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9. Apoptosis as a Mechanism of Developmental Neurotoxicity

Short title: 9. Apoptosis as a Mechanism of Developmental Neurotoxicity

Chapter 9

Apoptosis as a Mechanism of Developmental Neurotoxicity

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Abstract

Apoptosis is a tightly controlled physiological process in which individual cells die without damaging neighboring healthy cells. Apoptosis is crucial for normal neurodevelopment, and altered patterns of apoptotic cell death of neurons and glial cells during development are associated with functional deficits. Experimental and clinical evidence has established that neurological insults, including chemical exposures, can induce aberrant apoptosis in the developing nervous system. This chapter will discuss chemical-induced apoptosis as a mechanism of developmental neurotoxicity. The goals are as follows: (1) provide an overview of the molecular mechanisms of apoptosis and its role in the normally developing nervous system, (2) discuss the evidence implicating inappropriate apoptosis in adverse neurodevelopmental outcomes, and (3) illustrate mechanisms by which neurotoxic chemicals modulate apoptosis using pediatric anesthesia, developmental exposures to polychlorinated biphenyls, and zinc dyshomeostasis as examples.

Keywords: anesthetics; apoptosis; developmental neurotoxicity; PCBs; zinc

I Introduction

Apoptosis is derived from the Ancient Greek word “ἀπόπτωσις,” which literally translated means “falling off,” as in leaves falling off a tree. The term “apoptosis” was first used in 1972 by Kerr, Wyllie, and Currie¹ to describe a morphologically distinct form of cell death characterized by cell shrinkage, plasma membrane blebbing, and nuclear chromatin condensation and fragmentation, but maintenance of organelle integrity. Cells undergoing apoptosis ultimately break into multiple vesicles referred to as apoptotic bodies, which are phagocytosed by surrounding microglia and macrophages before the intracellular contents of the cell can spill out into the surrounding tissue. As a result, apoptosis enables individual cells to die without damaging neighboring cells or triggering an inflammatory response. These features are in sharp contrast to those associated with necrosis, which is a form of cell death characterized morphologically by rapid cell swelling, dilation of mitochondria and endoplasmic reticulum (ER), and formation of vacuoles in the cytoplasm. Necrotic cells eventually lyse, releasing their intracellular contents into the extracellular environment, which injures surrounding cells and provokes an inflammatory response. Thus, necrosis is a form of pathological cell death triggered by acute cellular injury that kills cells en masse, whereas apoptosis is a physiological process in which individual cells die without damaging neighboring healthy cells.

Apoptosis is a form of programmed cell death that is crucial for the normal development of the nervous system.^{2,3} However, experimental and clinical evidence demonstrate that diverse neurological insults can induce apoptosis in the developing nervous system, and that disruption of either the timing or the magnitude of apoptosis can alter neurodevelopmental trajectories to cause deficits in higher-order function even in the absence of obvious pathology.³⁻⁵ Whether neural injury triggers apoptosis or necrosis is thought to be largely determined by the intensity and/or duration of the insult: more severe and/or sustained insults tend to

cause necrosis, while less intense, more transient stresses induce apoptosis. However, it is not uncommon to observe features of necrosis and apoptosis in the same population of cells following neural injury.

This chapter will focus on apoptosis as a mechanism of developmental neurotoxicity. The goals are as follows: (1) provide an overview of the molecular mechanisms of apoptosis and its role in the normally developing nervous system; (2) discuss the evidence implicating altered patterns of apoptosis as a mechanism of pathogenesis in adverse neurodevelopmental outcomes; and (3) illustrate mechanisms by which neurotoxic chemicals modulate apoptosis using general anesthetics, polychlorinated biphenyls (PCBs), and zinc dyshomeostasis as examples.

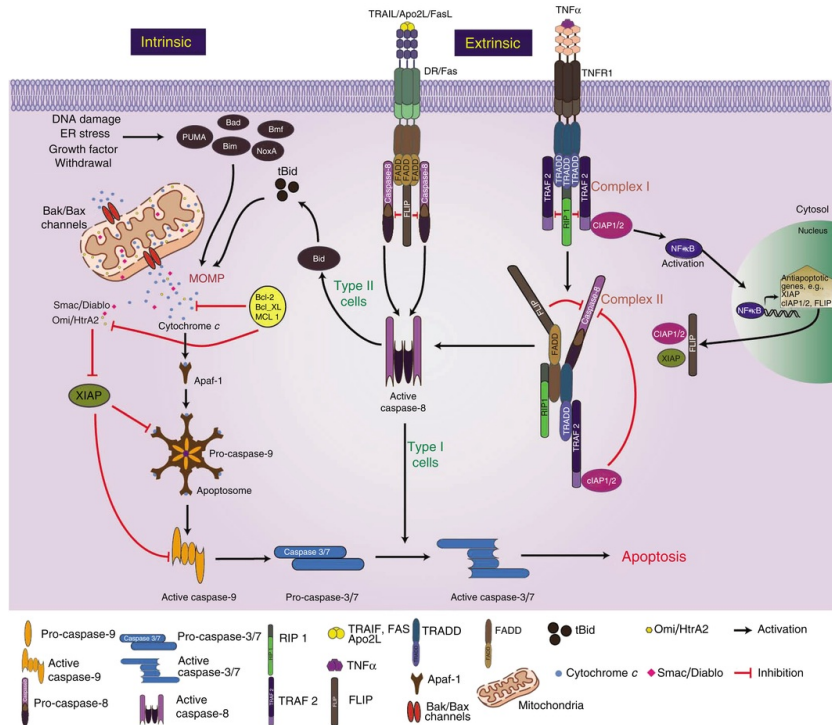
II Molecular Mechanisms of Apoptosis in the Developing Nervous System

A key biochemical event that defines apoptosis is the activation of caspases. Caspases comprise a family of evolutionarily conserved *cysteine aspartate proteases* with a conserved QACXG motif at the active site. At least 14 different mammalian caspases have been identified, which can be subdivided into three functional groups: (1) inflammatory caspases, (2) initiator caspases, and (3) effector caspases.⁶ It is the latter two groups, initiator and effector caspases, that mediate apoptosis in the developing nervous system. Initiator caspases are synthesized as zymogens, or proenzymes, and are characterized as having an N-terminal prodomain that contains protein-protein interaction motifs, such as the death effector domain (DED) found in caspase-8 and caspase-10, or the caspase recruitment domain (CARD) present in caspase-9.⁷ These domains enable initiator caspases to interact via homophilic interactions with like domains in specific adaptor proteins that control initiator caspase activation. Initiator caspases are capable of autocatalytic activation, but this is only triggered by proximity-induced self-dimerization, which is promoted by binding of initiator caspases to adaptor proteins. The primary function of initiator caspases is to activate effector caspases via proteolytic cleavage.

Effector or executioner caspases, which include caspase-3, -6, and -7, are also synthesized as zymogens but with a short prodomain.⁷ Proteolysis of the prodomain by initiator caspases is necessary to activate effector caspases, which in turn proteolytically cleave diverse substrates, including cytoskeletal proteins, nuclear and DNA-associated proteins, transcription factors, signal transduction proteins, kinases and phosphatases, and ion channels.⁸ Caspase-mediated proteolysis causes apoptotic cell death by compromising homeostatic and repair processes (e.g., DNA repair), inactivating antiapoptotic proteins, activating endonucleases and other proteases, disrupting the cytoskeleton, and triggering morphological changes in the nuclear and plasma membrane. One specific plasma membrane change, the translocation of phosphatidylserine from the inner leaflet of the plasma membrane bilayer to the cell surface, marks the cell for phagocytosis.⁸ Surface phosphatidylserine binds to a specific membrane receptor on phagocytic cells to trigger phagocytosis of the apoptotic body while its membrane is still intact.

Initiator caspases can be activated via diverse mechanisms, but the two best understood are the intrinsic and extrinsic apoptosis pathways (Fig. 9.1). The intrinsic pathway is triggered by intracellular signals generated in response to cellular stress. In the developing nervous system, stresses associated with activation of the intrinsic pathway include insufficient trophic support, oxidative and metabolic stress including ER stress, elevated levels of intracellular Ca^{2+} , DNA damage, and upregulation of DNA damage-responsive proteins. The intrinsic pathway is also known as the mitochondrial pathway because cell death depends on the release of proapoptotic factors from the intermembrane space of the mitochondria. Mitochondrial membrane permeability is influenced by many factors, but especially predominant in the context of apoptosis is the family of Bcl-2 proteins. Family members are characterized structurally by the presence of a Bcl-2 homology domain, and they can be subdivided into two functional groups: (1) those that exert antiapoptotic effects by stabilizing the mitochondrial membrane (e.g., Bcl-2, Bcl-XL, or Bcl-w) and (2) those that are proapoptotic and function to increase mitochondrial membrane permeability (e.g., Bid, Bad, Bim, NoxA, PUMA, Bax, and Bak). Activation of the intrinsic pathway of apoptosis alters Bcl-2 protein expression and/or activity via transcriptional and posttranslational modifications; the former downregulates expression of antiapoptotic Bcl-2 proteins and upregulate that of proapoptotic members of the Bcl-2 family, the latter triggers proapoptotic Bcl-2 proteins to oligomerize, and if they are cytosolic, to translocate to the outer mitochondrial membrane. These activities promote mitochondrial outer membrane permeabilization (MOMP).⁷ One example of a signaling molecule that regulates the expression and activity of Bcl-2 proteins is the tumor suppressor and transcription factor p53, which is activated by DNA damage.^{9,10} In response to DNA damage, nuclear p53 acts as a transcription factor to upregulate the expression of the proapoptotic Bcl-2 proteins, PUMA (p53-upregulated modulator of apoptosis), and NoxA,¹¹ while cytoplasmic p53 triggers Bax translocation to the mitochondrial membrane to cause MOMP.¹² Mitochondrial membrane permeability can also be influenced independent of Bcl-2 proteins by a number of factors including the following: (1) membrane-derived lipid mediators, such as ceramide and 4-hydroxynonenal, which are generated in response to oxidative stress and interact directly with the mitochondrial membrane to promote MOMP⁸; and (2) elevated

levels of intracellular Ca^{2+} that disrupt the resting mitochondrial membrane potential to induce mitochondrial membrane permeability transition pore opening.⁷



The intrinsic pathway of apoptosis, illustrated on the left side of the schematic, is mediated by the activation of proapoptotic proteins of the Bcl-2 family (e.g., Bid, Bad, PUMA, NoxA, Bmf, or Bim) and downregulation of antiapoptotic Bcl-2 proteins (e.g., Bcl-2, Bcl-XL, and Mcl-1). The increased ratio of pro- to antiapoptotic activity promotes mitochondrial outer membrane permeabilization (MOMP), which results in the release of cytochrome *c*, Smac/Diablo, and Omi/HtrA2 from the mitochondria. Once released into the cytoplasm, cytochrome *c* binds to apoptotic protease-activating factor-1 (Apaf-1), inducing a conformational change in Apaf-1 that leads to the formation of a heptamer that can then complex with procaspase-9 to form a multimeric complex known as the apoptosome. Formation of the apoptosome activates caspase-9, which proteolytically activates caspase-3 and caspase-7. Active caspase-3 and caspase-7 enzymatically cleave diverse intracellular macromolecules to give rise to the characteristic morphological features of apoptosis. Release of the mitochondrial intermembrane proteins Smac/Diablo and Omi/HtrA2 promotes apoptosis by inhibiting endogenous inhibitor of apoptosis proteins (IAPs), such as the X-linked inhibitor of apoptosis protein (XIAP), which inhibits caspases 3, 7, and 9. The extrinsic pathway of apoptosis, illustrated on the right in the schematic, is triggered by the binding of death ligands (TNF α , FASL, pro-NGF, and TRAIL) to transmembrane homotrimeric death receptors (TNFR, Fas, p75^{NTR}, and DR4/DR5, respectively). Activation of Fas, DR4, or DR5 recruits the death adaptor protein, Fas-associated death domain (FADD), which upon oligomerization recruits procaspase-8 into the death-inducing signaling complex (DISC), where caspase-8 is activated. In type I cells, activated caspase-8 directly activates caspases 3, 6, and 7 by enzymatic proteolysis, which is sufficient to cause apoptosis. In type II cells, activated caspase-8 cleaves Bid to form truncated Bid (tBid), which stimulates the proapoptotic proteins Bax and Bak to release cytochrome *c* and other proapoptotic proteins from the mitochondria to trigger the intrinsic pathway of apoptosis. Activation of the death receptor TNFR1 recruits TNFR-associated death domain (TRADD) and a complex of proteins containing receptor-interacting protein (Rip) and TNFR-associated factor 2 (TRAF2) to form Complex I. Complex I activates NF- κ B signaling, which results in rapid transcription of antiapoptotic genes, including FLIP and IAP1/2. FLIP ha:

been identified in the DISC. Dissociation of Complex I from TNFR1 enables TRADD to bind to FADD, which then recruits caspase-8 to form Complex II, which triggers caspase-8 activation and apoptosis unless sufficient FLIP and IAP1/2 are produced to block caspase-8 from self-activation.

MOMP releases cytochrome *c* from the mitochondrial intermembrane space into the cytoplasm. The synchronous release of cytochrome *c* from all mitochondria in the cell is thought to be controlled by Ca²⁺ released from IP₃-sensitive ER stores.⁸ Once released into the cytoplasm, cytochrome *c* binds to a cytosolic protein, apoptotic protease-activating factor-1 (Apaf-1), which contains a CARD domain at its N-terminus that binds to the CARD domain of procaspase-9. Interactions between Apaf-1, cytochrome *c*, and ATP cause Apaf-1 to undergo a conformational change to form an Apaf-1 heptamer, which can then recruit procaspase-9 via homophilic CARD interactions.⁷ Within this multimeric complex, known as the apoptosome, procaspase-9 self-activates, and subsequently proteolytically cleaves caspases 3 and 7 to activate these effector caspases. MOMP also releases the mitochondrial intermembrane proteins Smac/Diablo and Omi/HtrA2, which bind to and neutralize cytosolic inhibitors of apoptosis proteins (IAPs). In healthy cells, IAPs inhibit activation of caspases 9, 3, and 7; therefore binding of IAPs by Smac/Diablo and Omi/HtrA2 removes this inhibition to further enhance caspase activation.

The extrinsic pathway of apoptosis is also known as the death receptor (DR)-mediated pathway because it is activated by extracellular “death” ligands that bind to cell surface DRs. DRs comprise a subset of the tumor necrosis factor (TNF) receptor superfamily that includes TNF receptor-1 (TNFR1), Fas, the p75 neurotrophin receptor (p75^{NTR}), and death receptors 4 and 5 (DR4 and DR5). These receptors are synthesized as transmembrane homotrimers, which contain a death domain (DD) in their cytoplasmic domain. The DD is a conserved stretch of approximately 80 amino acids that upon receptor activation functions to recruit adaptor proteins that also express DDs, and its expression is essential for transducing apoptotic signals.¹³ DRs contain extracellular domains rich in cysteine that mediate binding of their cognate ligands, which for TNFR1 is TNF α ; Fas, Fas-Fas ligand (FasL); p75^{NTR}, proneurotrophins; and DR4/5, tumor necrosis factor-related apoptosis-induce ligand (TRAIL). Upon ligand binding, the death receptors Fas, DR4, and DR5 recruit the adaptor protein, Fas-associated death domain (FADD), which causes FADD oligomerization via homophilic interactions between DDs in the receptor and the adaptor protein. This oligomer then recruits procaspase-8 via DED homophilic binding to form a death-inducing signaling complex (DISC), where caspase-8 is autoproteolytically processed by proximity-induced dimerization to yield two subunits that form the active enzyme.⁷ In some cells, referred to as type I cells, active caspase-8 directly cleaves procaspases 3 and 7, and this is sufficient to induce apoptosis even in the absence of MOMP. In contrast, in other cells, referred to as type II cells, apoptosis is mediated by cross talk between the extrinsic and intrinsic pathways via caspase-8 cleavage of the proapoptotic Bcl-2 homology 3 (BH3)-interacting domain death antagonist (Bid). Cleavage of Bid forms truncated Bid (tBid), which interacts with and stimulates the proapoptotic proteins Bcl-2 associated X (Bax) and Bcl-2 homologous antagonist/killer (Bak) to promote MOMP.

The extrinsic pathway mediated by TNFR1 signaling is more complex in that TNFR1 activation can elicit both antiapoptotic and proapoptotic actions.^{7,13} Upon binding its cognate ligand, TNF α , TNFR1 recruits the DD-containing adapter protein, TNFR-associated DD (TRADD), which unlike the adaptor protein FADD, does not express a DED. TRADD interacts with receptor-interacting protein (Rip), another DD-containing protein, and with TNFR-associated factors (e.g., TRAF-2) to form Complex I. Binding of TRAF-2 and Rip to Complex I activates two sequential pathways: the NF- κ B pathway and the caspase-8 pathway. The former is activated by TRAF-2-mediated recruitment of the I κ B complex, which contains NF- κ B complexed to the inhibitor I κ B. Rip promotes the phosphorylation and subsequent proteasomal degradation of I κ B to release NF- κ B.¹³ NF- κ B translocates to the nucleus where it initiates transcription of a large number of antiapoptotic and prosurvival genes including FLICE inhibitory protein (FLIP) and various IAPs. In the second, proapoptotic pathway, TRADD recruits FADD via homophilic interactions between the DDs in each protein; experimental evidence suggests that this interaction occurs only after the TNFR1-Complex I multimeric assembly has been internalized by endocytosis.¹³ FADD-bound TRADD recruits procaspase-8 to form Complex II. Whether procaspase-8 self-activates upon complexing with the DED in FADD depends on relative levels of NF- κ B-induced antiapoptotic proteins, such as FLIP. FLIP, which has been found to coimmunoprecipitate with TNFR1/TRADD/FADD cytosolic complexes, can inhibit caspase-8 activation, thereby preventing apoptosis; however, under some conditions, FLIP may also activate caspase-8 by forming heterodimers with procaspase-8.¹³ Activated TNFR1 can also trigger an alternative proapoptotic pathway via the recruitment and binding to Rip of Rip-associated Ich-1/Ced-3-homologue protein with a death domain (RAIDD). The Rip-RAIDD complex recruits and activates procaspase-2 to cause apoptosis.⁷ Thus, apoptotic signaling through TNFR1 includes an NF- κ B-mediated prosurvival response that offsets the apoptotic response, so whether TNFR1 signaling induces apoptotic cell death depends on the relative balance of proapoptotic to antiapoptotic signals activated downstream of TNFR1 activation.

III Physiological Roles of Apoptosis in Neurodevelopment

Cell death occurs at specific developmental stages in response to extrinsic and intrinsic cues¹⁴ and this physiological event is referred to as programmed cell death. Apoptosis is the predominant mechanism of

programmed cell death in the developing nervous system and it is crucial for normal neurodevelopment.¹⁴ In rodents, nonhuman primates, and humans, apoptosis occurs in most regions of the brain and in the peripheral nervous system during embryonic and early postnatal development, resulting in the elimination of 50%-70% of all newly formed neurons.¹⁵ In the developing brain, there appear to be two major waves of apoptosis: one occurring prenatally and involving neuronal progenitor cells in proliferative zones, and the second occurring postnatally and involving postmitotic neurons in the process of forming synaptic connections with target cells.^{16,17} Glial cells (astrocytes and oligodendrocytes) also undergo programmed cell death via apoptosis,^{18,19} but this is less well understood than neuronal apoptosis.

Early appreciation of the physiological significance of apoptosis in neurodevelopment came from studies of the developing peripheral nervous system, which demonstrated that postganglionic sympathetic neurons are overproduced and their survival depends on competition for limited amounts of target-derived survival factors, such as nerve growth factor (NGF).^{20,21} This mechanism for eliminating redundant neurons became known as the neurotrophic hypothesis. The regulation of apoptosis in the central nervous system is more complex, involving not only neurotrophic factors, but also adhesion molecules, cytokines, neurotransmitters, and neuronal activity.^{8,14,22} Apoptosis also occurs normally in the adult brain, particularly in proliferative zones, such as the subventricular zone of the cerebral cortex and the subgranular layer of the dentate gyrus of the hippocampus.⁸

It has long been appreciated that programmed cell death is involved in morphogenetic processes in the embryo and the same is now known to be true in the developing nervous system. For example, many cells die by apoptosis in the boundary regions between the neural plate and nonneural ectoderm during and after neural tube closure.¹⁴ Live-imaging studies have revealed that inhibition of apoptosis prevents the normal progression of events in cranial neural tube closure in mice,²³ suggesting that apoptosis influences the dynamics of neuroepithelial morphogenesis. Apoptosis has also been implicated in sculpting and eliminating morphogen-signaling centers in the developing brain.¹⁴ In the developing mouse embryo, the anterior neural ridge (ANR), which lies between the neural plate and nonneural ectoderm at its most rostral boundary, exhibits massive apoptotic cell death before and after neural tube closure. Studies of apoptosis-deficient mutant mice (*caspase-9* or *apaf-1* knockouts) have revealed that apoptosis in the ANR selectively eliminates cells expressing fibroblast growth factor 8 (Fgf 8), which diffuses from the ANR and acts as a morphogen to regulate forebrain patterning.²⁴ Inhibition of apoptosis results in abnormally persistent Fgf 8 signaling in the ANR coincident with altered ventral forebrain development. Apoptotic regulation of signaling centers is thought to be an efficient and conserved mechanism for deleting cells or tissues that transiently function in a specific stage of normal neurodevelopment, but are not needed or may even be deleterious at other stages of neurodevelopment.¹⁴

Apoptosis plays a critical role in regulating cell number and in eliminating misspecified, mispositioned, and redundant neurons in the developing nervous system. Its role in regulating cell number is best exemplified by the neurotrophic theory, as described earlier. The neurotrophic theory is based on evidence demonstrating that experimentally increasing the amount of survival factor present in target tissues decreases neuronal apoptosis, thereby increasing the number of neurons innervating the target that survive. Conversely, decreasing the amount of target-derived survival factor increases apoptosis, thereby decreasing the number of surviving neurons.^{20,21} This mechanism ensures the quantitative matching of postmitotic neurons and glia with targets and afferents in the peripheral nervous system. Apoptosis also regulates the proper spacing of cells, as has been demonstrated during visual system development in invertebrates (*Drosophila*) and vertebrates (mouse). *Bax* knockout mice exhibit an approximate four-fold increase in the density of intrinsically photosensitive retinal ganglion cells in the eye, which is coincident with disrupted intrinsically photosensitive retinal ganglion cells spacing, abnormal dendritic stratification, ectopic synapses, and impaired rod/cone-driven photoentrainment.²⁵ These observations suggest that precise control of cell spacing by apoptosis is critical for establishing functional neural circuits in the eye. Apoptosis is also an important mechanism for eliminating cells that migrate to inappropriate places during neurodevelopment, as illustrated in the mouse brain, where genetic deletion of *bax* results in ectopic Purkinje cells in the cerebellum.²⁶ Classic studies of the developing avian visual system provided the first evidence to support the hypothesis that apoptosis also functions to eliminate neurons that project to the wrong site.²⁷ Subsequent genetic manipulations in mice corroborated this hypothesis by demonstrating that inhibition of apoptosis prevents the elimination of neurons with aberrant axonal projections in several neural systems.²⁸

More recent experimental evidence suggests that apoptosis may also be important in the pruning of nonproductive or faulty neurites or synapses in the

absence of cell death. Several studies have shown that local activation of caspases in axonal, dendritic, or synaptic subcellular compartments contributes to axonal degeneration, dendritic regression, and synaptic pruning without killing the neuron,^{29,30} thereby providing a mechanism for refining neural circuitry on a local level. Caspase activation has also been demonstrated to occur during synaptic remodeling associated with learning and memory.³¹ It is thought that locally activated caspases cause these effects by acting on cytoskeletal proteins and/or kinases and other signaling molecules within specific subcellular compartments. What prevents these local apoptotic signals from spreading to the soma to trigger apoptotic cell death remains an outstanding question. Two possibilities have been proposed: (1) the distance

separating distal axons and dendrites from the soma prevents interactions of local responses with downstream targets that trigger apoptotic cell death and/or (2) neurons have mechanisms for inhibiting the propagation of local apoptotic signaling to the rest of the cell to prevent local activation of caspases from causing cell death.^{8,14}

Apoptosis is also emerging as a central mechanism underlying the structural changes that occur in the brains of seasonally breeding songbirds and during puberty and adolescence in mammals.¹⁴ Massive apoptotic cell death has been documented during the steroid hormone-dependent global remodeling of the songbird brain that occurs during each reproductive season.³² In mammals, including humans, maturation of sexual behavior also depends on the action of gonadal steroid hormones in the brain during puberty and adolescence, which regulate rates of cell proliferation, migration, and survival to give rise to differences in the number of neurons found in sexually dimorphic nuclei of males vs. females.^{33,34} In mouse models, genetic deletion of *bax* largely abolishes sex-specific differences in neuronal cell number in these brain regions.³⁵

Clinical studies corroborate the importance of apoptosis in normal neurodevelopment. Altered patterns of apoptosis are associated with diverse neurodevelopmental disorders³⁶ including autism spectrum disorders^{37,38} and schizophrenia.^{39,40} There is also clinical evidence suggesting that the developing human brain has an increased propensity for apoptosis after traumatic brain injury⁴¹ and it is thought that increased apoptosis contributes to the cognitive deficits and acquired epilepsy often observed as a consequence of pediatric traumatic brain injury.^{41,42} Conversely, insufficient apoptosis has been posited as a pathogenic mechanism of pediatric brain cancer.⁸ Collectively, these observations suggest that nonphysiological shifts in the timing or spatial distribution of apoptosis in the developing nervous system can alter synaptic connectivity, which may contribute to adverse neurodevelopmental outcomes.

IV Chemical-Induced Apoptosis

Structurally and mechanistically diverse chemicals have been shown to induce neuronal and/or glial cell apoptosis in animal and cell culture models, as determined using morphological and biochemical criteria for apoptosis.^{2,18,43-46} Chemicals with proapoptotic activity include not only well-established developmental neurotoxicants, such as lead,^{47,48} methyl mercury,⁴⁹ ethanol,⁵⁰⁻⁵² and chlorpyrifos,^{53,54} but also drugs, notably pediatric general anesthetics.^{55,56} To date few, if any, chemicals have been shown to cause developmental neurotoxicity by inhibiting apoptosis. It is generally accepted that induction of apoptosis by exposure to chemicals during critical periods of neurodevelopment can alter neurodevelopmental trajectories, resulting in persistent cognitive and behavioral deficits.^{22,57} Perhaps the most compelling evidence in support of this hypothesis is the extensive experimental evidence linking ethanol-induced apoptosis to the adverse effects of prenatal ethanol exposure.^{18,58,59} Based on such data, Olney et al. proposed that ethanol-induced apoptosis provides a biologically plausible explanation for how in utero exposure of the developing human brain to ethanol causes the clinical manifestations of fetal alcohol syndrome (FAS), including reduced brain size and lifelong behavioral deficits.⁵⁶ It has been posited that chemical-induced apoptosis in the developing brain may also predispose the individual to neurodegenerative disease and/or increase susceptibility to subsequent stressors later in life that adversely impact neurological function.^{8,57} However, a causal relationship between increased apoptosis in the developing brain and functional deficits later in life has yet to be definitively established for most chemicals shown to have proapoptotic activity in vitro and/or in vivo.

Another research focus in the field involves defining the molecular mechanisms that link chemical exposure to apoptotic cell death of neuronal or glial cells. Given the structural and mechanistic diversity of chemicals that have been shown to promote neuronal and/or glial apoptosis in animal and cell culture models,^{2,18,43-46} it is perhaps not surprising that diverse molecular initiating events have been identified (Table 9.1). Despite chemical-specific differences in proximal molecular events that trigger apoptotic signaling, downstream events by which chemicals cause apoptotic cell death converge on three general mechanisms: (1) induction of cellular stress that activates the intrinsic pathway of apoptosis (e.g., decreased levels of neurotrophic survival factors, increased levels of intracellular reactive oxygen species (ROS) and/or Ca²⁺, disruption of the cytoskeleton, and DNA damage); (2) upregulated expression of DRs and/or death ligands that activate the extrinsic pathway of apoptosis; and (3) transcriptional or posttranslational mechanisms that increase the ratio of proapoptotic to

antiapoptotic factors. The remainder of this chapter discusses mechanisms by which chemical exposures induce apoptosis in the developing brain and the evidence linking chemical-induced apoptosis to functional deficits, using anesthetics, PCBs, and zinc dyshomeostasis as examples.

Table 9.1 Mechanisms By Which Chemicals Induce Apoptosis in the Developing Brain

Neurotoxic chemical	Mechanism(s) of apoptosis	Timing of exposure	Species	References
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Ethanol	<ul style="list-style-type: none"> • Zn availability • Cellular ROS, Ca²⁺ • Destabilize the cytoskeleton • Prosurvival proteins (PI3K/Akt, ERK, NF-κB, NFAT) 	Pre- and postnatal	Mouse Rat	60-64
Kainic acid	<ul style="list-style-type: none"> • Cellular ROS, Ca²⁺ • Lipid and protein oxidation • Excessive AMPAR activation • Cellular antioxidants (GSH activity and GSH/GSS) 	Prenatal	Rat	65,66
Anesthetics (propofol)	<ul style="list-style-type: none"> • Mature NGF levels • TNFR1 activation • p75^{NTR} activation • TNF-α and pro-NGF 	Early postnatal	Rat	67-69
Antiepileptic drugs	<ul style="list-style-type: none"> • Neurotrophins (BDNF, NT-3) • Prosurvival proteins (Akt, ERK) 	Early postnatal	Rat	70,71
Plasticizers (bisphenol A)	<ul style="list-style-type: none"> • Cellular ROS, Ca²⁺ • CREB phosphorylation • Cell cycle arrest • Death factors Bax and p53 • Cell proliferation biomarkers (PCNA and Ki67) 	E16 mouse, E13 rat primary neurons	Mouse, rat	72,73
Endotoxin (LPS)	<ul style="list-style-type: none"> • Zn availability • mTOR activation • TNF-α 	Prenatal	Rat	74-76

A Anesthetics

The induction of apoptosis in the developing brain by anesthetics was first reported by Olney and his coworkers. While studying cell death mechanisms following head trauma, they observed that traumatic brain injury triggered acute excitotoxic and delayed apoptotic cell death in the immature rodent brain.⁷⁷⁻⁷⁹ Subsequent studies to determine whether administration of drugs that block *N*-methyl-D-aspartate (NMDA) receptors or NMDARs protected against excitotoxicity-induced neurodegeneration revealed that while these drugs decreased head trauma-induced excitotoxic cell death as predicted,⁷⁷ unexpectedly, they also significantly increased apoptotic cell death.⁷⁹ Following up on this startling discovery, Olney and coworkers found that NMDAR antagonists promoted apoptotic neurodegeneration in not only the traumatized immature rodent brain, but also in the nontraumatized, normally developing rodent brain.⁸⁰ Evaluation of the neurotransmitter receptor specificity of the latter effect indicated that pharmacological agents that potentiated or mimicked the action of γ -aminobutyric acid (GABA) at GABA_A receptors, such as benzodiazepines and barbiturates,⁵² or drugs that blocked voltage-gated Na⁺ channels, such as the anticonvulsants phenytoin and valproate,⁷⁰ also elicited a robust apoptotic response in the developing rodent brain. Collectively, these data suggested that pharmacological agents that attenuated neuronal activity were capable of inducing apoptosis in the developing brain.

Many anesthetic drugs act to depress neuronal signaling, at least in part, by antagonism of NMDARs and/or positive allosteric modulation of GABA_A receptors,^{55,56} raising the possibility that pediatric anesthesia may trigger apoptosis in the developing brain. Olney and colleagues tested this hypothesis by maintaining postnatal day 7 rat pups in a light surgical plane of anesthesia for 6 h using a drug combination (nitrous oxide, midazolam, and isoflurane) commonly used in pediatric anesthesia.⁸¹ This anesthesia paradigm caused no signs of distress, did not change skin color or blood gases, and pups rapidly recovered from anesthesia, with no subsequent signs of obvious neurological impairments or differences in weight gain relative to unanesthetized litter mates. However, histological examination of brains obtained from exposed pups within hours after anesthesia indicated significantly increased, widespread apoptotic cell death in many brain regions, as determined by ultrastructural criteria and immunohistochemical analyses of caspase-3 activation. Exposed pups also exhibited persistent learning and

memory deficits in learning and memory tasks when tested as juveniles and adults.

Anesthetic-induced apoptosis in the developing brain has since been reported for a number of sedatives and anesthetics (e.g., ketamine, midazolam, propofol, isoflurane, sevoflurane, and nitrous oxide) administered alone or in combination in diverse preclinical models,⁸²⁻⁸⁶ including nonhuman primates.⁸⁷⁻⁹¹ Anesthetics trigger apoptosis in many brain regions, including the forebrain, midbrain, cerebellum, and visual system, although pyramidal neurons in the cortex appear to be particularly sensitive.⁸⁴ Apoptosis of interneurons and oligodendrocytes has also been observed in preclinical models following anesthetic administration.⁹²⁻⁹⁴ The peak apoptotic response is observed between 3 and 6 h after anesthesia, and the extent and spatial distribution of apoptotic neurodegeneration depends on the timing (with respect to developmental stage) of exposure, the dose of the anesthetic, and the duration of anesthesia.^{22,59,95} The influence of timing likely reflects the fact that the developing brain is most vulnerable to anesthetic-induced apoptosis during periods of peak synaptogenesis,⁹⁶ which varies between brain regions⁵⁷ and species.

Early mechanistic studies determined that anesthesia can induce apoptosis in the brain of postnatal rat pups via activation of intrinsic and extrinsic pathways of apoptosis. For example, within 2 h of nitrous oxide anesthesia, the intrinsic pathway of apoptosis is activated, as evidenced by western blot data indicating decreased levels of the antiapoptotic protein Bcl-XL and increased levels of cytochrome *c* in the cortex, and immunohistochemical data showing caspase-9 and caspase-3 activation in the cortex and the thalamus.⁹⁶ After 6 h of nitrous oxide anesthesia, the extrinsic pathway of apoptosis is activated in these same brain regions, as evidenced by Fas protein upregulation and caspase-8 activation.⁹⁶ But how do these anesthetic drugs activate apoptotic signaling pathways? As indicated earlier, many anesthetics act by antagonizing the NMDAR,^{80,97} which decreases the influx of calcium through NMDARs, thereby reducing activation of downstream Ca²⁺-dependent signaling cascades, notably, the extracellular regulated protein kinase (ERK1/2) signaling pathway (Fig. 9.2). ERK1/2 signaling activates the transcription factor, cAMP-responsive element binding protein (CREB), via phosphorylation, and pCREB transcriptionally upregulates expression of the antiapoptotic molecule Bcl-2.⁹⁷ Thus, reduced ERK1/2 signaling as a consequence of anesthesia decreases levels of pCREB, which in turn downregulates Bcl-2 expression. Experimental evidence confirms that anesthetic exposure reduces CREB activity in the developing rodent brain, and this is associated with reduced mRNA levels of the antiapoptotic molecule, Bcl-2, and a subsequent increase in the number of apoptotic cells in the brain.⁹⁸

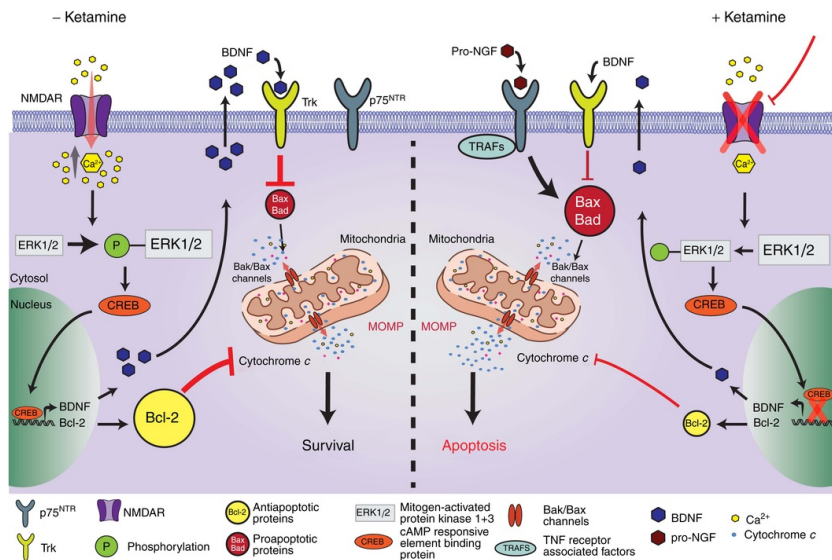


Figure 9.2 Mechanisms of neuronal apoptosis induced by anesthetics with NMDAR antagonist properties.

Under normal physiological conditions, as illustrated on the left side of the schematic, Ca^{2+} influx through the NMDAR activates the Raf1/MEK/ERK signaling pathway. Activated ERK (e.g., phosphorylated ERK) phosphorylates CREB, which triggers nuclear translocation of CREB and subsequent transcription of CREB-responsive genes, including genes that encode antiapoptotic (e.g., Bcl-2) and neuroprotective (e.g., BDNF) proteins. Bcl-2 prevents the release of cytochrome *c* from the mitochondria, thereby inhibiting the activation of intrinsic pathways of apoptosis. BDNF can act as an autocrine factor, binding to and activating the tropomyosin-related kinase-B (TrkB) receptor. Activation of TrkB triggers downstream signaling mechanisms that inhibit the proapoptotic proteins Bax and Bad. As illustrated on the right side of the schematic, anesthetics such as ketamine directly antagonize the NMDAR to inhibit Ca^{2+} influx, which downregulates the expression of antiapoptotic and neuroprotective genes. In addition, anesthetics can increase the expression of proneurotrophins, such as pronerve growth factor (pro-NGF), which have a high affinity for the p57 neurotrophin receptor (p75^{NTR}). Activated p75^{NTR} signals through TNF receptor-associated factors (TRAFs) to promote the activation of pro-apoptotic proteins (e.g., Bak and Bax), which trigger intrinsic apoptotic pathways via mitochondrial outer membrane permeabilization (MOMP).

In addition to the Bcl-2 family of antiapoptotic proteins, CREB positively regulates transcription of neurotrophins (Fig. 9.2), which play a key role in regulating neuronal survival in the developing brain.^{96,99} Neurotrophins are synthesized and released by neurons in an activity-dependent manner.¹⁰⁰ Developmental exposure to anesthetics disrupts neurotrophin signaling, and this often precedes apoptotic death of neurons across multiple brain regions.^{100,101} Neurotrophins control neuronal survival through interactions with two transmembrane receptor subtypes: tropomyosin receptor kinase (Trk) receptors and the TNFR family member, p75^{NTR}.^{100,102} Both receptor subtypes are coexpressed on all neurons and the mechanisms that control pro- or antiapoptotic cell fate rely on the affinity of these receptors for their cognate ligands.¹⁰³ Trk receptors have a greater affinity for mature neurotrophins, in which the prodomain has been proteolytically cleaved, whereas p75^{NTR} has a much higher affinity for the uncleaved precursor forms of neurotrophins. Binding of mature neurotrophins, such as NGF or brain-derived neurotrophic factor (BDNF), to the Trk family of receptors typically promotes cell survival, whereas direct activation of p75^{NTR} by binding of pro-NGF or pro-BDNF is believed to trigger apoptotic signaling pathways.¹⁰³ Thus, the balance between expression of Trk vs. p75^{NTR} receptors, and pro- versus mature neurotrophins, determines whether developing neurons survive or undergo apoptotic cell death. Anesthetic blockade of NMDAR activity can shift that balance toward apoptotic cell death via several mechanisms (Fig. 9.2). First, anesthetic-induced downregulation of CREB activity can decrease the transcription of prosurvival neurotrophins such as BDNF.¹⁰¹ Alternatively, anesthetics can increase pro-NGF levels and p75^{NTR} receptor expression, which has been shown to coincide with the activation of apoptotic pathways in preclinical models.⁶⁷ Activation of p75^{NTR} by pro-NGF triggers apoptosis by recruiting TRAF adaptor protein complexes^{102,103} that activate downstream apoptotic signaling cascades, resulting in oligomerization and translocation of proapoptotic proteins in the Bcl-2 family, such as Bad and Bax¹⁰⁴⁻¹⁰⁶ to the outer mitochondrial membrane to cause MOMP and release of cytochrome *c* into the cytoplasm (Fig. 9.2). Interestingly, the ontogenetic profile of p75^{NTR} expression in the brain, which peaks during early postnatal stages in rodents and then decreases with increasing age¹⁰⁷; aligns with the window of vulnerability of the developing brain to anesthetic-induced apoptosis. Alternatively, it has been hypothesized that exposure of developing neurons to NMDA antagonists/anesthetics (e.g., ketamine) for several hours produces, upon washout, a compensatory upregulation of NMDARs with subsequent overstimulation of the glutamatergic system by endogenous glutamate, triggering intracellular calcium overload, which enhances neuronal apoptosis.^{87,108,109}

The mechanisms by which anesthetics with GABA mimetic properties induce apoptosis in the developing brain are not as well understood, in part because early in development, GABA receptors mediate excitatory neurotransmission, but after birth they transition to mediating inhibitory neurotransmission, a developmental process known as the GABA switch.¹¹⁰ In rodents, the developmental GABA switch occurs during the first postnatal week. It is not clear if anesthetic effects on apoptosis are mediated via interactions with GABA_A receptors prior to or after the GABA switch. Agonism or positive allosteric modulation of the GABA_A receptor after the switch would attenuate neuronal activity, which would be expected to induce apoptosis via mechanisms similar to those described for NMDAR antagonism. In contrast, activation of GABA_A receptors prior to the switch would increase neuronal activity, which is proposed to induce apoptosis via a mechanism involving MOMP as a consequence of elevated intracellular Ca²⁺ levels.¹¹¹ Studies using a primary hippocampal cell culture model demonstrated that depolarizing agents blocked the proapoptotic activity of GABA mimetics,¹¹² suggesting that anesthetics that act as positive allosteric modulators of GABA_A receptors trigger apoptosis by reducing neuronal activity, that is, their apoptotic activity is mediated by interactions with the mature form of the GABA_A receptor.

Critical questions the field is addressing include whether anesthetic-induced apoptosis is causally linked to behavioral deficits and the potential for pediatric anesthesia to cause similar neurotoxic phenomena in the developing human brain.^{22,55} With respect to the former

issue, persistent sensorimotor, social, and cognitive deficits have been observed in preclinical models following exposure of the developing brain to anesthesia that lasted several hours or to repeated brief exposures to anesthetics over several days, but not after a single brief period of anesthesia.⁸⁴ While it has been proposed that apoptosis contributes to functional impairments associated with anesthesia,^{22,59} there is no experimental evidence that directly supports a causal link, and some experimental data argue against this possibility.⁴³ Studies in which short- and long-duration anesthetic exposures have been directly compared report outcomes suggestive of a disconnect between these two phenomena: (1) both exposure paradigms induced neuronal apoptosis, but cognitive deficits were observed only following long-duration exposure^{85,86}; (2) both exposures increased apoptotic neurodegeneration but neither were associated with cognitive deficits¹¹³; and (3) only the long-duration exposure caused significant increases in apoptosis, but both exposure paradigms elicited behavioral deficits. These findings do not rule out a causal link between anesthetic effects on apoptosis and behavioral deficits, but they do suggest that multiple factors, including biological (sex, age, and affected brain regions), chemical (type of anesthetic used), and experimental parameters (exposure paradigm, time after anesthesia when brains were collected, protocols used to measure apoptosis, and type of behavioral test used) will be important to consider in interpreting these data. The application of gene editing tools to genetically delete apoptotic-signaling molecules during targeted developmental stages may be the most definitive approach for addressing a causal link between anesthetic-induced apoptosis and functional deficits later in life.

The question of whether pediatric anesthesia causes apoptosis in the developing human brain is a significant public health concern. In humans, the period of maximal synaptogenesis, which preclinical studies indicate is the window of peak vulnerability to anesthetic-induced apoptosis, encompasses the last trimester of gestation and the first several years after birth.¹¹⁴ In the United States, over 75,000 pregnant women and 100,000 children under the age of 3 years are admitted to the hospital each year for surgical procedures requiring anesthesia.^{115,116} The results of published epidemiological studies that have investigated an association between early-life exposure to general anesthesia and increased risk of adverse neurodevelopmental outcomes are mixed (reviewed in the literature [22,84,117](#)). Much of the evidence is derived from retrospective cohort studies of academic achievement and/or diagnoses of neurobehavioral or other cognitive disorders in children who underwent surgery before the age of 3 years compared to age-matched healthy controls. Several metaanalyses of these epidemiological data^{118,119} have concluded that the weight-of-evidence suggests multiple exposures to general anesthesia before the age of 4 may moderately increase risk for adverse neurodevelopmental outcomes (reviewed in the literature [22,84,117](#)). There remains controversy, however, over the relative role of anesthesia vs. preexisting conditions that were the reason anesthesia was required, and perioperative risks (hypotension, hypoxia/ischemia, and hypocapnia).^{84,117} These issues may be addressed by ongoing prospective clinical trials, two of which reported no association between a single exposure to relatively short duration general anesthesia and adverse neurocognitive outcomes.^{120,121} There are also technical challenges in directly addressing the question of whether general anesthesia induces apoptosis in the developing human brain because, currently, unequivocal evidence of anesthetic-induced apoptosis relies on histological analyses of brain tissue collected within hours after anesthesia. This challenge may be overcome by the development of probes for in vivo imaging of apoptosis in the developing brain using positron emission tomography.¹²²

In summary, preclinical studies have established that anesthetics can induce apoptosis in the developing brain, and in many instances, this effect is coincident with sensorimotor, social, and cognitive deficits,⁸² although studies raise the question of whether these endpoints are causally related.⁴³ While the weight-of-evidence suggests that multiple,^{118,119} but not single short-duration,^{120,121} exposures to anesthetics have the potential to harm the developing human brain, the long-term outcomes are not definitive and causality has yet to be established.²²

B Polychlorinated Biphenyls

PCBs are a group of organic compounds classified according to their molecular structure and affinity for the arylhydrocarbon receptor as dioxin-like (DL) or non-dioxin-like (NDL). Congeners with >1 *ortho*-substituted chlorine are typically noncoplanar and NDL, while only 12 PCB congeners with <2 *ortho*-chlorine substituents are coplanar and DL in their toxicity.¹²³ Industrial PCB products, which were marketed in the United States as Aroclor mixtures, were used mostly as coolants and lubricants from the 1930s until 1977 when their production was banned in the United States. PCBs are still in use in the United States as dielectric fluids in transformers and capacitors. While environmental and human PCB levels dropped significantly between 1970 and 1995, PCBs remain a current and significant risk to the developing human brain. The latest NHANES study confirmed widespread exposure to PCBs among women of childbearing age living in the United States,¹²⁴ and studies detected PCBs in the indoor air of elementary schools in the United States at levels that exceed the Environmental Protection Agency's 2009 public health guidelines.¹²⁵

Legacy PCBs released from paint, caulking, and electrical transformers contribute to current human exposures; however, contemporary sources of NDL PCBs have been identified, most notably as unintentional byproducts of modern pigment manufacturing processes.^{126,127}

Critical reviews of epidemiological studies have concluded that the weight of evidence indicates a negative association between developmental exposure to environmental PCBs and measures of neuropsychological function in infancy or childhood.¹²⁸⁻¹³¹ Combined in utero and lactational PCB exposure correlates with decreased scores on IQ tests, impaired learning and memory, psychomotor difficulties, and attentional deficits. Discrepancies between studies with respect to the spectrum and persistence of adverse neurobehavioral outcomes, the confound of coexposures, and differences in congener profiles that comprise the exposures have caused some to question the causative role of PCBs in human developmental neurotoxicity¹³¹; however, experimental findings in animal models confirm that developmental PCB exposure causes deficits in learning and memory¹³²⁻¹³⁶ and sensorimotor functions.¹³⁷⁻¹³⁹ PCBs have been proposed as environmental risk factors for neurodevelopmental disorders.^{140,141} In support of this hypothesis, associations between developmental PCB exposure and increased risk of autism spectrum disorders^{142,143} and attention deficit/hyperactivity disorder (reviewed in the literature [144](#)) have been reported.

The predominant theory in the field is that PCB-induced neurobehavioral deficits reflect subtle organizational defects in neuronal connectivity.^{145,146} This hypothesis is derived in part from the lack of overt neuropathology or persistent neurochemical changes in PCB-exposed children or preclinical models of PCB developmental neurotoxicity¹⁴⁷ and from mechanistic studies indicating that PCBs, and in particular NDL PCBs, increase intracellular levels of Ca²⁺ and ROS in primary neurons cultured from embryonic rodent brains.¹⁴⁸⁻¹⁵¹ Collectively, these data pointed toward apoptosis as a potential mechanism contributing to PCB developmental neurotoxicity.¹⁵² In the initial test of this hypothesis, primary rat hippocampal neurons were exposed for 48 h to Aroclor 1254, a commercial mixture of NDL and DL PCB congeners whose congener profile is similar to that found in human tissues, including breast milk.¹⁵³ Quantification of DNA fragmentation in exposed vs. control cultures using morphometric and biochemical approaches demonstrated that Aroclor 1254 induced neuronal apoptosis and that this effect was blocked by pharmacological inhibition of caspase-3.¹⁵² This discovery has been replicated by a second laboratory that demonstrated Aroclors 1248 and 1260 induced apoptosis in primary rat cortical neurons coincident with increased caspase-3 activity, as well as increased Bax and decreased Bcl-2 proteins.¹⁵⁴ PCBs have also been shown to induce neuronal apoptosis in vivo.¹⁵⁵ Gestational and lactational exposure to Aroclor 1254 at 1 mg/kg/day in the maternal diet increases TUNEL staining and caspase-3 activity in the hippocampus, cortex, and cerebellum of newborn but not weanling rats.

To determine whether the proapoptotic activity of the Aroclor PCB mixture is due to NDL or DL congeners, apoptosis was quantified in primary neurons exposed to the single congener PCB 47, an NDL congener, or PCB 77, a DL congener.¹⁵² PCB 47, but not PCB 77, caused a caspase-3-dependent increase in neuronal apoptosis. This outcome indicates that PCB-induced apoptosis occurs independent of the arylhydrocarbon receptor, but may require ryanodine receptor (RyR) activity. RyRs are Ca²⁺-induced Ca²⁺ ion channels expressed in multiple regions of the mammalian brain whose function is to regulate Ca²⁺ release from the ER.¹⁵⁶ Binding of Ca²⁺ to the RyR stabilizes the channel in its open conformation, which increases the release of Ca²⁺ from the ER to increase intracellular levels.^{156,157} Increased RyR activity is a critical component of apoptotic signaling pathways¹⁵⁸⁻¹⁶² and a number of NDL PCBs, including PCB 47, have been shown to significantly increase RyR sensitivity to activation by nanomolar Ca²⁺ concentrations and attenuate their sensitivity to inhibitory feedback by millimolar Ca²⁺ and Mg²⁺ levels.^{163,164} Pharmacological inhibition of the RyR effectively blocked PCB 47-induced apoptosis in primary rat hippocampal neurons, while blocking other Ca²⁺ ion channels, including the IP₃ receptor, L-type calcium channel, and NMDAR was without effect.¹⁵² Collectively, these data suggest a model in which binding of NDL PCBs to RyRs triggers Ca²⁺ release from the ER and the resulting elevation of intracellular Ca²⁺ disrupts the resting mitochondrial membrane potential to induce mitochondrial membrane permeability transition pore opening,⁷ causing cytochrome *c* to be released into the cytosol ([Fig. 9.3](#)).

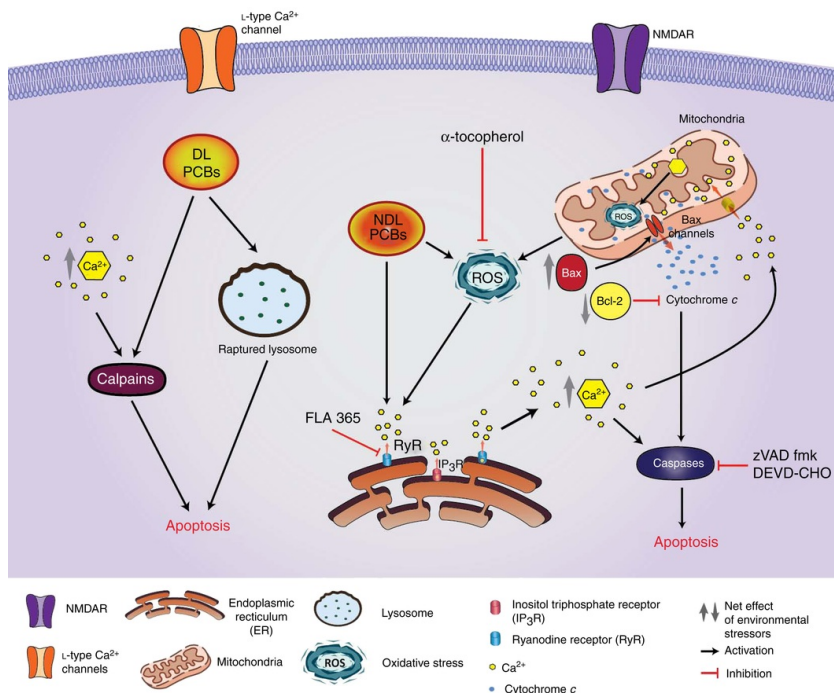


Figure 9.3 Mechanisms of PCB-induced neuronal apoptosis.

Nondioxin-like (NDL) PCBs can bind to and stabilize the ryanodine receptor (RyR) in its open configuration, thereby enhancing release of Ca^{2+} from endoplasmic reticular (ER) stores. PCB sensitization of RyRs can elevate cytoplasmic Ca^{2+} to levels that activate caspases, as evidenced by data showing that the proapoptotic activity of NDL PCBs in cultured hippocampal neurons can be blocked by pharmacological antagonism of RyRs (by FLA 365) or caspases (by zVAD fmk or DEVD-CHO). NDL PCB-induced neuronal apoptosis is also blocked by antioxidant treatment (α -tocopherol), suggesting a role for reactive oxygen species (ROS). Elevated cytoplasmic Ca^{2+} increases mitochondrial Ca^{2+} influx, which increases mitochondrial generation of reactive oxygen species (ROS); alternatively, PCBs may generate ROS directly, which then activate RyRs to increase cytoplasmic levels of Ca^{2+} . NDL PCBs have also been shown to upregulate proapoptotic proteins (Bax) and downregulate antiapoptotic proteins (Bcl-2) in neurons. Dioxin-like (DL) PCBs can also trigger neuronal apoptosis, as identified by morphological criteria, but this activity is not blocked by caspase inhibitors or by antioxidants; rather, it is prevented by pharmacological inhibitors of calpains and cathepsin D.

RyR activity is modulated by a diverse set of chemicals¹⁶⁵ and there is growing evidence that chemicals other than NDL PCBs, many of which also have noncoplanar structures, can sensitize and/or activate the RyR. Several examples include caffeine,¹⁶⁶ polybrominated diphenyl ethers (PBDEs),¹⁶⁷ triclosan,¹⁶⁸ and suramin.¹⁶⁹ Whether these RyR-active compounds trigger the same downstream key events as RyR-active PCBs has not been directly evaluated. However, the published literature suggests that at least a subset of these structurally diverse RyR-active chemicals phenocopy key events triggered by exposure to RyR-active PCBs. For example, developmental exposure to caffeine increases caspase-3 activation in multiple brain regions.¹⁷⁰ PBDEs, a class of halogenated flame retardants, sensitize RyRs via interactions with the FKBP12/RyR complex and this molecular effect is causally linked to enhanced Ca^{2+} oscillations and increased neuronal network activity.¹⁶⁷ PBDEs also

induce neuronal apoptosis in vivo and in vitro.^{45,171} In vivo, PBDE-induced apoptosis is associated with decreased expression of X-linked inhibitor of apoptosis protein (XIAP), and with deficits in learning and memory.¹⁷¹ In vitro, PBDE-induced apoptosis has been causally linked to oxidative stress.⁴⁵ Triclosan, an antimicrobial agent, and suramin, an antiparasitic drug, also sensitize RyRs.^{168,169} Triclosan induces apoptosis in cultured mouse cortical neurons via

activation of Ca²⁺-dependent caspases.¹⁷² Suramin decreases cell viability in neuronal cell lines and mouse primary neuronal cultures¹⁷³ and these neurotoxic effects are modulated by Ca²⁺ influx.¹⁷⁴ Whether the apoptotic activity of any of these RyR-active chemicals is mediated by RyR activity has yet to be addressed experimentally.

As observed with PBDEs,⁴⁵ PCB-induced apoptosis is associated with oxidative stress.^{152,155,175} In vivo, developmental exposures to doses of Aroclor 1254 that increase

neuronal apoptosis also increase levels of 3-nitrotyrosine and 4-hydroxynonenal in the same brain regions.¹⁵⁵ In vitro, hydroxylated metabolites of several PCB congeners (3, 14, 24, 35, 36, 39, and 68) induce ROS coincident with apoptosis in cultured cerebellar granule cells¹⁷⁵ and PCB 47-induced apoptosis in hippocampal neurons is blocked by treatment with the antioxidant α -tocopherol.¹⁵² Sensitization of RyRs by PCBs causes release of Ca²⁺ from the ER, which in turn can increase production of ROS,^{176,177} and increased ROS can directly modulate the channel activity of the RyR.^{178,179} Thus, whether PCB-induced ROS occurs upstream or downstream of RyR-mediated release of Ca²⁺, or is totally independent of PCB effects on RyRs is not clear (Fig. 9.3), but does suggest the possibility of a feed forward mechanism for amplifying apoptotic signals.

Additional mechanisms by which PCBs induce neuronal apoptosis independent of the intrinsic pathway (Fig. 9.3) have been described in a model of murine HT22 hippocampal cells exposed singly to PCB 153, an NDL congener, or PCB 126, a DL congener.¹⁸⁰ Apoptosis was triggered by both PCBs in the absence of mitochondrial release of cytochrome *c* or activation of caspase-3 and it was not blocked by pharmacological inhibition of caspase activity or antioxidant treatment.¹⁸⁰ Rather, PCB-induced apoptosis was associated with lysosomal disruption and was blocked by pharmacological inhibition of calpains or cathepsin D. Calpains are Ca²⁺-activated proteases, which exist as inactive zymogens in the cytosol. Intracellular Ca²⁺ overload triggers conversion of the procalpain to its active form, which then cleaves cytoplasmic and nuclear substrates, leading to apoptosis.¹⁸¹ Cathepsin D is a predominant lysosomal aspartic acid protease abundantly expressed in the brain that hydrolyzes protein substrates. Cathepsin D initiates caspase-8-dependent apoptosis.¹⁸¹ There are a number of discrepancies between this study and previous studies of PCB-induced neuronal apoptosis in cultured neurons (e.g., DL congeners induce apoptosis and NDL PCBs induce apoptosis via caspase-3 independent mechanisms in this study). Determining the reason for these differences may provide interesting insights regarding the influence of PCB structure or cell type on apoptotic mechanisms and outcomes that will inform risk assessment of exposures to complex PCB mixtures.

C Zinc

Zinc (Zn), one of the most abundant metals in the human body, is an essential metal required by all living organisms because the divalent zinc cation (Zn²⁺) is an essential cofactor for a large number of proteins (~300) that include enzymes, receptors, structural proteins, and transcription factors.^{182,183} Cellular zinc is maintained at physiological levels by the combined actions of transporter proteins, Zn²⁺-permeable ion channels, and intracellular zinc binding proteins. There are 24 known Zn²⁺ transporters, which are divided into two groups based on structure and function: (1) the Zn²⁺ transporter (ZnT) family, which consists of transmembrane H⁺-Zn²⁺ exchangers that primarily function to decrease intracellular Zn²⁺ concentrations by transporting Zn²⁺ into the extracellular space or intracellular organelles; and (2) the Zrt, Irt-like protein family, the members of which are responsible for increasing intracellular zinc levels by transporting Zn²⁺ from the extracellular space or from intracellular organelles into the cytoplasm.^{182,184} In the brain, the ZnT family member, ZnT-3, transports Zn²⁺ into synaptic vesicles in the presynaptic terminals of glutamatergic neurons, which represents a major source of chelatable or free Zn²⁺ in the brain.¹⁸³

Zn²⁺ stored in glutamatergic vesicles is released during synaptic transmission into the synaptic cleft, where it can bind to and inhibit NMDARs.^{182,184} Free Zn²⁺ in the synaptic space is cleared by zinc transport proteins or by influx through Zn²⁺ permeable ion channels, which include activated NMDARs—a subtype of α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) ionotropic glutamate receptors that lacks a GluR2 subunit in its tetrameric structure—and voltage-gated Ca²⁺ ion channels.^{185,186} Of these, the GluR2 lacking AMPA receptor seems to play a predominant role in mediating synaptic Zn²⁺ influx, particularly in the context of zinc-induced cell death.¹⁸⁵ Free Zn²⁺ in the cytoplasm is buffered by metallothioneins (MTs), which are low molecular weight proteins with numerous sulfhydryl groups that confer a high binding affinity for Zn²⁺.^{182,184} MT-bound zinc represents a major reservoir of zinc in neuronal cells that can be released as free Zn²⁺ under pathological conditions.¹⁸⁶

Zinc plays a key role in the physiology of the nervous system, functioning to regulate cellular redox balance¹⁸⁷ and to modulate synaptic transmission and intracellular signaling.¹⁸² Perturbations of zinc homeostasis are implicated in neurological and psychiatric disorders, including ischemic stroke, Alzheimer's disease, and depression^{182,184,185}. During brain development, zinc is critically involved in regulating neural cell proliferation, differentiation, and survival.¹⁸⁸ Consistent with its role in these neurodevelopmental processes, zinc dyshomeostasis is associated with adverse neurodevelopmental outcomes. Severe gestational zinc deficiency can cause overt fetal brain malformations: suboptimal zinc nutrition during gestation is associated with long-term neurological effects in the offspring. Excessive zinc can be neurotoxic.^{186,188}

Zinc status exerts significant influence on apoptosis in the developing brain, but its role is complex.^{186,189} In postnatal day seven rat pups, administration of the zinc chelator, *N,N,N',N'*-tetra-kis(2-pyridyl)methyl)

ethylenediamine (TPEN) significantly decreases the number of apoptotic neurons in the brain relative to control subjects,¹⁹⁰ suggesting that endogenous zinc mediates apoptotic cell death in the developing brain. However, other experimental data suggest that, at physiological levels, zinc functions to protect against apoptosis. The antiapoptotic effects of zinc are thought to be mediated by two of its biological activities: (1) zinc is a potent inhibitor of caspase-3¹⁹¹ with an IC₅₀ below 10 nM¹⁹²; and (2) zinc limits the damage caused by oxidative stress by protecting sulfhydryl groups in proteins and stabilizing lipids in cellular membranes.¹⁸⁶ Perturbations of zinc homeostasis that result in either deficiency or toxicosis have been shown to induce apoptosis, even in the same model system¹⁸² and some neurotoxic chemicals are thought to trigger apoptosis in the developing brain via zinc dyshomeostasis.^{186,193}

High levels of intracellular zinc have been shown to induce apoptosis in various tissues and cell types,¹⁸⁶ including neuronal precursor cells, neurons, and glial cells^{188,189,194,195} via multiple mechanisms (Fig. 9.4). In neurons, zinc-induced apoptotic cell death is associated with increased levels of ROS^{196,197} and attenuated by antioxidants,¹⁹⁸ suggesting that excessive Zn²⁺ triggers apoptosis via ROS-dependent mechanisms. High zinc levels can generate ROS via several mechanisms, including depolarization of mitochondria¹⁹⁷ and activation of ROS-generating cytosolic enzymes, such as NADPH oxidase.¹⁹⁹ High zinc levels can also induce ER stress and decrease energy production,^{200,201} which can increase intracellular Ca²⁺ to levels that cause MOMP. Elevated concentrations of intracellular Zn²⁺ can also directly induce mitochondrial permeability transition pore opening to release proapoptotic proteins from the intermembrane space.²⁰² Excessive zinc can additionally trigger the intrinsic pathway of apoptosis by upregulating the expression of Bax, which decreases the Bcl-2/Bax ratio,¹⁹⁸ and by activating p38, which enhances K⁺ efflux via Kv2.1 channels.²⁰³ High zinc levels also activate the extrinsic pathway of apoptosis, as evidenced by the induction of p75^{NTR} and the death executor-associated factor, early growth response factor 1,²⁰⁴ which together have been shown to mediate zinc-induced neuronal cell death.²⁰⁵

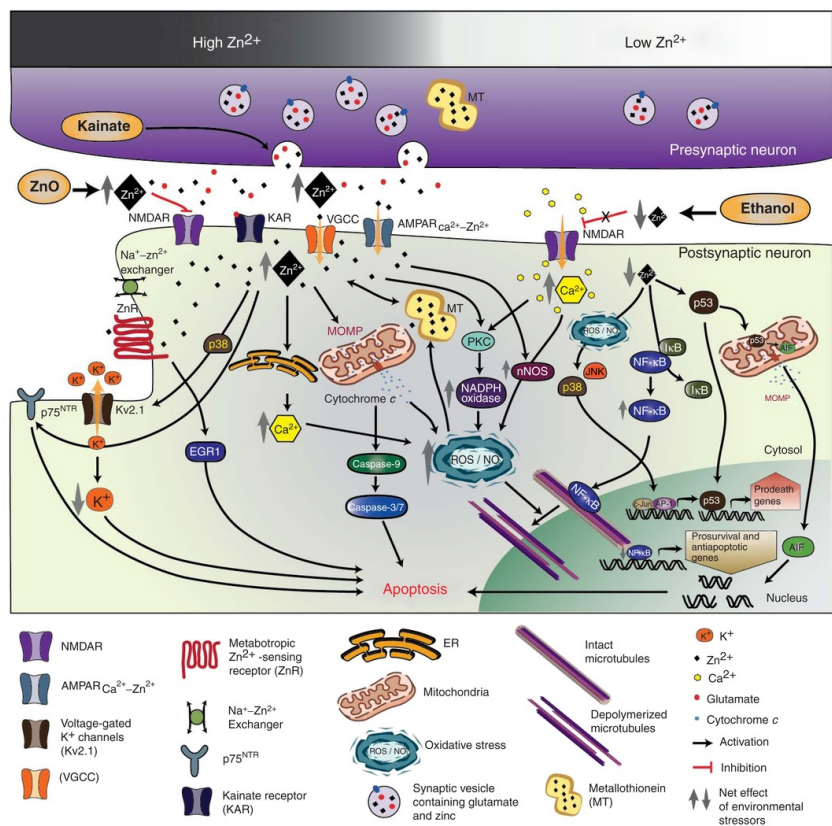


Figure 9.4 Mechanisms by which cellular zinc dyshomeostasis triggers neuronal apoptosis.

Neuronal apoptosis can be triggered by shifts in cellular Zn²⁺ toward levels that are either higher (left) or lower (right) than the physiological range. Environmental stressors that increase free Zn²⁺ include the following: (1) presynaptic kainate receptor agonists (e.g., kainite and domoic acid), which depolarize glutamatergic neurons to release Zn²⁺ sequestered with glutamate in synaptic vesicles; (2) zinc oxide nanoparticles, which release free Zn²⁺; and (3) elevated levels of reactive oxygen species (ROS), which promote release of Zn²⁺ from metallothionein (MT) proteins. Free Zn²⁺ can move from extracellular to intracellular compartments via the Zn²⁺-permeable GluR2-lacking AMPA receptors (AMPA_{Ca-Zn}), voltage-gated Ca²⁺ channels (VGCCs) and the transmembrane Na⁺-Zn²⁺ exchanger. Extracellular Zn²⁺ can also activate the metabotropic Zn²⁺-sensing receptor (ZnR). High levels of intracellular Zn²⁺ can trigger neuronal apoptosis via multiple mechanisms: (1) generation of ROS via depolarization of mitochondria or activation of ROS-generating cytosolic enzymes; (2) increased intracellular Ca²⁺ as a consequence of ER stress; (3) induction of p38-dependent K⁺ efflux via Kv2.1 channels; (4) mitochondrial outer membrane permeabilization (MOMP), which releases cytochrome *c* into the cytoplasm where it activates caspases; or (5) induction of death executor-associated factors, such as early growth response factor 1 (EGR1) and p75^{NTR}. Environmental stressors that decrease Zn²⁺ availability, such as ethanol, can also trigger neuronal apoptosis. Since the NMDAR is inhibited by Zn²⁺, Zn²⁺ deficiency increases NMDAR activation, which enhances Ca²⁺ influx. Increased intracellular Ca²⁺ activates protein kinase C (PKC) and calmodulin (CaM), which in turn activate NADPH oxidase and NO synthase to increase ROS. While low Zn²⁺ levels promote NF-κB activation, oxidative stress promotes microtubule depolymerization, thereby preventing nuclear translocation of NF-κB, resulting in decreased expression of prosurvival and antiapoptotic genes (e.g., cIAP and XIAP). Low intracellular Zn²⁺ levels also activate p38, JNK, and p53, which increase transcription of genes encoding proapoptotic proteins (e.g., Fas, Bid, Bax, Noxa, and PUMA).

Neurotoxic chemicals can increase free Zn²⁺ in the brain via several mechanisms (Fig. 9.4). First, since the release of Zn²⁺ sequestered in glutamatergic synaptic vesicles correlates with neuronal activity,²⁰⁶ presynaptic kainate receptor agonists (e.g., kainite and domoic acid) create Zn²⁺ overload in the extracellular space because of prolonged stimulation of presynaptic glutamatergic nerve terminals. Second, nitric oxide, oxidative stress, and acidosis promote release of Zn²⁺ from MT proteins.²⁰⁷ The physiological significance of this effect is suggested by studies demonstrating that the inhibition of nitric oxide synthase significantly reduces Zn²⁺ release from brain slices during oxygen and glucose deprivation coincident with decreased neuronal apoptosis.^{183,208} Thus, chemicals that promote acidosis or generation of nitric oxide and/or ROS (e.g., 3-nitropropionic acid and methamphetamine) can significantly increase intracellular concentrations of free Zn²⁺. Third, free Zn²⁺ in cells has been shown to be significantly increased by the release of free Zn²⁺ from zinc oxide (ZnO) nanoparticles,²⁰⁹ which trigger apoptotic cell death in neural stem cells.²¹⁰

Zinc toxicosis is uncommon in humans because the human body has extremely efficient systemic and cellular mechanisms for maintaining zinc homeostasis over a broad range of exposure levels.¹⁸⁶ Severe zinc deficiency is also relatively rare in humans, but moderate zinc deficiency is epidemic in human populations throughout the world. Approximately 82% of women worldwide and up to 100% of women in developing countries, are suspected to have insufficient zinc intake during pregnancy.²¹¹ Maternal zinc deficiency is associated with altered development of neurobehavior in humans²¹²⁻²¹⁵ and in animal models.^{216,217} Marginal zinc deficiency during gestation is not teratogenic, but it does affect signaling and gene expression in the developing brain^{60,218} and has been shown to cause long-term cognitive deficits.^{219,220} For example, the offspring of dams with marginal dietary zinc deficiency during gestation and lactation exhibit impaired performance in passive avoidance tasks as adults.²²¹

Zinc deficiency induces apoptotic cell death in various cell types, including neuronal precursor cells, neurons and glia, in vivo and in vitro.^{188,222,223} Incubation of human neuroblastoma IMR-32 cells and primary rat cortical neurons in culture medium depleted of zinc induces apoptosis via the intrinsic pathway. This is evidenced by increased levels of the proapoptotic protein, Bad, in the mitochondrial fraction of zinc-deficient cells, increased cytosolic levels of cytochrome *c*, caspase-3 activation and DNA fragmentation, and supplementation of the medium with zinc normalizes caspase-3 activity coincident with inhibition of apoptotic cell death.¹⁸⁹ The mechanisms by which low zinc levels activate the intrinsic pathway of apoptosis remain uncertain but it is proposed that activation of p53 and caspases, and perhaps oxidative stress, are involved^{188,223} (Fig. 9.4). A role for p53 in zinc deficiency-induced neuronal apoptosis is suggested by data from several labs.^{189,222-224} Neuronal precursor cells and neurons cultured in zinc-depleted medium exhibit significantly increased p53 protein levels^{189,222,223} and inhibition of p53 signaling by pharmacologic blockade or genetic deletion significantly attenuates apoptotic cell death in neuronal precursor cells treated with the zinc chelator TPEN.²²²⁻²²⁴ Zinc chelation by TPEN upregulates PUMA and NoxA expression via p53-dependent mechanisms, which consequently promote the release of cytochrome *c* from the mitochondria

and caspase activation.²²⁴ Low levels of cellular zinc may also enhance caspase activation by alleviating the inhibitory effects of physiological zinc levels on caspase activity.¹⁹¹ Zinc deficiency causes oxidative stress in neuronal

cells by increasing cell oxidant levels, particularly H₂O₂, and by decreasing concentrations of glutathione, a key antioxidant molecule in neurons.¹⁸⁷ However, treatment with catalase does not prevent caspase-3 activation induced by low zinc levels in cultured neurons, suggesting that oxidative stress is not the triggering event in zinc deficiency-induced neuronal apoptosis,¹⁸⁸ although it may exert proapoptotic actions via effects on signaling molecules (shown later).

Other signaling molecules that may be involved in zinc deficiency-induced apoptosis include prosurvival signaling molecules, such as ERK and Akt, and proapoptotic signaling molecules, such as p38 and JNK, whose activities are decreased and increased, respectively, by low zinc availability.^{186,188,225,226} Zinc deficiency may also induce neuronal apoptosis via modulation of NF-κB signaling (Fig. 9.4). NF-κB signaling is dysregulated in the developing brain⁶⁰ and in neuronal cell cultures²²⁷ under conditions of low zinc availability. In IMR-32 cells, zinc depletion increases the production of oxidants causing IκB phosphorylation and degradation,²²⁷ which are the initial steps in the activation of the NF-κB signaling pathway. However, active NF-κB is not efficiently translocated into the nucleus, as evidenced by decreased nuclear levels of NF-κB and NF-κB DNA binding in zinc-deficient neurons.²²⁸ This is thought to be due to the disruption of the microtubule network via oxidative damage caused by zinc deficiency,²²⁸ since functional microtubules are required for nuclear translocation of NF-κB in neurons.²²⁹ As a consequence, NF-κB-responsive genes, which include many prosurvival and antiapoptotic genes such as IAPs and antiapoptotic Bcl-2 and Bcl-XL,¹⁸⁹ are downregulated.¹⁸⁸

The developmental toxicity of several drugs and environmental toxicants can be partially attributed to deficiencies in maternal and embryonic zinc.^{186,193,230} Chemicals can cause zinc deficiencies by directly chelating zinc, upregulating cellular levels of zinc-binding proteins, such as MTs, or altering zinc metabolism. A classic example is ethanol. In wildtype mice, exposure to ethanol at a relevant concentration on gestational day 8 causes maternal liver MT concentrations to increase by 20-fold within 16 h.⁶¹ This is associated with redistribution of zinc from the maternal plasma to the maternal liver, as evidenced by increased zinc concentrations in maternal liver, decreased zinc concentrations in maternal plasma and the fetus, and increased fetal abnormalities. In contrast, the same ethanol exposure paradigm in MT knockout mice increases zinc concentrations in maternal plasma and the fetus.⁶¹ Other chemicals that similarly cause maternal zinc deficiencies by upregulating maternal liver MT include arsenic, melphalan, and α-hederin.¹⁹³ More recent studies implicate zinc deficiency as a mechanism contributing to the neurotoxic effects of lipopolysaccharide (LPS) in the developing brain,^{74,231} which include dysregulation of apoptosis.⁷⁵ How LPS decreases zinc availability remains unknown, but these observations suggest the intriguing possibility that zinc deficiency may contribute to the adverse neurodevelopmental outcomes associated with prenatal infection or exposure to inflammatory mediators.

V Conclusions

Apoptosis is a physiological process that plays a critical role in shaping neural circuitry in the developing brain. Perturbations of the spatiotemporal patterns or the extent of apoptosis are associated with adverse neurodevelopmental outcomes. Many chemicals have been shown to trigger the apoptosis of neuronal or glial cells in vitro or in vivo. While specific mechanisms of apoptosis induction vary between exposure paradigms, chemicals generally trigger apoptosis by increasing proapoptotic and/or decreasing antiapoptotic signaling secondary to oxidative stress, increased intracellular Ca²⁺ levels, or Zn²⁺ dyshomeostasis. It is widely posited that chemical-induced apoptosis is a mechanism of developmental neurotoxicity; however, a causal link between chemical-induced apoptosis and neurobehavioral deficits following developmental exposures has yet to be established in most cases. Establishing cause-effect relationships will likely require approaches that use modern gene editing tools, such as CRISPR-Cas, and the progress that has been made in the field thus far in identifying the molecular mechanisms underlying chemical-induced apoptosis will be required to inform the design of such studies. An exciting advance in the field is the development of probes for in vivo positron emission tomography imaging of apoptosis in the developing brain,^{122,232} which will enable the translation of preclinical evidence of chemical-induced apoptosis to the developing human brain.

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Queries and Answers

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