a 67 kDa flavoprotein with significant homology to bacterial and plant oxidoreductases located in the mitochondrial intramembranous 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 in a caspase-independent manner (Daugas et al. 2000; Penninger and Kroemer 2003). Recent findings in models of glutamate neurotoxicity in cultured neurons in vitro and cerebral hypoxia/ischemia in vivo suggested that AIF translocation from mitochondria to the nucleus requires Cyclophilin A (CypA), which seems to coordinate DNA binding and chromatinolysis through complex formation with histone H2AX (Artus et al., 2010; Baritaud et al., 2010; Doti et al., 2014; Zhu et al., 2007). Finally, the Dawson laboratory identified macrophage migration inhibitory factor (MIF) as the key nuclease mediating AIF-dependent DNA degradation in paradigms of parthanatos induced by oxidative stress and DNA-damage (Wang et al., 2016). Eliminating MIF's nuclease activity exerted sustained protective effects in a model of focal cerebral ischemia, both at the level of histology and behavior.

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, unlike the expression pattern of many other apoptotic proteins the expression of AIF 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 asphyxia 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 using 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 a 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 almost 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 1 h 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 inhibitory 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 (Figs. 6.2 and 6.3). Reduction of cell death was associated with a lack of nuclear AIF translocation, thereby demonstrating that AIF plays a causal role in excitotoxic and hypoxic-hypoglycaemic 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. 6.1c).

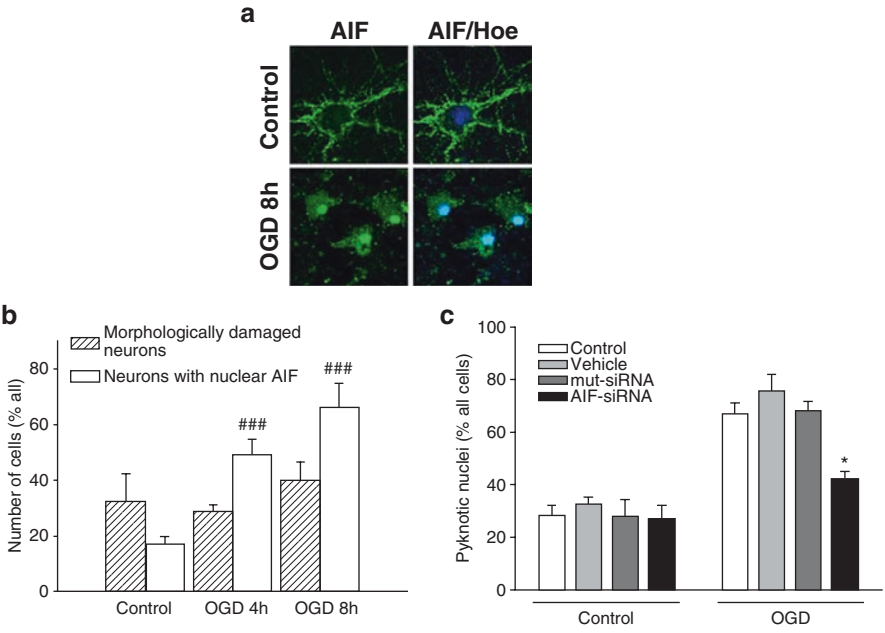

Fig. 6.2 AIF-siRNA knockdown attenuates glutamate-induced neuronal cell death in primary cultured neurons (**a**) Confocal laser scanning microscope images of AIF immunoreactivity (green) were obtained after 8 h of oxygene glucose deprivation (OGD). Co-staining with DAPI (dark blue) allowed the identification of nuclear translocation of AIF (AIF/DAPI, light blue) in damaged cells. (**b**) Number of damaged neurons and neurons displaying nuclear AIF 4 and 8 h after reoxygenation after 4 h of oxygen—glucose deprivation. AIF translocates to the nucleus before signs of morphological neuronal damage [as determined by nuclear morphology after DAPI/Hoechst staining or propidium iodide/calcein staining] become evident ($n = 4$; ###$p < 0.001$ vs. control). (**c**) Primary cultured neurons were pre-treated with vehicle (lipofectamine), non-functional mutant RNA (mut-siRNA), or AIF-siRNA for 48 h before exposure to OGD for 4 h. Cell death was quantified by counting of cells with pyknotic nuclei 24 h after re-oxygenation in medium containing glucose. 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)

Further analysis in vitro revealed that reduced AIF expression exerts preconditioning effects at the level of mitochondria, thereby preserving mitochondrial integrity and function in conditions of glutamate toxicity (Fig. 6.3) (Oexler et al. 2012). Whether such preconditioning effects at the level of mitochondria caused by reduced AIF expression levels account for protective effects against ischemic brain damage in vivo requires further investigation.

In vitro, nuclear AIF translocation was dependent on poly(ADP-ribose) polymerase 1 (PARP1) activation, as shown by using the specific PARP1 inhibitor PJ-34 (Culmsee et al. 2005). Accordingly, these results suggest that PARP1 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 (Lee et al.

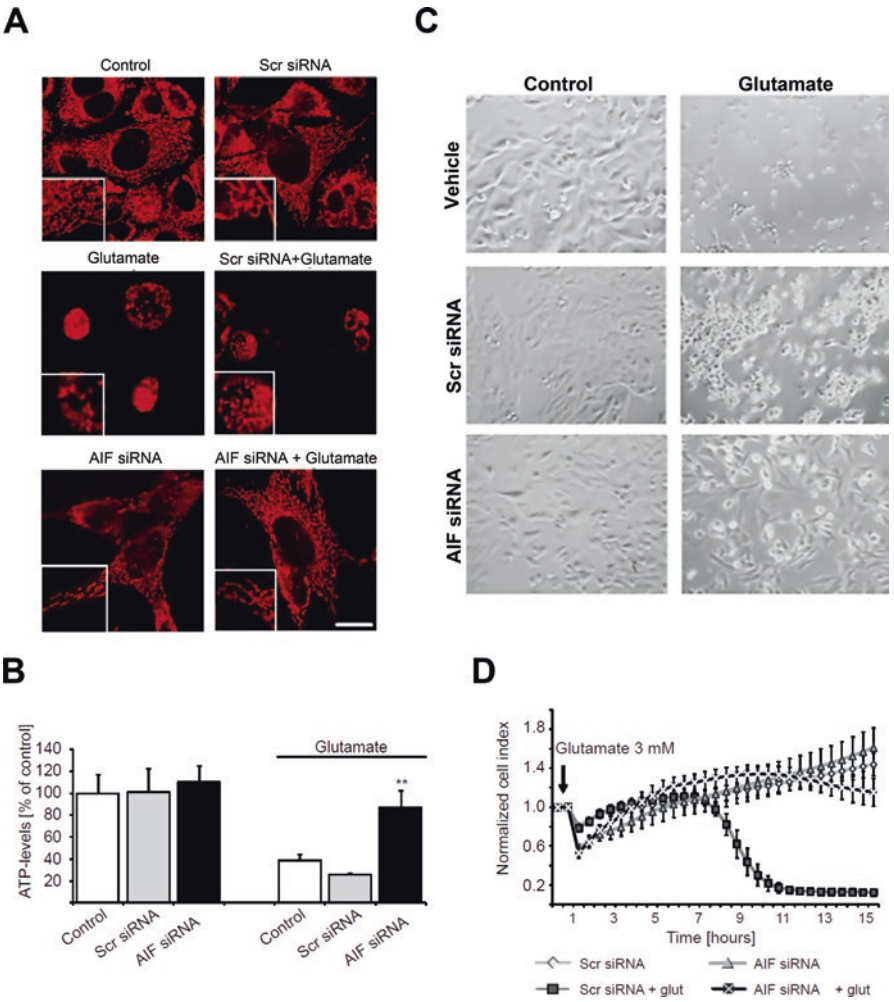

Fig. 6.3 AIF-siRNA preserves mitochondrial integrity and function, and cell viability. (**a**) Fluorescence photomicrographs show that AIF siRNA (20 nM) prevents the fission of mitochondria (stained with Mitotracker red) in glutamate-exposed (3 mM, 14 h) HT-22 cells compared to non-transfected control cells and cells transfected with scr siRNA. Scale bar 20 μm; insets show magnifications for better detection of mitochondrial morphology. (**b**) ATP levels from AIF siRNA transfected cells (20 nM) were protected from ATP depletion as determined 24 h after glutamate exposure (n = 6; **p\0.01 compared to glutamate treated control cells and scr siRNA; ANOVA, Scheffe' test). (**c**) AIF siRNA (20 nM) prevents glutamate-induced (5 mM, 12 h) cell death in neuronal HT22 cells compared to non-transfected control cells and cells transfected with scr siRNA. (**d**) xCELLigence real-time measurement: HT22 cells were treated with glutamate (glut) 72 h after transfection. AIF siRNA (20 nM) shows sustained protection over time (n = 8) (Oexler et al. 2012)

2007; Wei et al. 2007). More recently, Iduna was identified as an NMDA-receptor-induced survival protein which binds poly(ADP-ribose) polymers, thereby preventing AIF translocation to the nucleus in paradigms involving parthanatos in NMDA excitotoxicity in vitro and ischemic neuronal death in vivo (Andrabi et al. 2011). Further, activation of neuronal nitric oxide synthase (nNOS) and formation of ROS, particularly lipidperoxides, were linked to AIF-mediated neuronal cell death following experimental stroke (Li et al. 2007; Tobaben et al. 2011; Yigitkanli et al. 2017). Gene deletion of nNOS, application of a metalloporphyrin-based superoxide dismutase or inhibition of 12/15 lipidperoxidase (LOX) mimic reduced post-ischemic cell death, together with a reduction of the number of neurons displaying nuclear AIF, thereby suggesting that ROS 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 pro-apoptotic proteins of the bcl-2 family such as Bid interacting with regulators of mitochondrial fission such as Drp1 play an important role for this process. SiRNA-mediated knockdown and small molecule inhibitors of Bid or Drp1 fully preserved mitochondrial integrity and function, and 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; Grohm et al. 2010, 2012). Further, the small molecular inhibitors of Drp1, MDIVI-A and MDIVI-B reduced infarct size in a model of focal cerebral ischemia in mice (Grohm et al. 2012), similar to previously reported effects of genetic Bid deletion (Plesnila et al. 2001; Yin et al. 2002)

In conclusion, the current literature suggests that AIF-mediated caspase-independent signaling pathways are of major importance for delayed neuronal cell 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 inhibition of mitochondrial AIF release and subsequent AIF-dependent mechanisms of DNA damage may serve as novel targets for drug development aimed to mitigate cell death following stroke.

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Part IV
Transient Global Cerebral Ischemia

Chapter 7
Apoptosis-Inducing Factor Translocation to Nuclei After Transient Global Ischemia

Yang Sun, Tuo Yang, Jessica Zhang, Armando P. Signore, Guodong Cao, Jun Chen, and Feng Zhang

Abstract As a common human disorder, global ischemia causes long-term cognitive dysfunction. Selective death of hippocampal CA1 neurons underlies the cognitive impairment. After global ischemia, CA1 neuronal death occurs in a delayed manner, suggesting a type of programmed cell death. Apoptosis-inducing factor (AIF) is a mitochondrial protein with an important role in energy metabolism under physiological conditions. Following ischemia, AIF leaves mitochondria, translocates into nuclei, and induces DNA cleavage and chromatin condensation, therefore playing critical roles in inducing caspase-independent programmed cell death. In this chapter, we summarize the roles of AIF in CA1 nearonal death following global ischemia, highlighting recent progress.

Keywords AIF · Apoptosis · Stroke · Neuropretection

7.1 Introduction

In humans, global cerebral ischemia occurs following cardiac arrest and resuscitation, shock, or hypoxia, which produces neuronal cell death in the brain and reduced cognitive function if the patients recover. The loss of mitochondrial membrane integrity and the subsequent release of apoptogenic factors are critical in mediating the intrinsic, or mitochondrial, apoptotic pathway (Goto et al. 2002; Cao et al. 2003; Fujimura et al. 1999; Sugawara et al. 1999). Both caspase-dependent and -independent pro-death pathways can be initiated by the intrinsic pathway (Graham and Chen 2001; Zhang et al. 2004). The key signaling molecule to initiate the caspase-independent route is apoptosis-inducing factor (AIF), which is released by the mitochondria. AIF is a mitochondrial-specific flavoprotein that normally resides in the intermembrane space (Krantic et al. 2007). Following global ischemia, AIF is

Y. Sun · T. Yang · J. Zhang · A. P. Signore · G. Cao · J. Chen (✉) · F. Zhang (✉)
Department of Neurology, Pittsburgh Institute of Brain Disorders and Recovery, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: zhanfx2@upmc.edu; chenj2@upmc.edu

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truncated by calpain, allowing it to translocate from compromised mitochondria to the nucleus, where it degrades the nuclear genome (Cao et al. 2003, 2007).

Global ischemia can be induced in rodents using several models. The essential feature of these models is a delayed (48–72 h) but selective loss of neurons in the CA1 region of the hippocampus (Zhang and Chen 2008; Zhang et al. 2006, 2004). Neuronal death in these models occurs at least in part via an apoptotic mechanism (Jin et al. 1999; Endo et al. 2006; Xu et al. 2016). The traditional view of cell death in mammalian cells largely envisioned two distinct processes with mechanisms that shared little in common: programmed cell death (PCD) and unprogrammed cell death. PCD includes apoptosis, autophagy and paraptosis, and unprogrammed cell death mainly denotes necrosis (Krantic et al. 2007). In order to identify potential therapeutic targets and to develop successful treatments, it is crucial to understand the specific contribution of the signaling pathways activated by each of the cell death mechanisms in ischemic neuronal death.

All of the death processes mentioned above are involved in ischemic neuronal death (Xue et al. 2016; Zhao et al. 2016; Wei et al. 2015), whereas only apoptosis and necrosis are mediated by AIF (Cao et al. 2003, 2007; Xu et al. 2016). Apoptosis is a controlled, energy-dependent, and well-orchestrated degradation of cellular structures. It was first described in development, where apoptosis removes extraneous tissue to mold body and organ structures. Apoptosis is exemplified by the condensation of the nucleus and 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 (Boujrad et al. 2007; Bredesen et al. 2006; Golstein and Kroemer 2007; Xu et al. 2016).

Following cerebral ischemia and reperfusion, features of both apoptosis and necrosis appear (Muller et al. 2007; Pagnussat et al. 2007; Zhang et al. 2004). The pathological process of an individual neuron 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 released and actively involved in inducing cell death. This chapter now focuses on AIF activation and the mechanisms in the model of global cerebral ischemia.

7.1.1 Structures and Functions of AIF

The *AIF* gene is located at X chromosome, and AIF protein is synthesized as a ~67 kDa precursor, with N-terminal prodomain containing two mitochondrial localization sequences. After AIF precursor is imported into mitochondria, it is processed to a ~62 kDa mature protein (Susin et al. 1999). The mature form of AIF has three

structural domains: a FAD-binding domain, a NADH-binding domain, and a C-terminal domain (Mate et al. 2002). Under normal conditions, AIF is confined to the internal mitochondrial membrane, with its N-terminal region exposed to the matrix and the C-terminal to the inter-membrane space (Otera et al. 2005). Using multiple biochemical and immunogold electron microscopic analyses of mouse brain mitochondria, a recent study showed that about 30% of AIF loosely associates with the outer mitochondrial membrane (Yu et al. 2009). It is worth noting that the primary structure of AIF's C-terminal region shares about 30% identity with several bacterial NADH-oxidoreductases, suggesting its function (Miramar et al. 2001).

The discovery of AIF's normal functions was revealed by a mutation in the mouse. The AIF-deficient mouse, known as Harlequin, harbors 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 is a hallmark of the Hq/Hq mouse. Study of Hq/Hq mice determined 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 mitochondrial complex I dysfunction and impaired oxidative phosphorylation, evidenced by increased dependence on glycolytic glucose metabolism and progressive multifocal neuropathology (El Ghouzzi et al. 2007; Vahsen et al. 2004). Loss of central neurons was due to reduced neuronal survival during brain development and increased oxidative radical activity (Cheung et al. 2006). Increased sensitivity to peroxides occurs in Hq/Hq neurons, 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 does 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 the complex I and III subunits, most likely duo to post-translational mechanisms (Vahsen et al. 2004).

Given that caspase-independent cell death requires AIF activation, several studies have reported neuroprotective effects of AIF inhibition by either neutralizing intracellular AIF or genetically reducing the expression of AIF (Cao et al. 2007; Culmsee et al. 2005; Yu et al. 2002; Xu et al. 2016). In transient global ischemia, total AIF expression levels per se are not significantly altered (Cao et al. 2003; Xu et al. 2016). Instead, AIF is cleaved at position G102/L103 (in mouse) by activated calpains and/or cathepsins, resulting in a mature isoform via its N-terminal truncation (Otera et al. 2005; Cao et al. 2007). Due to a nuclear localization sequence at AIF's C-terminal domain, this isoform is then translocated into the nucleus, resulting in large-scale DNA fragmentation and chromatin condensation (Dalla Via et al. 2014). The discharge of AIF from mitochondria is also dependent on the pro-apoptotic Bcl-2 family members, Bax and Bid (Cregan et al. 2002; Culmsee et al. 2005; Van Loo et al. 2002). Through direct interaction with genomic DNA along with the activity of endonuclease G (EndoG), AIF causes chromatin condensation (Cande et al. 2002).

7.2 AIF Translocation Mechanism and Therapeutic Targets

7.2.1 The Time Course of AIF Translocation

AIF-mediated cell death is an energy-dependent process. After the period of greatest energy depletion during ischemia, neuronal death occurs during the recovery of energy after reperfusion (Pagnussat et al. 2007). The time course for AIF nuclear translocation after experimental stroke varies with the severity of injury. AIF translocation into the nucleus does 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 (Li et al. 2007; Plesnila et al. 2004). Interestingly, AIF is a larger protein than cytochrome c, but its translocation precedes cytochrome *c* release. The small pool (30%) of AIF on the outer mitochondrial membrane may play an important part in this phenomenon. It was discovered that the outer mitochondrial membrane accounts for the rapid release of a small pool of AIF, as 20% of the uncleaved AIF rapidly translocated to the nucleus and caused death following NMDA treatment (Yu et al. 2009). However, more studies are needed to determine the different mechanisms involved in the release of the two AIF pools.

7.2.2 Mechanism of AIF Release

7.2.2.1 Poly (ADP-ribose) Polymerase-1 (PARP-1) and AIF

The release of AIF after global ischemia occurs upon a variety of stimuli, and several cascades contribute to AIF neurotoxicity, which include the inappropriate activation of DNA reparative enzymes. Our study showed that DNA single-strand breaks is a form of DNA damage induced early in neurons following cerebral ischemia (Chen et al. 1997; Stetler et al. 2010). PARP-1 is an abundant and very active chromatin-associated enzyme involved in DNA repair, as well as 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; Ame et al. 2004; Ha 2004). Enzyme activity of PARP-1 is in fact activated by DNA strand breaks, and thus PARP-1 functions as a sensor of DNA damage (Demurcia and Demurcia 1994). Reperfusion following cerebral ischemia induces the generation of oxidative stimuli such as reactive oxygen species, which can lead to DNA damage, with activation of PARP-1 (Demurcia and Demurcia 1994; Eliasson et al. 1997). PARP-1 is a crucial part of both apoptosis and necrosis because its pathological over-activation can lead to cell death, generally by depleting 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).

Therefore, inhibition of PARP-1 over-activation should ameliorate cell death under these toxic conditions. Indeed, PARP-1 knockout animals demonstrate resistance to 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). 3-AB, a PARP-1 inhibitor, protects neurons against necrosis, which is dependent on the duration of the ischemic-reperfusion episode (Strosznajder and Walski 2004). Our previous work showed that when adequate cellular NAD+ levels were maintained (Nagayama et al. 2000) or supplied (Wang et al. 2008), inhibition of PARP-1 diminished neuronal survival in the transient global ischemia model or neuronal cultures (Nagayama et al. 2000; Wang et al. 2008). Since caspase-3-mediated cleavage of PARP-1 blocks DNA repair and concomitantly prevents a depletion of cellular NAD+ pool by PARP activity (Boulares et al. 1999; Herceg and Wang 1999), the exact role that PARP-1 plays in cell death remains contentious, and may depend on which pathway is preferentially activated in individual neurons.

Evidence indicates that the release of AIF from mitochondria is also dependent on PARP-1-initiated nuclear signals (Cipriani et al. 2005; Yu et al. 2002). Reperfusion accelerates the appearance of nuclear AIF after 1 h of transient focal brain ischemia compared with permanent ischemia, consistent with the possibility that early oxidant stress triggers the signaling pathways that stimulate AIF translocation (Li et al. 2007). In concert with it, inhibition of PARP-1 reduces nuclear AIF translocation (Culmsee et al. 2005). The PAR polymer, generated when PARP-1 is over-activated, is now known as a key signaling molecule in the PARP1 mediated cell death (Andrabi et al. 2006; Komjati et al. 2005). They reach a toxic level when PARP-1 becomes over-activated and translocates to the cytosol, inducing AIF nuclear translocation (Yu et al. 2006). AIF is a PAR polymer-binding protein and a physical interaction between PAR and AIF is required in inducing the release of AIF from the mitochondria (Gagne et al. 2008; Wang et al. 2011).

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 importance of PARP-1 is highlighted by recent reports, which showed that the activation of PARP-1 is necessary for calpain-mediated AIF cleavage (Cao et al. 2007; Vosler et al. 2009). In addition to PARP-1, a recent study showed that PARP-2 also contributed to nuclear translocation of AIF after transient focal cerebral ischemia in male mice (Li et al. 2010). The activation of AIF by PARP signaling may occur with a significant number of variables including 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.

7.2.2.2 Direct Activation of AIF: Truncation by Calpain

A number of cysteine proteases, including the caspases, cathepsins, and calpains, are activated in neurons after ischemic injury (Graham and Chen 2001; Windelborn and Lipton 2008; Vosler et al. 2009). In AIF-mediated neuronal death, two families of cysteine proteases play a key role: calpains and cathepsins. Calpain I, also known as u-calpain, requires micromolar order of calcium to be activated, while calpain II or m-calpain requires millimolar calcium, as measured *in vitro*. Nevertheless, calcium is not involved in the cathepsin-mediated control of AIF activation. The cellular distribution of mRNA for calpain I and calpastatin, the endogenous calpain inhibitor, are relatively uniform throughout the mouse brain. In contrast, 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 calpains are normal complements of cellular enzymatic activities, their inappropriate-activation or over-activation will lead to pathophysiological activity and contribute to cell death following cerebral ischemia. A major target of calpains is AIF. Calpain I cleaves AIF in a caspase-independent cell death manner in liver mitochondria (Polster et al. 2005; Cao et al. 2007) and the PC12 neuronal cell line (Liou et al. 2005). Our previous work shows that calpain I induces AIF release in the nervous system after both oxygen-glucose deprivation (OGD) *in vitro* and transient global ischemia *in vivo* (Fig. 7.1) (Cao et al. 2007). In fact, N-terminal truncation by calpain I was found to be required for AIF activation. When neurons express a mutant form of AIF that could not be cleaved by calpain, AIF was not released from mitochondria and was not found in the nucleus after OGD or global ischemia (Cao et al. 2007). This important finding shows that calpain I 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, there is ample evidence showing that a mitochondrial calpain exists and that it cleaves AIF, thus allowing AIF to leave the mitochondria (Garcia et al. 2005). A new form of calpains has been discovered and named calpain-10 (Arrington et al. 2006). Calpain-10 is located at the mitochondrial outer membrane, intermembrane space, inner membrane, and matrix region, and is an important mediator of mitochondrial dysfunction via the cleavage of Complex I subunits and activation of the mitochondrial-permeable transition pore (Arrington et al. 2006). The mitochondrial calpain most closely resembles m-calpain, as anti-m-calpain antibodies can also stain the mitochondrial calpain, and the calcium dependency of m-calpain and mitochondria calpain are similar (Ozaki et al. 2007). Thus, the discovery of calpain activity in the mitochondria itself 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 I can cleave Bid (Chae et al. 2007); and calpain II can trigger the ischemia-induced lysosomal release of cathepsins in brain (Windelborn and

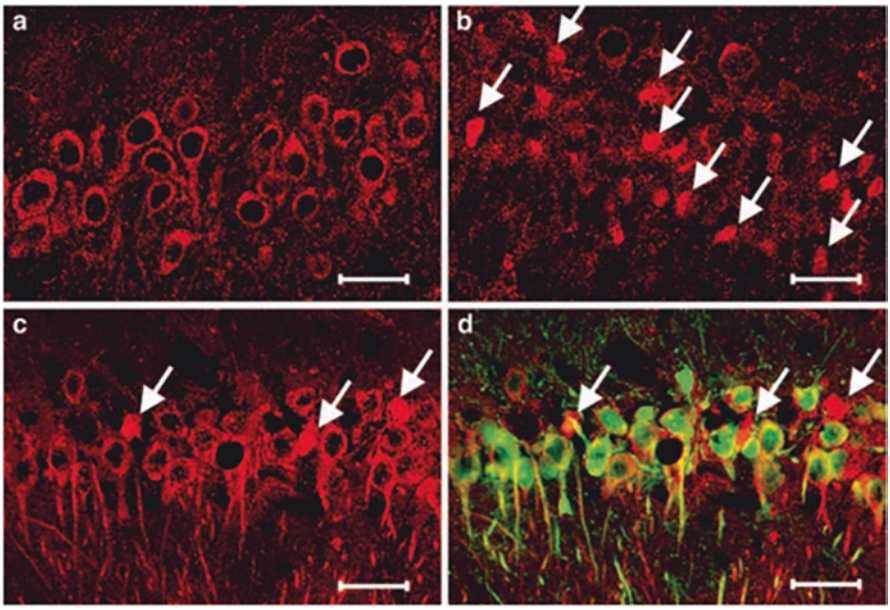

Fig. 7.1 AIF translocation in vivo following global ischemia is prevented by overexpression of calpastatin. Representative immunofluorescence of AIF (red) from non-ischemic CA1 (**a**) or 72 h after global ischemia (**b**–**d**). AAV–Cps (**c**, **d**) or the empty vector (**b**) was infused 14 days before ischemia, and brain sections were double-label immunostained for AIF (red) and hemagglutinin (HA) (green, **d**). Note that majority of CA1 neurons lost normal localization of AIF after ischemia (**b**, arrows), but AIF translocation was rare in Cps-overexpressed CA1 (**c**, **d**, arrows). Scale bars, 50 μm. From Cao et al. (2007)

Lipton 2008), which brings about the truncation of procaspase-3 into its active form (Blomgren et al. 2001; Mcginnis et al. 1999). This latter pathway is particularly interesting, as caspase-3 in turn reduces the activity of calpastatin, the endogenous calpain inhibitor, to form a positive feedback loop, which can result in the further activation of calpains and subsequent release of AIF (Kato et al. 2000; Porn-Ares et al. 1998; Wang et al. 1998).

7.2.2.3 Release of AIF from Mitochondria: Formation of the Mitochondrial Outer Membrane Pore

In addition to AIF and EndoG, mitochondria can release a variety of death-promoting molecules, including cytochrome c, Smac/Diablo, and Omi/HtrA2. The ability of mitochondria to release these molecules depends largely on the formation of large and nonselective pores or channels through the two layers of membrane systems in mitochondria. The inner membrane forms the mitochondrial-permeable transition

pore, which is a calcium-dependent process and uses the proteins cyclophilin (Cyp) D, voltage-dependent anion channel and adenylate nucleotide translocase. The Bcl-2 family proteins, including Bid, Bax, and Bak and very likely others as yet unidentified proteins, form the outer membrane permeabilization pore, termed the mitochondrial apoptosis channel (for complete review, see Belizario et al. 2007). Many of these proteins need to undergo proteolytic cleavage before they can form any type of channel or pore, and the cleavage is usually induced by many stressors, including ischemia. For example, Bid can be truncated by caspase-8 and translocates to the outer mitochondrial membrane in ischemic brain (Gross et al. 1999). The understanding of the mechanisms behind the formation of the mitochondrial death channels is still incomplete and under debate. In general, Bid is activated and leads to BAK and BAX oligomerization, mitochondrial outer membrane permeabilization, and AIF release following stressors (for complete review, see Chipuk et al. 2010). The prevention of unwanted mitochondrial channels during normal physiological conditions is critical for maintaining cellular function and health. Regulation occurs via several anti-apoptogenic members of the Bcl-2 protein family, including Bcl-2 and Bcl-xL (Breckenridge and Xue 2004). In fact, AIF translocation and cleavage is inhibited by Bcl-2 and Bcl-xL overexpression (Cao et al. 2003; Otera et al. 2005).

7.2.3 Regulation of AIF Activity in the Cytoplasm

Once released from mitochondria to the cytosol, AIF then translocates into the nucleus. This process is regulated positively by Cyp A and negatively by heat shop protein 70 (Hsp70).

7.2.3.1 Cyp A Induces AIF Nucleus Translocation

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; Cande et al. 2004; Zhu et al. 2007). For instance, CypA can form a complex with AIF to act as a co-factor for AIF nuclear translocation and AIF-dependent chromatinolysis following cerebral ischemia (Cande et al. 2004; Zhu et al. 2007). Elimination of Cyp A confers neuroprotection in vivo, suggesting that the lethal translocation of AIF to the nucleus requires interaction with Cyp A (Zhu et al. 2007).

7.2.3.2 Hsp70 Inhibits AIF Nucleus Translocation

Previous studies have shown that Hsp70 over-expression protects cells from death induced by various insults that cause either necrosis or apoptosis, including hypoxia and ischemia/reperfusion, by inhibiting multiple cell death pathways (Giffard and Yenari 2004). One of the mechanisms by which Hsp70 may be neuroprotective is the sequestration or neutralization of AIF by Hsp70, as evidenced by Hsp70 overexpression (Gurbuxani et al. 2003; Ravagnan et al. 2001; Wang et al. 2015). This process is associated with increased cytosolic retention of AIF when bound to Hsp70, limiting the entry of activated or cytotoxic AIF into the nucleus (Gurbuxani et al. 2003; Kroemer 2001).

7.2.3.3 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 cellular system (Reffey et al. 2001; Yamaguchi et al. 1999; Yang et al. 2000). Previous work shows that XIAP may participate in the ubiquitinization of AIF (Wilkinson et al. 2008), which leads to AIF proteosomal degradation (Wilkinson et al. 2008). Further study shows that lysine 255 of AIF is critical to bind DNA and degrade chromatin, and the target of XIAP is this lysine residue (Lewis et al. 2011).

7.2.4 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. 2000, 1999). 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 modulation 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).

EndoG is another death-promoting factor released from mitochondria along with AIF (Susin et al. 1999). In the cerebral cortex, 4 h after ischemia, endoG level is significantly increased in the nucleus, correspondingly with decreased mitochondrial endoG content. EndoG may also interact with AIF in the nucleus after cerebral ischemia (Lee et al. 2005). In these ways, AIF truncation and release lead to the disruption of neuronal DNA via both direct (DNA binding) and indirect (EndoG) mechanisms.

7.2.4.1 Cyclophilins

Cyp A has been stated as a co-factor for AIF nuclear translocation and AIF-dependent chromatinolysis. Cyp 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 Cyp 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.

7.2.4.2 Histone H2AX

Histone H2AX is another key factor in AIF-mediated apoptosis. As a member of the histone H2A family, H2AX participates in forming the histone nucleosome core. Previous studies have shown that the function of H2AX is primarily associated with DNA damage repair. On exposure of cells to inducers of double-strand breaks DNA damage, H2AX is phosphorylated at Ser139 in the nucleosomes surrounding the break point (Thiriet and Hayes 2005). Phosphorylated H2AX (γH2AX) renders damaged DNA sites accessible to repairing factors (Pilch et al. 2003). On the other hand, H2AX is crucial for AIF- mediated neuronal death. After DNA alkylating agent treatment, H2AX Ser139 phosphorylation is required for AIF mediated cell death (Artus et al. 2010; Baritaud et al. 2012).

7.3 Conclusion

Current knowledge about caspase-independent, AIF-induced neuronal death is incomplete. Though a large part 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 mediated by AIF provokes a compound 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, maybe a promising target for drug intervention using calpain-specific inhibitors.

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Chapter 8
Necroptosis in Cerebral Ischemia

Marta M. Vieira and Ana Luísa Carvalho

Abstract Necroptosis is a form of regulated necrotic cell death, which is mediated by receptor-interacting protein 1 kinase (RIPK1) and RIPK3, and the downstream effector mixed lineage kinase domain-like (MLKL). Necroptotic signals induced by death receptors, such as TNF receptor 1, Toll-like receptors or interferon receptors lead to the formation of the necrosome, and result in cell death with morphological features of necrosis. The execution of necroptosis involves oligomerization of MLKL upon phosphorylation by RIPK3, its translocation to the plasma membrane and consequent plasma membrane permeabilization. Necroptosis participates in physiological functions but is also involved in cell death associated with several pathophysiological conditions. Here, we discuss key features of necroptosis, and evidence implicating necroptotic cell death in brain ischemia.

Keywords Necroptosis · Brain ischemia · Receptor interacting protein kinase 1/3 · Mixed lineage kinase domain-like (MLKL) · Necrosome

8.1 General Considerations

Cell death has been classically divided into two major types, programmed (or regulated) cell death (PCD) and unregulated cell death. The archetypal form of PCD is apoptosis, whereas necrosis is generally considered a type of "accidental" cell death. The concept of PCD arises from the fact that upon specific stimuli (e.g. heat, radiation, hypoxia) cells activate a cascade of events that leads to a highly regulated cellular demise process. Apoptosis induces several cyto-architectural alterations,

M. M. Vieira
Receptor Biology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

A. L. Carvalho (✉)
Department of Life Sciences, CNC—Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
e-mail: alc@cnc.uc.pt

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including membrane blebbing, with formation of apoptotic bodies, chromatin condensation, DNA laddering and fragmentation of the nucleus, organelle fragmentation and release of protein content from the mitochondria to the cytoplasm. After cellular deconstruction, the debris are removed by phagocytes, avoiding activation of inflammatory responses and damage to the neighboring cells (Taylor et al. 2008). There are two major pathways of apoptosis, the extrinsic (or death receptor—DR—pathway) and the intrinsic (or mitochondrial pathway) one, which are thought to cross-talk and influence one another (Igney and Krammer 2002). In contrast, necrosis induces plasma membrane damage, swollen mitochondria and slow mitochondrial membrane depolarization, subsequent to nuclear and cytoplasmic morphological changes. Because the cellular debris are not so promptly removed as during the final stage of apoptosis, necrosis is thought to induce an inflammatory component and to have a propagating effect, causing damage to neighboring cells that may eventually die. In addition to these events, neuronal necrosis involves programmed, or regulated, biochemical cascades triggered by excessive calcium entry through NMDA-receptor-operated cation channels (Fujikawa 2015).

Interestingly, during the past two decades a novel concept of programmed necrosis has emerged. This notion arose from the observation that, in conditions of apoptosis inhibition, certain cells still initiate a process of regulated cell death, with necrotic phenotype. Indeed, this type of PCD, termed necroptosis, displays necrotic features, such as compromise of the cellular membrane, reactive oxygen species (ROS) production and mitochondrial damage, in the absence of typical apoptotic features, such as cytochrome c release or caspase-3-dependent cleavage of poly-ADP ribose polymerase (PARP), chromatin condensation or oligonucleosomal DNA degradation (He et al. 2009; Holler et al. 2000; Vandenabeele et al. 2010). Whether or not there are interactions between necroptosis and excitotoxic programmed necrosis has not been determined.

Mounting evidence has demonstrated that ischemic insults induce a combination of different processes of neuronal cell death, namely necroptosis and excitotoxic programmed necrosis (Meloni et al. 2011; Vieira et al. 2014; Xu et al. 2016; Vosler et al. 2009). Although we still lack a complete understanding of these mechanisms in neurons, the diversity of cell death processes activated following ischemia seems to imply a certain redundancy, possibly to ensure the demise of damaged neurons. Such hypothesis is further corroborated by the fact that necroptosis actually occurs in conditions of apoptosis inhibition (Degterev et al. 2005; Qu et al. 2016; Vieira et al. 2014), suggesting that this may be a backup pathway activated under unfavorable conditions.

In this chapter, we first describe necroptosis, with a focus on the mechanisms that trigger and mediate this form of regulated necrosis. We then explore the evidence supporting the implication of necroptosis in brain ischemia, and how this pathway could be therapeutically targeted.

8.2 Death Receptor Signaling

Death receptors (DRs) are ubiquitous cell membrane proteins whose signaling underlies complex cell fate events. The decision between life and death is initiated upon death ligand (DL) stimulation (classically, TNFα), with the recruitment of death domain (DD)-containing proteins to the vicinity of the TNFR1—complex I [for a review of the diverse DRs and their DLs see (Han et al. 2011)]. This complex comprises proteins such as RIPK1, TNFR-associated factor 2 (TRAF2) and TNFR-associated via death domain (TRADD) (Micheau and Tschopp 2003), among others. Cellular inhibitor of apoptosis protein 1 (cIAP1) may also be found in this complex, which leads to survival signaling mediated by nuclear factor kappa B (NF-κB) transcriptional activity, by acting as an inhibitor of DL-induced apoptosis (Gaither et al. 2007; Geserick et al. 2009; Wang et al. 1998; Yang and Du 2004). Increased NF-κB-mediated gene transcription contributes to cell survival and inflammation. However, depending on the molecular context of the cell, and upon impaired NF-κB signaling, DRs may also activate two distinct mechanisms of programmed cell death (Fig. 8.1). This outcome is achieved via the assembly of a

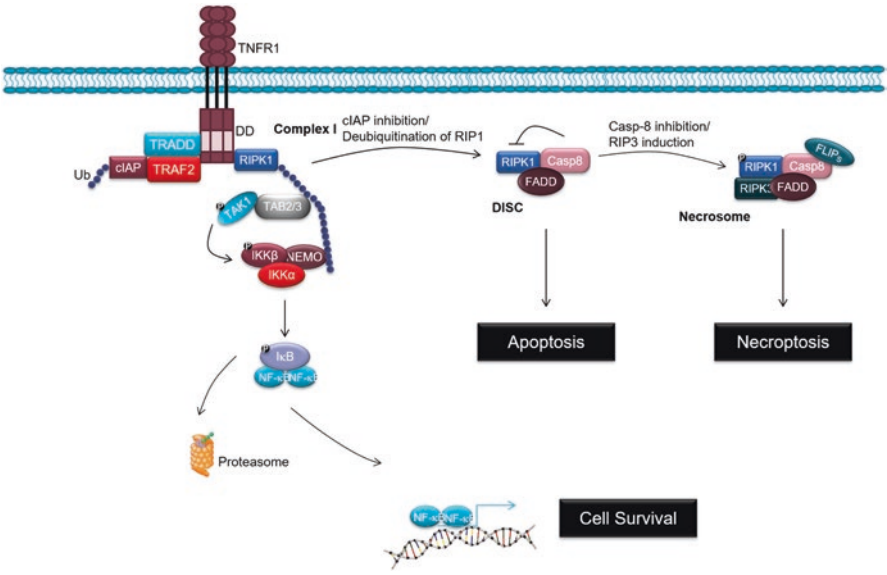

Fig. 8.1 DR signaling complexes. Upon DL binding, the DR recruits a complex of DD-containing proteins to its vicinity (complex I). In this complex, RIPK1 is ubiquitinated by cIAPs, initiating signaling to NF-κB activation. This contributes to cell survival via transcription of survival genes. Upon deubiquitination of RIPK1, the complex dissociates from the receptor and recruits FADD and procaspase-8, forming a complex called DISC. Caspase-8 auto-activates, cleaves RIPK1 inhibiting necroptosis, and initiates apoptosis. In conditions of low caspase-8 activity, RIPK3 is recruited to interact with RIPK1, assembling the necrosome, and together they activate necroptosis

second high molecular weight complex, complex II, also named death inducing signaling complex (DISC). This complex comprises RIPK1, TRADD, TRAF2, Fas-associated protein with death domain (FADD) and procaspase-8 and is dissociated from TNFR1, which is internalized. Assembly of the DISC generally leads to induction of apoptosis, through processing of caspases-8, −2 and −3 (Micheau and Tschopp 2003). In certain conditions, depending on the protein environment in this complex, additional proteins are recruited to assemble a third signaling complex—complex IIb or necrosome (Declercq et al. 2009). The necrosome then activates necroptosis via a distinct mechanism of PCD (Declercq et al. 2009; Weinlich et al. 2011).

8.3 RIPK1: A Multi-Talented DR Effector

Receptor interacting protein kinase 1 (RIPK1) is a 60 kDa ubiquitous protein that is recruited to complex I, following TNFα binding to TNFRs. The ubiquitination state of this protein seems to be determinant for the decision between life and death. This post-translational modification of RIPK1 is highly regulated by ubiquitinases and deubiquitinases (DUBs) in complex I. Indeed, cIAP1 and cIAP2 are able to ubiquitinate RIPK1 and upon inhibition of these enzymes the recruitment of RIPK1 to complex II is increased (Geserick et al. 2009). In addition, the DUB Cezanne leads to suppression of NF-κB signaling in response to TNFα activation by removing Lys63 polyubiquitin chains from RIPK1, in complex I. This contributes to increased stability of the inhibitor of kappa B (IκB) complex, which retains NF-κB in the cytoplasmic compartment (Enesa et al. 2008). Inhibition of both cIAP1 and transforming growth factor-β activated kinase-1 (TAK1) induces the formation of the necrosome and subsequent ROS production (Vanlangenakker et al. 2011). Thus, when ubiquitinated with Lys63 chains, RIPK1 seems to contribute to cell survival, by promoting NF-κB transcriptional activity. Once deubiquitinated, RIPK1 recruits proteins like FADD and procaspase8, thereby allowing DISC assembly.

The association between RIPK1 and caspase-8 in complex II is highly dependent on FADD availability (Micheau and Tschopp 2003). This protein can have an impact on the phenotype of cell death. Different domains of FADD have the ability to induce caspase-dependent apoptosis (death effector domain—DED) or necrosis (DD), in a caspase-independent manner, due to their ability to selectively interact with caspase-8 or RIPK-1, respectively (Vanden Berghe et al. 2004).

Autoactivation of caspase-8 is regulated by the cFLIP isoform ($FLIP_L$, $FLIP_S$ and $FLIP_R$) that is present in DISC (Kataoka et al. 1998; Scaffidi et al. 1999). The expression of $FLIP_L$, which is regulated by NF-κB activity, exerts a negative regulatory effect on complex II, through inhibition of caspase-8 (Micheau and Tschopp 2003). The $FLIP_L$ protein possesses caspase-like domains, but has no catalytic activity (Tschopp et al. 1998), thus it interacts with procaspase-8, allowing its allosteric activation and partial processing. This event inhibits induction of apoptosis due to caspase-8 limited activity, but allows this enzyme to process local substrates, like

RIPK1 (Kavuri et al. 2011; Krueger et al. 2001; Micheau et al. 2002), therefore facilitating concomitant inhibition of apoptosis and necroptosis (Oberst et al. 2011). When complex I displays limited activity, then the expression of $FLIP_L$ is lower and complex II has the ability to initiate apoptotic signaling (Micheau and Tschopp 2003). The $FLIP_S$ isoform, on the other hand, inhibits caspase-8 auto-activation (Golks et al. 2005; Krueger et al. 2001), thereby limiting the cleavage of RIPK1, which is then capable of initiating necroptosis signaling.

Necroptosis induction is achieved via the recruitment of a RIPK1-related protein—RIPK3—which has been shown to be specifically recruited to the necrosome (Cho et al. 2009; He et al. 2009; Zhang et al. 2009), implying that the presence of RIPK3 in complex II is specifically associated with necroptotic signaling. Although it is known that TNFα receptors are present in neuronal plasma membranes (Olmos and Llado 2014; Jara et al. 2007), whether TNFα activates the TNFα receptors in neurons, which then activate RIPK1 is not known. Thus, the factor or factors responsible for upregulation of RIPK3 and RIPK1 following oxygen-glucose deprivation of hippocampal neuronal cultures and upregulation of RIPK3 following transient global cerebral ischemia must be determined (Vieira et al. 2014).

8.4 Necroptosis Molecular Complexes

Recruitment of RIPK3 to complex II is thought to be the molecular prompt that shifts the cell death program to necroptosis. Accordingly, the pronecrotic complex II, called necrosome (Fig. 8.2), is distinct from the apoptotic DISC, in that the interaction between RIPK1 and RIPK3 appears to occur specifically under necroptotic-inducing conditions (Cho et al. 2009). Although the complete picture of the events downstream of the necrosome is still not clear, two molecular mechanisms seem to have a profound impact on necroptosis induction: RIPK1/RIPK3 interaction and their kinase activities (see Box 8.1 for a brief timeline of necroptosis discovery).

RIPK1 and RIPK3 were shown to associate via their RIP homotypic interaction motif (RHIM) domains (Sun et al. 2002), forming an amyloid complex upon induction of necroptosis (Li et al. 2012). Notably, viral proteins that disrupt this interaction have been shown to suppress necroptosis induction (Upton et al. 2010), and mutations in the RHIM domain of RIPK3 abolished its ability to induce necroptosis (He et al. 2009), thereby demonstrating the crucial role of RIPK1-RIPK3 interaction in this process of cell death. Additionally, RIPK3 was shown to autophosphorylate and phosphorylate RIPK1. Phosphorylation of the latter seems to act as a cue to shut-off NF-κB signaling, which contributes to cell death (Sun et al. 2002), and it may contribute to the stabilization of the association of RIPK1 within complex II (Cho et al. 2009). The intermediate domain (ID) of RIPK1 is important for induction of necroptosis, as deletion of this domain in the protein shifts the type of cell death from necroptosis to apoptosis, due to increased recruitment of RIPK1 and caspase-8 to FADD (Duprez et al. 2012). The RIPK3 kinase activity is required for

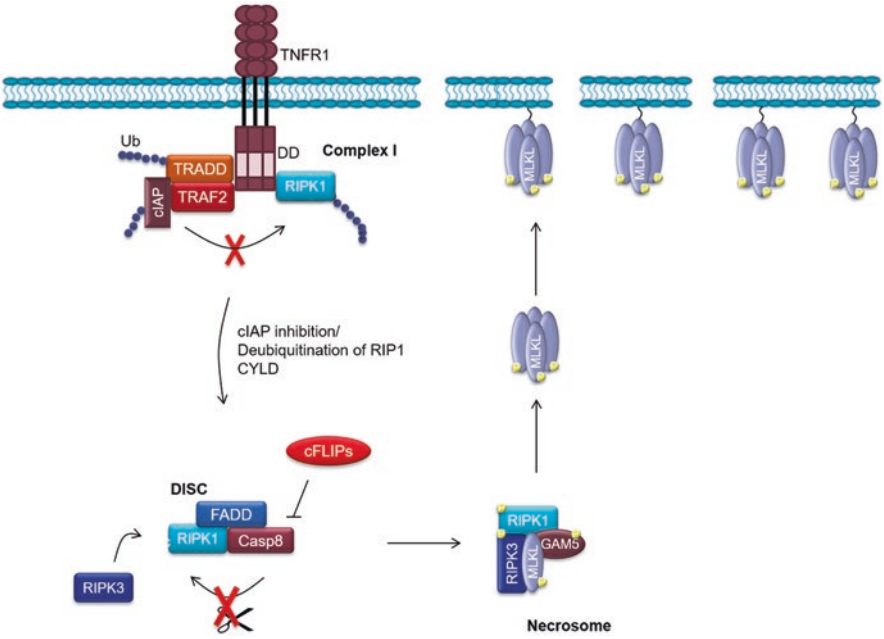

Fig. 8.2 Assembly of the necrosome. When caspase-8 is downregulated, RIPK1 is capable of recruiting RIPK3. RIPK1 and RIPK3 interact through their RHIM domains and autoactivate via phosphorylation. The necrosome then phosphorylates its substrates, including PGAM5 and MLKL. Once phosphorylated, MLKL oligomerizes and is recruited to the cellular membrane, where it interacts with phosphatidylinositol phosphates. This binding promotes formation of membrane pores, which is one of the hallmarks of necroptotic neuronal death

nuclear localization of the protein and for the induction of caspase-independent cell death (Feng et al. 2007).

Despite the prominent role of RIPK1 in scaffolding these complexes, RIPK1-independent (but RIPK3-dependent) necroptosis has been shown to occur upon knockdown of both RIPK1 and caspase-8, evidencing the negative regulation exerted by caspase-8 and suggesting that RIPK3 may be sufficient to induce necroptosis in certain cellular contexts (Vanlangenakker et al. 2011).

The cellular signaling network underlying the necroptotic process involves *de novo* protein synthesis (Yu et al. 2004), mitochondrial dysfunction, ROS accumulation and ATP depletion (Cho et al. 2009; He et al. 2009; Irrinki et al. 2011). These phenomena are promoted by the association between RIPK3 and a group of metabolism-related enzymes: phosphorylase-glycogen-liver (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1). The protein PYGL is the rate-limiting enzyme in glycogen degradation, while GLUL and GLUD1 are determinant enzymes for oxidative phosphorylation (Zhang et al. 2009). This association seems to contribute to mitochondrial damage (Kim et al. 2011).

Box 8.1: Timeline for the discovery of necroptosis

The identification of a form of caspase-independent cell death in response to death ligands (DL), such as tumor necrosis factor α (TNFα), was first reported in 1998 in L929 cells stimulated with this cytokine and treated with an array of caspase inhibitors. This cellular model is sensitized to a form of necrotic cell death, involving reactive oxygen species (ROS) formation, upon apoptosis inhibition (Vercammen et al. 1998). In 2000, a similar caspase-independent T-cell death mechanism downstream of Fas, TNF and TNF-related apoptosis-inducing ligand (TRAIL) ligands was identified (Holler et al. 2000). The type of cell death induced in these conditions was considered to have a necrotic phenotype and to induce late mitochondrial damage. In this same report, cells deficient in either FADD or RIPK1 were shown to be resistant to this form of caspase-independent cell death. Notably, the requirement for RIPK1 kinase activity to necrosis induction was first identified in the same study (Holler et al. 2000) and the ROS accumulation subsequent to necroptosis induction was confirmed a few years later (Lin et al. 2004).

The term necroptosis was first coined in 2005 by Degterev and colleagues (Degterev et al. 2005). They used this term to describe a type of non-apoptotic cell death that is mediated by Fas/TNF receptor activation, already observed in diverse cellular systems at the time. This cell death program was described to occur in the presence of the apoptotic inhibitor zVAD.fmk and as exhibiting necrotic morphology, such as plasma membrane integrity loss and mitochondrial dysfunction (Degterev et al. 2005). An important milestone in the understanding of necroptosis was achieved in this study, with the identification of the specific necroptosis inhibitor, Necrostatin-1 (Nec-1). This inhibitor was able to rescue cell viability following TNF/zVAD insults, but with no effect against purely apoptotic insults, like Fas ligand/cycloheximide treatment. The mechanism of Nec-1 inhibition was demonstrated to occur via RIPK1 kinase inhibition, confirming the requirement of kinase activity of this protein for necroptosis induction (Degterev et al. 2005).

Another important milestone was reached in 2009, with the identification of receptor-interacting protein kinase-3 (RIPK3) as a key mediator of necroptotic signaling by three independent groups using distinct approaches (Cho et al. 2009; He et al. 2009; Zhang et al. 2009). Cho et al. (2009) performed a siRNA screen in FADD-deficient Jurkat cells, which are known to die by necroptosis in response to TNFα. They identified both RIPK1 and RIPK3 as crucial mediators of this type of cell death. Interestingly, they also found that RIPK3 knockdown had no effect on TNF- and FasL-induced apoptosis or nuclear factor kappa B (NF-kB) activation, emphasizing the specific role of RIPK3 on the necroptotic mechanism. This protein was shown to be specifically recruited to complex II and, unlike RIPK1, was not present in complex I (Cho et al. 2009). He et al. (2009) performed a genome-wide siRNA screen and transfected HT-29 cells with different siRNA pools to assess which of

(continued)

Box 8.1 (continued)

these were protective against necroptotic stimulation. Besides RIPK1, which had been previously identified, they found RIPK3 to be important for this process. Importantly, this protein was specifically correlated with necroptosis since it did not interfere with induction of apoptosis. In fact, the ability of different cell lines to undergo necroptosis seemed to be correlated with RIPK3 expression. Accordingly, cells that expressed RIPK3 had the ability to induce necroptosis (for example Jurkat, U937, L929 and MEFs) while cells with no observable expression of RIPK3 were resistant to necroptotic stimulation, including HEK293T, MCF-7 and U2OS. Interestingly, when RIPK3 was expressed in "resistant" cells, like T98G cells, they were rendered susceptible to necroptosis (He et al. 2009). Finally, Zhang et al. (2009) studied the mechanism of necroptosis in two lines of NIH3T3 cells, N and A cells, which are, respectively, susceptible and resistant to necroptosis. A microarray analysis was performed to detect changes in genes that might be responsible for this differential vulnerability. RIPK3 was found to be responsible for induction of necroptosis in N cells, while A cells lack expression of this protein. Specifically, these authors demonstrated that knockdown of RIPK3 abolishes the component of necroptotic death in N cells, whereas expression of RIPK3 in A cells renders them susceptible to TNF-induced necroptosis (Zhang et al. 2009).

Despite the identification of a few necroptotic substrates downstream of the protein complex containing both RIPK1 and RIPK3, the complete understanding of the molecular signaling events underlying this type of cell death still remains elusive. However, it is increasingly clear that MLKL plays a central role as an executor of necroptosis. Phosphorylation of MLKL by RIPK3 leads to its homo-oligomerization and translocation to the plasma membrane (Cai et al. 2014; Wang et al. 2014a; Chen et al. 2014), where it forms membrane disrupting pores (Wang et al. 2014a; Ros et al. 2017) that likely mediate final plasma membrane disruption in necroptosis.

Despite the known role of RIPK1/3 kinase activities in necroptosis, few substrates have been identified so far. Mixed lineage kinase domain-like (MLKL) was shown to be a molecular target of the necrosome (Sun et al. 2012; Zhao et al. 2012). Its role in necroptosis is emphasized by complete abrogation of necroptosis upon MLKL knock down or in the presence of necrosulfonamide, a MLKL inhibitor. Phosphorylated MLKL forms homo-oligomers and translocates to the plasma membrane (Cai et al. 2014; Wang et al. 2014a; Chen et al. 2014). Phosphorylation of MLKL promotes its binding to phosphatidylinositol phosphates (PIPs), allowing plasma membrane recruitment (Dondelinger et al. 2014). Additional higher affinity PIP binding sites in MLKL are exposed upon binding to the membrane, resulting in binding stabilization (Quarato et al. 2016). At the plasma membrane,

oligomers of MLKL have been shown to form membrane disrupting pores (Wang et al. 2014a), and to cause Ca^{2+} influx mediated by the transient receptor potential melastatin related 7 (TRPM7) (Cai et al. 2014). In addition, a recent study demonstrated that osmotic forces mediate necroptotic plasma membrane rupture, which involves the formation of 4 nm diameter membrane pores (Ros et al. 2017). Accordingly, osmoprotectants reduce cell death in an in vivo renal model of ischemia-reperfusion injury that features necroptosis (Ros et al. 2017). The MLKL-dependent formation of membrane pores may mediate final plasma membrane disruption in necroptosis (Fig. 8.2).

The mitochondrial protein Ser/Thr-protein phosphatase (PGAM5) is also phosphorylated by RIPK3 and associates with the RIPK1/3-MLKL complex. The short isoform of PGAM5 (PGAM5S) is proposed to be an effector downstream of the necrosome since its RIPK3-mediated phosphorylation is abrogated by necrosulfonamide. PGAM5S then dephosphorylates the mitochondrial protein dynamin related protein 1 (Drp-1), resulting in its activation, which contributes to mitochondrial fragmentation (Wang et al. 2012).

A genome-wide siRNA screening allowed the identification of 432 genes related to necroptosis signaling (Hitomi et al. 2008). Interestingly, some of the genes identified in this study have enriched expression both in the immune and the nervous systems, both of which have been shown to undergo paradigms of necroptotic cell death. Additionally, a subset of genes that regulate both apoptosis and necroptosis, that included TNFR1a and CYLD, was also reported.

While a complex cascade of protein interactions from TNFRs to necrosome has been demonstrated to be required for necroptotic cell death, spontaneous formation of a protein module similar to complex II has also been described (Feoktistova et al. 2011; Tenev et al. 2011). Due to its independence from DR signaling, this high molecular weight complex comprising RIPK1, FADD and caspase-8, among other proteins, was called ripoptosome. The formation of this complex occurs following genotoxic stress (Tenev et al. 2011) or TLR3 stimulation (Feoktistova et al. 2011), both in conditions of cIAP depletion, suggesting that these proteins inhibit the assembly of the ripoptosome. This complex seems to have the ability to induce either apoptosis or necroptosis, depending on the cellular context (Tenev et al. 2011). Notably, in in vitro ischemia this complex does not seem to contribute to neuronal death, since treatment with a TNFα neutralizing antibody blocks induction of necroptosis in this cellular model (Vieira et al. 2014), suggesting that necroptosis in this context is TNFR-dependent and thereby induced by the necrosome, not by ripoptosome formation.

Overall, one of the most striking evidence from these diverse studies is the complementarity between apoptosis and necroptosis. Such notion is corroborated by occlusion of lethality observed in caspase-$8^{-/-}$/RIPK$3^{-/-}$ double knock out mice (Kaiser et al. 2011; Oberst et al. 2011). These results also emphasize the role of caspase-8 as a negative regulator of necroptosis. This regulation is achieved by the formation of catalytically active dimers of caspase-8 and $FLIP_L$, which inhibit the assembly of the necrosome, without inducing apoptosis (Micheau et al. 2002; Oberst et al. 2011).

8.5 Necroptosis in Disease

Deregulation of necroptosis contributes to the pathogenesis of various inflammatory, ischemic and neurodegenerative diseases characterized by excessive cell death and inflammation [for a recent review see (Galluzzi et al. 2017)]. Given the proinflammatory nature of necroptosis, it is also relevant in cancer conditions. However, it is still unclear how necroptosis influences tumorigenesis and metastasis, since while in some cancers there is loss of RIPK3 expression, associated with reduced necroptosis, in others inflammation associated with necroptosis in the tumor environment promotes cancer metastasis, but also cancer immunity [reviewed in Najafov et al. (2017)].

Necroptosis plays a role in the pathogenesis of several acute and chronic neurological diseases. Inhibition of necroptosis is neuroprotective in adult stroke and neonatal hypoxia/ischemia (see next section), and necroptosis was shown to occur in retinal neurons following ischemic damage. Necrostatin-1 was effective in reducing specifically this component of cell death, without affecting apoptotic neuronal death, and in improving the functional outcome in the ischemia challenged retina (Rosenbaum et al. 2010). Also, in a mouse model of retinal detachment, both inhibition of RIPK1 as well as RIPK3 ablation were shown to be neuroprotective, demonstrating the activation of necroptotic mechanisms upon apoptosis inhibition (Trichonas et al. 2010).

Certain neuronal death paradigms have been shown to induce necroptosis. Indeed, following treatment with 24(S)-hydroxycholesterol (24S-OHC), which is cytotoxic, both human neuroblastoma SH-SY5Y cells and cortical neurons were shown to die by necroptosis (Yamanaka et al. 2011). Additionally, menadione, a compound that induces superoxide production, with concomitant mitochondrial dysfunction and ATP depletion, was shown to induce caspase-independent necroptotic-like death in HT-22 cells, a cell line of immortalized mouse hippocampal neurons (Fukui et al. 2012). A recent study found that the environmental neurotoxicant polychlorinated biphenyl (PCB)-95 increases RIPK1, RIPK3 and MLKL expression in cortical neurons, while decreasing caspase-8 levels (Guida et al. 2017). Necrostatin-1 or siRNA-mediated knockdown of RIPK1, RIPK3 or MLKL expression significantly reduced neuronal death induced by PCB-95. In addition, this study showed that PCB-95 increases the levels of RE1-silencing transcription factor (REST), which represses caspase-8 and cAMP Responsive Element Binding Protein (CREB) expression. Since CREB binds to the promoter regions of the RIPK1, RIPK3 and MLKL genes, its down-regulation increases the levels of these proteins (Guida et al. 2017).

Necrostatin-1 was shown to be effective in reducing the damage associated with injury in a mouse model of traumatic brain injury (You et al. 2008), and in models of spinal cord injury (Wang et al. 2014b). Interestingly, necroptosis was proposed to have a neuroprotective effect by being activated in inflamed microglia, which results in less damage to neurons. Upon inflammatory stimulation, inhibition of caspase-8 activity is beneficial to neurons by promoting necroptosis in microglia, whereas

Nec-1 rescues neurodegeneration. This implies that caspase inhibitors could promote neuronal survival against certain insults by promoting microglial demise (Fricker et al. 2013).

Necroptosis has been recently implicated in amyotrophic lateral sclerosis (ALS) (Ito et al. 2016; Re et al. 2014). In this disease, motor neuron axonal degeneration occurs via RIPK1 and RIPK3-mediated mechanisms, suggesting that inhibiting RIPK1 may be protective and useful in the treatment of ALS. Accordingly, a RIPK1 inhibitor is in Phase I human clinical trials for the treatment of ALS (by Denali Therapeutics). In cortical lesions of human multiple sclerosis (MS) pathological samples, activation of RIPK1, RIPK3 and MLKL has been detected (Ofengeim et al. 2015). This study suggests that TNF α, implicated in MS, induces oligodendrocyte degeneration mediated by necroptosis, and shows that RIPK1 inhibition protects oligodendrocytes from cell death in animal models of MS.

8.6 Necroptosis Induction in Cerebral Ischemia

Cerebral ischemia is a leading cause of disability and death (Flynn et al. 2008). This pathology lacks effective treatments, a direct consequence of a limited time window for intervention before major neurodegeneration ensues, as well as of the lack of specificity of some of the treatments developed so far. The interruption in the blood supply to the brain causes a deprivation of oxygen and glucose, leading to a failure in ATP production, which is pernicious due to the brain high metabolic demand (Moskowitz et al. 2010). This results in neuronal depolarization and excessive glutamate release, accompanied by dysfunction of the glutamate reuptake mechanisms in glia cells and neurons, as a result of the dissipation of the Na^+ gradient, due to the depletion of ATP. Thus, an excessive glutamate accumulation at the synapse causes the overactivation of glutamate receptors leading to an intracellular Ca^{2+} overload that may trigger cytotoxicity, a phenomenon called excitotoxicity. Depending on the duration and intensity of the insult, other non-excitotoxic mechanisms are activated, contributing to Ca^{2+} overload (Besancon et al. 2008; Szydlowska and Tymianski 2010). Deregulation of Ca^{2+} homeostasis activates deleterious intracellular mechanisms, including mitochondrial dysfunction and oxidative and nitrosative stress, that induce inflammation and ultimately neuronal death (Iadecola and Anrather 2011; Moskowitz et al. 2010). In global ischemia and in the penumbra area, neuronal degeneration ensues within 24–72 h after the ischemic insult, a process called delayed neuronal death. This delay in neuronal demise onset suggests the reliance on transcriptional changes, leading to programmed cell death mechanisms.

Cerebral ischemia can be addressed experimentally, using several research models, both in vitro and in vivo. The most common in vivo models to study transient global ischemia are the four vessel occlusion (4-VO) and the 2-VO combined with hypotension, both in the rat. As for focal ischemia, the middle cerebral artery occlusion (MCAO) model is generally used. Regarding the in vitro models, the closest to *in vivo* ischemia is the oxygen and glucose deprivation (OGD) challenge, which is

most commonly used either in primary cultures of hippocampal or cortical neurons or in forebrain or organotypic hippocampal slices. Due to its characteristics, the OGD challenge of hippocampal neurons is a good model for global ischemia and allows for the dissection of molecular pathways underlying cerebral ischemia. This challenge consists of placing cultured neurons or slices in a glucose-free medium inside an anaerobic chamber, thereby combining the deprivation of these two factors which mimics, in a simplified system, what happens in the brain during the interruption of the blood flow.

The extent of pathways activated following brain ischemia underlies, as mentioned, a variety of cell death mechanisms, posing the difficulty of distinguishing the different types of cell death experimentally. For this purpose, a few methods have been advanced, which allow distinguishing apoptotic from necroptotic cells based on differential staining using AnnexinV/PI (apoptotic cells are AnnexinV positive/PI negative, while necroptotic cells are AnnexinV negative/PI positive), cell and nuclear morphology, and RIPK1 kinase activity, which has been correlated specifically to necroptosis induction (Degterev et al. 2014; Miao and Degterev 2009). Distinguishing between classical necrosis and necroptosis, however, poses other problems, since morphologically these two forms of cell death are very similar, despite one being a form of "accidental" cell death and the other a type of programmed cell death (Vanden Berghe et al. 2010). In this respect, identification of Nec-1 as a specific RIPK1 inhibitor (Degterev et al. 2008) was an important advancement in necroptosis research. As mentioned above, RIPK1 activity was demonstrated to be required solely for necroptotic cell death (Vanden Berghe et al. 2010), thus underscoring the importance of Nec-1 as a valuable tool in necroptosis studies. More recently, other necrostatins have been identified, with varying degrees of specificity to RIPK1 (Jagtap et al. 2007; Wang et al. 2007; Zheng et al. 2008).

The first evidence of necroptosis following a cerebral ischemic insult was reported by Degterev et al., in 2005. In this work, the authors described Nec-1 as a specific necroptosis inhibitor and observed significant neuroprotection by Nec-1 against a focal ischemic insult. Indeed, they reported a significant reduction in infarct size afforded by Nec-1 treatment, as well as improvement of neurological scores (Degterev et al. 2005). In recent years the neuroprotective effect afforded by Nec-1 treatment has also been observed following OGD (Vieira et al. 2014), as well as other in vivo ischemic paradigms (Degterev et al. 2005; Yin et al. 2015), even at 30d post-insult (Xu et al. 2016). These data argue for a component of necroptotic neuronal death following ischemic insults, both in vitro and in vivo, in addition to the apoptotic neuronal death previously described. However, a recent study found that brain injury following MCAO model of stroke or hypoxia-induced cerebral edema was not ameliorated in knock out mice for RIPK3 (Newton et al. 2016), despite the improvement observed by these mice in models of cardiac and kidney ischemia/reperfusion (reviewed in Galluzzi et al. 2017).

Several features of necroptosis were observed in the in vitro model of ischemia, OGD, including a DR-dependent component of neuronal death that is not inhibited by caspase inhibitors like zVAD.fmk. This component was sensitive, however, to Nec-1 treatment and displayed morphological features of necrosis, such as

cytoplasmic membrane rupture, as well as nuclear membrane compromise, allowing for positive PI staining (Vieira et al. 2014). Furthermore, following OGD, RIPK3 mRNA is upregulated, with concomitant downregulation of caspase-8 mRNA (Vieira et al. 2014), suggesting a cellular mechanism permissive to necroptosis induction, given the negative regulatory role of caspase-8 on necroptosis activation. At the protein level, RIPK3 expression is specifically upregulated following global ischemic insults, both in vitro and in vivo (Vieira et al. 2014), and RIPK3 translocates to the nucleus (Xu et al. 2016; Yin et al. 2015), where it interacts with AIF (Xu et al. 2016). The other necrosome component, RIPK1 protein, is also upregulated at least following in vitro insults (Qu et al. 2016; Vieira et al. 2014). Notably, overexpression of either component of the necrosome has a detrimental effect following OGD, whereas knockdown of RIPK3 or inhibition of RIPK1 are neuroprotective, reducing the extent of necroptotic neuronal death (Vieira et al. 2014).

Although the molecular mechanism downstream of the necrosome are still not completely resolved, in ischemic contexts, they involve MLKL. Indeed, the interaction between RIPK1-RIPK3 and MLKL increases following an OGD challenge, and downregulation of MLKL by siRNA exerts a neuroprotective effect against in vivo insults (Qu et al. 2016). PGAM5, another necrosome substrate, was initially described as a downstream target of RIPK1/3 contributing to necroptosis, but some recent evidence points to a protective role for PGAM5, by promoting mitophagy. Absence of this protein is proposed to have a deleterious effect by leading to an increase in the number of defective mitochondria and subsequent ROS production, which contributes to the necroptotic damage following heart and brain ischemia (Lu et al. 2016). The role of this protein is thus still not completely unveiled.

The MAPK ERK pathway has been suggested as a putative downstream signaling effector of necroptosis, since ERK inhibitors blocked glutamate-induced necroptosis in HT-22 cells, and Nec-1 reduced glutamate-induced ERK phosphorylation, thereby decreasing its activation, but not that of JNK or p38 MAPKs (Zhang et al. 2013). Furthermore, HDAC6, a histone deacetylase, was also found to contribute to necroptotic damage, since it is upregulated following in vitro ischemia, and inhibition of its activity decreases the extent of OGD-induced necroptosis, possibly via ROS production inhibition (Yuan et al. 2015).

Necroptotic cell death is implicated in brain ischemia, but also in ischemic injury in other organs, such as the kidney and the heart, with common features, namely the upregulation of the necrosome components RIPK1 and RIPK3, as well as the protective effect exerted by Nec-1, in the kidney (Linkermann et al. 2012), in cardiac tissue (Luedde et al. 2014; Tuuminen et al. 2016) and in retinal cells (Ding et al. 2015; Rosenbaum et al. 2010). These effects have also been observed in lung epithelial cells following ischemic insults associated with renal transplants, due to crosstalk between these organs (Zang et al. 2013; Zhao et al. 2015). Additionally, similar to what was observed in neurons, overexpression of the necrosome components is detrimental, whereas knockdown of RIPK3 is protective against ischemic insults in myocytes (Luedde et al. 2014) and retinal cells (Ding et al. 2015).

One of the challenges in stroke/global cerebral ischemia treatment is the fact that diverse modes of cell death seem to be activated concomitantly. Indeed, neurons

may die via necrosis, apoptosis, necroptosis or autophagy (Mehta et al. 2007; Meloni et al. 2011; Vieira et al. 2014). Thus, the best approach to ensure maximal neuronal survival may be the use of combinatorial strategies targeting the diverse modes of neuronal death that are induced by brain ischemia. As new data is reported, a more complete picture of the necroptosis process, as well as a better knowledge of the research models of cerebral ischemia, may shed light into the possibilities afforded by inhibition of an array of cell death programs in the treatment and management of the injury induced following ischemic paradigms.

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Chapter 9
Histological and Elemental Changes in Ischemic Stroke

M. Jake Pushie, Vedashree R. Meher, Nicole J. Sylvain, Huishu Hou, Annalise T. Kudryk, Michael E. Kelly, and Roland N. Auer

Abstract Stroke is a leading cause of serious long-term disability in adults and a leading cause of death in developed nations. Following an ischemic stroke the metabolic profile of the affected tissue is significantly altered, with the infarct representing the most severely affected tissue, and the surrounding penumbra, or peri-infarct zone (PIZ), containing a gradient of metabolic states progressing from severely impacted toward an otherwise healthy profile. The penumbra contains potentially salvageable tissue and is the focus in many stroke treatments. In this chapter, we employ the photothrombotic stroke model (a widely used animal model for studying focal ischemia) to study the histopathological and bioelemental changes that occur post-stroke. Synchrotron-based X-ray fluorescence imaging allows simultaneous measurement of multiple elements in situ within biological tissues, as their naturally-occurring concentrations. Images of elemental distributions are compared to conventional histopathological changes in the infarct and penumbra. Understanding the bioelemental changes associated with the post-stroke brain provides opportunities to expand our understanding of the underlying cellular and tissue changes associated with ischemic stroke and can ultimately be used to guide development of future treatment methods targeting the penumbra.

Keywords Ischemic stroke · Penumbra · Photothrombotic stroke · Excitotoxicity · X-ray fluorescence imaging · Elemental mapping

M. Jake Pushie · N. J. Sylvain · H. Hou · M. E. Kelly
Division of Neurosurgery, Department of Surgery, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

V. R. Meher
Department of Health Sciences, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

A. T. Kudryk
College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

R. N. Auer (✉)
Department of Pathology and Laboratory Medicine, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Department of Pathology, Royal University Hospital, Saskatoon, SK, Canada

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Stroke is a leading worldwide cause of serious long-term disability and death in developed countries (Thrift et al. 2017), and now developing countries (Feigin et al. 2009). Ischemic stroke incidence is roughly 6.6 million Americans, and the prevalence is 2.6%. Roughly 87% of strokes are ischemic (Mozaffarian et al. 2016), and once blood supply to the brain is reduced below an ischemic threshold (Heiss 1992), biochemical cascades, irreparable tissue injury and cell death can occur within only 2–4 min of global ischemia. In focal ischemia, the tolerable duration of occlusion is longer (Kaplan et al. 1991), but the region of tissue most severely impacted is the ischemic core. Surrounding the ischemic core (Hossmann 1994) is the peri-infarct zone, or penumbra, which contains tissue that is partially perfused by collateral arteries, and is therefore salvageable if blood flow is fully restored (Bandera et al. 2006; Latchaw et al. 2003). Due to the rapid loss of tissue viability in the ischemic core, the ischemic penumbra contains the only tissue that can potentially be rescued through intervention post stroke.

The ability to identify the ischemic penumbra and quantify dynamic changes in its size are critically important for many areas of stroke research, as therapeutic interventions and drug development rely on accurately identifying and quantifying penumbral salvageable tissue. A penumbra can be defined histologically by identifying regions of selective neuronal necrosis along the border of pan-necrotic tissue with normal brain. While this identifies a border rim of variable extent, the spatial extent of penumbra that can be salvaged can only be determined by shrinkage of an infarct using an effective treatment, retrospectively leaving the penumbra, perforce, as the tissue rim that was salvaged around an infarct that is reduced in size. Thus, new methods for identifying and defining the extent of the penumbra would aid studies that target the penumbra, and allow quantitative determination of the size and the neuroanatomical extent of the penumbra—critical metrics beneficial for determining treatment efficacy.

In this chapter we discuss the histopathology and elemental changes within tissue following induction of a focal ischemic stroke, and explore the tissue-level changes using the photothrombotic mouse model of stroke. Imaging multiple elemental distributions in tissue provides a means of measuring its metabolic status. For nervous tissue, elemental changes associated with altered membrane potential and excitotoxicity are particularly useful for studying ischemic stroke. Understanding cellular features that contribute to tissue vulnerability will help us better identify therapeutic options for stroke management and recovery.

9.1 The Photothrombotic Model

Our team employs the photothrombotic stroke model in mice (Labat-gest and Tomasi 2013), using isoflurane anesthesia, and head immobilization in a stereotactic frame. After skull is exposure via a midline scalp incision, and the area over the primary somatosensory cortex is identified (−1 to −2.5 mm midline to lateral, and +1 to −0.5 mm antero-posterior to bregma, right hemisphere) (Carmichael 2005;

Winship and Murphy 2008; Hackett et al. 2016). A photoactive dye, Rose Bengal, is injected intraperitoneally, which enters the circulation in under 5 min. A sterile blocking mask is applied over the area of interest and 5 min after the dye is injected, the area is photoexcited using a laser (532 nm) located 3 cm above the skull, for 20-min (Labat-gest and Tomasi 2013; Carmichael 2005; Winship and Murphy 2008).

Photoexcitation of the dye induces localized production of singlet O_2, endothelial damage, and platelet activation. This induces formation of a thrombus, resulting in a focal cortical stroke lesion which is highly reproducible. Blood flow to the affected region of the cortex ceases, mimicking an ischemic stroke, although the model is thought to contain little secondary oxidative damage due to the lack of reperfusion-associated production of free radicals (Carmichael 2005; Kim et al. 2000). Nevertheless, the model is popular due to its reproducibility and reliability (Bandera et al. 2006; Carmichael 2005; Watson et al. 1985), compared to other potentially more variable stroke models, such as the MCAO model (Fluri et al. 2015; Liu and McCullough 2011).

Following induction of the photothrombotic stroke, Marcaine anesthetic is applied to the incision area for pain relief. Twenty-four h after the laser illumination is ceased, the animals are sacrificed by decapitation and the heads are immediately frozen in liquid N_2 in order to preserve elemental distributions (Hackett et al. 2015) and to avoid artifacts introduced by conventional tissue processing methods (Hackett et al. 2011, 2012).

9.2 Tissue Preparation for X-Ray Fluorescence Imaging

Preservation of elemental distributions and concentration levels *in situ* is of paramount importance for elemental mapping (by "elemental" we are referring to the labile cations and anions, such as K^+ and Cl^-, as well as atoms bound within molecules, such as P, S, and Fe). Conventional animal euthanization and tissue processing methods, including perfusion, post-mortem interval, application of tissue fixative, and cryoprotection, can each alter the biodistribution of elements in tissue—particularly mobile ions and chelatable trace metals (Hackett et al. 2015, 2011). For this reason, careful consideration must be given to tissue preparation, and ideally as little sample handling as possible is preferred. We have developed a protocol which optimizes elemental preservation of brain tissue. This method, however, is at the expense of techniques which are commonly employed to improve the appearance of the tissue. Furthermore, due to the necrotic changes that take place in the stroke lesion, tears and cracks are not uncommon, as the underlying tissue is largely without structural integrity. Due to the absence of tissue fixative or cryoprotection, we also observe micro-cracks and bubbles throughout the tissue. These artifacts are typically small enough that they do not affect elemental imaging results and have no impact on the quantities of elements present.

Brains are chiseled out from the skull while still frozen, and are maintained at −20 °C during cryosectioning of 30 μm-thick sections, cut using Teflon-coated blades to minimize transfer of metals from the blade to the tissue. Cut sections are mounted on metal-free plastic coverslips for X-ray fluorescence imaging (XFI). Careful selection of coverslips is necessary for X-ray fluorescence experiments as most plastic or glass coverslips and microscope slides contain an abundance of unwanted elements related to their associated manufacturing processes, which will overwhelm detection of relatively weaker fluorescence signals from trace elements of interest in samples being analyzed. For example, the thermanox coverslips we use contain traces of cobalt, but since cobalt is not one of the elements of interest for our analysis, we use these particular coverslips.

9.3 Introduction to Synchrotron-Based X-Ray Fluorescence Imaging

With the current state of technology, XFI for biological specimens can only be performed at a synchrotron with beamlines that support such imaging capabilities. These are multi-user facilities where access is typically granted through a competitive proposal application system.

The experimental facilities for XFI can typically achieve beam spot sizes of a few microns, either through use of apertures, which reduce flux, or through the use of focusing optics. The sample is mounted on a mechanical stage in the path of the incident X-ray beam, which is typically tuned to an energy sufficient to excite a range of elements of interest. The energy of the X-ray fluorescence that is emitted is dependent on the fluorescing element. Acquisition of the full X-ray fluorescence spectrum, using an energy dispersive detector, provides the means to collect the fluorescence from all elements within the area of sample illuminated by the incident beam. By raster scanning the sample in the beam, a map of the fluorescence spectrum at each position (pixel) can be obtained. It is also important to point out that XFI data must be collected prior to any other staining techniques used for light microscopy. For a more complete review on the topic of XFI see reference (Pushie et al. 2014).

9.4 Histopathology

Normal brain tissue consists of cell bodies embedded in neuropil, the fine, bubbly, reticulated, and relatively structureless tissue between cell bodies as observed by light microscopy. Using electron microscopy, neuropil is resolved to axons, dendrites and glial processes. The axons and dendrites are neuronal processes, whereas

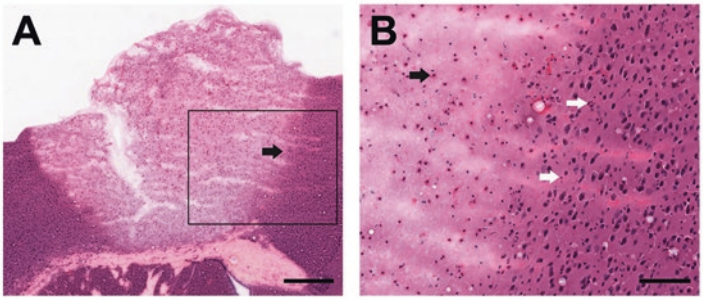

Fig. 9.1 Comparative hematoxylin and eosin (H&E) stained images showing pan-necrosis and selective neuronal necrosis of ischemic tissue. The presence of cracks and other artifacts is due to the lack of tissue preservatives which are undesirable for XFI analysis of the tissue. (**a**) Low magnification view of ischemic infarct boundary in the right somatosensory cortex, including the histological penumbra (black arrow), scale bar = 500 μm. (**b**) Higher magnification view showing pan-necrosis of neurons along the border of the infarct, which is indicated by pink-stained cytoplasm surrounding shrunken nuclei (black arrow) and selective neuronal loss in the penumbra indicated by scarce neuronal death where dead neurons are still surrounded by viable tissue (white arrows), scale bar = 100 μm

the glia, mostly astrocytes, fill the gaps between neurites, leaving little extracellular space (estimated to be 20% for diffusion of substances) (Nicholson et al. 2011). The cell bodies themselves are either neuronal or glial cells, with glia being further divided into macroglia (oligodendrocytes and astrocytes) and microglia. Microglia patrol the nervous system, subserving immune functions. Both astrocytes and oligodendrocytes, each have two forms. Astrocytes in the white matter tend to be fibrillated and are exposed to interstitial fluid, which contains metabolic waste products. It is thus not surprising that astrocytes within the white matter are more densely fibrillated (with glial fibrillary acidic protein) than those in the gray matter (termed protoplasmic astrocytes). Oligodendrocytes similarly have two localizations—one in the white matter, maintaining myelin, and the other in the cerebral cortex, where they surround and maintain neurons.

There are two forms of ischemic brain damage: selective neuronal necrosis (SNN) and pan-necrosis. SNN kills neurons but not glia or neuropil and is mediated by excitotoxicity of glutamate (Benveniste 1991). Pan-necrosis involves death of the entire tissue, affecting cellular elements indiscriminately (*i.e.* Fig. 9.1a). In the context of cerebral ischemia, pan-necrosis is termed infarction—where infarction is considered a tissue-level damage. Additionally, necrosis is often sharply demarcated, whereby neurons inside the infarct undergo pyknotic changes (black arrows in Fig. 9.1b). This sharp demarcation necessarily cuts cross axons, dendrites, and glial processes in the neuropil, thereby demonstrating this is a tissue-level mechanism as opposed to a cell-level mechanism. Another characteristic hallmark of necrosis includes mitochondrial flocculent densities, shown in the electron micrograph image in Fig. 9.2.

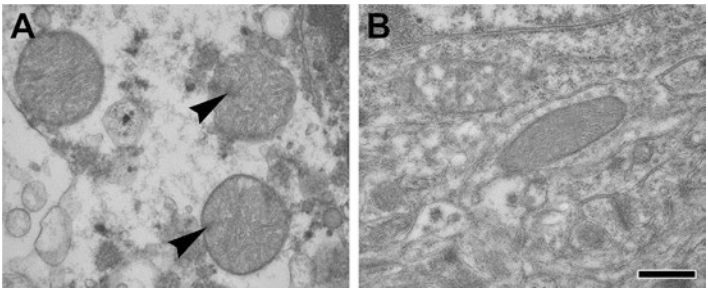

Fig. 9.2 Electron micrograph showing the morphology of necrotic changes in brain tissue following ischemia. The appearance of necrosis is marked by mitochondrial flocculent densities (indicated by the black arrows) inside the infarct (**a**) as opposed to normal non-necrotic tissue from the contralateral side, with normal mitochondria (**b**). Scale bar = 0.5 μm

9.5 Ischemic Cell Death Is Induced by Excitotoxicity

The brain is a highly perfused organ and neurons require a continuous supply of nutrients for proper functioning. Interruption of blood flow for just 2–4 min has been shown to be sufficient for neurons to die (Smith et al. 1984), whereas it can take from 20 to 40 min for other cell types, such as cardiomyocytes and kidney cells, to die as a result of ischemia (Lee et al. 2000). Excitotoxicity, the pathological process where over-excitation of receptors occurs by excitatory neurotransmitters with deleterious consequences, can also activate intracellular and extracellular pathways involved in cell death. Ischemia, hypoglycemia, epilepsy (Auer and Siesjö 1988), and domoic acid poisoning (Debonnel et al. 1989) are well-known examples of excitotoxicity-induced cell death.

There are also intracellular signalling pathways involved in information processing which are triggered by excitotoxicity, which cause cell death under ischemic conditions through induction of free radical production, membrane failure, and an inflammatory response (Lee et al. 2000).

Other important considerations during ischemia-induced cell death are acidosis, disruption of the blood brain barrier (BBB), and infiltration of leukocytes (Woodruff et al. 2011). Arterial occlusion leads to a reduction in tissue pO_2 and increase in pCO_2. Under hypoxic conditions, the tissues undergo an anaerobic glycolysis from the glucose supplied by the residual blood flow (Kalimo et al. 1981). A build-up of lactic acid triggers a drop in local tissue pH within the stroke lesion.

Due to bioenergetic failure under ischemic conditions, there is an increased influx of Na^+ and increased efflux of K^+ as well as glutamate (Paschen 1996), which results in anoxic depolarization (Hansen and Olsen 1980). Failure of glutamate uptake systems causes over- excitation of glutamate ionotropic receptors [*N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate], resulting in excessive intracellular influx of Ca^{2+}. High intracellular Ca^{2+} leads to mitochondrial failure, activates catabolic enzymes and production of reactive oxygen species (Kalogeris et al. 2012).

9.6 Elemental Imaging

Element-based imaging remains a growing research area applicable to a wide range of fields across the basic sciences and health research fields (Hackett et al. 2016; Caine et al. 2016). There are numerous experimental methods for mapping elemental distributions in tissue, including magnetically-susceptible nuclei for MRI (*e.g.* ^{23}Na), mass spectrometry-based imaging—specifically laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) (Egger et al. 2014), whereas matrix-assisted laser desorption and ionization (MALDI) imaging is applicable to molecules as opposed to individual elements, *per se* (Aichler and Walch 2015)—and fluorescent chelators designed to coordinate specific elements (Lindahl and Moore 2016; Karim and Petering 2016). While it is beyond the scope of this chapter to review these techniques, they are compared in a recent review (Pushie et al. 2014).The mass spectrometric techniques are destructive techniques which alter the specimen under analysis and require some level of pre-treatment. Fluorescence probes are often regarded as specific for a particular element; however their binding is, at best, selective for a particular element but may also bind chemically similar targets. Such probe-based approaches also rely on labile or exchangeable target ions, and the probe molecules themselves may have variable penetration into particular tissues or subcellular structures, or may mobilize an element into a region preferable to the chemical properties of the chelator.

The type of synchrotron-based XFI experiments described herein are particularly amenable to mapping elemental distributions within biological tissues at their naturally-occurring trace levels, and is not dependent on sample preparation method *per se*, but is best applied with minimal sample pre-treatment or manipulation in order to preserve the biodistribution in its original state. However, there are technical challenges with obtaining data for light elements below P and S, making observation of Na^+ beyond the ability of the technique to observe within biological specimens.

9.7 Results of X-Ray Fluorescence Imaging

XFI detects the intrinsic fluorescence produced by elements when photons of sufficient energy are absorbed, thereby promoting a ground state electron (*i.e.* a 1 s electron) into an excited state orbital. For XFI experiments synchrotrons are used to produce X-rays, as they are intensely bright sources of photons which can be tuned over a wide range to a desired energy (Egger et al. 2014). The X-ray beam spot can be tailored to the desired application and a range of elements can be imaged simultaneously using an energy dispersive detector combined with spectral fitting *in silico*. This affords the means to record contributions from many elements to the total fluorescence spectrum and generate element-specific maps by selectively displaying the fitted contribution of a specific element to the collected data.

Not to be confused with computed tomography-type imaging using a synchrotron light source (Lin et al. 2013; Zhang et al. 2014), synchrotron-based XFI is still a relatively new technique and there are only a small number of researchers at present investigating topics related to stroke (Caine et al. 2016).

9.8 Contrasting Elemental Images with the Elementary Dogma of Ischemic Stroke

With the benefit of new imaging modalities comes new insights and an opportunity to refine current dogma. The ischemic core of dead or dying tissue with low cerebral blood flow and high extracellular K^+ is regarded as a chief hallmark of ischemic cell damage. Although K^+ is no longer within the cell, XFI data, even as early as 1 h post-stroke, reveals that the infarct region is largely devoid of essential K^+. The infarct is readily identified histopathologically as the demarcated region with reduced hematoxylin staining, and appearance of eosinophilic neurons with shrunken cell body (Figs. 9.2 and 9.3) (Zille et al. 2012). XFI reveals these components are lost from the tissue almost entirely, indicating that the consequence of initial depolarization and eventual cell membrane degradation is not only loss of essential chemical messengers and redistribution of ions into the extracellular space. As the detected X-ray fluorescence is based on inherent atomic physics-based properties, the technique is sensitive to the elements themselves, and not to their chemical form, localization, or bioavailability. Due to concomitant disruption of the BBB during the early stages of ischemia the extracellular space of the affected tissue is in exchange with the blood, thereby providing a much larger reservoir for cellular components, like K^+ lost through depolarization or cell membrane breakdown. These components then follow their concentration gradient, such as from the affected tissue, to the blood, where the K^+ concentration is in the range of ~5 mM, compared to ~150 mM in cells of the brain. Comparing concentrations of key ions implicated in ischemic changes (Na^+, Cl^-, K^+, and Ca^{2+}), we observe that each of these ions follows its concentration gradient between the cell environment and blood (Fig. 9.4).

XFI data provides further evidence of this concentration-dependent exchange between the ischemic tissue and blood, revealing profound changes in Ca^{2+} and Cl^- content within the infarct (Fig. 9.5). Along with K^+, Na^+ and Ca^{2+} channels, Cl^- channels are also dysregulated in ischemic stroke. These channels allow passive diffusion of Cl^- into cells and decrease the membrane potential, resulting in tonic inhibition of excitatory neurotransmission, and lost Cl^- channel function is associated with neuropathologies such as epilepsy (Puljak and Kilic 2006). Ca^{2+} is a critically important chemical messenger within the brain, and is employed sparingly for functions such as activation of signaling pathways (Brini et al. 2014) and regulating gene expression (West et al. 2001). Ca^{2+}, while regarded as vital for neuronal health and function, is maintained at a very low concentration within the brain, with

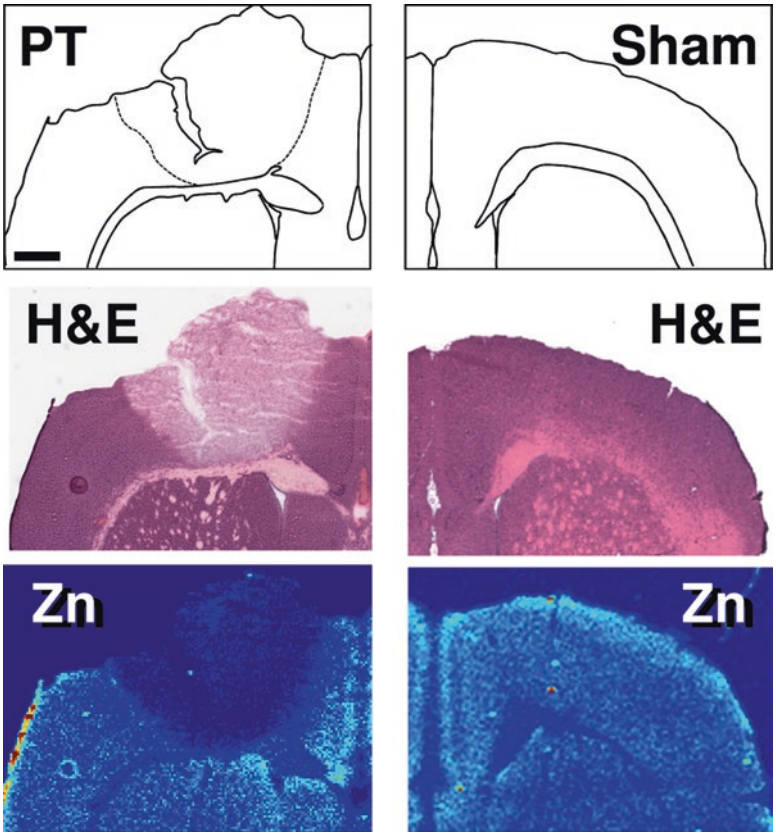

Fig. 9.3 Comparison of synchrotron X-ray fluorescence maps of Zn^{2+}, which best shows neuroanatomical detail, and conventional H&E staining of the same tissue. Zn^{2+} is present in all cells, and its concentration is dependent on cell type and tissue localization. Following ischemia, low molecular weight Zn^{2+} complexes are lost through depolarization, while the reduced pH of the infarct and competition by other divalent cations likely drives loss of Zn^{2+} from protein binding sites. The mouse photothrombotic model is compared to a sham mouse model. The stroke lesion demonstrates marked differences from normal tissue. The authors note that the cracking of the stroke lesion tissue is an artifact of the extent of necrosis and the tissue preparation method, which does not employ fixative or cryoprotectant, and requires that the head be snap-frozen. This procedure can lead to tissue cracking during the freezing procedure or during cryosectioning. Scale bar = 600 μm

average Ca^{2+} concentration in the 100 nM range. Following ischemic stroke the infarct demonstrates a significant elevation in Ca^{2+} and Cl^- content. Due to the nature of the X-ray fluorescence imaging, Na^+ (and other light element) images are not obtainable for these types of samples, due in part to low X-ray fluorescence yields, poor penetration of the relatively low energy Na^+ fluorescence, and absorption losses to the air in the sample-to-detector path length, mentioned earlier.

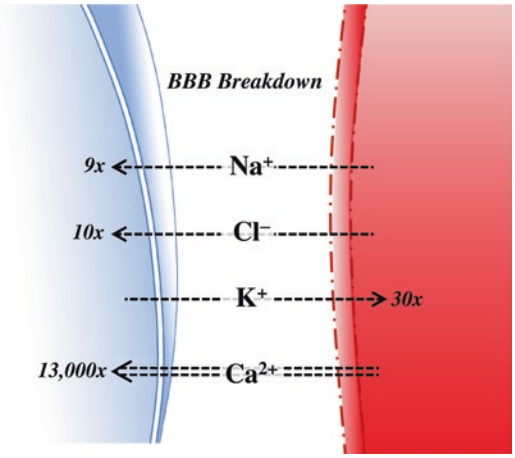

Fig. 9.4 Breakdown of the BBB allows exchange between the blood and the extracellular fluid, and between the extracellular and intracellular fluids. According to the zeroth law of thermodynamics, if two thermodynamic systems are each in equilibrium with a third, then they are also in equilibrium with each other. Therefore, as exchange between these compartments begins to reach equilibrium the small volume of the intracellular environment will more closely resemble that of blood. Comparing average concentrations for the most commonly implicated ions that undergo dysregulation in ischemic stroke (*i.e.* mobile ions) we find that the concentration differences will lead to a shift into (in the case of Na^+, Cl^- and Ca^{2+}) or out of (as in the case of K^+) the intracellular environment within the brain—an observation recapitulated in elemental maps (Fig. 9.5)

Nevertheless, the changes in Cl^- and Ca^{2+} reflect their significantly elevated concentrations in blood relative to the brain (Fig. 9.4).

With the exception of calcium-binding proteins, which have evolved high affinities for binding Ca^{2+} within specific coordination environments for signaling or as a cofactor for functionally requirements, solvation of the ions Cl^-, K^+ and Ca^{2+} is highly thermodynamically favourable. Upon breakdown of neuronal cell membrane potentials and disruption of the BBB these ions follow their chemical gradient, resulting in a redistribution of these ions from compartments of high concentration to compartments with lower concentration, which is highly entropically favourable. Other elements, however, including P and S, are associated with a wide range of biomolecules across a wide range of molecular weights. These elements are covalently bonded to multiple additional atoms, and in the case of proteins (Cys, Met, and P-Ser and P-Tyr), lipids, DNA, RNA, and other large molecules, the localization of these elements is dictated by the compartmentalization of their host molecule. P and S may also be components of small molecules, for example, taurine and GSH are sulfur-containing molecules which may also have relatively high mobility if membrane permeability of the host cell is disrupted. Taurine is a sulfur containing amino acid synthesized endogenously by metabolism of methionine and cysteine or exogenously by obtaining taurine rich food products. Under ischemic conditions, taurine provides neuroprotective effects against glutamate-induced excitotoxity, oxidative stress and inflammation (Menzie et al. 2013). Glutathione is a tripeptide

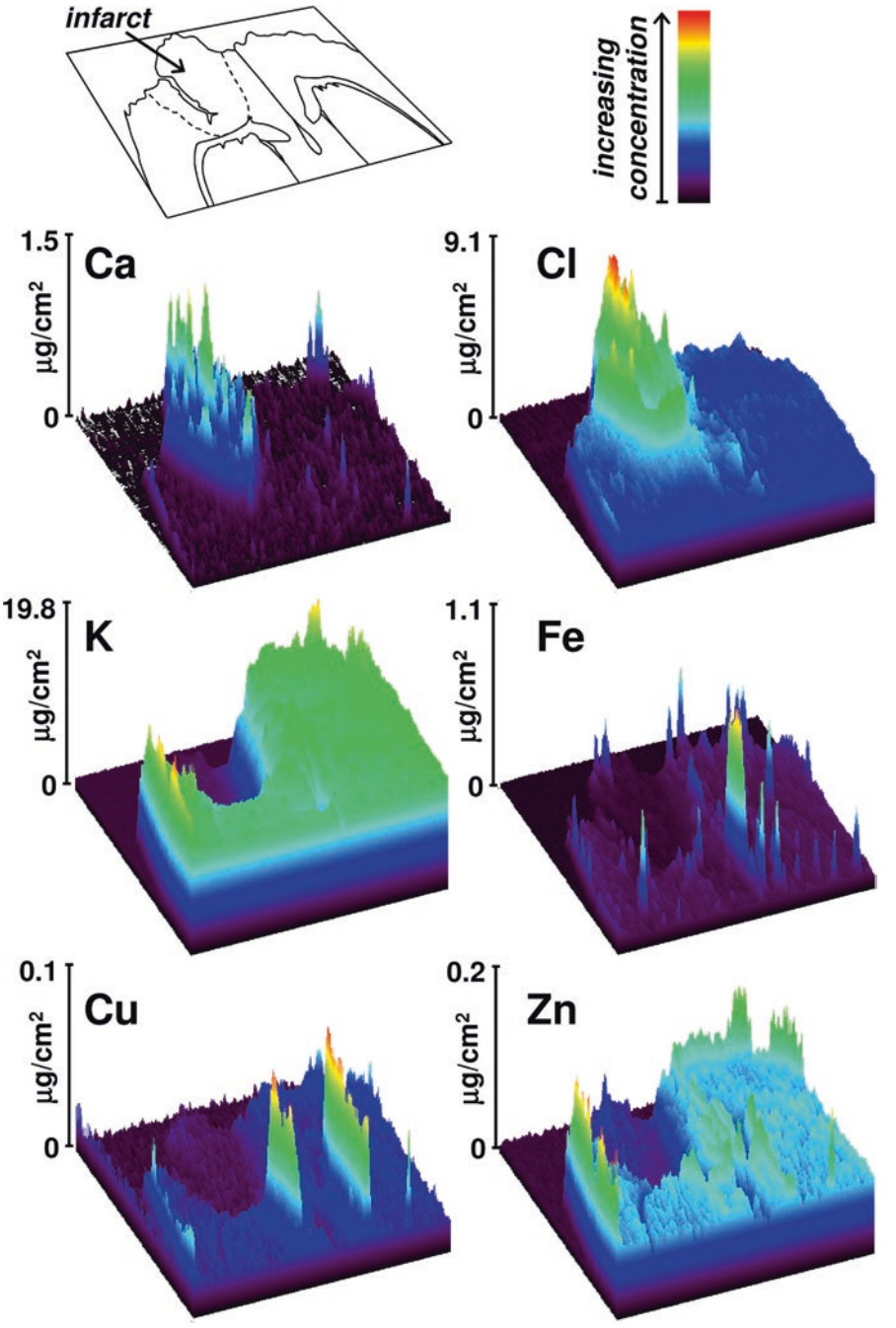

Fig. 9.5 Synchrotron XFI results for select elements rendered as 3D heat maps, demonstrating the quantitative changes that occur 24-h post- stroke in the photothrombotic model. The 2D neuroanatomical rendering is oriented with the same relative perspective as the 3D images. Normal levels for each element can be seen as the background signal within the brain section outside the stroke lesion

that exists in a reduced state (GSH) or oxidized state (GSSG). Ischemic reperfusion injury influences the generation of ROS. GSH donates either a proton or electron to the oxidant, thereby neutralizing it (Song et al. 2015).

Relative to most other organs the brain is highly enriched in trace metals, including Fe^{2+}, Cu^{2+}, and Zn^{2+}. While Zn^{2+}, by virtue of its spherically symmetric outer shell of electrons, has some similarities with the other ions discussed above, the transition metals generally possess substantially different binding properties with biomolecules and in many instances are tightly bound to host proteins under normal physiological conditions and are not typically regarded as being highly mobile and readily exchangeable. Zn^{2+} is again a notable exception, as neuronal cells maintain a pool of low molecular weight Zn^{2+}, likely complexed with glutamate and aspartate, in synaptic vesicles and is released during synaptic transmission and depolarization (Kitamura et al. 2006). Mn^{2+} is normally maintained at exceptionally low levels in tissue (Jensen and Jenson 2014); however, XFI provides sufficient sensitivity and high spatial resolution to detect Mn^{2+} traces (Robison et al. 2012). Both Fe^{2+} and Cu^{2+} are tightly regulated (Cu^{2+} exceptionally so, with estimates of less than 1 free atom of Cu^{2+} per cell) (Rae et al. 1999), and these comprise active sites in functionally-important proteins or are bound within storage proteins. Fe^{2+} and Cu^{2+} can readily partake in electron-transfer reactions, and while this property is capitalized by biological systems to catalyze reactions necessary for life, this same reactivity can promote production of deleterious reactive oxygen species (ROS) in the presence of O_2 when dysregulated. Fortunately, these storage proteins (ferritin in the case of Fe^{2+}, and MT3 in the case of Cu^{2+}), keep their metal payloads sequestered, thus preventing production of ROS. Interestingly, neurons do store a small amount of Cu^{2+} within synaptic vesicles and, like Zn^{2+}, Cu^{2+} is released during neurotransmission and depolarization (Bitanihirwe and Cunningham 2009). Within the small volume of the synaptic space the local Cu^{2+} concentration can approach 300 μM during synaptic depolarization. Zn^{2+} is found ubiquitously within the brain and is employed for a myriad of roles vital to cellular function, including proper protein folding and function, transcription, and signalling, to name a few (Bitanihirwe and Cunningham 2009). Zn^{2+} is therefore distributed relatively evenly throughout all tissues within the brain and Zn maps present the most approachable images to landmarking neuroanatomy. While Fe^{2+} and Cu^{2+} are also critical to cell survival, their roles are more specialized and are maintained at levels dictated by the tissue and cell type. In the case of Fe^{2+} the neuroanatomical distribution is largely overshadowed by the much more concentrated levels of Fe^{2+} within blood vessels. In some cases these spikes in Fe^{2+} concentration are displaced from the vessels during cryosectioning. Like Fe^{2+}, Cu^{2+} is also maintained at levels dictated by cell type; most notable are ependymal cells lining the ventricles, as well as elevated Cu^{2+} concentrations within periventricular regions, which are associated with specialized astrocytes which appear to serve a specialized role in the brain to store Cu^{2+} and likely supply neurons with essential Cu^{2+} (Pushie et al. 2011; Sullivan et al. 2017). These observations of elevated Cu^{2+} in the ependyma and periventricular regions of the rodent brain (mouse and rat models) have not been observed in similar human brain images, but may be an artifact of long post-mortem intervals, extended storage in fixative, or

natural loss of ependymal cells to gliosis, which begins in early adolescence (Del Bigio 2002). Under ischemic conditions we observe a significant decrease in Fe^{2+}, Cu^{2+}, and Zn^{2+} within the infarct (Fig. 9.5). Although there is diminished concentration of these metals following an ischemic stroke, some trace amount of metal content remains. We hypothesize that the chemical form of these trace amounts of metal ions that remain, largely represent dysregulated metal ions which are no longer bound to their normal targets and in the case of Fe^{2+} and Cu^{2+} likely to contribute to ROS-mediated damage upon reperfusion of tissue due to Fenton and Haber-Weiss reactions (Birben et al. 2012; Selim and Ratan 2004; Spasojevic et al. 2010).

We hypothesize that the reason for the decreases in elemental content shown in Fig. 9.5 are multi-factorial, and are a combination of (i) cell depolarization, (ii) reduced pH due to acidosis, which lowers the affinity of most metalloproteins for their target metals, (iii) oxidative modification and degradation of host proteins and biomolecules, and (iv) breakdown of the host cell membrane, resulting is loss of cellular contents and potential mobilization of higher molecular weight metalloproteins. These hypotheses are supported at least in part by observations that the region of the infarct is under acidic conditions following an ischemic (Tombaugh and Sapolsky 1993; Siesjö et al. 1996) episode and there is a concomitant reduction in protein and lipid content and an increase in misfolded proteins following ischemic stroke (Nowak and Jacewicz 1994).

Taken together the elemental mapping clearly reveals that the current dogma of ion relocalization from within neuronal cells into the extracellular space following ischemia-induced depolarization is insufficiently descriptive. In addition, it is clear that the influx of Cl^- and Ca^{2+} (and likely Na^+) does not involve extracellular ions alone, but significantly elevated levels beyond those that normally exist in the brain. In combination with the ischemia-induced increase in BBB permeability the extracellular contents of the ischemic lesion are consequently in exchange with blood (and vice versa), resulting in dilution of extracellular ions in the larger blood volume, while highly concentrated ions found in blood also permeate the lesion.

9.9 The Concept of the Ischemic Penumbra

The ischemic penumbra corresponds to tissue that has experienced limited blood flow and may be "potentially destined for infarction but not yet been irreversibly injured" (Fisher 1997). Cells within this region are generally regarded as being under metabolic stress, but otherwise maintain their membrane potential.

While the idea of the ischemic penumbra is conceptually straight forward, there are many definitions, related to various imaging modalities and other metrics, such as blood flow, that have been proposed to identify this region. Identification of the ischemic penumbra is critically important for quantitative assessment of potential post-stroke therapies and treatments, as previously mentioned.

Elemental maps of the region of tissue surrounding the infarct, reveals that there are consistent elemental changes that occur (Fig. 9.5). For some elements there is a

relatively abrupt border demarcating otherwise normal levels of the element from the dysregulated elemental levels found in the stroke lesion (*i.e.* P, S, Ca^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}). Even for these elements, there is a gradient that typically spans ~100 μm, which defines the border. For other elements like Cl and K this gradient may span 500 μm or more. These observations indicates that the tissue within this region is heterogeneous, with regions closer to the core of the infarct more closely mimicking the elemental levels seen in the necrotic region while regions farther away have elemental levels approaching normal levels. As the elemental levels are a measure of the metabolic state of the cells within the tissue, the elemental gradients can be collectively analyzed using soft clustering methods (Ward et al. 2013; Crawford 2015) to differentiate statistically similar regions (Fig. 9.6). The clustering shown in takes into account the elemental changes for P, S, Cl^{-}, K^{+} and Zn^{2+}, and reveals a statistically distinct band of tissue that surrounds the ischemic core. At early post-stroke time points (*i.e.* 1 h) it is possible to identify more than one narrow concentric band surrounding the central ischemic core.

An important finding in the elemental data and their statistical analysis is that the region of tissue demarcates metabolically distinct regions of tissue. It is clear from the elemental analysis that this region of tissue surrounding the infarct has altered elemental content, which would require that the membrane potential of the underlying cells be compromised. This paints a picture of a gradation of cell states, even in the region of the penumbra, ranging from tissue that is close to the normal healthy state, to tissue closely resembling the pan-necrotic tissue of the ischemic core. It is important to highlight that this band of tissue, that resembles the stroke penumbra, contains cells which are otherwise indistinguishable from those within otherwise healthy tissue. In Fig. 9.6, the penumbral zone does not show a clear region of selective neuronal death, indicating that in this model, the histological border is a demarcation between severely compromised pan-necrosis and histologically intact tissue, with no zone of selective neuronal death in between. Consideration of this indicates that in this model, any therapeutic benefit will be seen as a shrinkage of the infarct, not elimination of a rim of selective neuronal death, as the therapeutic gain.

The statistical differentiation of tissue regions, based on elemental content, provides a quantitative measure of tissue area corresponding to the penumbra and its elemental composition. Synchrotron-based XFI, combined with the application of advanced data analysis methods, is therefore a promising new tool to analyze efficacy and underlying metabolic changes in future stroke treatments and therapies in animal models. In combination with complimentary X-ray absorption spectroscopy, which can be performed at the same experimental facilities as XFI in some cases, it is possible to also obtain information on the chemical forms of any of the elements being investigated (Pushie et al. 2014, 2011; Egger et al. 2014; Puljak and Kilic 2006; Harris et al. 2008; Lins et al. 2016; Popescu et al. 2017), thereby providing a wealth of additional chemical and metabolic information. While this is still a relatively young field of research, the future of biological XFI, in combination with conventional histopathology, looks particularly bright.

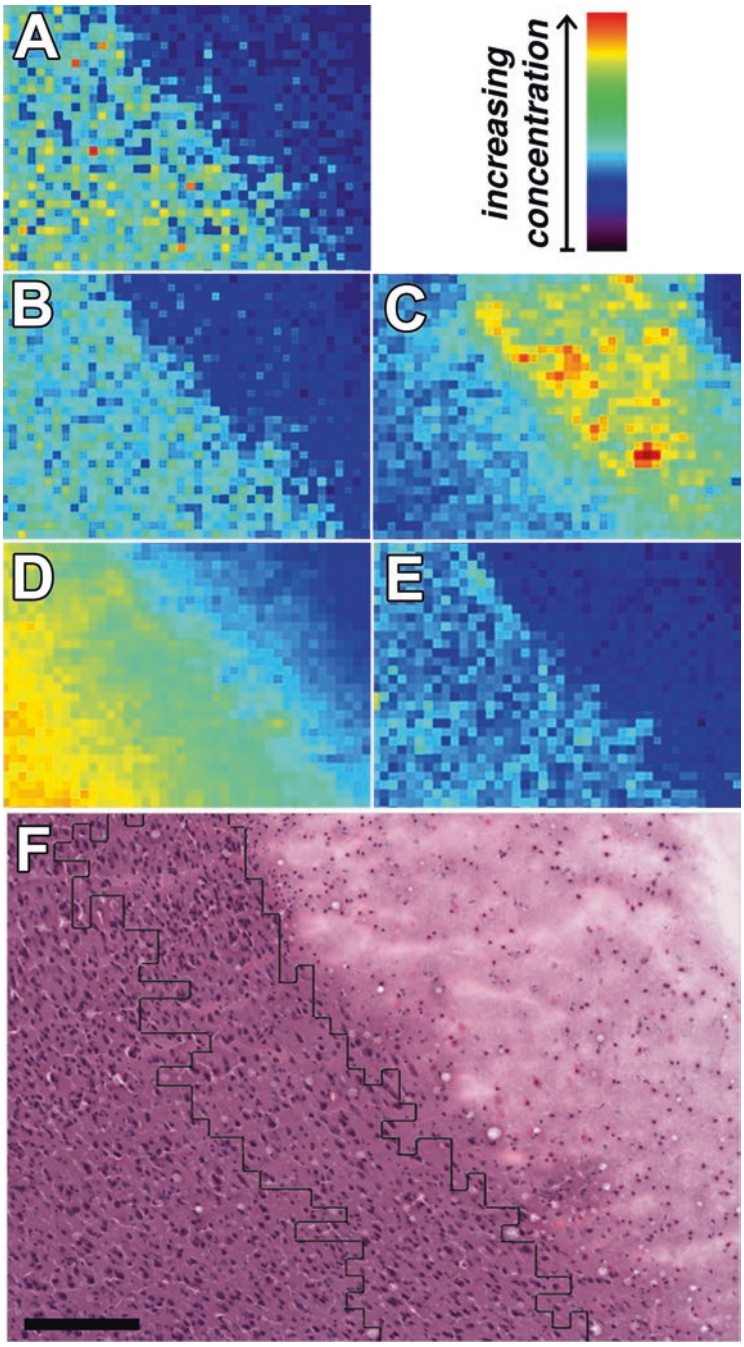

Fig. 9.6 Overlay of the border separating statistically distinct regions of tissue based on soft clustering of P (**a**), S (**b**), Cl^- (**c**), K^+ (**d**), and Zn^{2+} (**e**) distributions. These elements represent those most significantly altered across the stroke lesion and are the exemplar of elemental metabolic dysfunction between unaffected tissue and the infarct. The demarcated border overlaying the H&E image (**f**) shows (from left-to-right) unaffected tissue, the penumbra (outlined in black), and the infarct, as identified by cluster analysis. Scale bar = 200 μm; each pixel in the elemental maps = 30 μm

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Part V
Hypoglycemic Neuronal Injury

Chapter 10
Hypoglycemic Brain Damage

Roland N. Auer

Abstract Hypoglycemic brain damage is a different global brain insult from cardiac arrest encephalopathy. We here follow the path of glucose from blood to the brain interstitial space, into the cell, through glycolysis into the Krebs cycle, including the consequent new homeostasis in amino acid metabolism that gives rise to increased aspartic acid within cells. Leakage of aspartate massively floods the extracellular space to kill neurons, while continued turning of a truncated form of the Krebs cycle keeps most brain cells alive. Endogenous substrates are utilized, chiefly phospholipids and fatty acids. The duration of tolerable insult is much longer for hypoglycemia than ischemia, which also releases more glutamate than aspartate into the brain interstitium. The neuropathology in humans is not always distinguishable, but if there is dentate gyrus destruction, a very late event in global ischemia, the distinction of hypoglycemic from ischemic damage can be made. Hypoglycemic brain damage occurs in hospitals, attempted suicide and homicide.

Keywords Hypoglycemia · Clinical · Experimental · Glucose · EEG · Cortex · Hippocampus · Electron Microscopy

10.1 General Comparison of Hypoglycemic and Ischemic Brain Damage

Hypoglycemia was long considered to produce brain damage similar to ischemia, with respect to both mechanism and result. The former was based on the perceived similarity of oxygen and glucose deprivation on the brain. The latter was based on the similar neuropathology of global ischemia and hypoglycemic brain damage.

Within the hippocampus, hypoglycemic and ischemic nerve cell death, both cause neuronal death in the CA1 pyramidal cells of the hippocampus. The neocortex is also affected by both insults, and thus the structural effects of ischemia and

R. N. Auer (✉)
Department of Pathology and Laboratory Medicine, University of Saskatchewan, Saskatoon, SK, Canada

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hypoglycemia were considered identical (Brierley et al. 1971) until the 1980s (Auer and Siesjö 1988).

It is now clear that while the end result of brain damage due to global ischemia and hypoglycemia cannot always be distinguished in humans (Ng et al. 1989; Auer et al. 1989), under controlled experimental conditions in animals it can be easily shown that the distribution of hypoglycemic brain damage (Auer et al. 1984a) is distinct from both ischemic (Smith et al. 1984) and epileptic (Nevander et al. 1985) brain damage. Importantly, despite a common final death pathway and cytologic appearance of neuronal necrosis, the pathophysiology leading to neuronal killing in hypoglycemia is different in many ways from ischemic neuronal death.

10.2 A Brief History of Hypoglycemic Brain Damage

By hypoglycemic brain damage, we mean not the usual hypoglycemia that occurs say, in early metabolic syndrome or when meals are skipped. Hypoglycemic brain damage refers to the permanent brain damage that comes about when nerve cells die. It is an unusual brain insult, difficult to produce because of the requirement for massive amounts of insulin to overcome counter-regulatory hormones cortisol and adrenaline, hormones that increase blood glucose levels.

Prior to the discovery of insulin in 1921, the only endogenous source producing enough insulin to cause hypoglycemic brain damage was a pancreatic tumor, insulinoma, secreting large amounts of insulin into the bloodstream. With the discovery of insulin and its exogenous administration, overdoses came to be seen in the 1920s and 1930s due to over treatment of diabetes and medication error.

But in the late 1930s, the Viennese psychiatrist Dr. Manfred Sakel (1937) published on the use of hypoglycemia to treat schizophrenia. This was the only treatment available for an otherwise untreatable disease that had great life impact. 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.

Thus, 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 of 30 min EEG silence is on the cusp of accelerating hypoglycemic brain damage in the form of neuronal necrosis (Auer et al. 1984b, 1985a, b; Kalimo et al. 1985). 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.

Although easily considered barbaric by today's standards, such treatment for severe psychiatric illness was the only treatment 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 depolarization and coma itself, rather than the sparse neuronal necrosis that occurs after 30 min of hypoglycemic coma. Nevertheless, criticism of therapeutic

hypoglycemia mounted (Mayer-Gross 1951) and the development of phenothiazines in the 1950s sealed the fate of insulin shock therapy for schizophrenia.

10.3 Settings of Hypoglycemic Brain Damage

The three common settings remaining for hypoglycemic brain damage are over treatment of diabetes, suicide via insulin and homicide via insulin administration.

Insulin or sulfonylureas are occasionally given in error to the wrong patient (Auer et al. 1989; Kalimo and Olsson 1980) And in the context of treatment for type 1 or type 2 diabetes, overtreatment with insulin occurs (Anonymous 1993). Many hospitalized patients, especially those in renal failure, have symptomatic hypoglycemia that correlates with other causes of mortality without the hypoglycemia *per se* leading to brain damage or death (Fischer et al. 1986). A new cause of hypoglycemia in the modern era - gastric bypass surgery (Patti et al. 2005), leads to symptomatic hypoglycemia but not to brain damage. Likewise, starvation does not lead to cellular glucose deprivation, due to adaptive brain mechanisms that occur beyond the initial steps of blood-brain glucose transfer and glucose phosphorylation (Crane et al. 1985).

Besides exogenous sources of insulin, endogenous tumors, usually of the islets of Langerhans of the pancreas, account for most of the remaining cases of hypoglycemic brain damage (Auer 1986). Non-adenomatous tumors secreting insulin or congener molecules (Zhou et al. 2005; Lawson et al. 2009), and insulin antibody syndrome (Arzamendi et al. 2014) are rare causes.

With progressive hypoglycemia, the signs and symptoms go through well-defined stages as the blood sugar is progressively lowered. In any setting of hypoglycemia, these progress in an orderly fashion, and the onset of the biochemical changes leading to brain damage, outlined below, do not begin until the EEG goes flat (isoelectric EEG). The clinical counterpart to this flattening of the EEG is coma. We here outline these stages of hypoglycemia in a Table 10.1.

Table 10.1 The stages of hypoglycemia

Blood glucose (mM)[a]	5–8	2–3	≤1
Energy state (as % of normal)[b]	100	100	30
EEG	α and ß—Normal	δ—Slow waves	No waves
	8–20 Hz	1–4 Hz	Isoelectric
Clinical state	Normal	Stupor	Coma
[Aspartate] $_{ECF}$[c]	Normal	Normal	1600% of normal
[Glutamate]$_{ECF}$[c]	Normal	Normal	400% of normal

[a]To convert mM (millimolar or millimoles per liter) to mg/100 ml, multiply by 18, as the molecular weight of glucose is 180

[b]As defined by Atkinson (1968), energy charge = [ATP] + ½ [ADP]/Σ[ATP + ADP + AMP], normally 0.93

[c]Extracellular fluid

10.4 Delivery of Glucose and Cerebral Blood Flow

The pathophysiology of hypoglycemic brain damage has been detailed in several reviews (Auer and Siesjö 1993; Auer 2004; Suh et al. 2007). Briefly, low blood glucose levels are initially compensated for by an increase in cerebral blood flow (Kety et al. 1948; Abdul-Rahman et al. 1980; Gjedde et al. 1980), which can be conceptualized as the body's attempt to sustain delivery of glucose to the tissue, by increasing the flow of hypoglycemic blood.

Transport of glucose across the blood-brain barrier then occurs by a facilitated carrier, glucose transporter-1 (GLUT-1) (Mueckler et al. 1985; Lloyd et al. 2017), located on the cerebral endothelial cell (Cornford et al. 1993). Eventually, hypoglycemia leads to low interstitial brain levels of glucose, which lag behind blood glucose by roughly ~30 min in humans (Abi-Saab et al. 2002). The 1–2 mM concentration of glucose around neurons is lower than the 5–6 mM serum glucose levels, necessitating a neuronal glucose transporter termed GLUT-3 (Simpson et al. 2008); having roughly 5× the glucose transporting capacity of GLUT-1. The high rate of glucose transport directly into neurons by GLUT-3, perforce, challenges the astrocyte-neuron lactate shuttle hypothesis (Pellerin et al. 1998).

Low brain interstitial fluid levels of glucose then lead to decreased glucose phosphorylation and glycolytic flux, a low cerebral metabolic rate of glucose use (Abdul-Rahman and Siesjö 1980). Glucose is needed not only for synaptic activity, but also for development, evidenced by the microcephaly (Wang et al. 2005) seen in GLUT-1 deficiency, where blood-brain glucose transfer is impaired chronically during brain maturation.

10.5 Biochemistry of Hypoglycemic Brain Damage

Alkalosis, not acidosis, is a feature of hypoglycemia, again contrasting with ischemia. Glycolysis is decreased during hypoglycemia, giving rise to decreased lactate and pyruvate levels within the tissue. Release of any metabolic acid within tissue will cause the pH of the tissue to tend down toward the pK_a of that particular acid. The pK_a of lactic acid is 3.86, making it one of the most powerful metabolic acids reducing tissue pH. Thus, the kind of acidosis seen in *eg* ischemia, Wernicke's encephalopathy (Hakim 1984) and Leigh's disease is absent in hypoglycemic brain damage. This absence of both lactate and ischemia explains the absence of pannecrosis in hypoglycemic brain damage. The absence of a strong metabolic acid, together with the production of the strong base ammonia (Lewis et al. 1974; Agardh et al. 1978), account for the alkalosis of hypoglycemia (Pelligrino et al. 1981).

Energy failure occurs with the onset of EEG silence (Agardh et al. 1978; Feise et al. 1976), the latter being also the harbinger of neuronal necrosis (Auer et al. 1984b). The brain uses endogenous substrates almost immediately, which can be

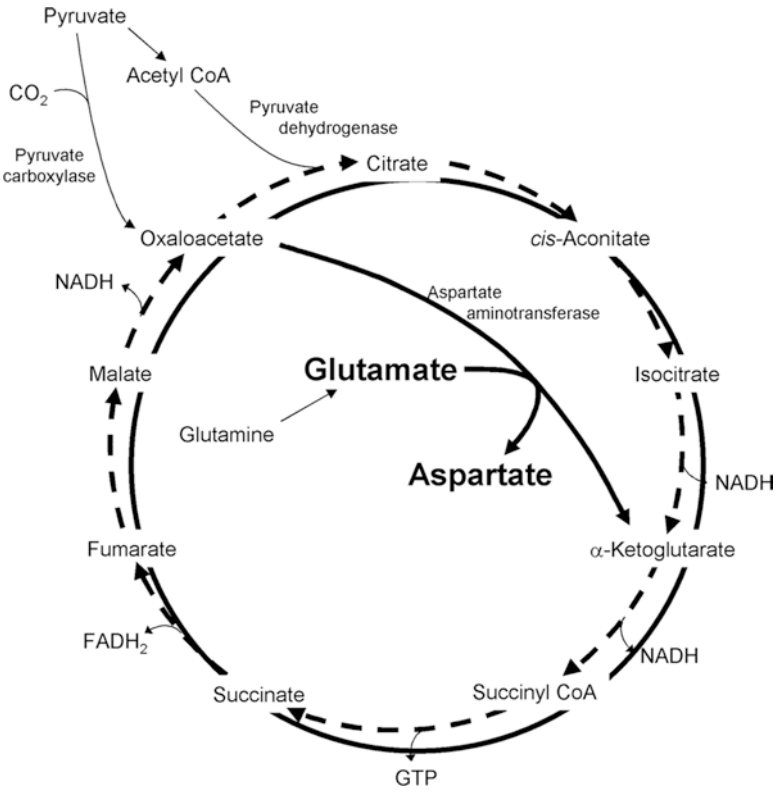

Fig. 10.1 Krebs cycle showing "short cut" from oxaloacetate to α-ketoglutarate due to transamination of glutamate to aspartate during hypoglycemia. The Krebs cycle continues to turn during hypoglycemia with EEG silence, explaining why the majority of brain cells survive

conceptualized as "throwing the furniture into the fire", literally brain fatty acids and phospholipids (Agardh et al. 1981).

Normally, pyruvate is decarboxylated to produce acetate, which enters the Krebs cycle. The release of one molecule of CO_2 from the 3-carbon pyruvate leaves the two carbon acetate, as acetyl coenzyme A to supply carbon atoms for the Krebs cycle which will be released eventually as CO_2. Pyruvate can also be carboxylated to produce the 4-carbon oxaloacetate, an anaplerotic reaction which builds up the molecules of the Krebs cycle itself (Fig. 10.1).

The decrease in glycolysis has consequences for amino acid metabolism. This occurs when reduced glycolytic flux leads to a shortage of acetyl coenzyme A entering the Krebs cycle to condense with oxaloacetate, leading to an increase in the latter. The increase in oxaloacetate drives the aspartate-glutamate transaminase reaction toward aspartate and away from glutamate, due to the law of mass action in chemistry, Le Chetalier's principle, driving the aspartate-glutamate transaminase reaction towards aspartate. When this transamination reaction is written across the

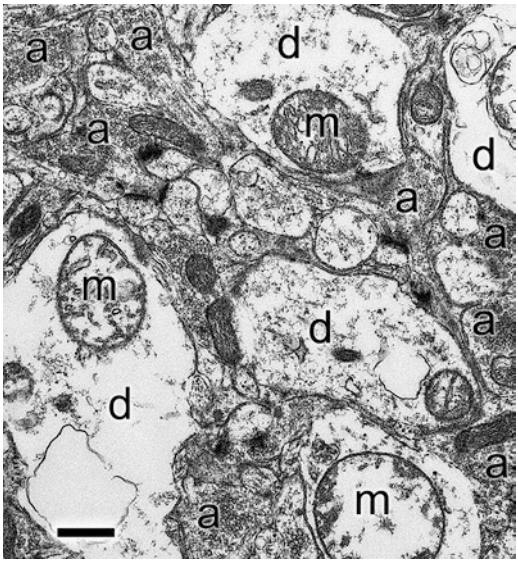

Fig. 10.2 Axon-sparing dendritic swelling characteristic of excitotoxins acting in CNS tissue. Axons (a) are not swollen and contain synaptic vesicles. Dendrites (d) however, are swollen and contain variably swollen mitochondria (m). Bar = 1 μm

Krebs cycle (Fig. 10.1), it is clear that a truncated Krebs cycle obtains in hypoglycemia, bypassing the 6-carbon intermediates citrate, *cis*-aconitate and isocitrate.

As a result, brain tissue aspartic acid levels rise in hypoglycemia (Lewis et al. 1974; Cravioto et al. 1951; Tews et al. 1965) as part of this new metabolic homeostasis. Due to the energy failure (Auer and Siesjö 1993), however, this metabolically derived aspartate leaks across the cell membranes to flood the extracellular space (Sandberg et al. 1986).

The extracellular aspartate then binds to dendritic receptors to produce characteristic axon-sparing lesions (Fig. 10.2). Essentially, the brain maintains a reduced but adequate energy state (Feise et al. 1976) by running a truncated version of the Krebs cycle in a new metabolic homeostasis (Sutherland et al. 2008).

Since the new, truncated, Krebs cycle still turns, fuel can be fed into the Krebs cycle as pyruvate (Suh et al. 2005) or lactate (Nemoto et al. 1974; Won et al. 2012), interconvertible metabolically by the enzyme lactate dehydrogenase (LDH), or as acetate (Urion et al. 1979). However, lactate can only replace one fourth of substrate use during hypoglycemia (Nemoto et al. 1974), and once glucose is given, changes in energy charge (Auer and Siesjö 1993), amino acids and ammonia are rapidly reversed (Agardh et al. 1978).

Thus, once the electroencephalogram goes flat (Auer et al. 1984b), the series of biochemical changes above allows a new homeostasis to occur. Energy failure is incomplete in hypoglycemia, at about 30% of normal (Auer and Siesjö 1993) due to the use of alternative substrates to generate ATP. These include fatty acids and proteins, once carbohydrate stores are exhausted (Agardh et al. 1981). A trickle of glucose supplements these neurochemical measures to maintain the brain's energy state, since glucose in the blood is never absolutely zero. Blood–brain glucose transport via GLUT-1 is maximized at these low blood glucose levels.

10.6 Neuropathology of Hypoglycemic Brain Damage

When over-treatment of diabetes patients with insulin began giving rise to coma and death, autopsies were performed in an attempt to elucidate whether brain damage had occurred (Auer 1986). The question at the time, in the 1920s and 1930s, was whether hypoglycemia had damaged the brain, or whether insulin itself caused brain damage. The therapeutic use of insulin in psychiatry indirectly shed some light on this problem. It was known that 30 min of insulin coma was reversible, whereas 1 h of coma was irreversible and the patient could never again be reawakened. This early clinical observation on the duration of coma has an electrical counterpart in the duration of a flat electroencephalogram, or isoelectric EEG ("flat EEG").

Without a flat EEG, no neuropathology is seen in hypoglycemia, *ie* no cell death, irrespective of the blood glucose levels. Experimentally, it can be shown that the blood glucose levels at which the EEG becomes flat vary by more than one order of magnitude, from 0.12 mM to 1.36 mM (2.16 mg/dL to 24.5 mg/dL) (Auer et al. 1984b).

Once the EEG goes flat, essentially a timer starts. That is to say, the number of dead neurons increases from a zero time point, marked by the onset of a flat EEG. Over time, the number of dead neurons increases in a monotone fashion as the duration of a flat EEG increases. In both animal experiments and in the history of the human therapeutic use of insulin coma in psychiatry, 30 min is a duration of insult that is easily recoverable. By 1 h, however, the number of dead neurons has increased to the point that coma becomes irreversible, explaining the clinical disasters in the historical use of therapeutic insulin-induced hypoglycemia (Auer 1986).

We note that unlike ischemia, the selective neuronal necrosis of hypoglycemia does not augment into infarction. Instead, the number of dead neurons increases, without a transformation of selective neuronal death into an infarct. This is because neither is blood flow occluded nor lactate produced (see above). Pan-necrosis results from either vascular occlusion or lactic acid production at a rate above that which can be cleared from the tissue.

Because 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. 10.3). Cysts are not seen.

The location of the gross atrophy is dictated by the brain areas that are selectively vulnerable in hypoglycemic brain damage. These constitute the cerebral cortex, the striatum, the hippocampus and the thalamus. The metabolic release of aspartate (Sandberg et al. 1986) outlined above, underlies the selective neuronal death in hypoglycemia. Glucose transport from blood to brain is better in the brainstem and cerebellum (LaManna and Harik 1985) leading to a relative sparing of the brainstem

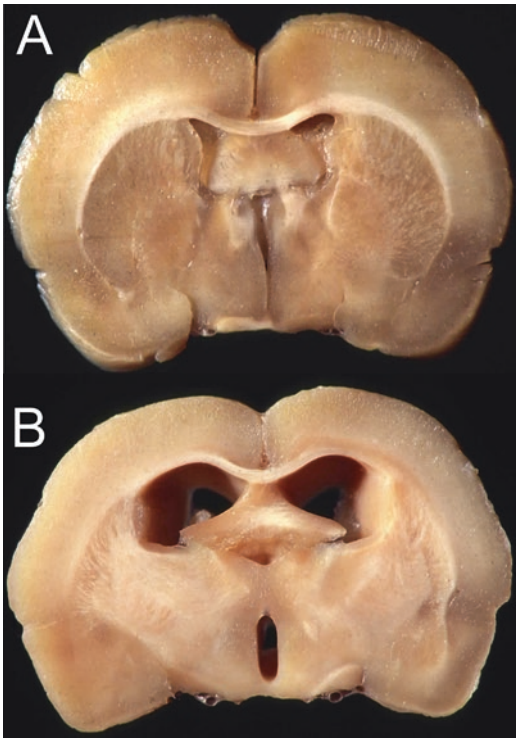

Fig. 10.3 Brain of a normal rat (**a**) compared to a rat with 60 min of flat EEG 1 week previously (**b**), showing hypoglycemia-induced atrophy due to widespread death of neurons and axons. Notably, no cysts are present despite the severe, 1 h insult of a flat EEG

and cerebellum from a metabolic insult during hypoglycemia. 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 a pure (without ischemia) primary insult of hypoglycemic brain damage to the nervous system.

Within the hippocampus, the CA1 pyramidal cells are vulnerable (Auer et al. 1985b), in common with ischemia. However, the signature lesion of dentate gyrus necrosis (Fig. 10.4) 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 (Auer et al. 1989). In rats, the lesion of the dentate is seen early, already after 10–20 min of coma, a comparably mild insult. The dentate gyrus lesions of rats and humans are compared (Fig. 10.4), both showing dentate necrosis (Fig. 10.5) as a feature of hypoglycemia. The CA1 necrosis is a feature in common with cerebral ischemia.

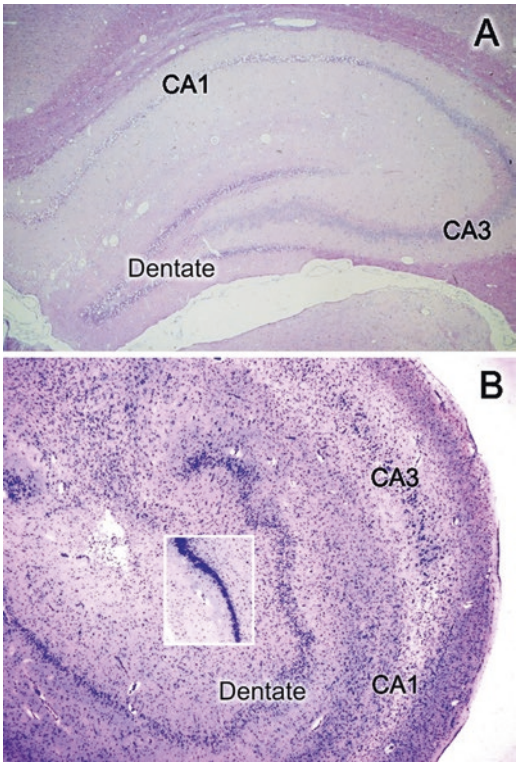

Fig. 10.4 Hippocampus in hypoglycemia. The rat (**a**) shows conspicuous necrosis of the dentate gyrus, as does the human (**b**) hippocampus. CA1 neuronal loss is common to both ischemia and hypoglycemia. The inset shows a stretch of normal dentate gyrus, a dark blue band of cells contrasting with the adjacent, depleted granule cells of the dentate in the surrounding hippocampus damaged by hypoglycemia

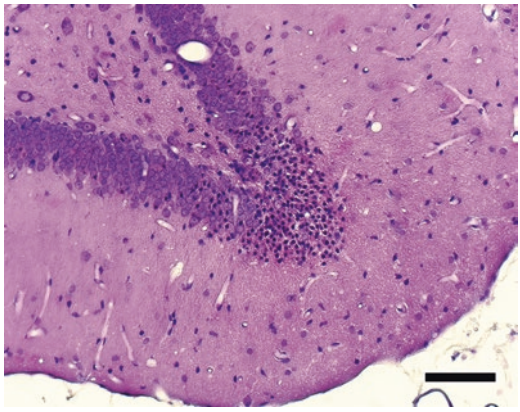

Fig. 10.5 Light microscopic detail of neuronal death in hypoglycemia. The cells of the crest dentate gyrus in the geographic area in the center of the photo are pyknotic, or shrunken. Both nuclei and cytoplasm are small compared to the normal cells continuous to the dead neurons in the band of neurons forming a "V". Neuronal cytoplasm is acidophilic, a sign of cell death. Bar = 100 μm

10.7 Electron Microscopy of Hypoglycemic Brain Damage

The discovery by John Olney of the phenomenon of excitotoxicity in the late 1960s and early 1970s was major (Olney 1969a, b, 1971). 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 examining the biochemistry of hypoglycemic brain damage outlined above.

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, when hypoglycemic neuronal death was blocked by an NMDA antagonist of excitatory amino acid receptors (Wieloch 1985) and excitotoxic lesions were seen in the dentate gyrus (Auer et al. 1985c). Historically remarkable is the prescient observation already in 1938 by Arthur Weil, who observed dentate gyrus in a geographic distribution in rabbits, presciently postulating at that time that a molecule in the extracellular space, perhaps a toxin, could kill nerve cells in hypoglycemic brain damage (Weil et al. 1938).

Electron microscopy of hypoglycemic neuronal death shows excitotoxic neuronal necrosis (Fig. 10.2). The lesion under the electron microscope is quite specific: dendritic swelling, sparing intermediate axons, was known to occur in glutamate neurotoxicity. The appearance is 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 dendritic neuronal death can be thought of as a brush fire in the dendritic tree, beginning where synaptic receptors are located and spreading towards the cell body or perikaryon. This is not characteristic of apoptotic cell death. Once the process of membrane leakage and breakage spreads to the perikaryon (cell body) of the neuron, cell death ensues.

The perikaryon of the neuron in hypoglycemic neuronal death shows features of necrosis, with a characteristic coarse chromatin having a tigroid appearance, mitochondrial flocculent densities (Trump et al. 1997) and cell membrane breaks (Fig. 10.6). Following these changes, cell death is fairly rapid, occurring in a few hours in both cortex (Auer et al. 1985a) and hippocampus (Auer et al. 1985b), again contrasting with the delayed neuronal death of ischemia (Kirino 1982).

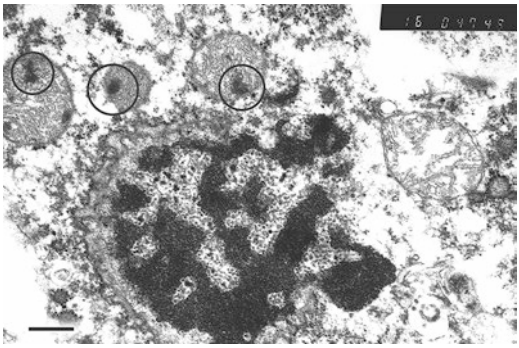

Fig. 10.6 Electron microscopy of neuronal cell death in hypoglycemia. Mitochondrial flocculent densities are seen (circled), representing denatured cytochrome proteins. The mitochondrion on the right is still swollen, but without flocculent densities. The nuclear chromatin has a coarse, tigroid appearance and the nuclear membrane is crenulated. Bar = 1 μm

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Part VI
Seizure-Induced Neuronal Death

Chapter 11
Activation of Caspase-Independent Programmed Pathways in Seizure-Induced Neuronal Necrosis

Denson G. Fujikawa

Abstract Prolonged epileptic seizures, or status epilepticus (SE), produce morphologically necrotic neurons in many brain regions. In contrast to prior notions of cellular necrosis being a passive process of cell swelling and lysis, SE-induced necrotic neurons show internucleosomal DNA cleavage (DNA laddering), a programmed process requiring endonuclease activation. The underlying mechanisms are triggered by excessive activation of NMDA receptors by glutamate, which allows calcium influx through their receptor-operated cation channels (excitotoxicity). Calcium-dependent enzymes are activated, such as calpain I and neuronal nitric oxide synthase (nNOS), the latter of which, through production of reactive oxygen species (ROS), activates poly(ADP-ribose) polymerase-1 (PARP-1). Calpain I and PARP-1 activation in turn cause translocation of death-promoting mitochondrial proteins and lysosomal enzymes that degrade cytoplasmic proteins and nuclear chromatin, creating irreversible cellular damage. Another programmed necrotic cell death pathway, necroptosis, has been described in cell culture following caspase inhibition, and activation of this pathway has been described following cerebral ischemia and traumatic brain injury *in vivo*. However, whether this pathway interacts with the excitotoxic pathway, while likely, and the specific mechanisms by which this occurs, are at present unknown. Based upon our knowledge of excitotoxic mechanisms, neuroprotective strategies can be devised that could ameliorate neuronal necrosis from refractory SE in humans.

Keywords Calpain I · Excitotoxicity · Glutamate · Necroptosis · Neuronal nitric oxide synthase (nNOS) · Poly(ADP-ribose) polymerase-1 (PARP-1) · Programmed pathways · Status epilepticus

D. G. Fujikawa (✉)
Department of Neurology, VA Greater Los Angeles Healthcare System, North Hills, CA, USA

Department of Neurology and Brain Research Institute, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA, USA
e-mail: dfujikaw@ucla.edu

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11.1 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 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, 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.

11.1.1 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 (Bruhn et al. 1992; Lallement et al. 1991; Lehmann et al. 1985; Millan et al. 1993; Smolders et al. 1997; Tanaka et al. 1996; Wade et al. 1987). At least part of the reason for finding a lack of 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 (Clifford et al. 1990; Fariello et al. 1989; Fujikawa 1995; Fujikawa et al. 1994).

11.2 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 non-ultrastructural 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 by 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 2000, 2002, 2005; Fujikawa et al. 1999, 2000, 2002, 2007).

Contrary to *in vitro* cell culture experiments, in which cells exposed to an overwhelming stimulus show cell swelling and then lyse, presumably thereafter to disappear, it was shown in the 1970s and 1980s (Evans et al. 1984; Auer et al. 1985a, b; Brown 1977; Griffiths et al. 1983, 1984), 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, all of which are markers of cellular necrosis (Fujikawa et al. 1999, 2000, 2002; Colbourne et al. 1999). 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*.

11.2.1 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 (Evans et al. 1984; Griffiths et al. 1983, 1984). The shrunken, electron-dense neurons showed nuclear pyknosis and cytoplasmic vacuoles, corresponding to what we now know is neuronal necrosis (Fujikawa et al. 1999, 2000, 2002; Colbourne et al. 1999) (Fig. 11.1). In addition, seizure-induced necrotic neurons show internucleosomal DNA cleavage, or DNA laddering, a programmed process requiring endonuclease activation (Fujikawa et al. 1999, 2000, 2002) (Fig. 11.1). 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.

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

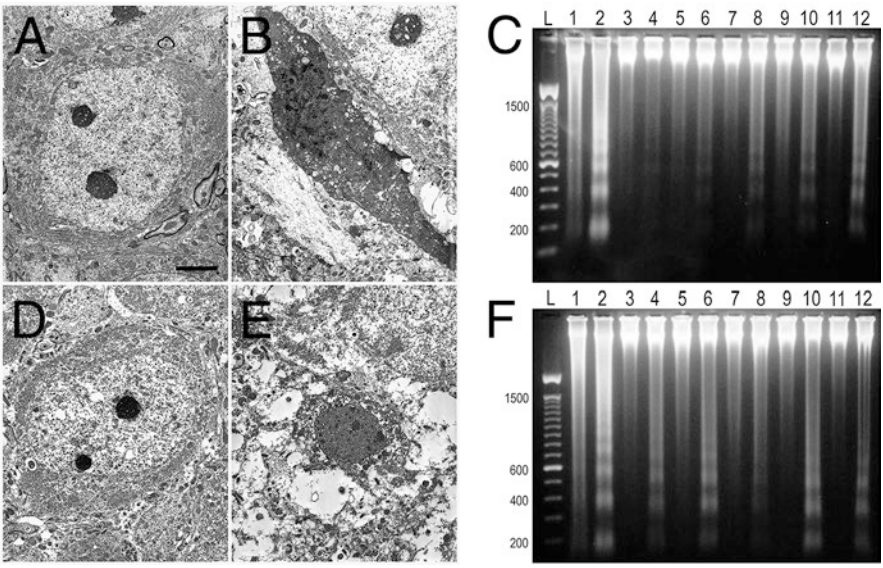

Fig. 11.1 Seizure-induced necrotic neurons show internucleosomal DNA cleavage (DNA laddering). **a** and **d** are electron photomicrographs of ventral hippocampal CA1 neurons in control rats given lithium chloride and normal saline instead of pilocarpine 24 (**a**) and 72 h (**d**) later. **b** and **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 h and 72 h time points. From Fujikawa (2006), with permission from MIT Press

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); Fig. 11.2b, c show microglia, Fig. 11.3b shows a microglial cell and Fig. 11.3c 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 (Figs. 2, 3, 5, and 6 in ref. Whalen et al. 2008) and ultrastructure (Fig. 9B and 9C in ref. Whalen et al. 2008).

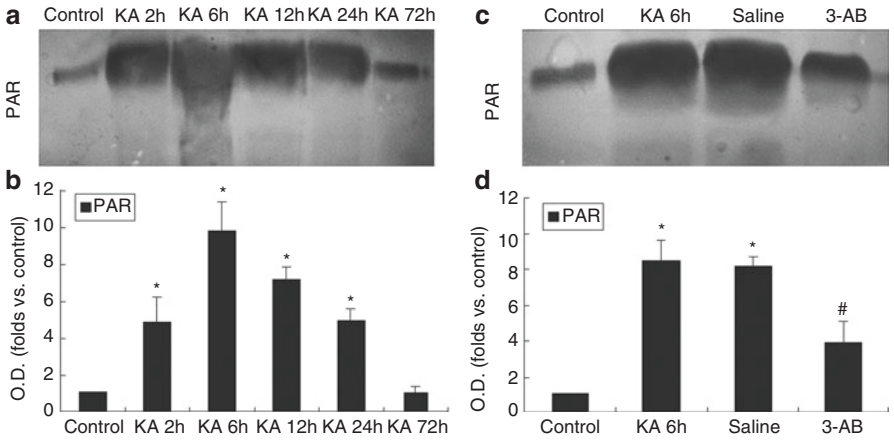

Fig. 11.2 Effects of PARP-1 inhibition on poly(ADP-ribose) (PAR) polymer formation in the rat hippocampus following 60-min SE. (**a**) Shows the time course of PAR formation in rat hippocampus from 2 to 72 h after 60-min SE, and (**c**) shows the effect of 3-aminobenzamide (3-AB), a PARP-1 inhibitor, 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

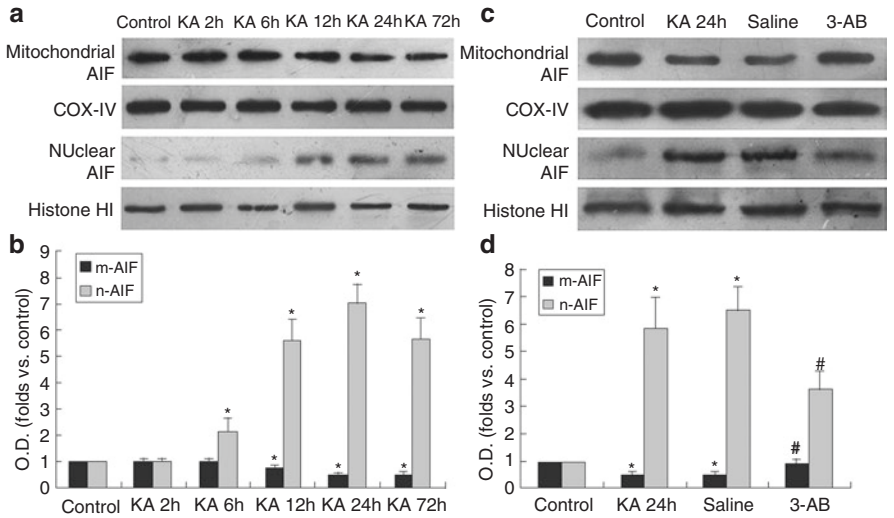

Fig. 11.3 Effects of the PARP-1 inhibitor 3-aminobenzamide (3-AB) on nuclear translocation of AIF following 60-min SE. (**a**) Shows the time course of AIF translocation to hippocampal nuclei from 2 to 2 h after SE, and (**c**) shows the effect of 3-AB, given i.p. 15 min before i.c.v. kainic acid injection, 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

11.3 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 that acidophilic neurons by H&E stain, the light-microscopic equivalent of ultrastructural neuronal necrosis (Fujikawa et al. 1999, 2000, 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.

11.4 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 (Bizat et al. 2005; Knoblach et al. 2004; Rozman-Pungerčar et al. 2003). 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; Araújo et al. 2008; Narkilahti et al. 2003; Takano et al. 2005).

11.5 Caspase-Independent Programmed Mechanisms in Necrotic Cell Death

Chapters by Rieckher and Tavernarakis, Krantic and Susin, Kang et al. and Liu et al. in the first edition of this book (Fujikawa 2010) 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 (Rieckher and Tavernarakis, Chap. 2, ref. Fujikawa 2010). 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 Chap. 10, by Tonchev and Yamashima, ref. Fujikawa 2010), 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 Chap. 9, Liu et al. in ref. Fujikawa 2010).

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 Chap. 3, by Krantic and Susin in ref. Fujikawa 2010).

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 (Andrabi et al. 2006; Yu et al. 2002, 2006) (also see Chap. 5, by Kang et al. in ref. Fujikawa 2010). 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, 2000, 2002). Olney's group did 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 called "excitotoxic neuronal death." They are one and the same.

11.5.1 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.

11.5.1.1 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. Araújo et al. (2008) showed that following kainic acid-induced seizures, there was Fluoro-Jade evidence of neuronal necrosis in hippocampal CA1 and CA3, calpain I activation and neuroprotection in CA1 with calpain I inhibition (Fig. 11.4). In the studies by Wang and colleagues (Wang et al. 2007, 2008), there was associated translocation of AIF to nuclei, with reduction of AIF translocation with PARP-1 and calpain I inhibitors (Figs. 11.2, 11.3 and 11.5). However, in the studies by Wang and colleagues 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 in almost exclusively in astrocytes, 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

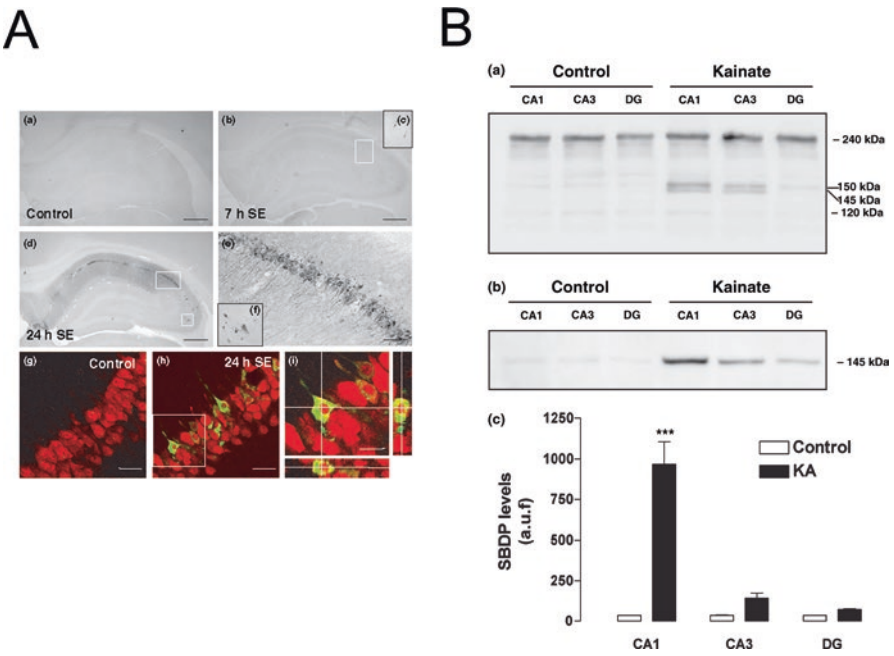

Fig. 11.4 Calpain activation 24 h after the onset of kainic acid-induced SE, assessed by expression of the calpain I-cleaved fragment of αII-spectrin (spectrin breakdown product, or SBDP). In **a**, SBDP expression occurred in dorsal hippocampal CA1 neurons 24 h after seizures began, but not in controls and only minimally 7 h after seizures began (*a*) through (*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

(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 d 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. Other

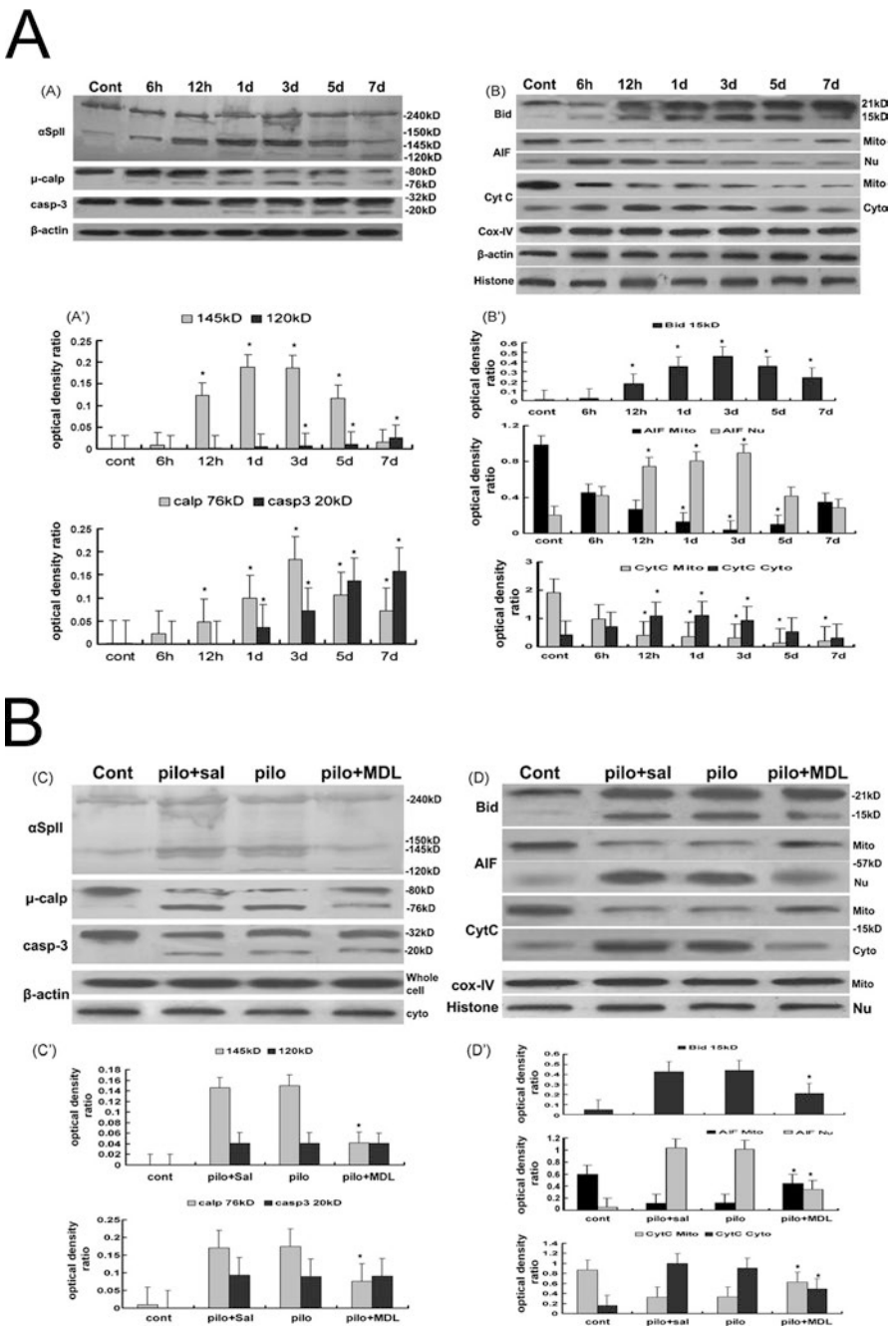

Fig. 11.5 In **a** (*A*) and (*B*) show western blots of αII-spectrin (αSpII), calpain I (μ-calp), caspase-3 (casp-3), Bid, AIF and cytochrome *c* (Cyt C) from 6 h to 7 d after 2-h lithium-pilocarpine-induced SE (LPCSE). (*A′*) Shows quantitation of (*A*) and *B′* shows quantification of (*B*). The calpain-I

studies have also noted a lack of caspase-3 activation following generalized SE (Fujikawa et al. 2002; Araújo et al. 2008; Narkilahti et al. 2003), and neither caspase-9 or 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 all 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 and Fujikawa 2010) (Figs. 11.6 and 11.7). We used our 0–3 grading scale, applying it to the degree of nuclear translocation, in which 0 = none, 0.5 = slight (<10%), 1.0 = mild (10–25%), 1.5 = mild-to-moderate (26–45%), 2.0 = moderate (46–54%), 2.5 = moderate-to-severe (55–75%), and 3.0 = severe (>75%) (Zhao and Fujikawa 2010). In the LPCSE seizure model, maximal neuronal necrosis occurs 24 h following 3-h SE (Fujikawa et al. 1999, 2000, 2002, 2007; Fujikawa 1996). Cytochrome *c* (*in vivo)* has not heretofore been shown to translocate to cellular nuclei, 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). Narkilathi et al. (2003) showed that in generalized SE that activated caspase-3 appeared primarily in astrocytes in hippocampus 2 and 7 d after SE. The increasing amount of activated caspase-3 from 1 to 7 d 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

Fig. 11.5 (continued) cleaved 145 kDa αSpII fragment increased significantly 12 h and 1, 3 and 5 d after SE, cleaved Bid (15 kDa) was significantly elevated 12 h and 1–7 d after SE, AIF translocated from mitochondria to nuclei 12 h and 1 and 3 d after SE, and Cyt C translocated from mitochondria to cytosol 12 h and 1 and 3 d after SE. Cleaved (activated) calpain I (76 kDa) appeared 12 h and 1–7 d after SE, with maximal expression 3 d after SE. Cleaved (activated) caspase-3 (20 kDa) appeared 1–7 d after SE, with maximal expression 7 d after SE. Narkilahti and colleagues (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

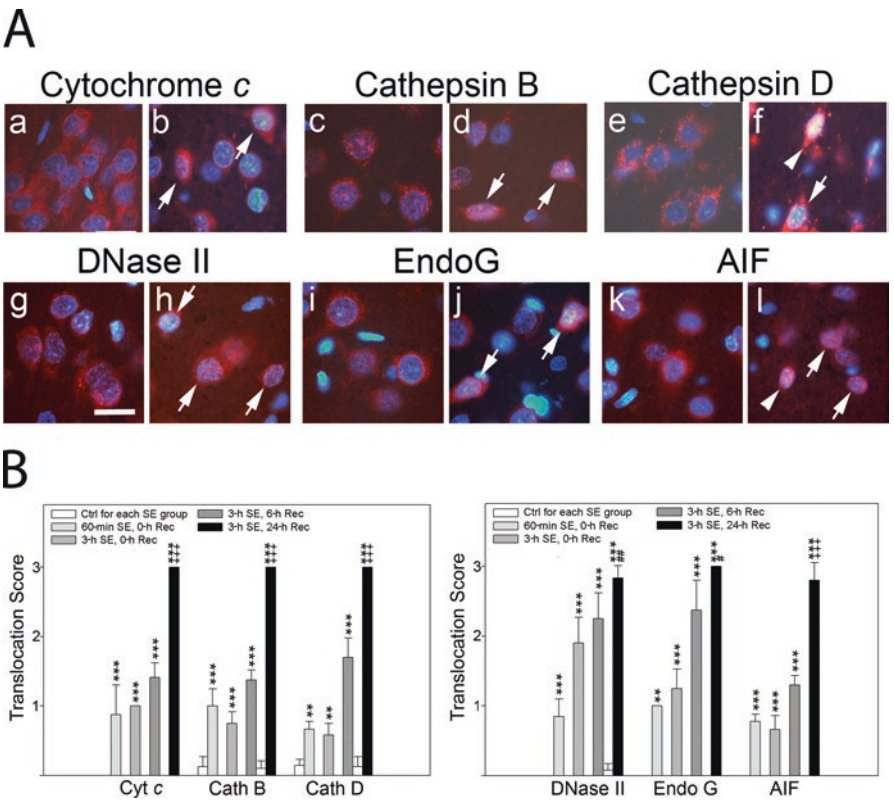

Fig. 11.6 Nuclear translocation of mitochondrial cytochrome *c*, endonuclease G and apoptosis-inducing factor (AIF) 60 min after the onset of LPCSE increases up to 24 h after 3-h SE. (**a**) Shows merged immunofluorescent images of control neurons (*a*, *c*, *e*, *g*, *i* and *k*) and neurons 60 min after the onset of SE in piriform cortex (*b*, *d*, *f*, *h*, *j* and *l*). Nuclei stained with DAPI are blue and cytoplasm staining the six proteins is red in control neurons. Following 60 min of SE, many neurons have purple to purplish-white nuclei, indicating nuclear translocation of the six proteins. Arrows point to nuclei showing translocation and arrowheads point to shrunken nuclei showing translocation. The scale bar is 10 μm. (**b**) Shows nuclear translocation scores for the six proteins in controls, 60-min SE, 3-h SE and 3-h SE with 6 h and 24 h recovery periods. See the text for a detailed description of the scoring method; in general the larger the number the greater the degree of nuclear translocation. ***$p < 0.001$ and **$p < 0.01$ compared to controls, +++$p < 0.001$ in the 24 h recovery group compared to the three other groups, and ##$p < 0.01$ and #$p < 0.05$ in the 24 h recovery group compared to the 60-min and 3-h SE groups. Abbreviations in **a** and **b** are EndoG (endonuclease G), AIF (apoptosis-inducing factor) and in B are cyt *c* (cytochrome *c*), cath B (cathepsin B) and cath D (cathepsin D). (**a**) Shows reformatted images from ref. Zhao and Fujikawa (2010) and in **b** both bar graphs are from ref. Zhao and Fujikawa (2010), which is in the public domain

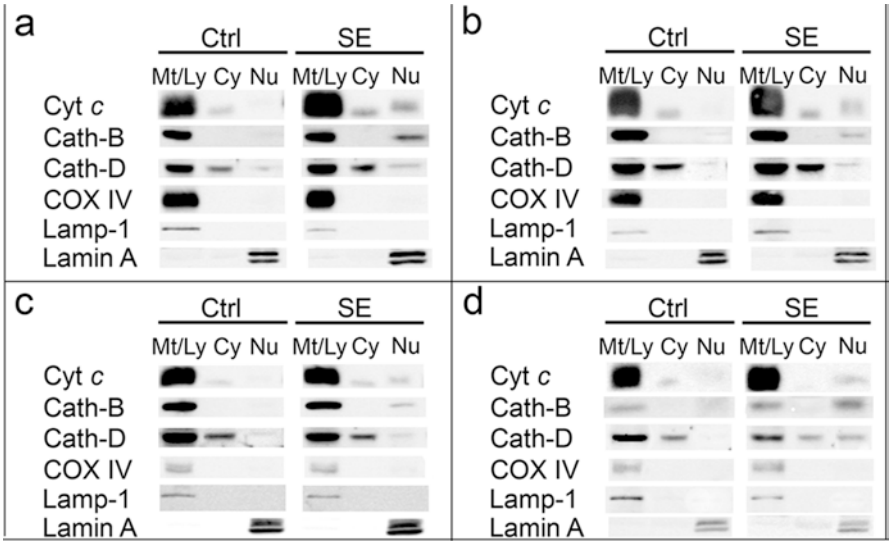

Fig. 11.7 Subcellular fractionation and western blots corroborate the nuclear translocation of cytochrome *c* (Cyt *c*), cathepsin B (Cath-B) and cathepsin D (Cath-D) after 60 min SE (**a**), 3 h SE (**b**), 3-h SE with a 6-h recovery period (**c**) and 3-h SE with a 24-h recovery period (**d**). COX IV is a mitochondrial marker (Mt/Ly), Lamp1 is a lysosomal marker (Mt/Ly) and Lamin A is a nuclear marker. Cath-D is present in the cytosol (Cy) of control and SE lanes at all four time points. Abbreviations are Ctrl (control), Mt/Ly (mitochondrial/lysosomal), Cy (cytosol) and Nu (nuclear). From ref. Zhao and Fujikawa (2010), which is in the public domain

generalized SE (Zhao and Fujikawa 2010). Nuclear translocation of cyt *c* points to a heretofore unexplored pathological mechanism for this mitochondrial respiratory chain protein.

11.5.1.2 Lysosomal Cathepsins, DNase II, Calpain I and Reactive Oxygen Species (ROS)

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 (Tsukada et al. 2001; Yamashima et al. 1996, 1998). 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 all 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 and Fujikawa 2010) (Figs. 11.6 and 11.7). We used the same grading scale and nuclear translocation scores for cathepsins B and D and DNase II as described above for AIF, cyt *c* and endoG (Zhao and Fujikawa 2010).

As mentioned previously, maximal neuronal necrosis occurs 24 h following 3-h LPCSE (Fujikawa et al. 1999, 2000, 2002, 2007; Fujikawa 1996). 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.

11.5.1.3 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 (Sakhi et al. 1994; Schreiber et al. 1993) (also see Chap. 15 by Tan and Schreiber in ref. Fujikawa 2010). p53 has also been shown to regulate the Bax-dependent, mitochondrial caspase cell death pathway (Chipuk et al. 2004). Since we have shown that KASE produces morphologically necrotic neurons, these findings suggest another programmed pathway activated by programmed necrosis.

11.5.1.4 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; Codogno and Meijer 2005; Shintani and Klionsky 2004; 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.

11.5.1.5 The Role of Necroptosis in Acute Neuronal Injury

Following caspase inhibition with the broad-spectrum caspase inhibitor zVAD.fmk in cell culture, cells still died, but with a necrotic instead of apoptotic morphology (Holler et al. 2000; Vercammen et al. 1998). This form of programmed necrosis was called "necroptosis" (a combination of "necrosis" and "apoptosis") (Degterev et al. 2005). In the same article, following transient focal cerebral ischemia, administration of the receptor interacting protein (RIP) inhibitor 7-Cl-necrostatin-1 (7-Cl-nec-1) reduced the volume of the focal infarct (Degterev et al. 2005). Necrostatin-1 was subsequently found to inhibit RIP-1 (Degterev et al. 2008), and the necroptotic pathway was found to involve phosphorylation of RIP3 by RIP1 (Zhang et al. 2009) and mixed lineage kinase domain-like protein (MLKL) by RIP3 (Sun et al. 2012).

Subsequent studies of transient global cerebral ischemia have shown RIP3 but not RIP1 was activated and translocated to neuronal nuclei (Yin et al. 2015), RIP3 phosphorylation (activation) and interaction with RIP1 (Miao et al. 2015) and nuclear translocation of RIP3 and AIF (Xu et al. 2016), resulting in necroptotic neuronal necrosis. In addition, in non-neuronal cell culture, nuclear translocation of RIP1, RIP3 and MLKL occurred prior to MLKL translocation to plasma membranes, and Nec-1 administration reduced the nuclear translocation and necroptosis (Yoon et al. 2016). This is of interest to us because of our finding of nuclear translocation of mitochondrial cyt-*c*, AIF and endoG and lysosomal cath-B, cath-D and DNase II within the first 60 min of SE (Zhao and Fujikawa 2010).

We have preliminary results showing nuclear translocation of RIP1, RIP3 and MLKL in the piriform cortex within the first 60 min of LPCSE and their persistence in necrotic nuclei 24 h after 3-h SE. We also have preliminary data showing that i.c.v. administration of 7-Cl-O-nec-1 results in a substantial reduction of acidophilic (necrotic) neurons in the dorsal hippocampal hilus 6 h after 3-h LPCSE. This suggests that both necroptotic and excitotoxic pathways are activated by LPCSE and points the way to further studies to determine the interaction between them and how the necroptotic pathway is activated by LPCSE.

11.6 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 post-synaptic 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 20 years ago, based upon their remarkable neuroprotective effects (Fujikawa 1995; Fujikawa et al. 1994). 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 (Clifford et al. 1990; Fariello et al. 1989; Fujikawa 1995; Fujikawa et al. 1994). 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 (Bleck 2005; Borris et al. 2000). Borris and colleagues (2000) showed that given after 60 min of spontaneous SE from electrical hippocampal stimulation in the rat that ketamine abolished seizure discharges, but 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 that SE continues.

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Concluding Remarks

Denson G. Fujikawa

The morphology of acutely injured neurons in the adult brain *in vivo* is necrotic, not apoptotic (Fujikawa 2000, 2002; Fujikawa et al. 1999, 2000; Auer et al. 1985a, b; Colbourne et al. 1999). Caspase-independent programmed mechanisms are important in the adult brain *in vivo* (Fujikawa 2010): For example, in status epilepticus (Fujikawa et al. 2002, 2007; Narkilahti et al. 2003; Gao and Geng 2013; Wang et al. 2008) and cerebral ischemia (Vosler et al. 2009; Hu et al. 2000) (see also Chap. 4 in the First Edition of this book and Chap. 8 in this book by Sun and colleagues).

Although this edition of *Acute Neuronal Injury* continues to emphasize the role of excitotoxic mechanisms in producing neuronal necrosis, new topics have been introduced. For example, the topic of synaptic vs. extrasynaptic NMDA receptors and their functions is addressed by Dr. Baudy and colleagues in Chap. 2. Also, in recent years a programmed necrotic cell death pathway other than excitotoxicity, called "necroptosis" (from "necrosis" and "apoptosis"), has been described (Degterev et al. 2005). This was discovered in non-neuronal cells *in vitro* when the broad-spectrum caspase inhibitor z-VAD.fmk to inhibit caspase activation; the cells still died, but with necrotic morphology (Degterev et al. 2005). However, investigators went on to investigate cerebral ischemia *in vivo*, and found that enzymes in the necroptotic pathway, receptor-interaction protein-1 (RIP-1), receptor-interacting protein-3 (RIP3) and mixed lineage kinase domain-like protein (MLKL), were activated, implicating the necroptotic pathway in acute neuronal injury *in vivo* (Yin et al. 2015; Miao et al. 2015; Xu et al. 2016; Liu et al. 2016; Vieira et al. 2014). Carvalho and Vieira address necroptosis in cerebral ischemia in Chap. 9, and Tao includes necroptotic mechanisms in in traumatic brain injury (TBI) in Chap. 5.

At present there is little information about the importance of the necroptotic pathway in acute neuronal injury. The first study of necroptosis in neuronal cells *in vitro* and in transient middle cerebral artery occlusion (MCAO) *in vivo* demonstrated that necrostatin-1 (Nec-1), given intraventricularly, reduced the size of a focal infarct produced by transient middle cerebral artery occlusion (MCAO) (Degterev et al. 2005). The same authors found later that Nec-1 inhibits RIP1 activation (Degterev et al. 2008). In another study, 20-min transient global cerebral ischemia

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(TGCI) produced RIP3 translocation to hippocampal CA1 pyramidal cell nuclei and Nec-1 protected CA1 neurons and reduced RIP3 nuclear translocation (Yin et al. 2015). They also showed that Nec-1 inhibited cathepsin B (cath-B) release from lysosomes, which is caused by activated calpain 1, which is one of the first studies to show an interaction between the necroptotic and excitotoxic pathways.

In 15-min TGCI, RIP3 S-nitrosylation and RIP3 phosphorylation interacting with RIP1 was associated with neuronal death (Miao et al. 2015). These investigators also found that the non-competitive NMDA-receptor antagonist MK-801 and 7-nitroindazole (7-NI), a neuronal nitric oxide synthase (nNOS) inhibitor, reduced RIP3 S-nitrosylation and neuronal damage, showing for the first time that an NMDA-receptor antagonist and an nNOS (nNOS) inhibitor had an effect on a necroptotic enzyme (Miao et al. 2015). In 20-min TGCI, RIP3 and apoptosis-inducing factor (AIF) translocated from cytosol and mitochondria, respectively, to hippocampal neuronal nuclei, and the RIP1 inhibitor Nec-1 inhibited the nuclear translocation and provided neuroprotection (Xu et al. 2016). This was the first time that RIP3 and AIF were shown to interact and to co-localize together in neuronal nuclei following ischemia and that Nec-1 prevented their interaction and nuclear translocation and was neuroprotective; it is another example of the interaction between the necroptotic and excitotoxic pathways.

Thus, it appears that there is an interaction between the necroptotic and excitotoxic pathways, but the relative contribution of each to the acute neuronal necrosis that is produced is unknown. This is an exciting area of inquiry about which much more is certain to be known in coming years.

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