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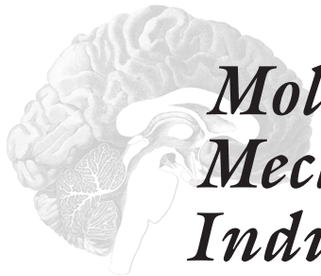
Chapter Forty-Two



Molecular and Cellular Mechanisms of Ischemia-Induced Neuronal Death

R. Suzanne Zukin, Teresa Jover, Hidenori Yokota, Agata Calderone, Monica Simionescu, and C. Geoff Lau

Ischemia is the condition or state in which a tissue such as brain is subjected to hypoxia or low oxygen because of an obstruction of the arterial blood supply or inadequate blood flow.[1] Brain ischemia can be broadly divided into two main classifications, global ischemia and focal ischemia. *Global ischemia* is the condition or state in which blood flow to the entire brain is transiently blocked, resulting in delayed, selective neuronal death. *Focal ischemia*, or cerebral infarction, is the condition or state in which a specific area of brain tissue undergoes injury as a consequence of a temporary or permanent obstruction of local blood supply. Focal ischemia results in death of both neurons and nonneuronal cells in contiguous areas of brain, usually representing a single vascular territory. In this chapter, we present the current understanding of the molecular and cellular mechanisms of neuronal death associated with brain ischemia. Table 42.1 defines the abbreviations used in the text.

GLOBAL ISCHEMIA

Global or brainwide ischemia arises most commonly in humans as a consequence of cardiac arrest, open-heart surgery, profuse bleeding, near-drowning, or carbon monoxide poisoning. Global ischemia associated with cardiac arrest affects 150,000 Americans each year and, in most cases, results in delayed onset of neurologic deficit.[1a–4] The most common neurologic deficits are cognitive impairments, of which memory loss is most notable. Although all forebrain areas experience oxygen and glucose deprivation (OGD) during the brief ischemic insult, only select neuronal populations degenerate and die in humans[1a–4] and in animals subjected experimentally to global ischemia.[5] Pyramidal neurons in the cornu ammonis 1 region of the hippocampus (CA1) are particularly vulnerable. Other neurons that may be damaged are hilar neurons of the dentate gyrus (DG), medium aspiny neurons of the striatum, pyramidal neurons in neocortical layers II, V, and VI, and Purkinje neurons of the cerebellum.[6,7] The molecular mechanisms underlying the cell-specific pattern of global ischemia–induced neuronal death are not well understood.

Histologic evidence of degeneration, or demonstration of the characteristics of apoptosis, is not observed until 2 to 3 days after ischemia in rats and 3 to 4 days in gerbils. At 1 week after induction of transient global ischemia, virtually complete ablation of the CA1 pyramidal cell layer can be observed. During the ischemic episode, cells exhibit a transient, early rise in intracellular calcium ion (Ca^{2+}), depolarize, and become inexcitable; ambient glutamate rises four-fold to ≈2μM. After reperfusion, cells appear morphologically normal, exhibit normal intracellular Ca^{2+}, and regain the ability to generate action potentials for 24 to 72 hours after the insult. Ultimately, there is a late rise in intracellular Ca^{2+} and zinc ion (Zn^{2+}), and death of CA1 pyramidal neurons ensues. Although the molecular mechanisms underlying ischemia-induced death are not yet completely understood, the substantial delay between insult and onset of death suggests that transcriptional changes play a critical role. Candidate transcription factors that are thought to direct programs of gene expression changes after global ischemia include the cyclic adenosine monophosphate (cAMP) response element–binding protein (CREB), the Forkhead family of transcription factors, and the restrictive element (RE)1 gene–silencing transcription factor, also known as REST/neuron-restrictive silencer factor (NRSF).

FOCAL ISCHEMIA

Focal or localized ischemia arises in humans most commonly as a result of stroke, cerebral hemorrhage, or traumatic brain injury. Most strokes are caused by clots that either form at the site of occlusion in a cerebral artery or travel there from the heart (classic stroke), and the remainder are caused by a bursting of a weakened blood vessel in the brain and bleeding into the surrounding tissue (cerebral hemorrhage or traumatic brain injury). Stroke is the third leading cause of death in the United States and the primary cause of disabilities in adults. Of the approximately 600,000 new victims each year, nearly 30% die and 20% to 30% become severely and permanently disabled. Others

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Table 42.1 Abbreviations Used in Chapter 42

AIF	Apoptosis-inducing factor
Akt	Serine-threonine kinase, a proto-oncogenic ser-thr kinase; also known as protein kinase B
AMP	Adenosine monophosphate
AMPARs	α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors
Apaf-1	Apoptotic protease–activating factor 1
ATP	Adenosine triphosphate
BAD	A proapoptotic member of the bcl-2 family of proteins
BCCO	Bilateral occlusion of the carotid arteries
BDNF	Brain-derived neurotrophic factor
BIR	Baculoviral IAP repeat
CA1	Cornu ammonis 1 region of the hippocampus
CaMKIV	Ca^{2+}-calmodulin–dependent kinase IV
CBP	CREB-binding protein
CREB	Cyclic AMP response element–binding protein
CREM	Cyclic AMP–response modulatory protein
CSD	Cortical spreading depression
DG	Dentate gyrus area of the hippocampus
DIABLO	Direct IAP–binding protein with low pI
DISC	Death-inducing signaling complex
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
ELK	Nuclear transcription factor
EPSC	Excitatory postsynaptic current
ERα	Estrogen receptor-α
ERE	Estrogen response element
ERK	Extracellular signal–regulated kinase
FADD	Fas-associated death domain
GABA	Gamma-aminobutyric acid
GluR	Glutamate receptor
GSK-3β	Glycogen synthase kinase-3β
HDAC	Histone deacetylase
HIF-1	Hypoxia-inducible factor-1
HSE	Heat shock element
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein
ICAM-1	Intercellular adhesion molecule-1
IEG	Immediate early gene
IGF	Insulin-like growth factor
IGF-IR	IGF-I receptor
IκB	Inhibitor of NF-κB
IL-1β	Interleukin-1β
IRS-1	Insulin receptor substrate-1
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
mGluR	Metabotropic GluR
MKP-1	MAPK phosphatase-1
MnSOD	Manganese superoxide dismutase
NAD^+	β-Nicotinamide adenine dinucleotide, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
Naspm	Naphthyl acetyl spermine
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NMDARs	*N*-methyl-D-aspartate (NMDA) receptors
nNOS	Neural NOS
NO	Nitric oxide
NOS	NO synthase
$\cdot O^{2+}$	Superoxide anion
NSRF	Neuron-restrictive silencer factor; also known as REST
OGD	Oxygen and glucose deprivation
$ONOO^-$	Peroxynitrite
PARP-1	Poly (ADP-ribose) polymerase-1
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PP1	Protein phosphatase 1
PSD-95	Postsynaptic density protein of 95 kDa
PV	Parvalbumin
REST	Repressor element gene silencing transcription factor; also known as NRSF
ROS	Reactive oxygen species
Rsk	Ribosomal S6 kinase
RT-PCR	Reverse transcription-PCR
SmaC	Second mitochondria–derived activator of caspases
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis–inducing ligand
VDAC	Voltage-dependent anion channel
VO	Vessel occlusion
ZnT-3	Zn^{2+} transporter-3

suffer paralysis, reduced coordination, and neurologic deficits including impaired cognition, visual disturbance, and loss of sensation. People older than 65 years experience almost three fourths of all strokes. "Brain attack" can be studied through the implementation of focal ischemia on animal models.

Tissues at risk of harm from occlusion of a cerebral artery are the *core* or center of the stroke, which contains cells that are highly dependent on the blocked artery and receive essentially no blood, and the *penumbra* or surrounding region, which contains cells that receive some blood from other arteries (Plate 42–1*C* following page 840). Cells in the core die from several overwhelming causes and probably cannot be salvaged by any treatment short of immediate removal of the clot. Although the infarct starts in the core, at its maximum it encompasses both core and penumbra, generally after 6 to 24 hours of permanent ischemia.[8] The duration of the ischemic episode determines the extent or grade of damage, assessed 1 to 2 days after reperfusion.[9] At 10 to 20 minutes after induction of focal ischemia, only a few scattered dead neurons are observed in the core. At 1 hour, infarct is observed in the core, and the infarct size is maximal. The mechanisms underlying death of cells in the core are complicated but most certainly include glutamate receptor–mediated necrotic cell death (see later). Brain edema (as studied with magnetic resonance imaging [MRI] and computed tomography [CT]) serves as one of the earliest markers for the ensuing pathophysiology and is a key determinant of whether a patient survives beyond the first few hours after stroke.

EXPERIMENTAL MODELS OF GLOBAL AND FOCAL ISCHEMIA

A number of experimental models are currently used to study brain ischemia. The three main paradigms involving

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intact animals (in vivo ischemia) are global ischemia, focal ischemia, and hypoxia-ischemia, a condition that shares properties with both focal and global ischemia. In vivo models of global ischemia enable neuronal death to mature in an intact animal in which neural circuitry is preserved. Therefore, these models have greater physiologic validity and clinical relevance to global ischemia associated with cardiac arrest in humans than in vitro models.

In vitro models are also used to examine molecular and biophysical mechanisms of neuronal death. These models are particularly useful for suppression or overexpression of genes of interest. In vitro models involving organotypically cultured brain slices are particularly useful in that they afford preservation of neural circuitry.

In Vivo Models

Global Ischemia

Global ischemic insults consist of brief but nearly complete cessation of cerebral blood flow produced by permanent occlusion of the vertebral arteries and transient occlusion of the common carotid arteries (rats) or by transient occlusion of the common carotid arteries (gerbils and mice), followed by reperfusion.[10,11] The most commonly used models of global ischemia are (1) the four-vessel occlusion (4-VO) model in rats[12]; (2) the two-vessel occlusion (2-VO; also known as temporary bilateral common carotid occlusion, or BCCO) in gerbils[7,13] or (less commonly) mice[13,14]; and (3) two-vessel occlusion in combination with hypotension in rats.[10] Global ischemia can also be induced in large mammals such as monkeys[15] and goats.[16] Global ischemic insults are typically short (on the order of 5 to 20 minutes). During the ischemic episode, blood flow to the entire brain is reduced (to < 1%) essentially immediately and remains blocked until reperfusion. Adenosine triphosphate (ATP) is depleted in cells throughout the brain essentially immediately but recovers to near physiologic levels by the time of reperfusion.[17]

The 4-VO model in rats and the 2-VO model in gerbils differ from more severe models involving hypotension, in that neuronal death is extremely delayed and highly specific. Although all forebrain areas experience OGD during a brief ischemic insult, neuronal death elicited by a brief episode (10 minutes for 4-VO in rats; 5 minutes for 2-VO in gerbils) is largely restricted to pyramidal neurons of the hippocampal CA1 and hilar neurons (Plate 42–1*B*).[18] Inhibitory interneurons of the CA1 and most neurons in the nearby CA2 or transition zone, CA3, and DG survive. With the exception of a few scattered hilar neurons or pyramidal neurons in the cortex, no other neurons exhibit cell death. Although these models afford virtual ablation of the hippocampal CA1 by 7 days, the onset of histologically detectable neuronal death is not manifested until more than 48 hours in rats,[10,19] or more than 72 hours in gerbils,[7,20,21] after insult. Longer insults induce more widespread damage to areas such as medium aspiny striatal neurons, pyramidal neurons in neocortical layers II, V, and VI, and cerebellar Purkinje neurons.[6,7]

Advantages of the in vivo models of global ischemia are as follows:

1. They have clinical relevance to global ischemia associated with cardiac arrest in humans.
2. Neural circuitry is preserved.
3. The substantial delay between insult and neuronal death enables detailed molecular studies.
4. The specificity of cell death allows comparison of molecular changes in CA1 with those in CA3.
5. The cranium is completely blocked (rather than reduced by hypotension); thus, monitoring of blood flow is not necessary.
6. Animals exhibit no obvious behavioral manifestations, and the mortality rate is low.

The rare animals that do exhibit obvious behavioral manifestations (abnormal vocalization when handled, generalized convulsions, loss of greater than 20% of body weight by 3 to 7 days, hypoactivity) are excluded from the study.

Four-Vessel Occlusion Model in Rats

The 4-VO model is a well-established model of neuronal insult in which neuronal death is largely restricted to pyramidal neurons of the hippocampal CA1 and does not manifest until 3 to 4 days after insult.[12] A specific advantage of the rat model is that many available complementary DNA (cDNA) and RNA probes are directed to rat RNA, and many antibodies exhibit high specificity for rat tissue and may not recognize epitopes in other species. Age-matched male Sprague Dawley or Wistar rats, weighing 100 to 125 g are fasted overnight; the next day, animals are anesthetized with halothane. The vertebral arteries are exposed through a small incision in the neck and subjected to permanent electrocauterization. The common carotid arteries are exposed and isolated with a 3-0 silk thread, and the wound is sutured. Twenty-four hours later, the wound is reopened, and the common carotid arteries are subjected to temporary occlusion with surgical clasps (4 minutes for sublethal ischemia and 10 minutes for global ischemia), and anesthesia is discontinued (Plate 42–1*A*).[12,22] At the time of occlusion of the carotid arteries, blood flow is typically reduced to less than 3% of normal in the hippocampus, striatum, and neocortex.[23] The electroencephalogram (EEG) generally becomes isoelectric[24] and spontaneous cortical activity is abolished within 1 minute.[25] For sham operation, animals are subjected to the same anesthesia and surgical exposure procedures, except that the carotid arteries are not occluded. Although anesthesia is typically administered until occlusion of the carotid arteries, it is not essential to the surgical procedure.

Two-Vessel Occlusion with Hypotension in Rats

An alternative model of global ischemia in rats involves ligation of the common carotid (but not vertebral) arteries, together with systemic hypotension (50 mm Hg). Under these conditions, blood flow falls to 1% in the hippocampus, striatum, and neocortex,[26,27] and the EEG becomes isoelectric within 15 to 25 seconds.[28] Animals are subjected to anesthesia for the entire duration of the ischemic episode. Models of global ischemia involving systemic hypoxia and/or hypotension are more severe than the 4-VO model. These models cause a more rapid onset of generalized neuronal death, particularly in the cortex, striatum, and hippocampus, major behavioral manifestations, and a considerable death rate.

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Two-Vessel Occlusion in Gerbils

Gerbils are advantageous for studies of global ischemia in that they lack posterior communicating arteries, structures that in humans and rats are necessary to complete the circle of Willis and permit collateral blood flow. Thus, global ischemia can be induced in gerbils by the relatively simple 2-VO model. In gerbils, 2-VO (5 minutes) elicits highly selective, extremely delayed neuronal death, with a pattern of cell specificity virtually identical to that in rats; neuronal death is not manifested until more than 72 hours after onset.[10,21] The 2-VO model is the model of global ischemia most commonly used for testing neuroprotective agents. Within 20 seconds of 2-VO, blood flow falls to 1% in neocortex and to 4% in hippocampus,[29] and the EEG becomes isoelectric.[30] Anesthesia is administered until occlusion of the carotid arteries.

Two-Vessel Occlusion in Mice

Mice offer advantages in that some strains (C57/BL6 and related strains) exhibit global ischemia in response to the relatively simple 2-VO model, enabling comparisons between animals with null mutations in a gene of interest and their wild-type littermates. However, strain differences in vulnerability to ischemic damage can complicate results.[31] In mice, 2-VO (20 minutes) elicits somewhat selective, delayed cell death. At 72 hours after ischemia, the majority of animals exhibit no detectable cell loss in the hippocampus; in one study, about 17% of animals exhibited minor cell loss and another 17% showed moderate cell loss in the CA1.[14] At 7 days after ischemia, nearly all animals exhibited marked loss in the pyramidal cell layer of CA1. In the majority of animals, CA3 exhibited at most slight cell loss, and the DG exhibited no cell loss at 7 days.

Focal Ischemia

Focal ischemia is the animal model that most nearly approximates stroke or cerebral infarction in humans.[32–34] Focal ischemia is produced experimentally by occlusion of the middle cerebral artery. Arterial occlusion can be permanent (arterial blockade maintained throughout the experiment) or temporary (occlusion for up to 3 hours, followed by reperfusion) and either proximal or distal (see later). These procedures induce a necrotic core of cells that are irreversibly damaged and a penumbra of cells that can be revived (see Plate 42–1*C*).[35] Focal ischemia is typically performed in rodents such as rats or mice. For rats, a preferred strain is the spontaneously hypertensive rat, which exhibits reduced collateral circulation during the ischemic episode.[36–38]

Proximal Occlusion

In the case of proximal occlusion, the middle cerebral artery (MCA) is subjected to occlusion close to its branching from the internal carotid, before the origin of the lenticulostriate arteries.[11,36] Proximal MCA occlusion (MCAO) is most commonly induced by ligation of the common carotid and external carotid arteries, followed by insertion of a suture into the internal carotid artery at the bifurcation of the common carotid and external carotid arteries. The suture is advanced intraluminally beyond the origin of the posterior communicating artery and past the origin of the MCA (see Plate 42–1*A*).[10,11,33] After MCAO, blood flow is nonuniformly reduced throughout the affected region. The center of the stroke, or core, is defined as the region in which blood flow is reduced to less than 15%; it encompasses the lateral portion of the caudate putamen and the parietal cortex. The penumbra, defined as the region in which blood flow is reduced to less than 40%, encompasses the remainder of the neocortex, the entorhinal cortex, and the medial caudate-putamen.

Distal Occlusion

In distal MCAO, blood flow to the basal ganglia is not interrupted; thus, damage is restricted to the neocortex. This type of occlusion can be induced surgically by means of a clip[37] or by inducing thrombotic clots[38,39] in combination with transient unilateral occlusion of the common carotid arteries.[28,40,41] The reduction of blood flow achieved in the core and penumbra with distal MCAO is similar to that achieved in the proximal model.

Hypoxia-Ischemia

The hypoxia-ischemia model involves transient unilateral occlusion of the common carotid artery in combination with systemic hypoxia, such that oxygen flow to the brain is reduced to 3% in adult rats or to 8% in neonates.[42–44] After 15 to 30 minutes of hypoxia, delayed neuronal death occurs in the hippocampal CA1 and CA3, striatum, and layer V of the neocortex in adults.[45] Young rats show delayed development of infarct, which can be induced by subjecting them to low levels of oxygen (8% of normal) for 60 minutes.[46]

In Vitro Models

Oxygen and glucose deprivation (OGD) of cell cultures or brain slices provides an in vitro model of global ischemia (Plate 42–2).[47–49] In vitro models require longer periods of OGD to induce cell death, and ATP levels do not fall as much as in in vivo models. The absence of blood vessels and blood flow simplifies interpretation of the results but renders the model less relevant than a model using an intact animal. Advantages of the in vitro OGD model are as follows:

1. Manipulations of the microenvironment can be more precise.
2. The model is amenable to patch clamp recording and detailed electrophysiologic analyses.
3. Prolonged survival of cultures permits molecular and genetic manipulations as, for example, ease of antisense suppression of a protein of interest by administration of antisense oligonucleotides.
4. It allows optical monitoring of changes in the same slice over days.
5. It enables internal control of a number of slices that can be obtained from the same animal.

Oxygen-Glucose Deprivation of Dissociated Neurons in Culture

In vitro OGD is performed in primary cultures of neurons or glia from the neocortex, hippocampus, cerebellum, and hypothalamus of embryonic or early postnatal rats or mice. Mixed neocortical specimens containing both neurons and glia are typically cultured from embryonic day 15 (E15) rats.[50] At 14 days in vitro (DIV), the culture medium is

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exchanged with deoxygenated, glucose-free salt solution to induce OGD. Cultures are deprived of oxygen and glucose for 90 to 100 minutes and then transferred to an oxygenated serum-free medium containing glucose and propidium iodide. Cell death is assayed at 24 and 48 hours.

Oxygen-Glucose Deprivation in Cultured Hippocampal Slices

Ischemic damage is also studied in organotypic hippocampal slice cultures from perinatal rats. Typically, hippocampal slices are obtained from rat pups (postnatal day 8, or P8) and maintained in vitro for 14 to 21 days.[51] Briefly, hippocampi are removed from rat brains, and transverse slices are cut with a tissue chopper in a sterile environment. Isolated slices are placed in ice-cold Hanks balanced salt solution supplemented with glucose and Fungizone and then transferred to humidified semiporous membranes. Slices are maintained in culture medium at 37°C and 95% air/5% CO_2. At 14 to 21 DIV, hippocampal slices are subjected to OGD by exposure to a serum-free medium devoid of glucose and saturated with 95% N_2/5% CO_2 for 30 to 60 minutes, then transferred to an oxygenated serum-free medium containing glucose and propidium iodide. Cell death is assayed at 48 and 72 hours. A 30-minute insult elicits selective death of CA1 neurons by 48 hours (see Plate 42–2). Neuronal death is typically assessed from permeability to dyes such as trypan blue and propidium iodide.[52] In vitro ischemia impairs synaptic transmission, protein synthesis, ATP production, and neuron morphology.

MODALITIES OF ISCHEMIC CELL DEATH

Cell death occurs by necrosis or apoptosis.[53,54] These two mechanisms have distinct histologic and biochemical signatures. In necrosis, the stimulus of death (e.g., ischemia) is itself often the direct cause of the demise of the cell. In apoptosis, however, the stimulus of death activates a cascade of events that orchestrate the destruction of the cell. Unlike necrosis, which is a pathologic process, apoptosis is part of normal development; however, aberrant apoptosis occurs in response to injurious stimuli.

Global ischemia induces neuronal death with hallmarks of both necrosis and apoptosis.[55] Ultrastructural studies indicate that global ischemia induces many of the morphologic features of necrotic cell death in CA1 neurons (see later) but do not detect critical hallmarks of apoptosis such as apoptotic bodies.[56] Thus, apoptosis as defined by stereotypic morphologic changes, especially evident in the nucleus where the chromatin condenses to compact an apparently simple geometric figure, does not occur. These and other studies cast doubt as to whether global ischemia elicits any of the morphologic features of apoptosis. Strong evidence in support of apoptosis, defined as activation of specific intracellular signaling cascades that result in cellular suicide,[53,57] comes from molecular studies that show mitochondrial release of cytochrome c and activation of the caspases, in particular caspase-3 (see later).

Focal ischemia also induces neuronal death with hallmarks of both necrosis and apoptosis.[11] Focal ischemia elicits early cell shrinkage and swelling of mitochondria, followed by cell dispersal, shrinkage of the nucleus, the formation of cytoplasmic projections, and, ultimately, a shrunken, pyknotic nucleus without surrounding cytoplasm (the last remnant of the dead neuron). The early mitochondrial swelling and loss of integrity of the plasma membrane, with preservation of the nuclear membrane, are hallmarks of necrotic cell death. Evidence in support of apoptotic death in the penumbra has surfaced, including DNA fragmentation, activation of death receptors, mitochondrial release of cytochrome c, and activation of the caspase death cascade (see later).

Necrotic Cell Death

Necrosis is the death of a circumscribed area of tissue as a result of a wide variety of injuries. At the light-microscopic level, the morphologic hallmarks of necrotic cell death are early mitochondrial swelling and loss of integrity of the plasma membrane, with preservation of the nuclear membrane. At the ultrastructural level, the hallmarks of necrotic cell death are proliferation of endoplasmic reticulum, disaggregation of polyribosomes, selective swelling of dendrites, and dilation of organelles and intranuclear vacuoles. The necrotic tissue morphology is to a large extent due to postmortem events occurring after cell lysis. Necrotic cell death can be divided into two main states, edematous death, characterized by edema or organelle swelling, and ischemic death. The edematous state is characterized by swollen cytoplasm, the absence of dynamic plasma membrane blebbing of a dying cell (zeiosis), absence of microtubules, and presence of the endoplasmic reticulum, Golgi apparatus, and polysomes as incomplete structures. Although the nucleus appears nearly normal, irregular clumping of chromatin is seen.[58,59] These characteristics are observed for CA1 neurons undergoing delayed death in the rat and gerbil models of global ischemia.[60,61] By contrast, the ischemic state is characterized by darkening and shrinkage of the nucleus and cytoplasm[62–64]; the plasma and nuclear membranes become highly irregular, and the cell assumes a triangular shape. Cells undergoing ischemic cell change are acidophilic. In models of global ischemia, CA1 neurons exhibit edematous changes in the end stages of degeneration. In focal ischemia, cortical neurons in the core and penumbra exhibit morphologic changes before their demise.

Apoptotic Cell Death

Apoptosis, or programmed cell death (PCD), is the evolutionarily conserved process by which cells die as a result of an internally programmed series of events mediated by a dedicated set of gene products. Apoptotic cell death is essential during development (embryogenesis) and tissue homeostasis; when dysregulated, apoptotic cell death can result in cancer, abnormal neuronal death, or autoimmunity. A variety of injurious stimuli, including focal ischemia and global ischemia, can induce apoptosis if the insult is mild but will induce necrosis with a stronger insult. Apoptotic neurons exhibit characteristic morphologic features that differentiate them from necrotic neurons—cytoplasmic shrinkage, chromatin condensation, zeiosis (dynamic membrane blebbing), and apoptotic bodies. Unlike

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necrotic cells, in which plasma membrane is damaged early, apoptotic cells exhibit intact plasma membranes until the last stages of death, at which time the membranes become permeable to normally retained solutes. A number of specific apoptotic death cascades involving downstream signaling molecules have now been identified. Molecular hallmarks of apoptosis include phosphatidylserine exposure (translocation from the inner leaflet to the outer surface of the plasma membrane), activation of the cell surface receptors such as Fas/CD95, a member of the tumor necrosis factor (TNF) family of death receptors,[65] mitochondrial release of cytochrome c,[66] activation of the caspases, notably caspase-3,[67] and DNA fragmentation.[68–70]

The Caspase Death Cascade

Caspases are a family of structurally related cysteine proteases that cleave target proteins just after an aspartate residue.[53] Because caspases are constitutively expressed as biologically inactive precursors or procaspases in most cells and can cleave their own procaspases, the caspase cascade is self-amplifying.

It had been thought that a key event (and "point of no return") in the execution of apoptosis was activation of the essential "terminator" protein, caspase-3. However, two recent studies provide compelling evidence that neurons can survive in the face of caspase-3 activation.[70a–b] In global ischemia, caspase-3 upregulation and activation occur 2 to 3 days before the onset of histologically detectable neuronal death.[71] The importance of early caspase-3 activation to global ischemia–induced neuronal death is underscored by the finding that Z-DEVD-FMK, a selective caspase-3 inhibitor, is neuroprotective if administered at the time of ischemia but not at 24 hours or later.[72] Thus, neurons become "committed" to die early in the postischemic period. Caspase-3 promotes cell death through proteolytic cleavage of downstream target proteins such as poly (ADP-ribose)polymerase (PARP), nuclear lamins, DNA-dependent protein kinase, and the inhibitory subunit of DNA fragmentation factor.[73,74] DNA fragmentation results in cell disintegration followed by engulfment by surrounding cells. The caspase cascade can be activated by either of two reversible and interacting pathways, an extrinsic or death receptor–dependent route (Plate 42–3) and an intrinsic (death receptor–independent) or mitochondrial route (Plate 42–4).

In the extrinsic or death receptor–dependent pathway, apoptotic stimuli trigger activation of death ligand–death receptor systems such as the FasL/Fas (also known as the Apo-1 or CD95) system.[75] Fas is a death domain-containing receptor and member of the TNF superfamily of cytokine receptors. Forkhead1 (FOXO-3A1 or FOXO-3A), a member of the Forkhead family of transcription factors, induces expression of target genes, such as the cytokine FasL (Fas ligand), that are implicated in the extrinsic receptor pathway of caspase activation.[76] FasL initiates apoptosis by binding to its cognate receptor Fas, triggering formation of a death-inducing signaling complex (DISC) within seconds of receptor engagement. Fas acts via its death domain to recruit the adaptor protein FADD (Fas-associated death domain). FADD, in turn, acts via its death domain to recruit procaspase-8 into the DISC. The signaling complex catalyzes the proteolytic cleavage and transactivation of procaspase-8 to generate the "instigator" caspase, caspase-8. Once activated, caspase-8 is released from the DISC into the cytoplasm as a heterotetramer composed of two small subunits and two large subunits. Activated or processed caspase-8 can directly activate other members of the caspase family, paving the way for the execution phase of apoptosis. Caspase-8 additionally acts to induce the translocation of Bcl-2 family member BID to the mitochondria. When the abundance of BAX, BIM, BAD and BID exceeds that of anti-apoptotic Bcl-2 family members, cytochrome c is released from the mitochondria (see Plate 42–3).

In the death receptor–independent or mitochondrial route, apoptotic and necrotic death stimuli trigger the mitochondrial release of cytochrome c into the cytoplasm, an event that is blocked by Bcl-2 and its anti-apoptotic family members.[77] Once in the cytoplasm, cytochrome c assembles with the apoptotic protease activating factor 1 (Apaf-1), procaspase-9 and deoxy ATP to form a protein-signaling complex termed the *apoptosome*. Formation of the apoptosome promotes transactivation of procaspase-9 by Apaf-1. Activated caspase-9, in turn, cleaves procaspase-3 to generate the downstream "terminator" protein caspase-3. Thus, the apoptosome enables cytochrome c to jump-start the caspase self-amplifying cascade of proteolysis independently of ligand-activated death receptors (see Plate 42–4).

Both focal ischemia and global ischemia trigger the caspase death cascade in neurons destined to die. Global ischemia induces activation of death receptors, such as Fas[78] and $p75^{NTR}$ [79–81] and terminators, such as caspase-9 and caspase-3,[72,80,82] and the onset of DNA fragmentation, a marker for apoptotic cell death.[79,80,83] The observation that global ischemia triggers early release of cytochrome c from the mitochondria,[84] activation of caspase-9[82,84] and caspase-3,[71,80] and relatively late activation of Fas/FasL[78] provides strong support for activation of the caspases via an intrinsic pathway. In the case of focal ischemia, delayed neuronal death with many of the hallmarks of apoptosis are observed in the penumbra, including evidence of DNA fragmentation,[68–70] activation of the FasL/Fas receptor,[65] release of cytochrome c from the mitochondria,[66] and activation of caspase-3.[67]

Inhibitors of Apoptosis

The inhibitor of apoptosis proteins (IAPs) are a family of structurally related proteins that confer protection from death-inducing stimuli by potently binding and inhibiting activated caspases. IAPs, originally identified in the genome of baculovirus on the basis of their ability to suppress apoptosis in infected host cells, suppress apoptosis in mammalian cells by halting the caspase death cascade.[85–87] To date, eight human IAPs have been identified, including XIAP, c-IAP, c-IAP2, and survivin, and all exhibit anti-apoptotic activity in cell culture. The best-characterized IAP family member is the X-chromosome–linked protein XIAP. XIAP is an extremely potent suppressor of apoptosis, an effect mediated at least in part by its ability to bind and suppress active caspases. XIAP binds caspases -3, -7, and -9 reversibly and with high affinity, thereby masking the caspase active site. The main functional unit in the IAPs is the BIR (or baculoviral IAP repeat) domain, which contains about 80 amino acids folded around a zinc atom.

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Most IAPs have multiple BIR domains that mediate specialized functions. For example, the linker region between BIR1 and BIR2 binds caspase-3 and caspase-7, BIR2 selectively targets caspase-7, and BIR3 targets caspase-9.

Under physiologic conditions, IAPs are present in mammalian cells, where they act as buffers or dampeners that suppress spurious spontaneous caspase activation. The actions of IAPs on neuronal survival are, however, not limited to caspase inhibition. Compelling data support additional roles for IAPs in protein degradation, cell cycle regulation and caspase-independent signaling cascades.[86] Emerging data indicate that the presence of a zinc-binding motif or RING domain at the distal end of the carboxy-termini in a subset of IAPs confers protein degradation activity.[88] These IAPs catalyze degradation of select target proteins via ubiquitylation. In this process, IAPs catalyze the sequential covalent addition of ubiquitin (a 76–amino acid protein) onto select lysine residues within target proteins. The modified residues can form multimeric poly-ubiquitin chains, which tag the protein and mark it for destruction.[88]

Injurious stimuli such as global ischemia elevate the expression of IAP proteins, which bind and reversibly inhibit the caspases. At the same time, injurious stimuli that are sufficiently potent promote the mitochondrial release of Smac (second mitochondria–derived activator of caspases)/DIABLO (direct IAP-binding protein with low pI) and cytochrome c. Whereas cytochrome c directly activates Apaf-1 and caspase-9 and forms the apoptosome, Smac/DIABLO binds to IAP family members and neutralizes their anti-apoptotic activity. Smac forms an elongated arch-shaped dimer more than 130 Å in length. The Smac dimer forms a stable complex with the BIR2 and BIR3 domains of XIAP. Structural studies involving nuclear magnetic resonance (NMR) and X-ray analyses reveal that the Smac *N*-terminal tripeptide (Ala-Val-Pro-Ile) recognizes a surface groove composed of highly conserved residues on the BIR3 domain.[89] The balance between the IAPs and Smac/DIABLO establishes a threshold for "lethal" caspase-3 activity. Only under conditions in which Smac/DIABLO is released from the mitochondria is activated caspase-3 liberated from IAPs and free to execute apoptotic cell death.

An alternative pathway to inhibit activated caspase-3 is via the anti-apoptotic protein Bcl-2.[90] Evidence indicates that the mammalian cell-death inhibitors Bcl-2 and Bcl-x_L, by analogy to the *Caenorhabditis elegans* cell-death inhibitor CED-9, might function as a pseudosubstrate inhibitor of activated caspase-3.[91] Studies of programmed cell death in *C. elegans* indicate that the CED-9 protein prevents apoptosis by direct inhibition of CED-3. CED-9 can be cleaved by CED-3 at two sites near its amino-terminus, at least one of which is important for complete protection by CED-9 against cell death. Ischemic preconditioning induces expression of Bcl-2 in CA1 neurons.[92] There is as yet no hard evidence, however, that mammalian Bcl-2 functions as an active site-directed inhibitor of caspase-3.

Poly(ADP-ribose)polymerase-1 and Apoptosis-Inducing Factor

Poly(ADP-ribose)polymerase-1 (PARP-1) is an abundant nuclear enzyme involved in DNA damage surveillance and DNA repair and is a critical downstream target of caspase-3. Under physiologic conditions, PARP-1 is critical for maintaining genomic integrity and may play a role in DNA replication and regulation of gene expression.[93] PARP acts via an amino-terminal DNA-binding motif to recognize nicks and breaks in the double-stranded nucleic acid. Upon binding to DNA strand breaks, PARP catalyzes the polyribosylation of β-nicotinamide adenine dinucleotide (NAD^+), generating branched polymers of ADP-ribose that it transfers to a subset of nuclear proteins, including histones, topoisomerases I and II, DNA polymerases, and PARP itself.[94–96] The obligatory triggers of PARP-1 activation are nicks and breaks in double-stranded DNA. Once activated, PARP-1 transfers between 50 and 200 molecules of ADP-ribose to target proteins, which may activate or inhibit their function. In the case of histones, poly(ADP-ribosyl)ation promotes chromatin relaxation.

Neuronal insults and other types of environmental stress effect the production of free radicals and oxidants. These agents induce DNA damage, which frequently triggers cell death by apoptosis. Apoptosis culminates in activation of caspase-3, which catalyzes the cleavage and activation of downstream targets including PARP-1. A major trigger for DNA damage in cerebral ischemia is peroxynitrite ($ONOO^-$), a cytotoxic oxidant formed by reaction between nitric oxide and superoxide (see Plate 42–4). Excessive activation of PARP-1 depletes the entire cell of its substrate NAD^+ (Plate 42–5*B*). NAD^+ depletion in mitochondria causes pronounced slowing of glycolysis, electron transport, and ATP formation, leading to energy failure and neuronal death. Observations that PARP-1 inhibition or gene inactivation may prevent the neuronal death associated with cerebral ischemia,[97,98] myocardial infarction,[99] inflammatory injury, reactive oxygen species–induced injury,[96] and glutamate excitotoxicity[100,101] have triggered an explosion of interest in the process of poly(ADP-ribosyl)ation.

Our understanding of the underpinnings of PARP-1-induced cell death was significantly advanced by the discovery that massive PARP-1 activation triggers apoptotic cell death mediated by apoptosis-inducing factor (AIF) (see Plate 42–5*A*).[102,103] AIF is a powerful cytotoxin that under normal conditions is confined to the mitochondrion together with cytochrome c and other pro-apoptotic molecules. Massive activation of PARP-1 promotes production of poly(ADP-ribose) and depletion of NAD^+, which appear to signal the release of AIF from the mitochondria.[103] Once released, AIF rapidly translocates to the cytoplasm and nucleus. Nuclear AIF promotes chromatin condensation, chromatin fragmentation, and, ultimately, apoptotic cell death via a caspase-independent pathway. Cytosolic AIF acts on the mitochondria to collapse the mitochondrial membrane potential and initiate the release cytochrome c, which activates caspase-3. However, caspase activation is apparently not required for PARP-1–initiated cell death, because caspase inhibitors do not afford protection.[103] Thus, PARP-1 activation is required for AIF translocation during cell death and AIF is essential for PARP-1–mediated cell death. Moreover, AIF-mediated cell death is caspase-independent.

Important unanswered questions are: Does NAD^+ depletion play a role in AIF release? Is the first step in

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bidirectional signaling between the nucleus and mitochondria mediated by poly(ADP-ribosyl)ated molecules? and How does block of AIF afford protection in the presence of caspase-3 activation?

TRIGGERS OF ISCHEMIC CELL DEATH

Glutamate Excitotoxicity

Excitotoxicity refers to the ability of glutamate (and other excitatory amino acids) to destroy neurons by excessive activation of excitatory amino acid receptors.[104] Knowledge that glutamate is potentially toxic dates to observations by Lucas and Newhouse[105] nearly a half century ago that glutamate administered to animals in vivo caused death of retinal neurons. The concept of excitotoxic death was significantly advanced by Olney and Ho,[106] who used ultrastructural analysis to analyze the cytopathology of neurons exposed to glutamate. These studies revealed a characteristic pattern of glutamate-induced neuronal death in which postsynaptic structures such as dendrites and somata were destroyed but axons, presynaptic terminals, and nonneural cells survived. Other excitatory amino acids and glutamate analogues induced neuronal death with a rank order of potency similar to that for their ability to elicit excitatory transmission. With the advent of selective excitatory amino acid receptor antagonists in the 1980s and their application to studies of glutamate actions, excitatory amino acids were accepted as the major excitatory transmitters of the central nervous system (CNS) and, in high concentrations, as excitotoxins, capable of excessive activation of excitatory amino acid receptors and excitotoxic cell death.

The concept that glutamate plays a critical role in the pathogenesis of global and focal ischemia originated with observations that raising extracellular magnesium markedly reduced the vulnerability of cultured hippocampal neurons to anoxia[107,108] and that glutamate antagonists reduced neuronal injury in both in vitro and in vivo models of ischemia.[109,110] Over the past 15 to 20 years, accumulating evidence has mounted that glutamate antagonists afford neuroprotection in global and focal ischemia. It is now widely accepted that excitotoxicity plays a critical role in the neuronal death associated with these and many other neuronal insults and disorders.[104]

During the ischemic episode, anoxic depolarization triggers the massive release of synaptic glutamate.[111] Synaptically released glutamate activates the ionotropic glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs) and kainate receptors, which mediate the influx of Na^+ and thus further depolarize the postsynaptic membrane, and *N*-methyl-D-aspartate receptors (NMDARs) and GluR2-lacking AMPARs, which directly flux Ca^{2+} and Na^+ into postsynaptic cells.[112,113] Glutamate also activates group I metabotropic glutamate receptors (mGluRs). Group I mGluRs, consisting of mGluR1 and mGluR5, act via phospholipase C and inositol 1,4,5-triphosphate ($InsP_3$) to trigger release of Ca^{2+} from intracellular stores.[114] The massive rise in cytosolic Ca^{2+} causes cells to further depolarize and become inexcitable. Anoxic depolarization also drives the reverse operation of glutamate transporters in astrocytes, contributing to the rise in extracellular glutamate. Glutamate toxicity induces reverse operation of the Na^+-Ca^{2+} exchanger in neurons and astrocytes, exacerbating the buildup of Ca^{2+}.[104,115] During global ischemia, extracellular glutamate rises from about 0.6 μM to between 1 and 2μM.[116] During focal ischemia, glutamate rises to between 16 and 30μM in the core.[117,118] A major consequence of the rise in extracellular glutamate is activation not only of synaptic but also of extrasynaptic ionotropic glutamate receptors (AMPARs, NMDARs, and kainate receptors), with consequent influx of toxic Ca^{2+} and shutoff of the CREB-initiated program of cell survival (see later).

NMDA Receptors

For nearly two decades, intense interest focused on NMDARs as the candidate mediator of Ca^{2+} entry into neurons destined to die.[119] NMDARs mediate the influx of toxic Ca^{2+} in a number of neurologic disorders, insults, and neurodegenerative diseases. Although it is well established that NMDARs are a critical player in focal ischemia–induced neuronal death, AMPARs appear to mediate the cell death associated with global ischemia. Although not completely understood, the following two factors are thought to reduce the contribution of NMDARs in postischemic neurons: (1) the rise in extracellular acidity, which inhibits NMDAR functional activity, and (2) the rise in extracellular Zn^{2+}, which potentiates currents mediated by AMPARs and inhibits those mediated by NMDARs.

Injurious stimuli, including hypoxia and transient ischemia, cause excessive activation of NMDARs, excessive Ca^{2+} influx, and excitotoxic cell death. Activation of NMDARs leads to neuronal death via nitric oxide (NO) signaling. NO is implicated as an important downstream mediator of NMDAR-induced excitotoxicity in postischemic neurons. At excitatory synapses, neuronal NO synthase (nNOS) is physically anchored to the NMDAR through the scaffolding protein postsynaptic density protein of 95kDa (PSD-95). NMDARs provide a direct route for entry of Ca^{2+} into the cell; once in the cell, Ca^{2+} binds calmodulin and rapidly activates nNOS. Upon stimulation, nNOS converts arginine to NO and citrulline. Overproduction of NO from excessive or inappropriate stimulation of nNOS is thought to mediate a major component of excitotoxic damage and focal ischemic cell death.

Under conditions of excessive glutamate release, activation of NMDARs further contributes to neuronal death via shutoff of the CREB-initiated program of gene expression. Under physiologic conditions, restricted Ca^{2+} influx via synaptic NMDARs activates CREB. CREB activates prosurvival target genes such as the neurotrophin brain-derived neurotrophic factor (BDNF). Ca^{2+} influx via synaptic NMDARs activates Ca^{2+}-calmodulin-dependent kinase IV (CaMKIV) and downstream kinases, which act in a coordinated manner to induce robust, sustained phosphorylation of Ser133 and activation of CREB.[120,121] In addition, CaMKIV phosphorylates and activates the coadaptor protein CREB-binding protein (CBP) at Ser301. Upon activation, CREB recruits phosphorylated CBP to the promoter region of target genes, and the coactivator complex induces gene transcription.

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Findings reported by Hardingham and colleagues[120] reveal that the location of Ca^{2+} entry into cells critically influences the fate of neurons. Whereas Ca^{2+} influx via synaptic NMDARs induces activation of CREB, Ca^{2+} influx via extrasynaptic NMDARs elicits dephosphorylation and inactivation of CREB.[122–124] Interestingly, contemporaneous activation of synaptic and extrasynaptic NMDARs by bath-applied NMDA also shuts off CREB, suggesting that extrasynaptic NMDARs act via a dominant, cell-death signal to override the CREB-promoting effects of synaptic NMDARs, L-type calcium channels and protein kinase A (PKA), or both.[122] Ca^{2+} influx via extrasynaptic NMDARs (and CREB shutdown) causes breakdown of the mitochondrial membrane potential, ATP depletion, and necrotic cell death. Thus, the cellular penalty of excess Ca^{2+} entry through extrasynaptic NMDARs is not only dysfunction of CREB signaling but also neuronal death.

The link between extrasynaptic NMDARs and mitochondrial dysfunction and neuronal death is particularly relevant to the pathophysiology of ischemia. Injurious stimuli such as focal ischemia and global ischemia cause excessive glutamate release and spillover; high extracellular glutamate stimulates extrasynaptic NMDARs, leading ultimately to cell injury or death. This model would explain severe, necrotic cell death in the ischemic core. Neurons experiencing shorter, less severe hypoxic-ischemic episodes or neurons in the penumbra may suffer only a transient and incomplete depolarization of the mitochondria. Their health could be compromised because of stimulation of extrasynaptic NMDARs. This model is consistent with findings of CREB shutoff and apoptosis in cells in the penumbra. Stroke-surviving neurons have sustained concentrations of CREB and elevated concentrations of BDNF.

An additional link between NMDARs and mitochondrial dysfunction has emerged. Mounting evidence suggests that efflux of K^+ leads to reduced intracellular K^+ concentrations and may be a critical driver of apoptosis.[55] NMDARs are an important route of K^+ efflux in cells undergoing apoptosis. Whereas NMDAR-mediated necrosis primarily involves influx of Na^+ and Ca^{2+}, NMDAR-mediated apoptosis primarily involves efflux of K^+.[125] Under normal conditions, excessive activation of NMDARs in cultured cortical neurons triggers necrotic cell death, characterized by prominent, acute swelling of cell bodies, little or no DNA laddering, and insensitivity to protein synthesis inhibitors. In contrast, under conditions of reduced extracellular Na^+ and Ca^{2+}, activation of NMDARs in the same cells induces apoptotic cell death, characterized by cell shrinkage, nuclear condensation, internucleosomal fragmentation, and sensitivity to protein synthesis inhibitors. The last is particularly relevant in the presence of low extracellular Na^+ and Ca^{2+}, as is observed after brain ischemia in vivo.[55]

Calcium-Permeable AMPA Receptors

AMPARs mediate fast synaptic transmission at excitatory synapses and play important roles in synaptic remodeling, activity-dependent synaptic plasticity, and excitotoxic cell death. The GluR2 subunit governs the biophysical properties of AMPARs, including Ca^{2+} permeability,[126,127] voltage-dependent block by intracellular polyamines, single channel conductance, and activity-dependent AMPAR recycling and targeting to the synapse.[128] Thus, an acute change in the level of GluR2 expression would be expected to have profound effects on synaptic activity and neuronal survival. The relative expression of GluR2 in neurons is not static but is regulated in a cell-specific manner during development and is remodeled by activity, anti-psychotics, drugs of abuse and corticosteroids, and after seizures[129,130] or ischemic insult.[131]

Considerable evidence implicates GluR2-lacking AMPARs in the neuronal death associated with global ischemia.[131] AMPAR antagonists, but not NMDAR antagonists, protect against global ischemia–induced cell death, even when administered hours after the ischemic insult.[132–134] GluR2-lacking AMPARs are an important route of Ca^{2+} and Zn^{2+} entry into insulted neurons.[135] In adult brain, principal neurons of the hippocampus express high levels of GluR2 and exhibit relatively low Ca^{2+} influx via AMPARs. Injurious stimuli such as global ischemia, severe limbic seizures, and spinal cord injury trigger suppression of GluR2 messenger RNA (mRNA) and protein expression in vulnerable CA1 neurons in a subunit-specific and cell-specific manner before onset of cell death (Plate 42–6). In hippocampal slices, CA1 neurons with robust action potentials exhibit greatly enhanced AMPA-elicited Ca^{2+} rises before onset of cell death.[136] Excitatory postsynaptic currents (EPSCs) at Schaffer collateral–CA1 synapses exhibit an enhanced Ca^{2+}-dependent component that may be mediated by GluR2-lacking AMPARs and marked inward rectification at late times after ischemia.[134a] These findings suggest an important role for GluR2-lacking AMPARs in ischemia-induced neuronal death. Consistent with this concept, acute gene suppression of GluR2 by in vivo administration of antisense oligonucleotides, even in the absence of an ischemic insult, causes selective death of pyramidal neurons.[137] A number of these events are replicated in in vitro models of hypoxia-ischemia. In hippocampal neurons, OGD triggers downregulation of GluR2 mRNA expression and a rise in intracellular Ca^{2+} that is blocked by 1-naphthyl acetyl spermine (Naspm, a channel blocker selective for GluR2-lacking AMPARs).[138]

Kainate Receptors

Although little attention has focused on the role of kainate-type glutamate receptors in global ischemia–induced neuronal death, one study provides convincing evidence that these receptors are also critical players. The kainate-selective drug decahydroisoquinoline LY377770 (a novel, soluble, systemically active Glu5 antagonist) affords robust protection against global ischemia–induced and focal ischemia–induced cell death, even when administered after occlusion.[139] A mechanism by which LY377770 may contribute to neuronal survival is by preventing glutamate release in focal ischemia. These findings suggest that kainate receptors play a central role in ischemic brain damage. Because NMDAR antagonists have a number of side effects, including psychotomimetic effects in humans, increased glucose utilization, and morphologic changes in the rat cingulate cortex, non-NMDAR antagonists may be better candidates for clinical use.

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Calcium

The universal second messenger Ca^{2+} is a neuronal signaling molecule and critical player in ischemia-induced neuronal death. During the ischemic episode, CA1 pyramidal cells depolarize. Depolarization activates voltage-sensitive Ca^{2+} channels, which flux Ca^{2+} into the interior of cells, and triggers a massive release of synaptic glutamate. Synaptically released glutamate activates AMPARs and kainate receptors, which further depolarize the postsynaptic membrane, and NMDARs and GluR2-lacking AMPARs, which directly flux Ca^{2+} (as well as Na^{+}) into postsynaptic cells.[112,113] Glutamate also activates group I mGluRs. As already mentioned, group I mGluRs (mGluR1 and mGluR5) act via phospholipase C and $InsP_3$ to trigger release of Ca^{2+} from intracellular stores.[114] The massive rise in cytosolic Ca^{2+} causes cells to further depolarize and become inexcitable. Glutamate toxicity induces reverse operation of the Na^{+}-Ca^{2+} exchanger, exacerbating the buildup of Ca^{2+}.[104,115] At the same time, energy-dependent processes, such as presynaptic uptake of excitatory amino acids, are impeded, further contributing to the rise of glutamate in the extracellular space. Extracellular Ca^{2+} is depleted to less than 90% of its physiologic concentration. After reperfusion, Ca^{2+} homeostasis is restored; cells appear morphologically normal, exhibit normal intracellular Ca^{2+}, and regain the ability to generate action potentials for 24 to 72 hours after the insult. Ultimately, ambient glutamate elicits a late rise in intracellular Ca^{2+} and Zn^{2+} and death of CA1 neurons ensues, exhibiting hallmarks of apoptosis and necrosis.

Neuronal homeostasis requires that the intracellular concentration of Ca^{2+} be maintained in the range of 50 to 300 nM (or about four times lower than that of extracellular Ca^{2+}).[111] The massive rise in intracellular Ca^{2+} during the ischemic episode initiates a series of cytoplasmic and nuclear events that impairs cellular activity and damages tissue profoundly. High cytosolic Ca^{2+} activates Ca^{2+}-ATPase, which depletes the energy stores of the cell, and uncouples mitochondrial oxidative phosphorylation, which causes acute swelling of dendrites and cell bodies and subsequent cell death. Additionally, high Ca^{2+} activates Ca^{2+}-sensitive transcription factors, phospholipases, endonucleases, and proteases.[111] Proteases destroy cytoskeletal proteins such as actin and spectrin[140] as well as extracellular matrix proteins such as laminin.[141] High cytosolic Ca^{2+} also causes a derangement in signaling. A notable example is excessive activation of nitric oxide synthase, which promotes generation of free radicals. Free radicals destroy cell membranes by inhibition of critical membrane proteins, initiation of lipid peroxidation (see later),[115,142–145] damage of DNA, and induction of apoptosis.[115,146]

More severe insults elicit massive elevations in cytosolic Ca^{2+} and necrotic cell death,[115] and milder insults elicit less dramatic elevations in Ca^{2+} and may trigger apoptosis.[125] The "calcium set-point hypothesis" posits that whereas substantial elevations in cytosolic Ca^{2+} inhibit apoptosis,[147] lowering of cytosolic Ca^{2+} as, for example, by block or inactivation of voltage-sensitive Ca^{2+} channels, can induce apoptosis in otherwise healthy cells. Modest elevations in Ca^{2+} during the reperfusion period are implicated in the execution of apoptosis; possible targets of a late rise in Ca^{2+} include the cysteine proteases, such as caspase-3,[148] and endonucleases such as NUC18.[149]

Zinc

The transition metal Zn^{2+}, like Ca^{2+}, is a neuronal signaling molecule and critical player in ischemic cell death.[150] Zn^{2+} is present in cells throughout the body and serves as a tightly bound, functionally important component of many metalloenzymes and transcription factors. Free, chelatable Zn^{2+} is present at high concentrations in the presynaptic terminals of a subset of glutamatergic neurons and is particularly abundant in presynaptic vesicles. Zn^{2+} is colocalized with glutamate in a subset of vesicles in the presynaptic terminals of excitatory synapses of the neocortex layers I through III and V, hippocampus, subiculum, amygdala, thalamus, and striatum. Zn^{2+} is particularly abundant in vesicles at mossy fiber synapses of the hilar and CA3 region of the hippocampus (Plate 42–7*A*). Zn^{2+} is loaded into synaptic vesicles via a high-affinity transporter, Zn^{2+} transporter-3 (ZnT-3), which is highly expressed in hippocampus and cortex.[151] Zn^{2+} is released from nerve terminals in response to synaptic activity[152] or by exposure to high K^{+} or kainate.[153–155] Upon release from presynaptic terminals, Zn^{2+} modulates the functional activity of excitatory and inhibitory amino acid receptors, including NMDARs, AMPARs and gamma-aminobutyric acid A ($GABA_A$) receptors. Whereas Zn^{2+} blocks currents mediated by NMDARs and $GABA_A$ receptors, it potentiates currents mediated by AMPARs.[150]

The notion that Zn^{2+}, like glutamate, might be neurotoxic emerged from findings that perforant path stimulation releases Zn^{2+} and that Zn^{2+} damages postsynaptic target hilar interneurons and CA3 pyramidal cells both in vivo[156,157] and in vitro.[158] Exposure of cortical neurons in culture to 300 μM Zn^{2+} for 15 minutes or to 1 mM Zn^{2+} for 5 minutes kills virtually all neurons. Moreover, vulnerability to Zn^{2+} is substantially enhanced by concurrent membrane depolarization. Studies also show that Zn^{2+} is a critical mediator of neuronal death associated with global ischemic insults[159] and prolonged seizures.[160] At late times after global ischemia (48 to 72 hours) or after status epilepticus (16 to 24 hours), Zn^{2+} accumulates in degenerating hilar and selectively vulnerable hippocampal neurons (see Plate 42–7). Studies with Zn^{2+} indicator dyes show that toxic levels of Zn^{2+} may reach as high as 0.5 μM. Channels that mediate this Zn^{2+} influx into CA1 neurons include voltage-sensitive Ca^{2+} channels, the Na^{+}-Zn^{2+} antiporter (exchanger), NMDARs, and GluR2-lacking AMPARs.[150] Administration of the membrane-impermeant Zn^{2+} chelator ethylenediaminetetraacetic acid (EDTA) substantially reduces the rise in intracellular Zn^{2+} in CA1 and hilar neurons and affords robust neuroprotection.[159] These observations implicate Zn^{2+} as a critical mediator of the neuronal death associated with global ischemia. Unresolved issues are (1) the pattern of global ischemia–induced neuronal death does not parallel the distribution of synaptic Zn^{2+} and (2) Zn^{2+} translocation precedes neuronal death in rodents (and probably humans) by 48 to 72 hours.

Mechanisms by which Zn^{2+} induces neurotoxicity include production of free radicals, disruption of mito-

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chondrial function, including disruption of glycolysis and energy production and inhibition of respiration, and potentiation of AMPAR-mediated currents.[135] The ability of Zn^{2+} to shift excitotoxic injury from NMDAR-mediated injury and toward AMPAR-mediated injury is illustrated by preferential death of neurons with high NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) diaphorase. These neurons exhibit resistance to NMDA toxicity and high susceptibility to AMPA toxicity,[161] consistent with the presence of GluR2-lacking AMPARs.[162] Ultimately, Zn^{2+} induces apoptotic cell death, necrotic cell death, or both, depending on the intensity of exposure. Zn^{2+} may also play a role in the cell death associated with focal ischemia.

MECHANISMS OF ISCHEMIC CELL DEATH

Metabolic Stress

A hallmark of cells undergoing ischemia is energy depletion with altered energy dynamics. Neurons and glia have relatively high consumptions of oxygen and glucose and depend almost exclusively on oxidative phosphorylation for energy production. During the ischemic episode, impairment of cerebral blood flow restricts the delivery of substrates, particularly oxygen and glucose, and impairs the energetics required to maintain ionic gradients.[49] Anoxic depolarization triggers the release of synaptic glutamate, which acts via ionotropic and metabotropic receptors to induce a massive rise in cytosolic Ca^{2+} (see previous discussion) and a resultant loss of extracellular Ca^{2+}.[163] At the same time, energy-dependent processes, such as presynaptic uptake of excitatory amino acids, are impeded, further contributing to the rise of glutamate in the extracellular space. Glutamate toxicity induces reverse operation of the Na^+-Ca^{2+} exchanger, exacerbating the buildup of Ca^{2+}.[104,115] Na^+ and Ca^{2+} influx drives a massive efflux of K^+, which flows out of neurons via NMDARs, leading to a rise in extracellular K^+.[164,165]

In global ischemia, ATP is depleted in cells throughout the brain essentially immediately but recovers to near physiologic levels by the time of reperfusion.[17,69] Low ATP levels encourage cells to die by necrosis by inducing depolarization of neurons, leading to synaptic release of glutamate from neurons and reverse operation of glutamate transporters in astrocytes, swelling of cells (edema), and rupture of the plasma membrane.[166] Because ATP is required for formation of the apoptosome that activates the caspase death cascade, ATP depletion prevents caspase activation and shifts the balance of death cascades in favor of necrosis. Nevertheless, insults such as global ischemia ultimately induce apoptosis. Apoptosis culminates in activation of caspase-3, which catalyzes the cleavage and activation of downstream targets, including PARP-1. Excessive PARP-1 activation depletes the entire cell of its substrate NAD^+. NAD^+ depletion in mitochondria causes pronounced slowing of glycolysis, electron transport, and ATP formation, resulting in further energy failure and cell demise.

Focal ischemia elicits different patterns of metabolic changes in the core and penumbra. Within 1 to 3 minutes, cells in the core exhibit a dramatic decline in ATP[94,95] as well as anoxic depolarization, which triggers release of synaptic glutamate. Na^+ enters neurons via NMDARs, AMPARs, and other channels permeable to monovalent ions. K^+ flows out of cells via NMDARs. Water follows passively, driven by the influx of Na^+ and Cl^-, which greatly exceeds the efflux of K^+. At the same time, as cells lose energy, energy-requiring pumps that normally force ions into and out of the cell in an attempt to maintain a concentration gradient either fail or operate in reverse. These factors induce a rise in extracellular K^+[164,165] and a reduction in extracellular Ca^{2+}.[163,167] By 2 hours after temporary focal ischemia, extracellular K^+ is restored to a physiologic concentration.[164] The ensuing edema negatively affects perfusion of cells in the penumbra and affects more remote regions via long-range changes, including intracranial pressure, vascular compression, and herniation.

In contrast, cells in the penumbra experience a decline in energy[94] but do not exhibit anoxic depolarization or a rise in extracellular K^+.[94,95] In these cells, low ATP promotes necrosis by inducing failure or reverse operation of ion pumps, swelling of cells, and rupture of the plasma membrane.[166] ATP depletion inhibits formation of the apoptosome and tends to oppose caspase activation and the onset of apoptotic cell death. Nevertheless, apoptosis ensues in the penumbra. As in global ischemia, activated caspase-3 activates PARP-1, leading to NAD^+ depletion and further energy failure.

Mitochondrial Demise

Mitochondria house not only proteins involved in oxidative phosphorylation, but also pro-apoptotic proteins, including cytochrome c. Under physiologic conditions, cytochrome c is localized to the outer compartment of the mitochondria, where it serves as an electron carrier and participates in oxidative phosphorylation. Apoptotic (and necrotic) stimuli such as global ischemia disrupt the integrity of the outer mitochondrial membrane and cause the release of cytochrome c (see Plate 42–4). Upon release into the cytoplasm, cytochrome c forms the apoptosome (in a deoxy-ATP–mediated reaction), the signaling complex required for activation of caspase-9. Although the precise mechanisms by which the integrity of mitochondrial membrane breaks down are unknown, Bcl-2 family members are known to play a critical role.[75,168] For example, addition of pro-apoptotic Bcl-2 family members to purified mitochondria is sufficient to induce release of cytochrome c, and overexpression of anti-apoptotic Bcl-2 family members is sufficient to prevent it.

Many of the Bcl-2 family members are anchored in the outer membranes of mitochondria but oriented toward the cytosol. BAD, a pro-apoptotic member of this family, can heterodimerize with anti-apoptotic family members Bcl-2 and Bcl-x_L to initiate apoptosis. Injurious stimuli such as global ischemia induce sustained high cytoplasmic Ca^{2+}, which triggers activation of the serine-threonine phosphatase calcineurin.[169] Upon activation, calcineurin dephosphorylates BAD, promoting its release from cytosolic retention factor 14-3-3 and its translocation to the mitochondria, where it heterodimerizes with Bcl-x_L and initiates apoptosis.[169] Both apoptotic and necrotic stimuli

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are thought to converge upon Ca^{2+}-dependent destabilization of the mitochondrial membrane.[168] In contrast, growth factors and other pro-survival agents promote phosphorylation of BAD, impairing its binding to Bcl-x_L and abrogating its pro-apoptotic effect in cells. Ca^{2+}-dependent activation of calcineurin and dephosphorylation of BAD are thought to be critical to the change in mitochondrial membrane permeability and demise of mitochondria.[169]

How do Bcl-2 family members cause disruption of the outer mitochondrial membrane? One hypothesis is that Bcl-x_L, which is structurally similar to the pore-forming subunit of diphtheria toxin, might act by inserting into the outer mitochondrial membrane, where Bcl-2 family members could form channels or even large holes.[168] It is known that Bcl-2 family members insert into synthetic lipid bilayers, oligomerize, and form channels with discrete conductances. It is not clear, however, whether the holes are sufficiently large as to allow large proteins such as cytochrome c to pass through. In a second model, Bcl-2 family members would recruit other outer mitochondrial membrane proteins, such as voltage-dependent anion channel (VDAC), into forming a large pore channel. Such recruitment would likely involve a substantial conformational change in VDAC, which normally is permeable to much smaller molecules.

Nitric Oxide

The reactive gas NO is a diffusible signaling molecule present throughout cells of the body. In neurons, NO acts as an "aberrant transmitter," in that it is a small signaling molecule released by one cell that acts on another cell but is not released from vesicles and does not act via a classic membrane receptor.[170,171] NO is synthesized by nitric oxide synthetase nNOS, an enzyme that converts arginine to NO and citrulline. At excitatory synapses, nNOS is physically anchored to NMDARs in the postsynaptic membrane via PSD-95 (also known as synapse-associated protein-90, SAP-90). Activation of NMDARs triggers a rise in postsynaptic Ca^{2+}; intracellular Ca^{2+} binds calmodulin and rapidly activates nNOS, which synthesizes NO.[171,172] Thus, NO is an important downstream mediator of NMDARs and is critical to many forms of neuronal signaling and synaptic plasticity. Neuronal NOS is expressed in a small population of neurons throughout the CNS, with high densities in the accessory olfactory bulb and granule cells of the cerebellum.

NO is also implicated as an important downstream mediator of NMDA-induced excitotoxicity (see previous discussion). Injurious stimuli such as global and focal ischemia cause excessive stimulation of NMDARs and production of NO.[173] Some studies implicate the free radical form of NO and the superoxide anion ($\cdot O^{2-}$) in the oxidative damage of cellular DNA, lipid peroxidation (see later), and excitotoxic cell death.[174] NO reacts with the superoxide anion to form peroxynitrite, a cytotoxic oxidant that induces DNA damage (see Plate 42–4). DNA damage is a trigger of apoptotic cell death. Apoptosis culminates in activation of caspase-3, which catalyzes the cleavage and activation of downstream targets, including PARP-1. In addition to DNA damage, peroxynitrite contributes to cellular damage by several other mechanisms. It can nitrosylate cysteine residues in target proteins such as the NMDAR and impair NMDAR-mediated synaptic transmission. Peroxynitrite oxidizes and destroys other critical neuronal proteins, such as mitochondrial cytochrome c oxidase complex,[175] and reacts with unsaturated fatty acids in membranes to initiate lipid peroxidation (see later).

NO contributes to cell damage via several additional mechanisms. It interferes with superoxide dismutase, thereby reducing its antioxidant action, and complexes with non-heme iron present in the form of iron-sulfur clusters within enzymes critical to DNA replication and mitochondrial energy production. NO is thought to damage DNA by diffusing from the mitochondria and cytoplasm to the nucleus, where it is cleaved to form hydroxyl radicals or singlet oxygen. Important evidence that NO is a critical mediator of neuronal death comes from studies that show that mice that either have a knockout of nNOS or have been treated with 7-nitroindazole, an inhibitor of nNOS, are protected against neuronal death in experimental models of stroke.[176]

Free Radicals and Lipid Peroxidation

Neuronal insults and other types of environmental stress induce the formation of free radicals such as the superoxide anion, hydroxyl radical, singlet oxygen, radical nitric oxide, and oxidants such as peroxynitrite. These agents, collectively termed *reactive oxygen species* (ROS), are implicated as critical mediators of neuronal injury.[174,177,178] NMDAR-mediated Ca^{2+} influx promotes production of free radicals by stimulation of NO, which reacts with superoxide free radical ($\cdot O^{2-}$) to form peroxynitrite (see earlier discussion). NMDAR-mediated influx of Ca^{2+} also activates phospholipase A_2. Phospholipase A_2 liberates arachidonic acid, an unsaturated fatty acid, and promotes production of free radicals via activation of the lipoxygenase and cyclooxygenase pathways.[179] Cyclooxygenase catalyzes the addition of two molecules of O_2 to arachidonic acid to produce prostaglandin PGG_2, which is rapidly peroxidized to PGH_2 with concomitant release of superoxide anion.[180] Metabolism of free arachidonic acid is thought to be a major source of superoxide anion. Zn^{2+} influx via GluR2-lacking AMPARs also promotes production of free radicals, such as mitochondrial superoxide, in injured neurons.[181]

Short exposure (10 to 90 minutes) to free radicals damages protein, lipids, and nucleic acids. In neurons, free radicals inactivate and damage critical membrane proteins such as Na^+ and Ca^{2+} pumps, creatine kinase and mitochondrial dehydrogenases, and promote oxidation of the Na^+-K^+-ATPase exchanger, rendering it susceptible to calpain-mediated proteolysis.[182] Free radicals damage proteins by oxidation of side chains and modification of disulfide bonds. Free radicals also inactivate and damage nucleic acids. Free radicals effect oxidative damage by causing single- and double-stranded breaks in DNA, chemically modifying nucleic acid bases, breaking the glycosylic bond between ribose and individual bases and crosslinking of protein to DNA strands.[174] If not repaired, such oxidative lesions disrupt nucleic acid elongation, alter the coding of DNA, or both, thus impairing DNA replication and transcription and contributing to the demise of cells.[183]

Free radicals also oxidize and damage unsaturated fatty acids. *Lipid peroxidation*, the oxidative deterioration of

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membrane unsaturated fatty acids, is caused by reaction of free radicals with unsaturated bonds in the side chains of polyunsaturated fatty acids.[184,185] These reactions spark a chain reaction leading to formation of peroxides, hydroperoxides, and aldehydes. Although superoxide anion is not itself a potent oxidizer, it promotes oxidation of ferric ion and release of ferrous iron from ferritin.[186] In the presence of transition metals such as copper and ferrous iron, these chain reactions can expand geometrically. Glial cells in the brain have abundant stores of oxidized iron,[187] mostly in the form of ferritin and transferrin.[117] Ultrastructural studies show that excessive generation of oxygen radicals, followed by lipid peroxidation, accelerates the structural damage of neurons.[188] Lipid peroxidation compromises the integrity of the neuronal plasma membrane by altering membrane permeability and fluidity and allowing ions such as Ca^{2+} to leak into the cell. Disruption of the membrane compromises the function of receptors, channels, transporters, and ion exchangers, adding further to the demise of injured cells. Specific populations of neurons are thought to be especially vulnerable to free radical–induced damage during reperfusion, either because they are deficient in glutathione peroxidase or because they are surrounded by iron-laden supporting cells that release iron during and after ischemia.

Transcription Factors

Injurious stimuli such as ischemia trigger a number of transcriptional pathways. Candidate transcription factors that are thought to direct programs of gene expression changes after global ischemia include (1) CREB and nuclear factor kappa B (NF-κB), which direct pro-survival programs, and (2) the Forkhead family of transcription factors and REST/NRSF, which direct pro-death pathways in adult neurons.

CREB is a stimulus-induced transcription factor that activates transcription of pro-survival (and pro-adaptive) target genes in response to a wide array of external stimuli, including NMDAR-mediated Ca^{2+} influx at synaptic sites.[121] Immediate early genes (IEGs), such as c-fos,[189] Bcl-2, IAPs, nNOS,[190] and BDNF,[191] are important to neuronal survival and are gene targets of CREB.[121] Upon activation, CREB plays an important role in promoting neuronal survival and adaptation in response to environmental cues. Consistent with this notion, targeted deletion of the genes encoding CREB and the cAMP-response element modulator (CREM) in neurons of the developing CNS elicits apoptosis. Postnatal ablation of CREB and CREM results in progressive neuronal degeneration in the adult brain.[121]

A member of the leucine-zipper superfamily of transcription factors, CREB is activated in response to external stimuli that activate intracellular signaling cascades, culminating in phosphorylation of CREB. Upon phosphorylation, CREB forms a functionally active dimer that binds the *cis*-acting CRE within the promoters of target genes.[121] As already mentioned, Hardingham and colleagues[120] have found that the location of Ca^{2+} signaling in neurons critically controls CREB activity. Ca^{2+} influx via synaptic NMDARs promotes the activation of Ca2+-calmodulin–dependent kinase IV (CaMKIV) and the Ras/mitogen–activated protein kinase (MAPK) pathway, which phosphorylates and activates the MAPK-activated kinases, the pp90 ribosomal S6 kinase (Rsk) family members. Rsks regulate gene expression by phosphorylating transcription factors such as CREB. These kinases act in a coordinated manner to induce robust, sustained phosphorylation of Ser133 and activation of CREB.[120,121] Whereas CaMKIV mediates the early phase of Ser133 phosphorylation, the MAPK pathway mediates prolonged Ser133 phosphorylation.[192] In addition, CaMKIV phosphorylates and activates the coadaptor protein CBP at Ser301. Upon activation, CREB recruits phosphorylated CBP to the promoters of CREB target genes, and the coactivator complex induces gene transcription. Considerable evidence indicates that phosphorylation of Ser142 and Ser143 also contributes to CREB activation, but inhibits the interaction of CREB with CBP.[192]

Although influx of Ca^{2+} via synaptic NMDARs induces activation of CREB and neuronal survival, influx of Ca^{2+} via extrasynaptic NMDARs elicits CREB shutoff.[122–124] At extrasynaptic sites, Ca^{2+} influx via NMDARs activates protein phosphatase 1 (PP1) and PP2A, which dephosphorylate and inactivate CREB. Interestingly, contemporaneous activation of synaptic and extrasynaptic NMDARs by bath-applied NMDA also shuts off CREB, suggesting that extrasynaptic NMDARs act via a dominant cell-death signal. Ca^{2+} influx via extrasynaptic NMDARs (and CREB shutdown) causes mitochondrial dysfunction, ATP depletion, and neuronal death.

NF-κB is expressed in nearly all mammalian cells. Under physiologic (resting) conditions, NF-κB exists as an inactive form composed of the transcription factor dimer and IκB (inhibitor of NF-κB) protein, which maintains NF-κB in an inactive form.[193] NF-κB is activated in response to a diverse range of external stimuli, including the cytokine TNF-α, neurotrophic factors such as nerve growth factor (NGF), neurotransmitters, cell adhesion molecules, and various types of stress. These stimuli activate NF-κB by phosphorylation and proteosomal degradation of IκB, releasing the active, dimeric NF-κB. Upon activation, NF-κB translocates to the nucleus, where it binds to upstream regulatory elements in κB-responsive genes. These include the Ca^{2+}-binding protein calbindin, cytokines such as TNF-α and interleukin-2β (IL-β2) the antioxidant enzyme manganese superoxide dismutase (MnSOD), the anti-apoptotic proteins Bcl-x_L and Bcl-2, the IAPs, and BDNF.[193] Upon activation, NF-κB plays an important role in regulating cell survival and synaptic plasticity in neurons. Focal ischemia activates NF-κB and relocalizes it to the nucleus of ischemic neurons, where it binds target genes.[194] Targeted deletion of NF-κB significantly reduces ischemic damage, suggesting a cell death–promoting role of NF-κB in focal ischemia.[194]

As already mentioned, Forkhead1 (FOXO-3A or FKHRL-1), a member of the Forkhead family of transcription factors (FOXO-3A, FOXO-1, and AFX), induces expression of pro-apoptotic target genes.[76,195]). Under physiologic conditions, FOXO-3A resides in the cytosol away from target genes and is thus inactive. The neurotrophins (NGF, BDNF) and insulin-like growth factor-1 (IGF-I) act via the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway to promote FOXO-3A

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phosphorylation.[196]). PI3K promotes neuronal survival by phosphorylation and activation of the serine-threonine kinase Akt. Akt promotes cell survival by phosphorylation and inactivation of target genes implicated in apoptotic cell death.[196] FOXO-3A is a critical target of Akt phosphorylation and inactivation.[197] Phosphorylation of FOXO-3A promotes its binding to the retention factor 14-3-3, which retains FOXO-3A in the cytoplasm, away from target genes such as FasL (Plate 42–8).[196]

Injurious stimuli trigger FOXO-3A dephosphorylation and nuclear translocation. Putative targets of FOXO-3A are the death cytokines FasL, TNF-α, and TRAIL (TNF-related apoptosis–inducing ligand) and their cognate death receptors, which are members of the TNF family of death receptors.[198] The death cytokines act via the extrinsic or death receptor–mediated pathway to initiate the caspase death cascade. Global ischemia triggers expression of FasL, which is implicated in the extrinsic or death-receptor pathway of caspase activation.[198] FasL, by binding to its cognate death receptor Fas, recruits caspase-8, which initiates the caspase death cascade.

The gene silencing transcription factor REST/NRSF is widely expressed during embryogenesis and plays a strategic role in terminal neuronal differentiation.[199,200] In neural progenitor cells and nonneural cells, REST actively represses a large array of neural-specific genes important to synaptic plasticity and synaptic remodeling, including synaptic vesicle proteins, structural proteins, voltage-sensitive ion channels, and neurotransmitter receptors.[201,202] Examples are synapsin I, superior cervical ganglion (SCG)—the protein also known as "SCG10", nicotinic acetyl choline receptor, muscarinic acetylcholine m4 receptor, μ-opioid receptor, the neuronal nicotinic acetylcholine receptor subunit β2, and the AMPAR subunit GluR2. As neural progenitors differentiate and migrate out of the ventricular zone, REST downregulation is essential for induction and maintenance of the neural phenotype. Perturbation of REST expression during embryogenesis causes cellular apoptosis, aberrant differentiation and patterning, and embryonic lethality.[203]

REST is a member of the Gli-Krüppel family of zinc-finger transcriptional repressors; it contains nine non-canonical zinc-finger motifs through which it binds the *cis*-acting RE1 (neuronal repressor element) within the promoter region of target genes.[201] REST associates with the co-repressors Sin3A and coREST, which in turn recruit histone deacetylase (HDAC) to the promoters of target genes.[204] The co-repressor complex silences gene transcription by deacetylation of core histone proteins and tightening of the core chromatin complex, thus restricting access of the transcription machinery required for gene activation.[201] Chromatin remodeling is a universal mechanism of transcriptional repression and is implicated in other histone-modulated processes, including DNA replication, recombination, and repair.

Dysregulation of REST and its target genes is implicated in the pathogenesis of Down's syndrome,[205] Alzheimer's disease,[206] a subset of medulloblastoma cells,[207] and global ischemia.[208] Global ischemia triggers a pronounced upregulation of REST mRNA and protein in selectively vulnerable CA1 neurons (Plate 42–9). Consistent with induction of REST, core histone proteins over the GluR2 promoter exhibit pronounced deacetylation, indicative of reduced GluR2 promoter activity, and GluR2 mRNA and protein expression are suppressed in CA1 neurons. Because the GluR2 subunit governs AMPAR Ca^{2+} permeability and AMPARs are implicated in the excitotoxic death associated with global ischemia, these changes are expected to affect neuronal survival. Consistent with this concept, acute knockdown (suppression) of the REST gene by administration of antisense oligonucleotides directed to the REST mRNA administration rescues neurons from ischemic death. These findings suggest a causal relation between REST induction and neuronal death and implicate REST-dependent gene silencing and chromatin remodeling in transcriptional repression of GluR2 in neurons subjected to insult.

Inflammation

Considerable evidence indicates that inflammation exacerbates ischemic injury.[209,210] Ischemia-hypoxia triggers activation of transcription factors such as NF-κB, hypoxia-inducible factor-1 (HIF-1), interferon regulatory factor-1, and signal transducers and activators of transcription (STATs) STAT3. These, in turn, orchestrate expression of an array of proinflammatory target genes, such as platelet-activating factor and the cytokines TNF-α and IL-1β.[211] Cytokines are critical mediators of inflammation and are expressed within the first 2 hours after onset of ischemia. The first inflammatory response is the unleashing of resident immune cells such as microglia. Microglia become activated and exhibit characteristic ameboid morphology, owing to retraction of their processes by 24 hours.

Subsequently, invasion and infiltration of nonresident cells occur. Ischemia-hypoxia triggers expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), P-selectins, and E-selectins, by endothelial cells on their luminal surfaces. The adhesion molecules interact with cognate receptors on neutrophils, guiding their migration across the vascular wall and inside the brain parenchyma to the site of injury. Neutrophils are present in high numbers in ischemic brain by 24 to 48 hours. Infiltration of lymphocytes, macrophages, and monocytes follows next. Lymphocytes release inflammatory cytokines such as TNF-α, which triggers production of chemokines such as IL-8 and monocyte chemoattractant protein-1[212] and thereby initiates the inflammatory reaction. Chemokines are a family of small, soluble adhesion molecules that rapidly recruit leukocytes (blood-borne inflammatory cells) from the circulation across the endothelial barrier to the site of injury by promoting their adhesion and chemotaxis. Macrophages appear in large quantity between 1 and 5 days after MCAO; by 5 to 7 days, they become the predominant cell type in the injured region, where they phagocytose dead cells. The triggering of inflammatory cascades and release of cytokines is thought to cause astrocyte death and exacerbate neuronal death.

Critical evidence that inflammatory responses are involved in the pathogenesis of ischemia-induced neuronal death comes from studies that show that ischemic injury is attenuated by preischemic induction of systemic neutropenia, pharmacologic block of adhesion molecules or their receptors, deletion of the ICAM-1 gene, anti-inflammatory steroids or antibody block of inflammatory mediators such as IL-1β or the transcription factor interferon regulatory

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factor-1.[210] Mounting evidence indicates that, in addition to its role as perpetrator of neuronal death, NO is a catalyst for microglial activation (see earlier discussion).

MECHANISMS OF NEUROPROTECTION

Immediate Early Genes

IEGs are early response genes that are coordinately activated (dynamically regulated) in response to neuronal activity and neuronal insults. Whereas some IEGs function as a network of constitutively expressed (and coordinately regulated) proteins that are upregulated in response to activity or insult, other IEGs are activated only in response to external stresses (stimuli). As such, IEGs are markers of neural activity. A striking feature of the IEG response is the broad functional repertoire of the molecules. These include growth factors, transcription factors, enzymes that synthesize neurotransmitters, synaptic vesicle proteins, and ion channel and structural proteins (scaffolding, adaptor, and cytoskeletal proteins). Examples are the proinflammation gene cyclooxygenase-2 (Cox-2), an enzyme implicated in synthesis of prostaglandins; neuronal activity-regulated pentraxin (Narp), implicated in the aggregation of AMPARs at excitatory synapses; activity-regulated cytoskeleton-associated protein (Arc), a component of the synaptic junctional complex; Homer, an adaptor protein that binds mGluRs via their carboxy-terminal tails and localizes them to excitatory synapses; and c-fos. A transcription factor, c-fos forms a heterodimer with a member of the Jun family of transcription factors and binds to the activator protein-1 (AP-1) promoter element to regulate gene expression. More than half of IEGs are transcription factors that act collectively to orchestrate expression of delayed response genes involved in neuronal plasticity.

Neuronal insults such as ischemia trigger activation of CREB, which in turn drives transcription of IEGs. Within minutes of ischemia, IEGs such as c-fos, c-jun, and zif268 are expressed in the entire hemisphere ipsilateral to the occluded MCA.[209] Focal ischemia also induces expression of nerve growth factors I-A, IB, and I-C, NF-κB, Nurr-1-activating transcription factor, and erg-2 and erg-3 within minutes of the ischemic episode.[213] Eng 1 is a member of the ether-a-gogo-related genes (ERG) family which encodes K^+ channel subunits. During the inflammation response, cytokines induce expression of proinflammatory genes such as cox-2. Global ischemia induces expression of a number of stress genes, including the IEGs c-fos, c-jun, junB, knox, and zif268, within the first 30 minutes. In rats and gerbils, global ischemia induces IEG expression throughout the hippocampus, including vulnerable CA1 neurons.[214,215] Thus, whether or not IEGs play a protective role in ischemia is unclear.[213]

Heat Shock Protein and Stress Genes

Heat shock proteins (HSPs) are a highly conserved family of molecular chaperone proteins that play a role in the aggregation, assembly, transport, and folding of proteins. Under physiologic conditions, HSPs are critical to cell growth and maintenance and are thought to play a role in neuronal signaling, differentiation, and migration. Harmful stresses such as ischemia trigger expression (or upregulation) of HSPs, which act collectively to sustain survival by limiting cellular damage and accelerate recovery by rescuing denatured proteins from degradation (the "stress response."[216] Upon activation, HSPs act to prevent aggregation of denatured proteins and aid in their refolding to the correct tertiary structure.

HSPs are typically classified according to their molecular weight. Examples are HSP10, HSP27, HSP32, HSP47, HSP60, HSP70/72, HSP90, HSP100/105, and ubiquitin. Some heat shock proteins, such as HSP60 and HSP90, are constitutively expressed and associated with specific intracellular organelles. HSP70 and Hsc70 are cytosolic. HSP75 is associated with mitochondria, and the glucose-regulated protein (GRP78) gene with the endoplasmic reticulum. Others, such as HSP27 and HSP70, are rapidly induced in response to cellular stresses (see later).

HSPs are endowed with a modular structure that facilitates their regulatory actions on protein translocation, import, and folding.[216] HSPs act via a carboxy-terminal peptide-binding domain to bind stretches of hydrophobic residues exposed in unfolded proteins. The energy for these interactions is provided by an amino-terminal ATPase domain, which binds and hydrolyzes ATP. HSPs are a target of the serine-threonine kinase Akt, which phosphorylates and activates them by promoting trimerization. Trimeric HSPs binds to heat-shock elements (HSEs) within the promoter regions of target genes and stimulate transcription of pro-survival genes.

Injurious stimuli such as hyperthermia, oxidative stress, radiation, and ischemia can engage either or both of two fundamental responses, apoptosis and the HSP stress response, which functions to sustain survival by limiting cellular damage and accelerating recovery. Research indicates that a fine balance between these two opposing pathways may determine cellular susceptibility to damaging stresses.[217] Ischemia-hypoxia triggers pronounced upregulation of HSPs such as HSP70.[217,218–221] Focal ischemia induces a dramatic upregulation of HSP70 protein expression in cells of the ischemic penumbra but not in the core.[222,223] The induction of HSPs in the penumbra is correlated with tolerance to subsequent ischemic insults. Global ischemia triggers a massive upregulation of HSP70 mRNA and protein expression, although the cellular specificity of expression and its relation to neuronal vulnerability are unclear.[213] Thus, whether or not HSP70 is a major determinant of neuronal death associated with global ischemia remains unsettled.[213]

Evidence shows that HSP27, HSP70, and HSP90 exert their cytoprotective effects by halting the self-amplifying caspase death cascade and preventing apoptosis.[217] Heat shock proteins 70, 90, and 27 each possess the ability to prevent caspase-3 processing and activation. HSP70 and HSP90 interact with Apaf-1 to prevent the recruitment of caspase-9 to the apoptosome by directly associating with Apaf-1 and preventing its oligomerization (see Plate 42–4). HSP27 binds and sequesters cytosolic cytochrome c away from its target, Apaf-1.[224–226] Consistent with its presumptive role in neuroprotection, overexpression of HSP70 provides efficient protection against cell death triggered by harmful stresses. Transgenic mice that overexpress HSP70 exhibit significantly reduced infarct volumes compared with wild-type mice in models of focal ischemia,[227] and

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HSP70.1 knockout mice exhibit significantly greater infarct volumes than wild-type mice.[228] HSP72 overexpression is neuroprotective, even when implemented after the onset of global ischemia.[229]

Spreading Depression

Cortical spreading depression (CSD) is a wave of sustained depolarization (neuronal inactivation) moving through intact brain tissue and associated with brain ischemia, migraine aura, and seizures.[230] Spreading depression elicits a temporary, but major, redistribution of ions between intracellular and extracellular compartments without causing irreversible damage. The ion redistribution becomes clinically significant under conditions of impaired brain metabolism, such as global or focal ischemia. Injurious stimuli such as hypoxia trigger spreading depression. As cells lose energy, energy-requiring pumps that normally force ions into and out of the cell in an attempt to maintain a concentration gradient either fail or operate in reverse. As previously described, these factors induce a rapid efflux of K^+, which flows out of neurons via NMDARs and a rise in extracellular K^+. The rapid rise in extracellular K^+ elicits neuronal excitation, followed by excessive depolarization and a period of electrical silence during which the potential at the brain surface becomes negative. Ca^{2+} ions flow in as the depolarization opens voltage-dependent Ca^{2+} channels and extracellular Ca^{2+} falls to abnormally low levels. Na^+ and Cl^- enter neurons. Water follows passively, driven by the influx of Na^+ and Cl^-, which greatly exceeds the efflux of K^+. The extracellular space is reduced, and edema ensues. Critical evidence that CSD is NMDAR dependent comes from studies that show that NMDAR antagonists block spreading depression completely in human neocortical tissue and delay the onset of spreading depression in rat hippocampus.[230]

Astrocytes are thought to play a critical role in the energy-dependent restitution of ion gradients that restores normal neuronal activity. Astrocytes actively take up glutamate during normal neuronal activity. They are coupled via gap junctions, which mediate spatial buffering of K^+. Cooperation between glia and neurons maintains normal extracellular ion and transmitter levels during neuronal activity. Under conditions of spreading depression, glutamate transporters operate in reverse and astrocytes release glutamate, prolonging the spreading depression. Ion fluxes are enhanced and highly synchronized. Astrocytic gap junctions are implicated in the synchronization of neuronal firing and propagation of spreading depression. Consistent with this notion, gap junction blockers prevent the formation and propagation of CSD, and astrocytes cultured from Cx43 knockout mice exhibit slow propagation of Ca^{2+} waves.[230] Curiously (and inexplicably), mice with a conditional Cx43 knockout in brain exhibit accelerated propagation of spreading depression.[231]

Growth Factors and Neurotrophins

Growth factor–induced signal transduction proceeds via a cascade of protein phosphorylation events that serve to relay environmental cues into cellular responses. These events ultimately culminate in activation of multiple nuclear transcription factors with a diverse range of target genes, many of which are involved in orchestrating cell survival and proliferation. Growth factors can initiate signaling via either the PI3K/Akt (protein kinase B) pathway, the Ras/MAPK signaling pathway, or both.[197]

The PI3K/Akt Pathway

Glutamate receptor activation can elicit the production and release of BDNF, which can signal through activation of the PI3K/Akt or Ras/MAPK pathway. The survival-promoting effects of neurotrophins (NGF, BDNF) and other growth factors (IGF-I) are executed, at least in part, through the PI3K/Akt pathway.[232–236] PI3K enzymes reside in the cytosol and catalyze the formation of the lipid 3′-phophorylated phosphoinositides, which regulate the localization and activity of a key component in neuronal survival, the serine-threonine kinase Akt. PI3K acts via 3′-phosphoinositol to phosphorylate and activate the serine-threonine kinase Akt (Plate 42–10). Upon activation, Akt is translocated to the nucleus, where it exerts its actions on gene expression. Akt promotes cell survival by suppressing genes implicated in apoptotic cell death.[196] In each case, Akt phosphorylates and thereby inactivates its target. Targets of Akt include the pro-apoptotic protein BAD (an inhibitor of Bcl-2), pro-caspase-9, which is processed to generate caspase-9 (an initiator of the caspase death cascade), and the transcription factor Forkhead-1 (FOXO-3A) (see Plate 42–8).[196,197]

Akt is uniquely endowed with a modular structure that enables it to translocate to the nucleus and phosphorylate target proteins in response to external stimuli. In addition to a centrally located kinase domain, Akt has an *N*-terminal pleckstrin homology domain, which mediates its interactions with proteins and phospholipids. Upon binding to lipids, Akt is translocated from the cytoplasm to the inner surface of the plasma membrane, which brings the kinase into close proximity with its activators. PI3K is also regulated by phospholipids. Thus, the lipid products generated by PI3K enzymes control the activity of Akt by regulating its location and activation. The ability of neurotrophins to promote neuronal survival requires functional PI3K/Akt signaling in the cell body and in distal axons that are in contact with the dendrites of target neurons. Critical evidence that Akt mediates neuronal survival comes from studies that show that Akt supports neuronal survival even in the absence of trophic factors and that a dominant mutation of Akt inhibits neuronal survival even in the presence of survival factors.[237]

Ras/MAPK Pathway

Neurotrophins and other growth factors also promote cell survival via the Ras/MAPK signaling pathway. The small guanosine triphosphate–binding protein Ras is a mitogen and key mediator of growth factor–dependent cell survival. Growth factors act via their cognate cell surface receptors to activate the MAPK signaling pathway, which entails a series of sequential phosphorylation events leading ultimately to phosphorylation of the MAPK-activated kinases known as Rsks.[238] The Rsks promote cell survival by a dual mechanism; they phosphorylate and inactivate the pro-apoptotic factor BAD and phosphorylate and potently activate the transcription factor CREB, which promotes

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transcription of pro-survival genes.[239] BDNF, a neurotrophin, acts via the Ras/MAPK pathway and extracellular signal–regulated kinases 1 and 2 to induce neuroprotection in an in vivo model of neonatal hypoxic-ischemic brain injury.[240]

In the CNS, the best-characterized targets of growth factor signaling are the extracellular signal–regulated kinases 1 and 2 (ERK1 and ERK2). ERK phosphorylates and activates nuclear transcription factors such as nuclear transcription factor ELK-1 and CREB. Other ERK targets are cytoskeletal proteins, cell adhesion molecules, ion channels, and transcription factors. CREB stimulates cell survival directly by activating transcription of bcl-2. In addition, CREB stimulates transcription of immediate early response genes, which in turn induce the delayed response genes that influence neuronal activity, including growth factors, enzymes that synthesize neurotransmitters, synaptic vesicle proteins, and ion channel and structural proteins. Thus, although there is a divergence in the survival signaling pathways downstream of neurotrophin receptors, both the PI3K and MAPK pathways converge on the same set of proteins, BAD and CREB, to inhibit apoptosis.

Interneurons

GABA-releasing inhibitory interneurons (or "local circuit" neurons) of the hippocampus and cortex are also thought to contribute to the selective spatial patterns of neuronal death in brain ischemia. Whereas pyramidal (or principal) neurons of the hippocampal CA1 die because of global ischemia, inhibitory interneurons of the CA1 (and other subfields) survive. Inhibitory interneurons are typically characterized by axons that make short-range projections and release GABA onto their targets.[241] Interneurons comprise a diverse array of anatomical and neurochemical subtypes. Interneurons have typically been characterized according to their neurochemical content (by calcium-binding proteins such as parvalbumin [PV], calbindin, and calretinin or by neuromodulator-transmitters such as somatostatin, neuropeptide Y, and nNOS). Many PV-positive interneurons are classified anatomically as basket cells or interneurons that send their axons to the cell body of the postsynaptic cell, surrounding it with a structure akin to a basket. Many somatostatin-positive interneurons are anatomically characterized as chandelier cells, a sub-class of inhibitory interneurons whose morphology resembles a chandelier, but there are many exceptions to the rule. Yet a third classification is by action potential firing patterns—for example, fast-spiking cells, low-threshold cells, and regular-spiking cells. Global ischemia kills pyramidal neurons of the hippocampal CA1, but interneurons in the CA1 survive. Studies have provided evidence for electrical coupling between like interneurons mediated via gap junctions.[242]

Gap junctions are conductive channels that connect the interiors of coupled cells.[242] Their large internal diameters (≈1.2 nm) allow the exchange of small ions and intracellular signaling molecules between neighboring cells. As a result, gap junctions synchronize activity of coupled cells and are thought to play an important role in intercellular signaling in brain development, morphogenesis, and pattern formation.[243–246] Gap junctions are composed of *connexins*, integral membrane proteins encoded by a gene family of at least 20 structurally related members in mammals. Although connexins share sequence similarity and a common membrane topology, they assemble to form channels that differ in gating and permeability properties and in temporal and spatial patterns of expression. Three gap junctional proteins, Cx32, Cx36, and Cx43, are expressed abundantly in mammalian brain but with differing cellular specificity. Whereas Cx43 is the most abundant connexin expressed by astrocytes, Cx32 is expressed predominantly in oligodendrocytes[247,248] and interneurons; Cx36 protein expression is neuron-specific.[249,250]

In CA1, PV-positive interneurons form a vast dendrodendritic network extending many hundreds of microns and connected by anatomically identified electrical and mixed electrical-chemical synapses. This network of GABA-ergic interneurons is a candidate for the generator of synchronized oscillations in hippocampus. Electrophysiologic evidence indicates the presence of electrical coupling between GABA-ergic interneurons in the hippocampus and in visual cortex.[251,252] Reverse transcription–polymerase chain reaction (RT-PCR) studies indicate that electrical coupling between interneurons in these regions is likely to be mediated by Cx36 (and possibly Cx32), which exhibits high expression in bipolar interneurons in layers 2 and 3 of visual cortex, interneurons, and spiny stellate cells, and in layer 4 of barrel cortex and basket cells in DG.[252] Electrical coupling between interneurons is thought to mediate synchronous firing and thereby promote inhibitory transmission. These observations raise the possibility that Cx36 or Cx32 gap junction or both might play a role in the survival of hippocampal interneurons or the death of pyramidal neurons after ischemia.

A 2001 study[252a] indicates that global ischemia induces a selective upregulation of Cx36 (and Cx32) protein expression in PV-positive inhibitory interneurons in the vulnerable CA1 before the onset of neuronal death, consistent with a role in the survival of GABA-ergic interneurons.[253] Moreover, transgenic Cx32 null–mutant mice exhibit enhanced vulnerability to global ischemia–induced neuronal death. These findings provide a basis for understanding a role for neuronal gap junctions in defining cell-specific patterns of neuronal injury after global ischemia. Because global ischemia targets pyramidal neurons of the CA1 while sparing inhibitory interneurons, the findings just enumerated suggest the novel possibility that enhanced expression of Cx36 may play a critical role in the protection and survival of CA1 interneurons after global ischemia. One possibility is that coupling of inhibitory interneurons promotes their survival by mediating intercellular metabolic cooperation. Another possibility is that enhanced coupling of inhibitory interneurons represents a failed attempt of such interneurons to rescue CA1 neurons. The greater vulnerability of CA1 pyramidal cells in Cx32 knockout mice is consistent with observations that neocortical cells in these mice display enhanced intrinsic excitability and prolonged paroxysmal depolarizations, indicating dysfunction of inhibitory synaptic transmission.[253] Moreover, late-depolarizing glutamatergic excitatory postsynaptic potentials are enhanced, indicating reduced inhibitory input. Enhanced excitatory transmission and deficient inhibitory transmission would be

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expected to increase pyramidal cell excitability and enhance vulnerability to excitotoxic cell death.

Astrocytes

Astrocytes are abundant glia (nonneural cells) present throughout the brain, where they are positioned in close association with neurons.[254,255] Astrocytes can enwrap synapses and play a critical role in transmitter uptake and release during normal neuronal activity. In the hippocampus, 57% of the synapses are associated with the process of an astrocyte. Astrocytes also have extensive contacts with endothelial cells from capillaries. Thus, astrocytes are well positioned to mediate signaling between neurons, between neurons and astrocytes, and between neurons and capillaries. Astrocytes respond to a variety of synaptically released transmitters, including glutamate, noradrenaline, histamine, acetylcholine, ATP and GABA. Application of any of these transmitters to astrocytes elicits sustained or oscillating elevations of intracellular Ca^{2+} concentration in the astrocytes. Ligands that evoke Ca^{2+} elevations in astrocytes can cause the release of glutamate from astrocytes in a Ca^{2+}-dependent manner. The three mechanisms that have been proposed to mediate the Ca^{2+}-dependent release of glutamate from astrocytes are reverse operation of glutamate transporters, an anion-channel dependent pathway induced by swelling, and Ca^{2+}-dependent exocytosis. Mounting evidence supports exocytosis as the key mechanism mediating glutamate release.

Astrocytes are thought to play a critical role in the neuronal death associated with focal ischemia.[254–256] Swelling of astrocytes contributes to infarct size in focal ischemia. Four to 6 hours after focal ischemia, astrocytes become hypertrophic and displace a greater volume. Both anti-excitotoxic and anti-apoptotic interventions reduce infarct volume, not just neuronal cell death, in animal models of focal ischemia. Astrocyte death may occur as a secondary consequence of neuronal death, possibly as a result of release of cytokines and the triggering of inflammatory cascades. In animal models of global ischemia, astrocytes survive and play an important role in reactive gliosis after neuronal loss.

Evidence supports a role for interastrocytic gap junctions in the spread of secondary injury associated with focal ischemia.[257–259] Astrocytic gap junctions remain functional in postischemic brain, and gap junction blockers limit the secondary expansion of infarcts after focal ischemia. In cultures, dying glia propagate cell death to healthy bystanders in proportion to their expression of gap junctions and functional coupling. Presumably, the "metabolic cooperation" mediated by gap junctions between healthy and dying cells provides too great a metabolic stress for the healthy cells. These findings suggest that astrocytic gap junctions might propagate and amplify neuronal death after global ischemia as well, and cooperativity in death of CA1 neurons after global ischemia has been proposed. Coupling of astrocytes can also attenuate neuronal death in some paradigms and appears to play a neuroprotective role in helping neurons to maintain Ca^{2+} homeostasis in the presence of oxidative stress. Administration of uncouplers of gap junctions, such as 18α-glycyrrhetinic acid (AGA), to slice cultures exacerbates neuronal death induced by oxidative stress.

A number of mechanisms have been proposed to explain the neuroprotective role of coupling between astrocytes; they include (1) enhanced synthesis and release of protective signaling molecules from astrocytes, (2) reduced synthesis and release of neurotoxic substances, (3) enhanced removal of glutamate from the intercellular space, and (4) spatial buffering of K^+. Astrocytes synthesize and release neurotrophic factors and cytokines, such as nerve growth factor, TNF-α, and basic fibroblast growth factor,[260] and are rich in the antioxidant enzyme catalase, which may increase survival after global ischemia.

NEUROPROTECTIVE STRATEGIES

Ischemic Tolerance

Ischemic tolerance is a well-known phenomenon in which brief ischemic insults (ischemic preconditioning) confer robust neuroprotection to hippocampal CA1 neurons against a subsequent severe ischemic challenge.[240,261–266] Ischemic tolerance can also be induced in vivo by spreading depression, hypoxia, and activation of A1 adenosine and inhibitors of oxidative phosphorylation and in vitro by exposure to excitotoxins. Although the molecular mechanisms underlying ischemic tolerance are not yet fully delineated, the considerable delay from the preconditioning stimulus until onset of ischemic tolerance is consistent with a role for transcriptional changes in adaptation. Ischemic preconditioning enhances expression of the anti-apoptotic factor Bcl-2, which can act upstream of caspase-3 to prevent initiation of the caspases and downstream to directly bind activated caspase-3 to halt the self-amplifying caspase death cascade.[90]

Ischemic tolerance is thought to require activation of NMDA-type ionotropic glutamate receptors and enhanced Ca^{2+} as well as opening of ATP-sensitive K^+ channels via activation of adenosine A_1 receptors. Ischemic preconditioning is known to activate c-fos and c-jun and a number of survival factors, HSP70, superoxide dismutase, NO, BDNF, and the anti-apoptotic factors Bcl-2 and Bcl-x_L.

Spreading depression can induce ischemic tolerance. CSD preconditioning induces expression of pro-survival genes such as nNOS, Ca^{2+}-independent protein kinase C, c-fos, junB, c-jun, and MAP kinase phosphatase-1 (MKP-1), and reduces infarct volume in animal models of focal ischemia.[267] The rise in intracellular Ca^{2+} associated with CSD activates nNOS, which promotes NO formation.[268] The rise in extracellular glutamate associated with spreading depression promotes phosphorylation and activation of ERK1 and ERK2.[267] Upon activation, ERK phosphorylates the synaptic vesicle protein synapsin I, which promotes transmitter release and a rise in extracellular glutamate.

Neuroprotection by Estrogen in Experimental Models of Stroke

Estradiol, the primary estrogen produced and secreted by the ovaries, has widespread actions on the brain.[269,270] Estradiol increases spine density, synapse number, and NMDA receptor NR1 subunit expression and potentiates kainate-elicited currents in CA1 pyramidal neurons. Moreover, estrogen affords neuroprotection in several experimental models of stroke.[269,271] Acute exogenous estrogen at

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physiologic levels protects against focal ischemia–induced cortical injury in estrogen-deprived female and male rats and mice.[271-277] To afford protection, estradiol must be present at physiologic concentrations at least 48 hours before focal ischemia. Clues as to the molecular mechanisms by which estrogen affords protection come from studies showing that estrogen acts via estrogen receptor-α (ERα) to afford neuroprotection in the focal ischemia model[278] and induces upregulation of ERα and the anti-apoptotic factor Bcl-2 neurons (Plate 42–11).[279]

Estrogen also provides neuroprotection in animal models of global ischemia. Acute estrogen at supraphysiologic concentrations protects against global ischemia–induced hippocampal injury[280,281] and improves behavioral outcome after ischemia in male gerbils with ischemia.[282] Moreover, long-term administration of estrogen at more physiologic concentrations typically used in hormone replacement therapy (HRT) gives robust protection.[80] In addition to direct actions on hippocampal neurons, estrogen may affect hippocampal neurons indirectly via ERs on basal forebrain cholinergic neurons, which innervate the CA1 or via a receptor-independent anti-oxidative mechanism to inhibit oxygen radical–induced lipid peroxidation. The importance of postmenopausal estrogen replacement therapy for protection against the neuronal death associated with cardiac arrest or stroke, however, remains controversial.

The classic mode of estrogen action on neurons involves binding to the intracellular estrogen receptors ERα and ERβ, which function as ligand-activated transcription factors.[269,271] Estrogen binding to ERα or ERβ induces a conformational change leading to release of HSPs and formation of a dimer with high affinity for specific DNA sequences known as estrogen response elements (EREs), which are located at the promoter region of target genes. EREs occur in a diverse set of genes involved in cell survival and cell proliferation. EREs include the growth factors NGF, BDNF, and IGF-I receptor (IGF-IR) and the anti-apoptotic factors Bcl-2 and Bcl-x_L. The estrogen receptor can also interact with transcription factors such as c-jun and c-fos, with transcriptional enhancers and repressors, and with other DNA elements, such as AP1 sites.[283,284] AP1 is a dimeric transcription factor complex AP1, whose key components are the proteins Fos and Jun.

In addition to its genomic actions, estrogen signals rapidly via the PI3K/Akt signaling pathway, the Ras/MAPK signaling pathway, or both.[285] PI3K phosphorylates and activates Akt, which promotes cell survival by suppression of pro-apoptotic target genes. These genes include the pro-apoptotic proteins BAD and pro-caspase-9 and FOXO-3A. FOXO-3A induces apoptosis by transactivation of the Fas ligand, which acts via the Fas receptor to initiate the caspase cascade. Akt phosphorylates FOXO-3A, eliciting its relocalization from the nucleus to the cytoplasm, away from target genes. Estrogen acts via the PI3K/Akt signaling pathway (see earlier discussion) to afford protection of cortical neurons in culture against glutamate toxicity. Findings also indicate that estrogen blocks apoptotic signaling cascades in a gerbil model of global ischemia.[80] An intriguing possibility is that estrogen acts via Akt to halt the caspase death cascade at the point of Forkhead activation.

Estrogen also signals rapidly via Ras/MAPK and the downstream kinases ERK1 and ERK2. MAPK signaling promotes cell survival via phosphorylation and inactivation of BAD and via phosphorylation and activation of CREB, which promotes transcription of pro-survival target genes. Estrogen rapidly phosphorylates ERK1 and ERK2, which phosphorylate and inactivate the downstream kinase glycogen synthase kinase-3β (GSK-3β). Estrogen is a powerful inducer of MAPK in various models, including neocortical explants, primary cortical neurons, and neuroblastoma cells.[269] Estrogen acts via the Ras/MAPK pathway and ERK1 and ERK2 to promote neuronal survival of cortical neurons against glutamate toxicity.[286,287]

Considerable evidence implicates crosstalk between ERα and IGF-I signaling in the actions of estrogen on neurons.[269,271] ERα and ERβ are colocalized with IGF-IRs in neurons and astrocytes throughout the brain. Estrogen and IGF-I each engage the PI3K/Akt and MAPK signaling cascades. Estrogen neuroprotection in vivo and in vitro requires growth factor signaling. Research has shed light on the molecular mechanisms underlying this unique codependence. Upon activation, ERα interacts with IGF-IR to form a supramolecular complex that includes ERα, IGF-IR, the p85 subunit of PI3K, and IRS-1.[288] Formation of such a supramolecular complex is likely to prove critical to the protective actions of estrogen against ischemic death.

Neuroprotection by Zinc Chelation

Considerable evidence indicates that a rise in intracellular Zn^{2+} to toxic levels in CA1 neurons contributes to global ischemia–induced neuronal death. Zn^{2+} chelation affords robust protection against global ischemia-induced neuronal death.[289] Koh and colleagues[159] were the first to demonstrate that Zn^{2+} translocation from presynaptic terminals to postsynaptic target neurons occurs relatively early after neuronal insult and is not restricted to CA3 and hilar neurons (see Plate 42–7). Moreover, exposure of cortical neurons to Zn^{2+} elicited a rise in intracellular Zn^{2+}, as assessed by the selective fluorescent indicator dye TSQ. To examine whether the rise in Zn^{2+} was causally related to neuronal death, Koh and colleagues injected the cell membrane impermeant chelator calcium disodium ethylenediaminetetraacetate (CaEDTA) into the lateral ventricles 30 minutes before global ischemia markedly reduced the rise in Zn^{2+} in CA1 (and hilar) neurons and abolished neuronal death. Although CaEDTA is not specific for Zn^{2+}, it has a higher affinity for Zn^{2+} than for Ca^{2+} or Mg^{2+}. Thus, CaEDTA can bind Zn^{2+} without significantly altering intracellular Ca^{2+} or Mg^{2+}. Moreover, CaEDTA blocks Zn^{2+} neurotoxicity in vitro but not the neurotoxicity of Cu^{2+} or Fe^{2+}. These findings suggest strongly that Zn^{2+} is a critical mediator of ischemia-induced cell death.

These observations raise the possibility of novel targets for neuroprotection. A major problem inherent with membrane-impermeant Zn^{2+} chelators is achieving adequate access of membrane-impermeable zinc chelators to the CNS. Alternative strategies include block of channels that mediate entry of Zn^{2+} into neurons; block or attenuation of release of synaptic Zn^{2+}; upregulation of transporters responsible for Zn^{2+} extrusion, sequestration, or both; upregulation of metallotransporters; and counteraction of downstream disturbances involved in neuronal death (e.g., block of mitochondrial respiration). Promising leads along

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these lines include dietary manipulation of synaptic zinc stores, intake of pyruvate to compensate for Zn^{2+}-induced blockade of glycolysis, and pharmacologic block of Zn^{2+}-induced apoptosis.[289]

Hypothermia

Hypothermic therapy (lowering of the body temperature during or after ischemic insult) affords robust neuroprotection against global ischemia induced neuronal death.[290] Prolonged, postischemic hypothermia provides robust and sustained neuronal survival and greatly reduces ischemia-induced cognitive deficits in rats and gerbils.[56,290,291] Brief forebrain ischemia kills more than 98% of CA1 pyramidal neurons. Prolonged, delayed hypothermia (induced 1 hour after ischemia and maintained for 48 hours) persistently (for as many as 60 days) preserves the ultrastructure of and protects more than 90% of CA1 neurons from ischemia-induced death. Hypothermia can be initiated as late as 12 hours after ischemia, although the neuroprotective effect is significantly less than when hypothermia is initiated 1 hour after ischemia. These findings support the potential clinical usefulness of postischemic hypothermia as a mode of intervention after global ischemia associated with cardiac arrest or cardiac surgery in humans.

Hypothermia decreases cerebral blood flow, intracranial pressure, and brain metabolism. It also reduces cerebral metabolic rate, which results in decreased consumption of glucose and oxygen. The reduction in energy demand slows the rate of high-energy phosphate (ATP) depletion and lactate accumulation and thereby lessens oxidative stress. Curiously, although hypothermia protects against global ischemia–induced neuronal death, it exacerbates neuronal injury associated with focal ischemia.

There are a number of mechanisms by which hypothermia protects against hypoxia. Only a few, however, are understood. During ischemia, there is a rise in extracellular K^+, in part due to efflux of K^+ from vulnerable neurons via NMDARs and in part due to breakdown and reverse operation of ion pumps such as the Na^+-K^+-ATPase.[292] Hypothermia reduces synaptic activity (the rate of neuronal firing) and, in effect, relieves the cell of its excessive extracellular K^+.[292] Findings also indicate that hypothermia promotes survival of neurons by accelerating recovery of ATP and other important sources of cellular energy in the mitochondria to physiologic levels.[293] Future studies will more completely elucidate the molecular mechanisms by which hypothermia affords protection.

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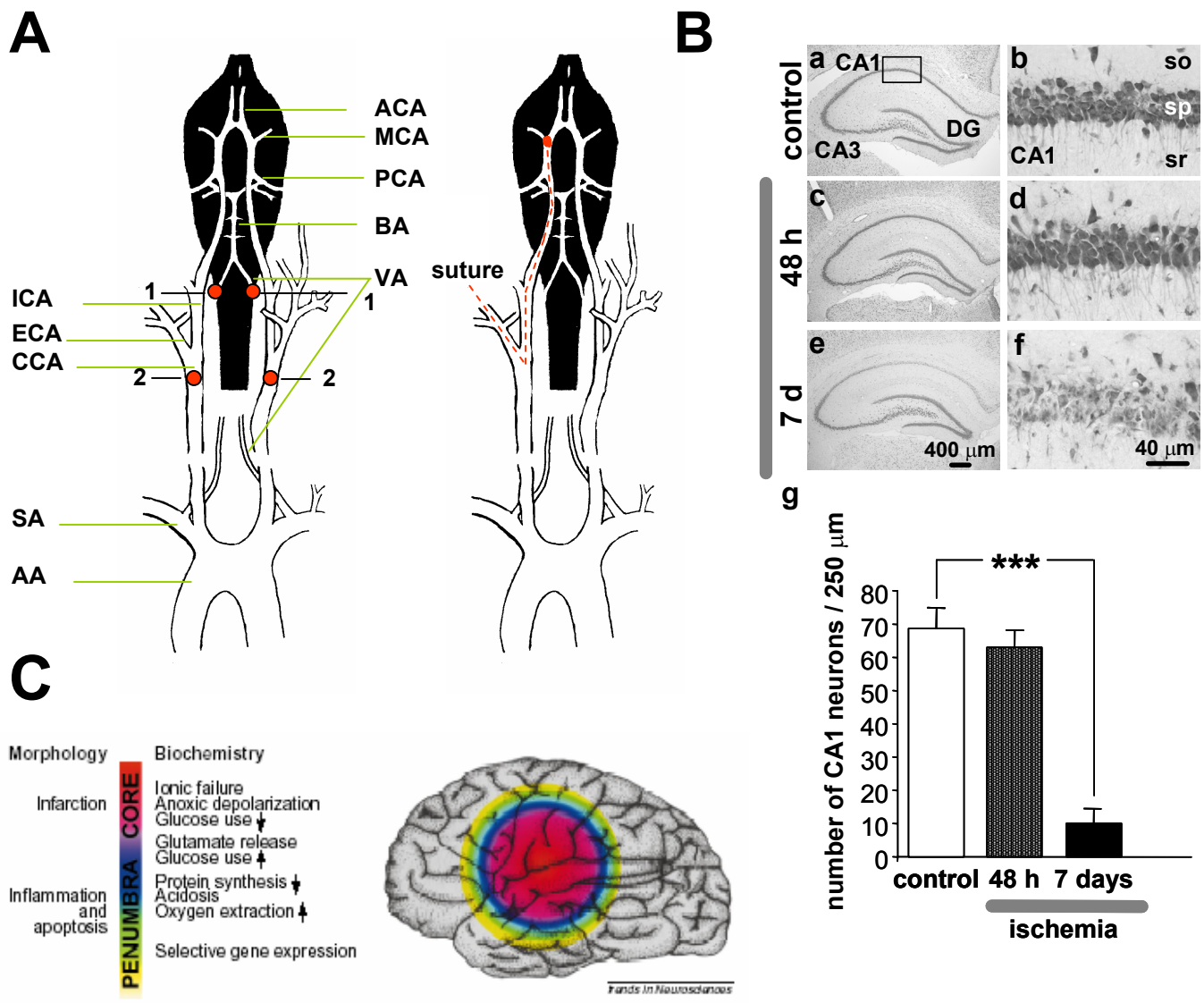

A
ACA
MCA
PCA
BA
VA
ICA
ECA
CCA
1
2
SA
AA
suture
B
control
48 h
7 d
a CA1
DG
CA3
b
so
sp
CA1
sr
c
d
e
f
400 µm
40 µm
g
number of CA1 neurons / 250 µm
***
80
70
60
50
40
30
20
10
0
control
48 h
7 days
ischemia
C
Morphology
Biochemistry
Infarction
Ionic failure
Anoxic depolarization
Glucose use
Glutamate release
Glucose use
Protein synthesis
Acidosis
Oxygen extraction
Inflammation and apoptosis
Selective gene expression
CORE
PENUMBRA

Figure 1

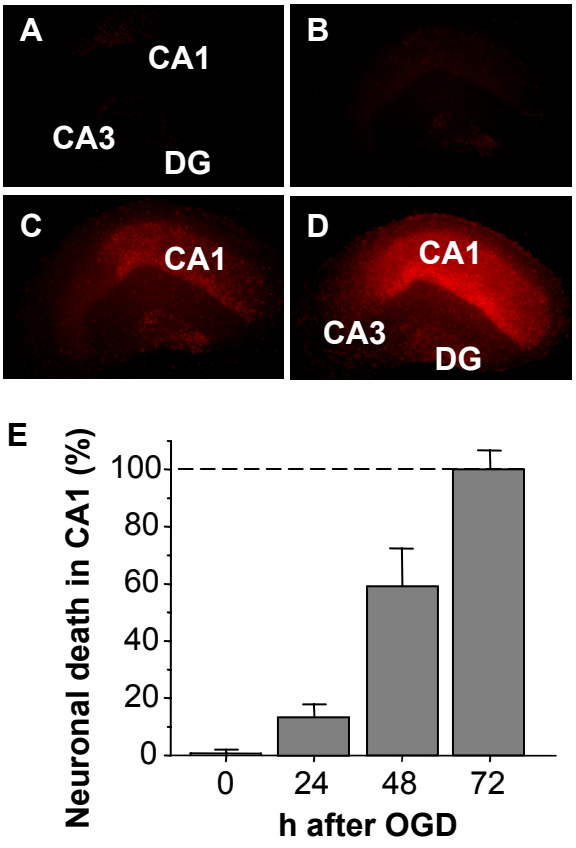

A
CA1
CA3
DG
B
C
CA1
D
CA1
CA3
DG
E
Neuronal death in CA1 (%)
100
80
60
40
20
0
0
24
48
72
h after OGD

Figure 2

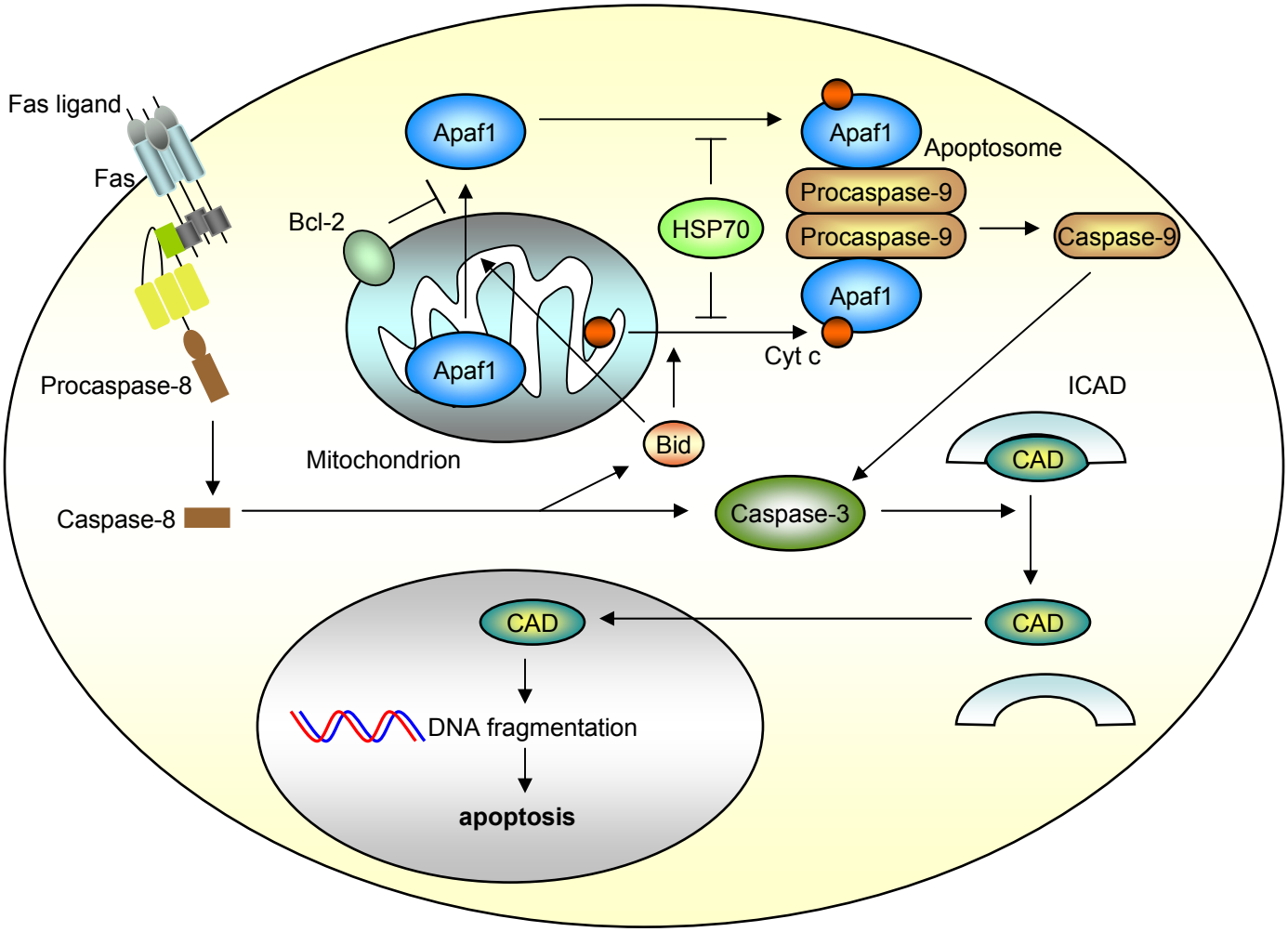

Fas ligand
Fas
Procaspase-8
Caspase-8
Bcl-2
Apaf1
Apaf1
Mitochondrion
Bid
HSP70
Apaf1
Apoptosome
Procaspase-9
Procaspase-9
Caspase-9
Apaf1
Cyt c
ICAD
CAD
Caspase-3
CAD
CAD
DNA fragmentation
apoptosis

Figure 3

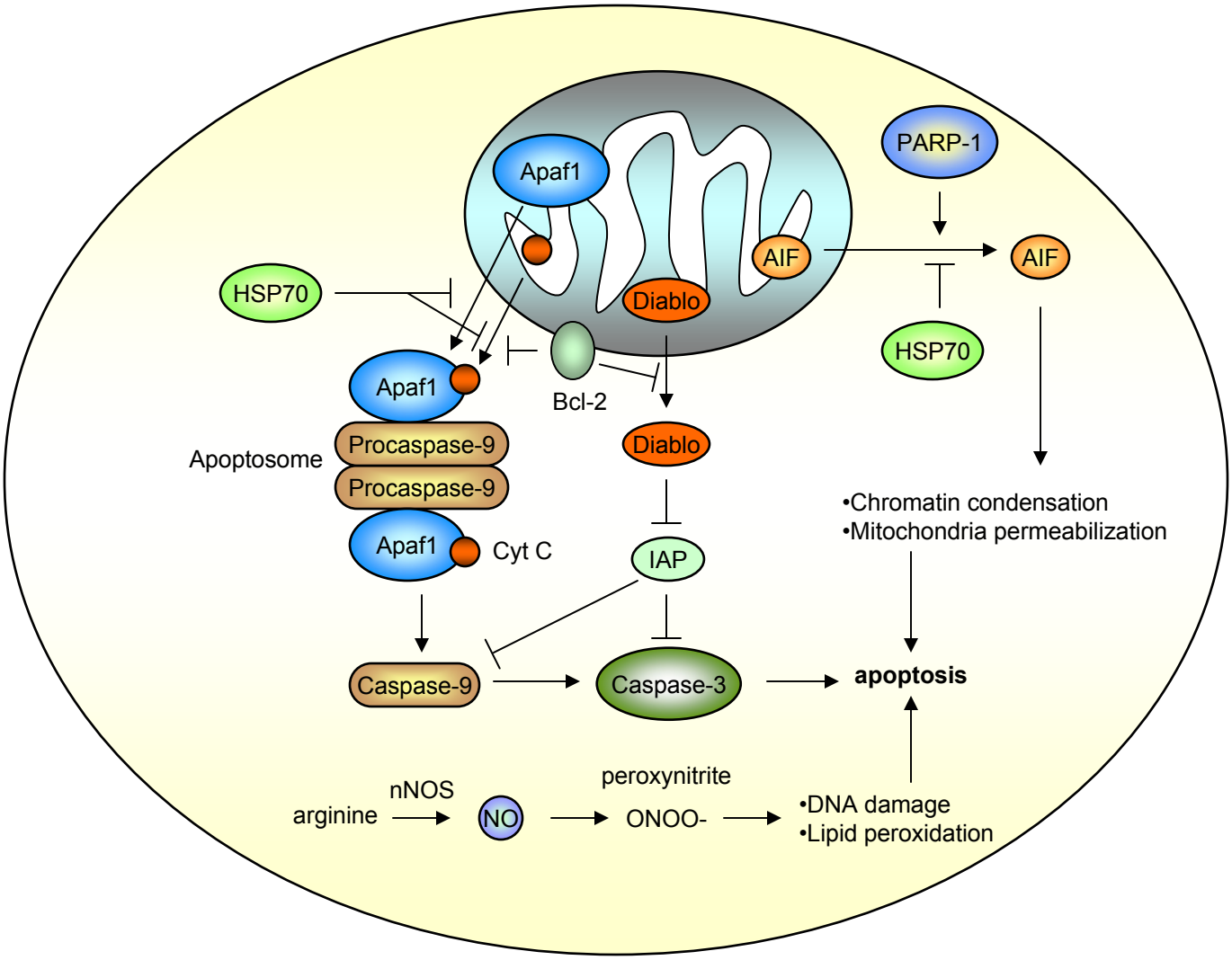

Apaf1
PARP-1
AIF
AIF
HSP70
Diablo
HSP70
Apaf1
Bcl-2
Apoptosome
Procaspase-9
Diablo
Procaspase-9
•Chromatin condensation
•Mitochondria permeabilization
Apaf1
Cyt C
IAP
Caspase-9
Caspase-3
apoptosis
peroxynitrite
nNOS
arginine
NO
ONOO-
•DNA damage
•Lipid peroxidation

Figure 4

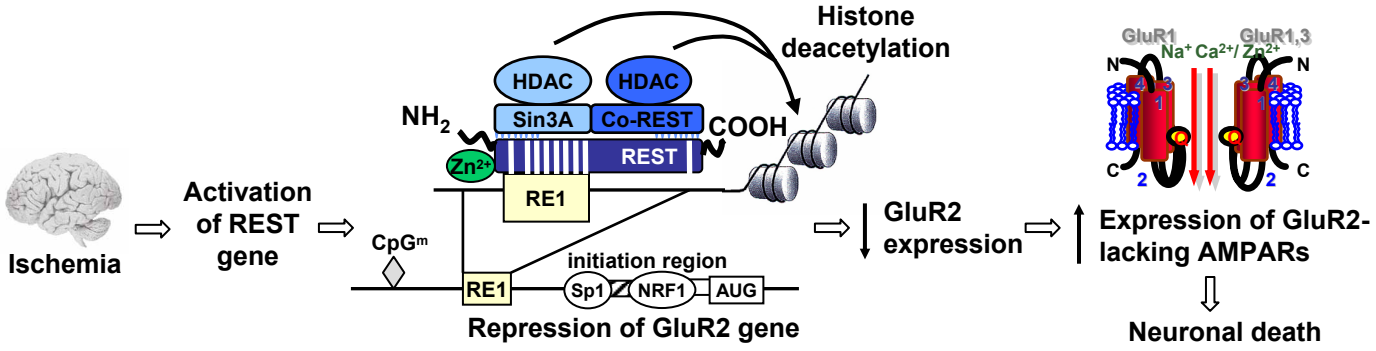

Ischemia
Activation of REST gene
CpGm
RE1
initiation region
Sp1
NRF1
AUG
Repression of GluR2 gene
HDAC
HDAC
Sin3A
Co-REST
REST
NH2
COOH
Zn2+
RE1
Histone deacetylation
GluR2 expression
GluR1
GluR1,3
Na+ Ca2+/ Zn2+
N
C
Expression of GluR2-lacking AMPARs
Neuronal death

Figure 5

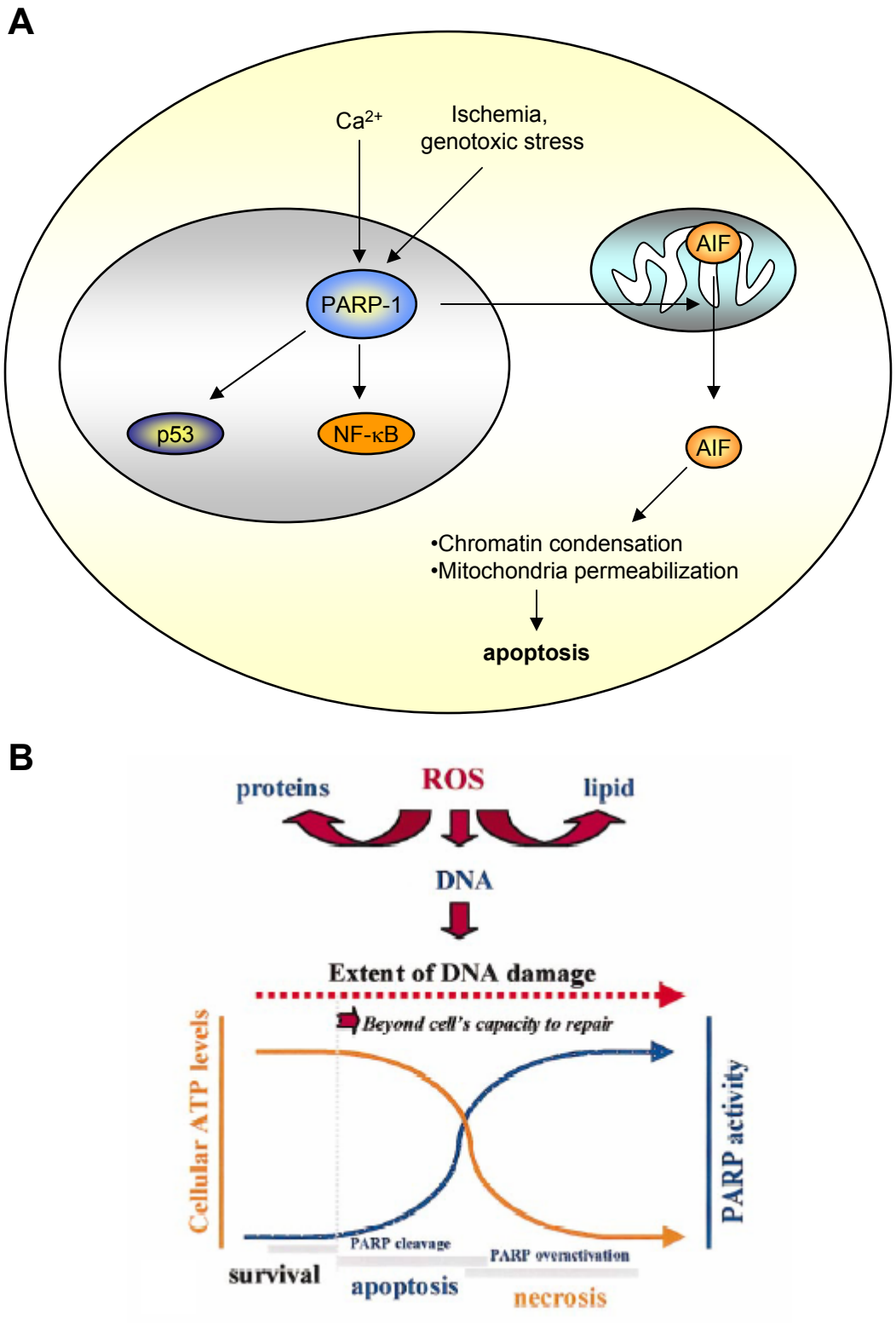

A
Ca2+
Ischemia,
genotoxic stress
PARP-1
AIF
p53
NF-κB
AIF
•Chromatin condensation
•Mitochondria permeabilization
apoptosis
B
proteins
ROS
lipid
DNA
Extent of DNA damage
Beyond cell's capacity to repair
Cellular ATP levels
PARP activity
PARP cleavage
PARP overactivation
survival
apoptosis
necrosis

Figure 6

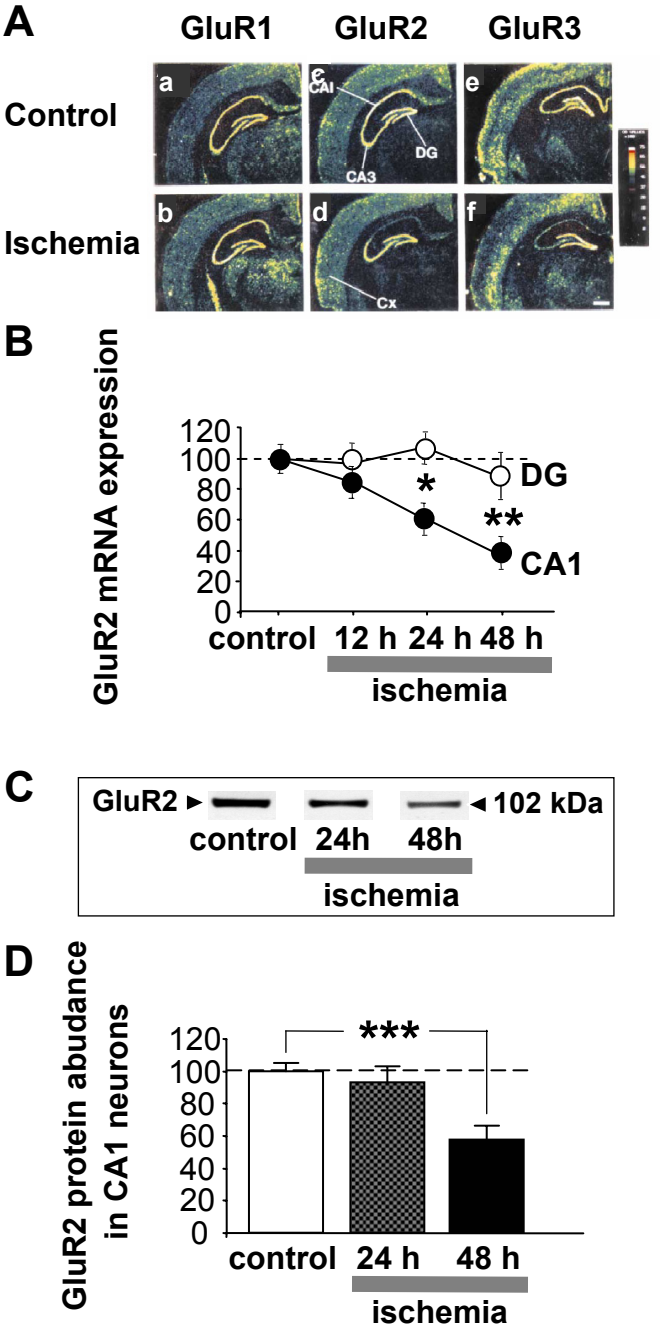
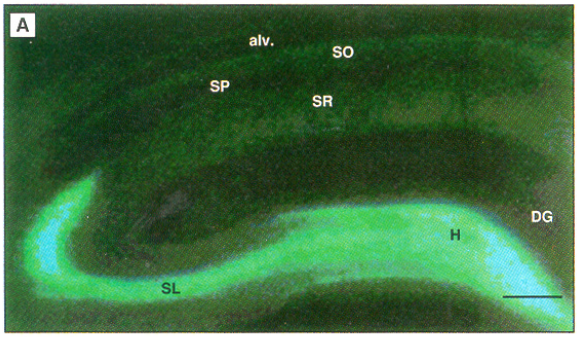
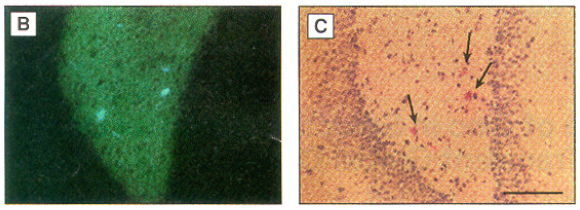

A
GluR1
GluR2
GluR3
Control
Ischemia
a
b
c
d
e
f
CAI
DG
CA3
Cx
B
GluR2 mRNA expression
120
100
80
60
40
20
0
*
**
DG
CA1
control
12 h
24 h
48 h
ischemia
C
GluR2
102 kDa
control
24h
48h
ischemia
D
GluR2 protein abudance
in CA1 neurons
***
120
100
80
60
40
20
0
control
24 h
48 h
ischemia

Figure 7

Control



24 h after 10 min global ischemia; hilus



72 h after 10 min global ischemia; CA1 stratum pyramidale

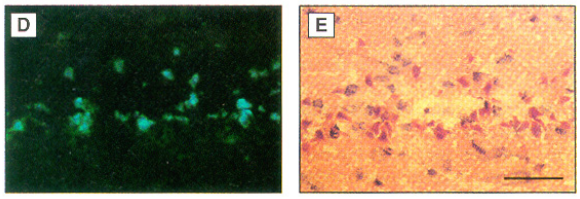


Figure 8

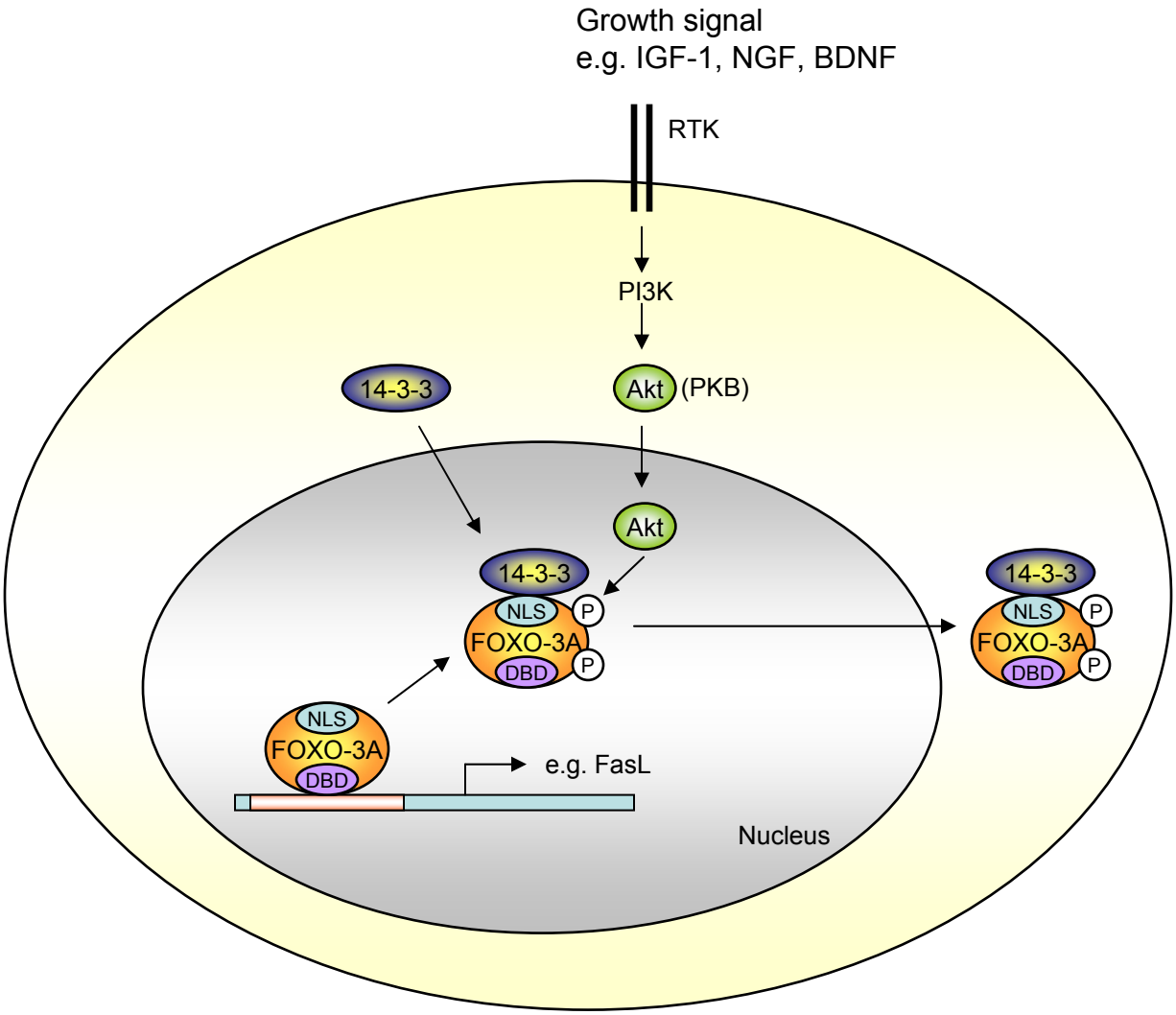

Growth signal
e.g. IGF-1, NGF, BDNF
RTK
PI3K
14-3-3
Akt
(PKB)
Akt
14-3-3
NLS
P
FOXO-3A
DBD
P
14-3-3
NLS
P
FOXO-3A
DBD
P
NLS
FOXO-3A
DBD
e.g. FasL
Nucleus

Figure 9

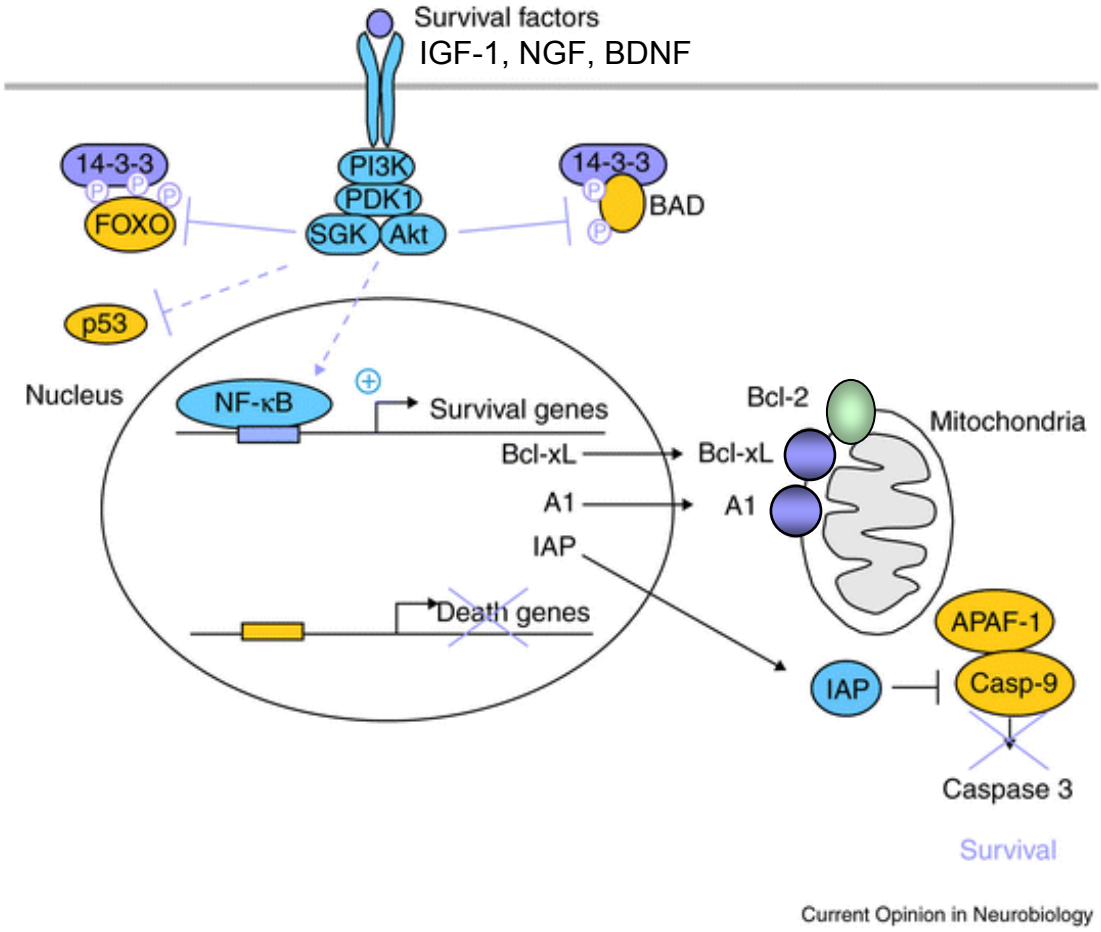

Survival factors
IGF-1, NGF, BDNF
14-3-3
FOXO
PI3K
PDK1
SGK
Akt
14-3-3
BAD
p53
Nucleus
NF-κB
Survival genes
Bcl-xL
A1
IAP
Death genes
Bcl-2
Mitochondria
Bcl-xL
A1
APAF-1
IAP
Casp-9
Caspase 3
Survival
Current Opinion in Neurobiology

Figure 10

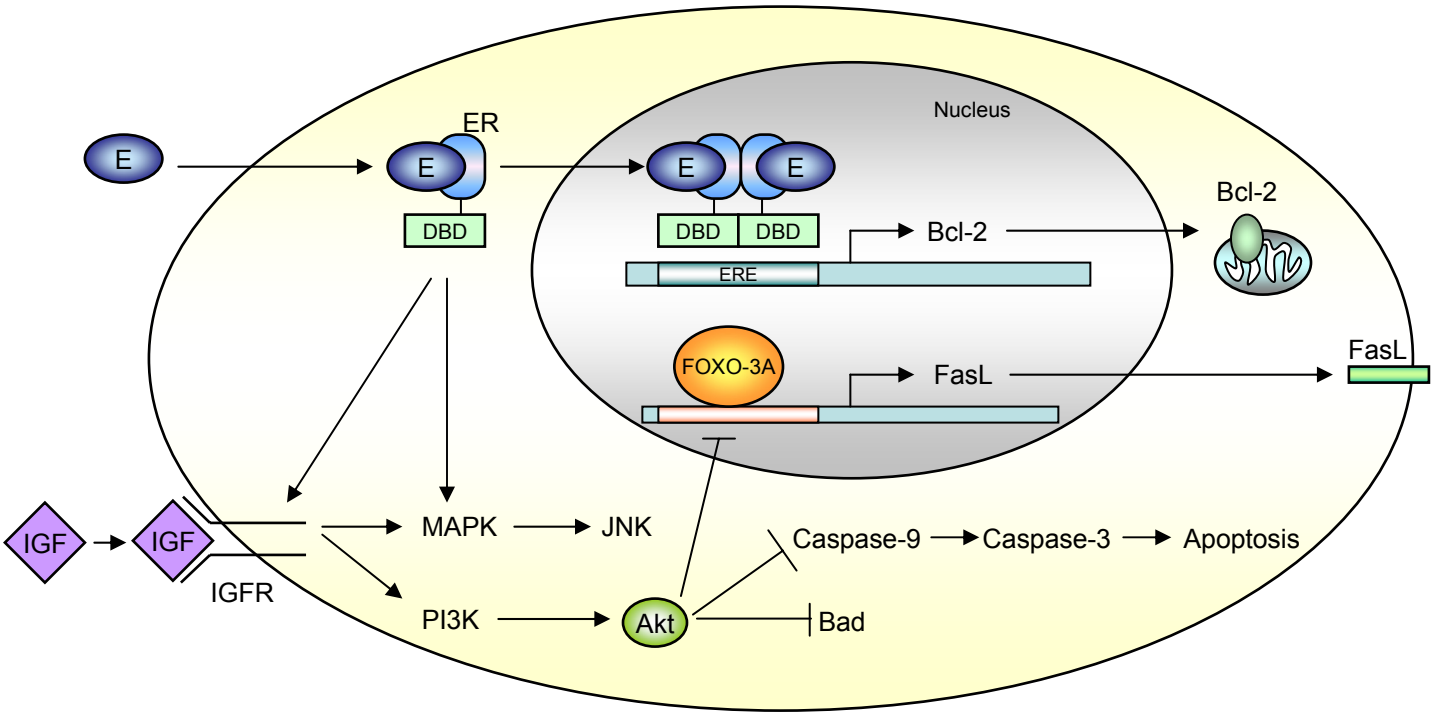

E
ER
E
DBD
Nucleus
E
E
DBD
DBD
Bcl-2
ERE
Bcl-2
FOXO-3A
FasL
FasL
IGF
IGF
IGFR
MAPK
JNK
Caspase-9
Caspase-3
Apoptosis
PI3K
Akt
Bad

Figure 11