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Chapter 9 Apoptosis as a Mechanism of Developmental Neurotoxicity

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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 to: (1) provide an overview of 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

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 Currie[1] 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 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 both 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 to: (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.

I. 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 fourteen 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 pro-enzymes, and are characterized as having an N-terminal pro-domain 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 pro-domain[7]. Proteolysis of the pro-domain 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 anti-apoptotic 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 (**Figure 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 release of pro-apoptotic

factors from the intermembrane space of 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 (BH) domain, and they can be subdivided into two functional groups: (1) those that exert anti-apoptotic effects by stabilizing the mitochondrial membrane (e.g., Bcl-2, Bcl-XL or Bcl-w); and (2) those that are pro-apoptotic and function to increase mitochondrial membrane permeability (e.g., Bid, Bad, Bim, NoxA, PUMA, Bax, Bak). Activation of the intrinsic pathway of apoptosis alters Bcl-2 protein expression and/or activity via both transcriptional and post-translational modifications: the former downregulate expression of anti-apoptotic Bcl-2 proteins and upregulate that of pro-apoptotic members of the Bcl-2 family; the latter trigger pro-apoptotic 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 expression of the pro-apoptotic 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: (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 MOMP[8]; and (2) elevated levels of intracellular Ca^{2+} that disrupt the resting mitochondrial membrane potential to induce mitochondrial membrane permeability transition pore opening[7].

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 Ca^{2+} released from IP_3-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 pro-caspase-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 pro-caspase-9 via homophilic CARD interactions[7]. Within this multimeric complex, known as the apoptosome, pro-caspase-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-mediated pathway because it is activated by extracellular "death" ligands that bind to cell surface death receptors (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 TNFα; Fas, Fas-Fas ligand (FasL); $p75^{NTR}$, pro-

neurotrophins; 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 pro-caspase-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 pro-caspases 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 pro-apoptotic Bcl-2 homology 3 (BH3)-interacting domain death antagonist (Bid). Cleavage of Bid forms truncated Bid (tBid), which interacts with and stimulates the pro-apoptotic 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 anti-apoptotic and pro-apoptotic actions[7,13]. Upon binding its cognate ligand, TNFα, 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-κB pathway and the caspase-8 pathway. The former is activated by Traf-2-mediated recruitment of the Iκκ complex, which contains NF-κB complexed to the inhibitor IκB. Rip promotes the phosphorylation and subsequent proteasomal degradation of IκB to release NF-κB[13]. NF-κB translocates to the nucleus where it initiates transcription of a

large number of anti-apoptotic and pro-survival genes including FLICE inhibitory protein (FLIP) and various IAPs. In the second, pro-apoptotic 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 pro-caspase-8 to form Complex II. Whether pro-caspase-8 self-activates upon complexing with the DED in FADD depends on relative levels of NF-κB-induced anti-apoptotic proteins, such as FLIP. FLIP, which has been found to co-immunoprecipitate 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 pro-caspase-8[13]. Activated TNFR1 can also trigger an alternative pro-apoptotic 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 pro-caspase-2 to cause apoptosis[7]. Thus, apoptotic signaling through TNFR1 includes an NF-κB-mediated pro-survival response that offsets the apoptotic response, so whether TNFR1 signaling induces apoptotic cell death depends on the relative balance of pro-apoptotic to anti-apoptotic signals activated downstream of TNFR1 activation.

II. Physiological roles of apoptosis in neurodevelopment

Cell death occurs at specific developmental stages in response to extrinsic and intrinsic cues[14] 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 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 post-mitotic 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 post-ganglionic 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 non-neural 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 non-neural 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 mis-specified, mis-positioned and redundant neurons in the developing nervous system. Its role in regulating cell number is best exemplified by the neurotrophic theory, as described above. 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 post-mitotic 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 4-fold increase in the density of intrinsically photosensitive retinal ganglion cells (ipRGCs) in the eye, which is coincident with

disrupted ipRGC 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[29-34], 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[35]. 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 in order 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[36]. 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[37,38]. In mouse models, genetic deletion of *bax* largely abolishes sex-specific differences in neuronal cell number in these brain regions[39].

Clinical studies corroborate the importance of apoptosis in normal neurodevelopment. Altered patterns of apoptosis are associated with diverse neurodevelopmental disorders[40] including autism spectrum disorders[41,42] and schizophrenia[43,44]. There is also clinical evidence suggesting that the developing human brain has an increased propensity for apoptosis after traumatic brain injury[45] 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[45,46]. Conversely, insufficient apoptosis has been posited as a pathogenic mechanism of pediatric brain cancer[8]. Collectively, these observations suggest that non-physiological 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.

III. 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[2,18,47-50]. Chemicals with pro-apoptotic activity include not only well-established developmental neurotoxicants, such as lead,[51,52] methyl mercury,[53] ethanol[54-56], and chlorpyrifos[57,58], but also drugs, notably pediatric general anesthetics[59,60]. 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[22,61]. 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[18,62,63]. Based on such data, Olney and colleagues 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[60]. More recently, 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[8,61]. 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 pro-apoptotic 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[2,18,47-50], it is perhaps not surprising that diverse molecular initiating events have been identified (**Table 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 ROS and/or Ca^{2+}, disruption of the cytoskeleton, DNA damage); (2) upregulated expression of DRs (death receptors) and/or death ligands that activate the extrinsic pathway of apoptosis; and (3) transcriptional or post-translational mechanisms that increase the ratio of pro-apoptotic to anti-apoptotic 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, polychlorinated biphenyls (PCBs) and zinc dyshomeostasis as examples.

A. Anesthetics

The induction of apoptosis in the developing brain by anesthetics was first reported by John Olney and his colleagues. 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[64-66]. Subsequent studies to determine whether administration of drugs that block N-methyl-D-aspartate (NMDA) receptors protected against excitotoxicity-induced neurodegeneration revealed that while these drugs decreased head trauma-induced

excitotoxic cell death as predicted[64], unexpectedly, they also significantly increased apoptotic cell death[66]. Following up on this startling discovery, Olney and colleagues found that NMDA receptor antagonists promoted apoptotic neurodegeneration in not only the traumatized immature rodent brain, but also in the non-traumatized, normally developing rodent brain[67]. 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[56], or drugs that blocked voltage-gated Na^+ channels, such as the anticonvulsants phenytoin and valproate[68], 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 NMDA receptors and/or positive allosteric modulation of $GABA_A$ receptors[59,60], 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 hours using a drug combination (nitrous oxide, midazolam and isoflurane) commonly used in pediatric anesthesia[69]. 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, nitrous oxide), administered alone or in combination in diverse preclinical models[70-74], including nonhuman primates[75-79]. 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[72]. Apoptosis of interneurons and oligodendrocytes has also been observed in preclinical models following anesthetic administration[80-82]. The peak apoptotic response is observed between 3 and 6 hours 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[22,63,83]. The influence of timing likely reflects the fact that the developing brain is most vulnerable to anesthetic-induced apoptosis during periods of peak synaptogenesis[84], which varies between brain regions[61] 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 hours of nitrous oxide anesthesia, the intrinsic pathway of apoptosis is activated, as evidenced by western blot data indicating decreased levels of the anti-apoptotic 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[84]. After 6 hours 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[84]. But how do these anesthetic

drugs activate apoptotic signaling pathways? As indicated above, many anesthetics act by antagonizing the NMDA receptor[67,85], which decreases the influx of calcium through NMDA receptors, thereby reducing activation of downstream Ca^{2+}-dependent signaling cascades, notably, the extracellular regulated protein kinase (ERK1/2) signaling pathway (**Figure 2**). ERK1/2 signaling activates the transcription factor, cAMP-responsive element binding protein (CREB), via phosphorylation, and pCREB transcriptionally upregulates expression of the anti-apoptotic molecule Bcl-2[85]. 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 anti-apoptotic molecule, Bcl-2 and a subsequent increase in the number of apoptotic cells in the brain[86].

In addition to the Bcl-2 family of anti-apoptotic proteins, CREB positively regulates transcription of neurotrophins (**Figure 2**), which play a key role in regulating neuronal survival in the developing brain [84,87]. Neurotrophins are synthesized and released by neurons in an activity-dependent manner[88]. Developmental exposure to anesthetics disrupts neurotrophin signaling, and this often precedes apoptotic death of neurons across multiple brain regions[88,89]. Neurotrophins control neuronal survival through interactions with two transmembrane receptor subtypes: tropomyosin receptor kinase (Trk) receptors and the TNFR family member, $p75^{NTR}$ [88,90]. Both receptor subtypes are co-expressed on all neurons and the mechanisms that control pro- or anti-apoptotic cell fate rely on the affinity of these receptors for their cognate ligands[91]. Trk receptors have a greater affinity for mature neurotrophins, in which the pro-domain 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 nerve growth factor (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[91]. Thus, the balance between expression of Trk *vs*. $p75^{NTR}$ receptors, and pro- *vs*. mature neurotrophins, determines whether developing neurons survive or undergo apoptotic cell death. Anesthetic blockade of NMDA receptor activity can shift that balance towards apoptotic cell death via several mechanisms (**Figure 2**). First, anesthetic-induced downregulation of CREB activity can decrease the transcription of pro-survival neurotrophins such as BDNF[89]. 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[92]. Activation of $p75^{NTR}$ by pro-NGF triggers apoptosis by recruiting TRAF adaptor protein complexes[90,91] that activate downstream apoptotic signaling cascades, resulting in oligomerization and translocation of pro-apoptotic proteins in the Bcl-2 family, such as Bad and Bax[93-95] to the outer mitochondrial membrane to cause MOMP and release of cytochrome *c* into the cytoplasm (**Figure 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 age[96]; 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 NMDA receptors with subsequent over-stimulation of the glutamatergic system by endogenous glutamate, triggering intracellular calcium overload, which enhances neuronal apoptosis[75,97,98].

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[99]. 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 NMDA receptor 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 Ca^{2+} levels[100]. Studies using a primary hippocampal cell culture model demonstrated that depolarizing agents blocked the pro-apoptotic activity of GABA mimetics[101], suggesting that anesthetics that act as positive allosteric modulators of $GABA_A$ receptors trigger apoptosis by reducing neuronal activity, i.e., their apoptotic activity is mediated by interactions with the mature form of the $GABA_A$ receptor.

Critical questions the field is currently 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[22,59]. 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[72]. While it has been proposed that apoptosis contributes to functional

impairments associated with anesthesia[22,63], there is no experimental evidence that directly supports a causal link, and some experimental data argue against this possibility[47]. 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[73,74]; (2) both exposures increased apoptotic neurodegeneration but neither were associated with cognitive deficits[102]; 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, 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, 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[103]. 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[104,105]. 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 [22,72,106]]. 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 meta-analyses of these epidemiological data[107,108], 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 [22,72,106]]. There remains controversy, however, over the relative role of anesthesia vs. pre-existing conditions that were the reason anesthesia was required, and perioperative risks (hypotension, hypoxia/ischemia, hypocapnia)[72,106]. These issues may be addressed by ongoing prospective clinical trials, two of which recently reported no association between a single exposure to relatively short duration general anesthesia and adverse neurocognitive outcomes[109,110]. 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 recent development of probes for *in vivo* imaging of apoptosis in the developing brain using positron emission tomography (PET)[111].

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[70], although recent studies raise the question of whether these endpoints are causally related[47]. While the weight-of-evidence suggests that multiple[107,108], but not single short duration [109,110], 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)

PCBs are a group of organic compounds classified according to their molecular structure and affinity for the arylhydrocarbon receptor (AhR) as dioxin-like (DL) or non-dioxin-like (NDL). Congeners with >1 *ortho*-substituted chlorine are typically non-coplanar and NDL, while only twelve PCB congeners with <2 *ortho*-chlorine substituents are co-planar and dioxin-like in their toxicity[112]. 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[113] and recent studies detected PCBs in the indoor air of elementary schools in the United States at levels that exceed the EPA's 2009 public health guidelines[114]. 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[115,116].

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[117-120]. 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 co-exposures and differences in congener profiles that comprise the exposures have caused some to question the causative role of PCBs in human developmental neurotoxicity[120]; however, experimental findings in animal models confirm that developmental PCB exposure causes deficits in learning and memory[121-125] and sensorimotor functions[126-128]. More recently, PCBs have been proposed as environmental risk factors for neurodevelopmental disorders[129,130]. In support of this hypothesis, associations between developmental PCB exposure and increased risk of autism spectrum disorders (ASD)[131,132] and attention deficit/hyperactivity disorder (ADHD) [reviewed in [133]] have been reported.

The predominant theory in the field is that PCB-induced neurobehavioral deficits reflect subtle organizational defects in neuronal connectivity[134,135]. 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 neurotoxicity[136] and from mechanistic studies indicating that PCBs, and in particular NDL PCBs, increase intracellular levels of Ca^{2+} and ROS in primary neurons cultured from embryonic rodent brains[137-140]. Collectively, these data pointed towards apoptosis as a potential mechanism contributing to PCB developmental neurotoxicity[141]. In the initial test of this hypothesis, primary rat hippocampal neurons were exposed for 48 hours 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[142]. 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[141]. 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 increased Bax and decreased Bcl-2 proteins[143]. PCBs have now also been shown to induce neuronal apoptosis *in vivo*[144]. 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 pro-apoptotic 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[141]. 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 AhR, but may require ryanodine receptor (RyR) activity. RyRs are Ca^{2+}-induced Ca^{2+} ion channels expressed in multiple regions of the mammalian brain whose function is to regulate Ca^{2+} release from the ER[145]. Binding of Ca^{2+} to the RyR stabilizes the channel in its open conformation, which increases release of Ca^{2+} from the endoplasmic reticulum to increase intracellular levels[145,146]. Increased RyR activity is a critical component of apoptotic signaling pathways[147-151] and a number of NDL PCBs, including PCB 47, have been shown to significantly increase RyR sensitivity to activation by nanomolar Ca^{2+} concentrations and attenuate their sensitivity to inhibitory feedback by millimolar Ca^{2+} and Mg^{2+} levels[152,153]. Pharmacological inhibition of the RyR effectively blocked PCB 47-induced apoptosis in primary rat hippocampal neurons, while blocking other Ca^{2+} ion channels, including the IP_3 receptor, L-type calcium channel and NMDA receptor was without effect[141]. Collectively, these data suggest a model in which binding of NDL PCBs to RyRs triggers Ca^{2+} release from the ER and the resulting elevation of intracellular Ca^{2+} disrupts the resting mitochondrial

membrane potential to induce mitochondrial membrane permeability transition pore opening[7], causing cytochrome *c* to be released into the cytosol (**Figure 3**).

RyR activity is modulated by a diverse set of chemicals[154] and there is growing evidence that chemicals other than NDL PCBs, many of which also have non-coplanar structures, can sensitize and/or activate the RyR. Several examples include caffeine[155], polybrominated diphenyl ethers (PBDEs)[156], triclosan[157] and suramin[158]. 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[159]. 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[156]. PBDEs also induce neuronal apoptosis *in vivo* and *in vitro*[49,160]. *In vivo*, PBDE-induced apoptosis is associated with decreased expression of XIAP, and with deficits in learning and memory[160]. *In vitro*, PBDE-induced apoptosis has been causally linked to oxidative stress[49]. Triclosan, an antimicrobial agent, and suramin, an antiparasitic drug, also sensitize RyRs[157,158]. Triclosan induces apoptosis in cultured mouse cortical neurons via activation of Ca^{2+}-dependent caspases[161]. Suramin decreases cell viability in neuronal cell lines and mouse primary neuronal cultures[162] and these neurotoxic effects are modulated by Ca^{2+} influx[163]. 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[49], PCB-induced apoptosis is associated with oxidative stress[141,144,164]. *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[144]. *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 cells[164] and PCB 47-induced apoptosis in hippocampal neurons is blocked by treatment with the antioxidant α-tocopherol[141]. Sensitization of RyRs by PCBs causes release of Ca^{2+} from the ER, which in turn can increase production of ROS[165,166] and increased ROS can directly modulate the channel activity of the RyR[167,168]. Thus, whether PCB-induced ROS occurs upstream or downstream of RyR-mediated release of Ca^{2+}, or is totally independent of PCB effects on RyRs is not clear (**Fig 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 (**Figure 3**) have been described in a model of murine HT22 hippocampal cells exposed singly to PCB 153, a NDL congener, or PCB 126, a DL congener[169]. 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[169]. Rather, PCB-induced apoptosis was associated with lysosomal disruption and was blocked by pharmacological inhibition of calpains or cathepsin D. Calpains are Ca^{2+}-activated proteases, which exist as inactive zymogens in the cytosol. Intracellular Ca^{2+} overload triggers conversion of the pro-calpain to its active form, which then cleaves cytoplasmic and nuclear substrates, leading to apoptosis[170]. 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[170]. 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 (Zn^{2+}) is an essential cofactor for a large number of proteins (~300) that include enzymes, receptors, structural proteins and transcription factors[171,172]. Cellular zinc is maintained at physiological levels by the combined actions of transporter proteins, Zn^{2+}-permeable ion channels and intracellular zinc binding proteins. There are 24 known Zn^{2+} transporters, which are divided into two groups based on structure and function: (1) the Zn^{2+} transporter (ZnT) family, which consists of transmembrane H^{+}- Zn^{2+} exchangers that primarily function to decrease intracellular Zn^{2+} concentrations by transporting Zn^{2+} into the extracellular space or intracellular organelles; and (2) the Zrt, Irt-like protein (ZIP) family, the members of which are responsible for increasing intracellular zinc levels by transporting Zn^{2+} from the extracellular space or from intracellular organelles into the cytoplasm[171,173]. In the brain, the ZnT family member, ZnT-3, transports Zn^{2+} into synaptic vesicles in the presynaptic terminals of glutamatergic neurons, which represents a major source of chelatable or free Zn^{2+} in the brain[172].

Zn^{2+} stored in glutamatergic vesicles is released during synaptic transmission into the synaptic cleft, where it can bind to and inhibit NMDA receptors[171,173]. Free Zn^{2+} in the synaptic space is cleared by zinc transport proteins or by influx through Zn^{2+} permeable ion channels, which include activated NMDA receptors--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 Ca^{2+} ion channels[174,175]. Of these, the GluR2 lacking AMPA receptor seems to play a predominant role in mediating synaptic Zn^{2+} influx, particularly in the context of zinc-induced cell death[174]. Free Zn^{2+} 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 Zn^{2+}[171,173]. MT-bound zinc represents a major reservoir of zinc in neuronal cells that can be released as free Zn^{2+} under pathological conditions[175].

Zinc plays a key role in the physiology of the nervous system, functioning to regulate cellular redox balance[176] and to modulate synaptic transmission and intracellular signaling[171]. Perturbations of zinc homeostasis are implicated in neurological and psychiatric disorders, including ischemic stroke, Alzheimer's disease and depression[171,173,174]. During brain development, zinc is critically involved in regulating neural cell proliferation, differentiation and survival[177]. 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[175,177].

Zinc status exerts significant influence on apoptosis in the developing brain, but its role is complex[175,178]. In postnatal day 7 rat pups, administration of the zinc chelator, *N*,*N*,*N*′,*N*′-tetra-

kis(2-pyridylmethyl) ethylenediamine (TPEN) significantly decreases the number of apoptotic neurons in the brain relative to control subjects[179], 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 anti-apoptotic effects of zinc are thought to be mediated by two of its biological activities : (1) zinc is a potent inhibitor of caspase-3[180] with an IC_{50} below 10 nM[181]; and (2) zinc limits the damage caused by oxidative stress by protecting sulfhydryl groups in proteins and stabilizing lipids in cellular membranes[175]. Perturbations of zinc homeostasis that result in either deficiency or toxicosis have been shown to induce apoptosis, even in the same model system[171] and some neurotoxic chemicals are thought to trigger apoptosis in the developing brain via zinc dyshomeostasis[175,182].

High levels of intracellular zinc have been shown to induce apoptosis in various tissues and cell types[175], including neuronal precursor cells, neurons and glial cells[177,178,183,184], via multiple mechanisms (**Figure 4**). In neurons, zinc-induced apoptotic cell death is associated with increased levels of ROS[185,186] and attenuated by antioxidants[187], suggesting that excessive Zn^{2+} triggers apoptosis via ROS-dependent mechanisms. High zinc levels can generate ROS via several mechanisms, including depolarization of mitochondria[186] and activation of ROS-generating cytosolic enzymes, such as NADPH oxidase[188]. High zinc levels can also induce ER stress and decrease energy production[189,190], which can increase intracellular Ca^{2+} to levels that cause MOMP. Elevated concentrations of intracellular Zn^{2+} can also directly induce mitochondrial permeability transition pore opening to release pro-apoptotic proteins from the intermembrane space[191]. Excessive zinc can additionally trigger the intrinsic pathway of apoptosis by upregulating expression of Bax, which decreases the Bcl-2/Bax ratio[187], and by

activating p38, which enhances K^+ efflux via Kv2.1 channels[192]. High zinc levels also activate the extrinsic pathway of apoptosis, as evidenced by induction of $p75^{NTR}$ and the death executor-associated factor, early growth response factor 1 (EGR1)[193], which together have been shown to mediate zinc-induced neuronal cell death[194].

Neurotoxic chemicals can increase free Zn^{2+} in the brain via several mechanisms (**Figure 4**). First, since release of Zn^{2+} sequestered in glutamatergic synaptic vesicles correlates with neuronal activity[195], presynaptic kainate receptor agonists (e.g., kainate, domoic acid) create Zn^{2+} overload in the extracellular space as a consequence of prolonged stimulation of presynaptic glutamatergic nerve terminals. Second, nitric oxide, oxidative stress and acidosis promote release of Zn^{2+} from MT proteins[196]. The physiological significance of this effect is suggested by studies demonstrating that inhibition of nitric oxide synthase significantly reduces Zn^{2+} release from brain slices during oxygen and glucose deprivation coincident with decreased neuronal apoptosis[172,197]. Thus, chemicals that promote acidosis or generation of nitric oxide and/or ROS (e.g., 3-nitropropionic acid, methamphetamine) can significantly increase intracellular concentrations of free Zn^{2+}. Third, free Zn^{2+} in cells has been shown to be significantly increased by the release of free Zn^{2+} from zinc oxide (ZnO) nanoparticles[198], which trigger apoptotic cell death in neural stem cells[199].

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[175]. 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[200]. Maternal zinc deficiency is associated with altered development of neurobehavior both in humans[201-204] and in animal models[205,206]. Marginal zinc deficiency during gestation is not teratogenic, but it does affect signaling and gene expression in the developing brain[207,208] and has been shown to cause long-term cognitive deficits[209,210]. For example, offspring of dams with marginal dietary zinc deficiency during gestation and lactation exhibit impaired performance in passive avoidance tasks as adults[211].

Zinc deficiency induces apoptotic cell death in various cell types, including neuronal precursor cells, neurons and glia, *in vivo* and *in vitro*[177,212,213]. 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 pro-apoptotic 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[178]. 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[177,213] (**Figure 4**). A role for p53 in zinc deficiency-induced neuronal apoptosis is suggested by data from several labs[178,212-214]. Neuronal precursor cells and neurons cultured in zinc-depleted medium exhibit significantly increased p53 protein levels[178,212,213] 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[212-214]. Zinc chelation by TPEN upregulates PUMA and NoxA expression via p53-dependent mechanisms, which consequently promote release of cytochrome *c* from the mitochondria and caspase activation[214].

Low levels of cellular zinc may also enhance caspase activation by alleviating the inhibitory effects of physiological zinc levels on caspase activity[180]. Zinc deficiency causes oxidative stress in neuronal cells by increasing cell oxidant levels, particularly H_2O_2, and by decreasing concentrations of glutathione, a key antioxidant molecule in neurons[176]. 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[177], although it may exert pro-apoptotic actions via effects on signaling molecules (see below).

Other signaling molecules that may be involved in zinc deficiency-induced apoptosis include pro-survival signaling molecules, such as ERK and Akt, and pro-apoptotic signaling molecules, such as p38 and JNK, whose activities are decreased and increased, respectively, by low zinc availability[175,177,215,216]. Zinc deficiency may also induce neuronal apoptosis via modulation of NF-κB signaling (**Figure 4**). NF-κB signaling is dysregulated in both the developing brain[208] and in neuronal cell cultures[217] under conditions of low zinc availability. In IMR-32 cells, zinc depletion increases production of oxidants causing Iκ-B phosphorylation and degradation[217], which are the initial steps in 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[218]. This is thought to be due to disruption of the microtubule network via oxidative damage caused by zinc deficiency,[218] since functional microtubules are required for nuclear translocation of NF-κB in neurons[219]. As a consequence, NF-κB-responsive genes, which include many pro-survival and anti-apoptotic genes, such as IAPs and anti-apoptotic Bcl-2 and Bcl-XL[178], are downregulated[177].

The developmental toxicity of several drugs and environmental toxicants can be partially attributed to deficiencies in maternal and embryonic zinc[175,182,220]. 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 hours[221]. 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[221]. Other chemicals that similarly cause maternal zinc deficiencies by upregulating maternal liver MT include arsenic, melphalan and α-hederin[182]. More recent studies implicate zinc deficiency as a mechanism contributing to the neurotoxic effects of lipopolysaccharide (LPS) in the developing brain[222,223], which include dysregulation of apoptosis[224]. 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.

IV. 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 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 pro-apoptotic and/or decreasing anti-apoptotic signaling secondary to oxidative stress, increased intracellular Ca^{2+} levels or Zn^{2+} 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* PET imaging of apoptosis in the developing brain[111,225], which will enable the translation of preclinical evidence of chemical-induced apoptosis to the developing human brain.

Table 1: Mechanisms by which chemicals induce apoptosis in the developing brain

Neurotoxic chemical	Mechanism(s) of Apoptosis	Timing of Exposure	Species	References
Ethanol	• ↓ Zn availability • ↑ Cellular ROS, Ca^{2+} • Destabilize the cytoskeleton • ↓ Pro-survival proteins (PI3K/ Akt, ERK, NF-κB, NFAT)	pre- and postnatal	mouse rat	208,221,226-228
Kainic acid	• ↑ Cellular ROS, Ca^{2+} • ↑ Lipid and protein oxidation • Excessive AMPAR activation • ↓ Cellular antioxidants (↓GSH activity and ↓GSH/GSSH)	prenatal	rat	229,230
Anesthetics (propofol)	• ↓ mature NGF levels • ↑TNFR1 activation • ↑ $p75^{NTR}$ activation • ↑TNF-α and pro-NGF	early postnatal	rat	92,231,232
Antiepileptic drugs	• ↓ neurotrophins (BDNF, NT-3) • ↓ Pro-survival proteins (Akt, Erk)	early postnatal	rat	68,233
Plasticizers (bisphenol A)	• ↑ Cellular ROS, Ca^{2+} • ↓ CREB phosphorylation • ↑ Cell cycle arrest • ↑ Death factors Bax and p53 • ↓ Cell proliferation biomarkers (PCNA and Ki67)	E16 mouse, E13 rat primary neurons	mouse, rat	234,235
Endotoxin (LPS)	• ↓ Zn availability • ↑ mTOR activation • ↑ TNF-α	prenatal	rat	222,224,236

References

1. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer.* 1972;26(4):239-257.
2. Dikranian K, Ishimaru MJ, Tenkova T, et al. Apoptosis in the in vivo mammalian forebrain. *Neurobiol Dis.* 2001;8(3):359-379.
3. Martin LJ. Neuronal cell death in nervous system development, disease, and injury (Review). *Int J Mol Med.* 2001;7(5):455-478.
4. Barone S, Jr., Das KP, Lassiter TL, White LD. Vulnerable processes of nervous system development: a review of markers and methods. *Neurotoxicology.* 2000;21(1-2):15-36.
5. Sastry PS, Rao KS. Apoptosis and the nervous system. *J Neurochem.* 2000;74(1):1-20.
6. Galluzzi L, Lopez-Soto A, Kumar S, Kroemer G. Caspases Connect Cell-Death Signaling to Organismal Homeostasis. *Immunity.* 2016;44(2):221-231.
7. Ribe Garrido EM, Heidt L, Beaubier N, Troy CM. Molecular mechanisms of neuronal death. In: Blass JP, ed. *Neurochemical mechanisms in disease*. New York, NY: Springer; 2011:17-47.
8. Mattson MP, Bazan NG. Apoptosis and necrosis. In: Brady ST, Siegel GJ, Albers RW, Price DL, eds. *Basic Neurochemistry: Principles of Molecular, Cellular and Medical Neurobiology*. 8th ed. Oxford, UK: Academic Press; 2012:663-676.
9. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature.* 1993;362(6423):847-849.
10. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* 1991;51(23 Pt 1):6304-6311.
11. Villunger A, Michalak EM, Coultas L, et al. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science (New York, NY).* 2003;302(5647):1036-1038.
12. Culmsee C, Mattson MP. p53 in neuronal apoptosis. *Biochem Biophys Res Commun.* 2005;331(3):761-777.

13. Ricci MS, El-Deiry WS. The extrinsic pathway of apoptosis. In: Gewirtz DA, Holt SE, Grant S, eds. *Apoptosis, Senescence, and Cancer*. New York City, NY: Humana Press; 2007:31-54.
14. Yamaguchi Y, Miura M. Programmed cell death in neurodevelopment. *Developmental cell.* 2015;32(4):478-490.
15. Oppenheim RW. Cell death during development of the nervous system. *Annual review of neuroscience.* 1991;14:453-501.
16. Blaschke AJ, Staley K, Chun J. Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. *Development.* 1996;122(4):1165-1174.
17. Blaschke AJ, Weiner JA, Chun J. Programmed cell death is a universal feature of embryonic and postnatal neuroproliferative regions throughout the central nervous system. *J Comp Neurol.* 1998;396(1):39-50.
18. Guizzetti M, Zhang X, Goeke C, Gavin DP. Glia and neurodevelopment: focus on fetal alcohol spectrum disorders. *Front Pediatr.* 2014;2:123.
19. Barres BA, Hart IK, Coles HS, et al. Cell death and control of cell survival in the oligodendrocyte lineage. *Cell.* 1992;70(1):31-46.
20. Barde YA. Trophic factors and neuronal survival. *Neuron.* 1989;2(6):1525-1534.
21. Purves D, Snider WD, Voyvodic JT. Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature.* 1988;336(6195):123-128.
22. Creeley CE. From Drug-Induced Developmental Neuroapoptosis to Pediatric Anesthetic Neurotoxicity-Where Are We Now? *Brain sciences.* 2016;6(3).
23. Yamaguchi Y, Shinotsuka N, Nonomura K, et al. Live imaging of apoptosis in a novel transgenic mouse highlights its role in neural tube closure. *J Cell Biol.* 2011;195(6):1047-1060.
24. Nonomura K, Yamaguchi Y, Hamachi M, et al. Local apoptosis modulates early mammalian brain development through the elimination of morphogen-producing cells. *Developmental cell.* 2013;27(6):621-634.
25. Chen SK, Chew KS, McNeill DS, et al. Apoptosis regulates ipRGC spacing necessary for rods and cones to drive circadian photoentrainment. *Neuron.* 2013;77(3):503-515.

26. Jung AR, Kim TW, Rhyu IJ, et al. Misplacement of Purkinje cells during postnatal development in Bax knock-out mice: a novel role for programmed cell death in the nervous system? *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2008;28(11):2941-2948.
27. Clarke PG. Neuron death in the developing avian isthmo-optic nucleus, and its relation to the establishment of functional circuitry. *Journal of neurobiology.* 1992;23(9):1140-1158.
28. Buss RR, Sun W, Oppenheim RW. Adaptive roles of programmed cell death during nervous system development. *Annual review of neuroscience.* 2006;29:1-35.
29. Raff MC, Whitmore AV, Finn JT. Axonal self-destruction and neurodegeneration. *Science (New York, NY).* 2002;296(5569):868-871.
30. Williams DW, Kondo S, Krzyzanowska A, Hiromi Y, Truman JW. Local caspase activity directs engulfment of dendrites during pruning. *Nat Neurosci.* 2006;9(10):1234-1236.
31. Schoenmann Z, Assa-Kunik E, Tiomny S, et al. Axonal degeneration is regulated by the apoptotic machinery or a NAD+-sensitive pathway in insects and mammals. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2010;30(18):6375-6386.
32. Nikolaev A, McLaughlin T, O'Leary DD, Tessier-Lavigne M. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature.* 2009;457(7232):981-989.
33. Kuo CT, Zhu S, Younger S, Jan LY, Jan YN. Identification of E2/E3 ubiquitinating enzymes and caspase activity regulating Drosophila sensory neuron dendrite pruning. *Neuron.* 2006;51(3):283-290.
34. Cusack CL, Swahari V, Hampton Henley W, Michael Ramsey J, Deshmukh M. Distinct pathways mediate axon degeneration during apoptosis and axon-specific pruning. *Nat Commun.* 2013;4:1876.
35. D'Amelio M, Cavallucci V, Cecconi F. Neuronal caspase-3 signaling: not only cell death. *Cell Death Differ.* 2010;17(7):1104-1114.
36. Thompson CK. Cell death and the song control system: a model for how sex steroid hormones regulate naturally-occurring neurodegeneration. *Dev Growth Differ.* 2011;53(2):213-224.

37. Ahmed EI, Zehr JL, Schulz KM, Lorenz BH, DonCarlos LL, Sisk CL. Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. *Nat Neurosci.* 2008;11(9):995-997.
38. Juraska JM, Sisk CL, DonCarlos LL. Sexual differentiation of the adolescent rodent brain: hormonal influences and developmental mechanisms. *Horm Behav.* 2013;64(2):203-210.
39. Forger NG. Control of cell number in the sexually dimorphic brain and spinal cord. *J Neuroendocrinol.* 2009;21(4):393-399.
40. Johnston MV. Clinical disorders of brain plasticity. *Brain & development.* 2004;26(2):73-80.
41. Avino TA, Hutsler JJ. Abnormal cell patterning at the cortical gray-white matter boundary in autism spectrum disorders. *Brain Res.* 2010;1360:138-146.
42. Wei H, Alberts I, Li X. The apoptotic perspective of autism. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience.* 2014;36:13-18.
43. Jarskog LF, Glantz LA, Gilmore JH, Lieberman JA. Apoptotic mechanisms in the pathophysiology of schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry.* 2005;29(5):846-858.
44. Margolis RL, Chuang DM, Post RM. Programmed cell death: implications for neuropsychiatric disorders. *Biological psychiatry.* 1994;35(12):946-956.
45. Giza CC, Mink RB, Madikians A. Pediatric traumatic brain injury: not just little adults. *Curr Opin Crit Care.* 2007;13(2):143-152.
46. Wasterlain CG, Gloss DS, Niquet J, Wasterlain AS. Epileptogenesis in the developing brain. *Handb Clin Neurol.* 2013;111:427-439.
47. Yu D, Li L, Yuan W. Neonatal anesthetic neurotoxicity: Insight into the molecular mechanisms of long-term neurocognitive deficits. *Biomed Pharmacother.* 2017;87:196-199.
48. Song B, Zhou T, Liu J, Shao L. Involvement of Programmed Cell Death in Neurotoxicity of Metallic Nanoparticles: Recent Advances and Future Perspectives. *Nanoscale Res Lett.* 2016;11(1):484.
49. Costa LG, de Laat R, Tagliaferri S, Pellacani C. A mechanistic view of polybrominated diphenyl ether (PBDE) developmental neurotoxicity. *Toxicol Lett.* 2014;230(2):282-294.

50. Flaskos J. The developmental neurotoxicity of organophosphorus insecticides: a direct role for the oxon metabolites. *Toxicol Lett.* 2012;209(1):86-93.
51. Fox DA, Campbell ML, Blocker YS. Functional alterations and apoptotic cell death in the retina following developmental or adult lead exposure. *Neurotoxicology.* 1997;18(3):645-664.
52. Oberto A, Marks N, Evans HL, Guidotti A. Lead (Pb+2) promotes apoptosis in newborn rat cerebellar neurons: pathological implications. *J Pharmacol Exp Ther.* 1996;279(1):435-442.
53. Nagashima K. A review of experimental methylmercury toxicity in rats: neuropathology and evidence for apoptosis. *Toxicologic pathology.* 1997;25(6):624-631.
54. Olney JW, Tenkova T, Dikranian K, et al. Ethanol-induced caspase-3 activation in the in vivo developing mouse brain. *Neurobiol Dis.* 2002;9(2):205-219.
55. Olney JW, Tenkova T, Dikranian K, Qin YQ, Labruyere J, Ikonomidou C. Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. *Brain Res Dev Brain Res.* 2002;133(2):115-126.
56. Ikonomidou C, Bittigau P, Ishimaru MJ, et al. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science (New York, NY).* 2000;287(5455):1056-1060.
57. Yu F, Wang Z, Ju B, Wang Y, Wang J, Bai D. Apoptotic effect of organophosphorus insecticide chlorpyrifos on mouse retina in vivo via oxidative stress and protection of combination of vitamins C and E. *Exp Toxicol Pathol.* 2008;59(6):415-423.
58. Roy TS, Andrews JE, Seidler FJ, Slotkin TA. Chlorpyrifos elicits mitotic abnormalities and apoptosis in neuroepithelium of cultured rat embryos. *Teratology.* 1998;58(2):62-68.
59. Olney JW, Young C, Wozniak DF, Ikonomidou C, Jevtovic-Todorovic V. Anesthesia-induced developmental neuroapoptosis. Does it happen in humans? *Anesthesiology.* 2004;101(2):273-275.
60. Olney JW, Young C, Wozniak DF, Jevtovic-Todorovic V, Ikonomidou C. Do pediatric drugs cause developing neurons to commit suicide? *Trends Pharmacol Sci.* 2004;25(3):135-139.

61. Rice D, Barone S, Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environmental health perspectives.* 2000;108 Suppl 3:511-533.
62. Nikolic M, Gardner HA, Tucker KL. Postnatal neuronal apoptosis in the cerebral cortex: physiological and pathophysiological mechanisms. *Neuroscience.* 2013;254:369-378.
63. Olney JW. Focus on apoptosis to decipher how alcohol and many other drugs disrupt brain development. *Front Pediatr.* 2014;2:81.
64. Ikonomidou C, Qin Y, Labruyere J, Kirby C, Olney JW. Prevention of trauma-induced neurodegeneration in infant rat brain. *Pediatr Res.* 1996;39(6):1020-1027.
65. Bittigau P, Sifringer M, Pohl D, et al. Apoptotic neurodegeneration following trauma is markedly enhanced in the immature brain. *Annals of neurology.* 1999;45(6):724-735.
66. Pohl D, Bittigau P, Ishimaru MJ, et al. N-Methyl-D-aspartate antagonists and apoptotic cell death triggered by head trauma in developing rat brain. *Proc Natl Acad Sci U S A.* 1999;96(5):2508-2513.
67. Ikonomidou C, Bosch F, Miksa M, et al. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science (New York, NY).* 1999;283(5398):70-74.
68. Bittigau P, Sifringer M, Genz K, et al. Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proc Natl Acad Sci U S A.* 2002;99(23):15089-15094.
69. Jevtovic-Todorovic V, Hartman RE, Izumi Y, et al. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2003;23(3):876-882.
70. Stratmann G, Sall JW, May LD, Loepke AW, Lee MT. Beyond anesthetic properties: the effects of isoflurane on brain cell death, neurogenesis, and long-term neurocognitive function. *Anesth Analg.* 2010;110(2):431-437.
71. Wang C, Liu F, Patterson TA, Paule MG, Slikker W, Jr. Preclinical assessment of ketamine. *CNS neuroscience & therapeutics.* 2013;19(6):448-453.

72. Vutskits L, Xie Z. Lasting impact of general anaesthesia on the brain: mechanisms and relevance. *Nature reviews Neuroscience.* 2016;17(11):705-717.
73. Loepke AW, Soriano SG. An assessment of the effects of general anesthetics on developing brain structure and neurocognitive function. *Anesth Analg.* 2008;106(6):1681-1707.
74. Lin EP, Soriano SG, Loepke AW. Anesthetic neurotoxicity. *Anesthesiol Clin.* 2014;32(1):133-155.
75. Slikker W, Jr., Zou X, Hotchkiss CE, et al. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. *Toxicological sciences : an official journal of the Society of Toxicology.* 2007;98(1):145-158.
76. Zou X, Patterson TA, Divine RL, et al. Prolonged exposure to ketamine increases neurodegeneration in the developing monkey brain. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience.* 2009;27(7):727-731.
77. Zou X, Liu F, Zhang X, et al. Inhalation anesthetic-induced neuronal damage in the developing rhesus monkey. *Neurotoxicol Teratol.* 2011;33(5):592-597.
78. Paule MG, Li M, Allen RR, et al. Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys. *Neurotoxicol Teratol.* 2011;33(2):220-230.
79. Raper J, Alvarado MC, Murphy KL, Baxter MG. Multiple Anesthetic Exposure in Infant Monkeys Alters Emotional Reactivity to an Acute Stressor. *Anesthesiology.* 2015;123(5):1084-1092.
80. Brambrink AM, Back SA, Riddle A, et al. Isoflurane-induced apoptosis of oligodendrocytes in the neonatal primate brain. *Annals of neurology.* 2012;72(4):525-535.
81. Istaphanous GK, Ward CG, Nan X, et al. Characterization and quantification of isoflurane-induced developmental apoptotic cell death in mouse cerebral cortex. *Anesth Analg.* 2013;116(4):845-854.
82. Creeley C, Dikranian K, Dissen G, Martin L, Olney J, Brambrink A. Propofol-induced apoptosis of neurones and oligodendrocytes in fetal and neonatal rhesus macaque brain. *Br J Anaesth.* 2013;110 Suppl 1:i29-38.
83. Drobish JK, Gan ZS, Cornfeld AD, Eckenhoff MF. From the Cover: Volatile Anesthetics Transiently Disrupt Neuronal Development in Neonatal Rats.

Toxicological sciences : an official journal of the Society of Toxicology. 2016;154(2):309-319.

84. Yon JH, Daniel-Johnson J, Carter LB, Jevtovic-Todorovic V. Anesthesia induces neuronal cell death in the developing rat brain via the intrinsic and extrinsic apoptotic pathways. *Neuroscience.* 2005;135(3):815-827.
85. Hansen HH, Briem T, Dzietko M, et al. Mechanisms leading to disseminated apoptosis following NMDA receptor blockade in the developing rat brain. *Neurobiol Dis.* 2004;16(2):440-453.
86. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science (New York, NY).* 1999;286(5443):1358-1362.
87. Yon JH, Carter LB, Reiter RJ, Jevtovic-Todorovic V. Melatonin reduces the severity of anesthesia-induced apoptotic neurodegeneration in the developing rat brain. *Neurobiol Dis.* 2006;21(3):522-530.
88. Lewin GR, Barde YA. Physiology of the neurotrophins. *Annual review of neuroscience.* 1996;19:289-317.
89. Lu LX, Yon JH, Carter LB, Jevtovic-Todorovic V. General anesthesia activates BDNF-dependent neuroapoptosis in the developing rat brain. *Apoptosis.* 2006;11(9):1603-1615.
90. Teng KK, Felice S, Kim T, Hempstead BL. Understanding proneurotrophin actions: Recent advances and challenges. *Dev Neurobiol.* 2010;70(5):350-359.
91. Dechant G, Barde YA. The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat Neurosci.* 2002;5(11):1131-1136.
92. Milanovic D, Pesic V, Popic J, et al. Propofol anesthesia induces proapoptotic tumor necrosis factor-alpha and pro-nerve growth factor signaling and prosurvival Akt and XIAP expression in neonatal rat brain. *J Neurosci Res.* 2014;92(10):1362-1373.
93. Ye X, Mehlen P, Rabizadeh S, et al. TRAF family proteins interact with the common neurotrophin receptor and modulate apoptosis induction. *J Biol Chem.* 1999;274(42):30202-30208.

94. Nykjaer A, Willnow TE, Petersen CM. p75NTR--live or let die. *Current opinion in neurobiology.* 2005;15(1):49-57.
95. Young C, Klocke BJ, Tenkova T, et al. Ethanol-induced neuronal apoptosis in vivo requires BAX in the developing mouse brain. *Cell Death Differ.* 2003;10(10):1148-1155.
96. Nykjaer A, Lee R, Teng KK, et al. Sortilin is essential for proNGF-induced neuronal cell death. *Nature.* 2004;427(6977):843-848.
97. Shi Q, Guo L, Patterson TA, et al. Gene expression profiling in the developing rat brain exposed to ketamine. *Neuroscience.* 2010;166(3):852-863.
98. Wang C, Fridley J, Johnson KM. The role of NMDA receptor upregulation in phencyclidine-induced cortical apoptosis in organotypic culture. *Biochem Pharmacol.* 2005;69(9):1373-1383.
99. Leonzino M, Busnelli M, Antonucci F, Verderio C, Mazzanti M, Chini B. The Timing of the Excitatory-to-Inhibitory GABA Switch Is Regulated by the Oxytocin Receptor via KCC2. *Cell Rep.* 2016;15(1):96-103.
100. Patkai J, Zana-Taieb E, Didier C, Jarreau PH, Lopez E. [Basic aspects of the potential toxicity of anesthetic drugs]. *Arch Pediatr.* 2013;20(9):1059-1066.
101. Mennerick S, Zorumski CF. Neural activity and survival in the developing nervous system. *Molecular neurobiology.* 2000;22(1-3):41-54.
102. Liang G, Ward C, Peng J, Zhao Y, Huang B, Wei H. Isoflurane causes greater neurodegeneration than an equivalent exposure of sevoflurane in the developing brain of neonatal mice. *Anesthesiology.* 2010;112(6):1325-1334.
103. Bayer SA, Altman J, Russo RJ, Zhang X. Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. *Neurotoxicology.* 1993;14(1):83-144.
104. Goodman S. Anesthesia for nonobstetric surgery in the pregnant patient. *Semin Perinatol.* 2002;26(2):136-145.
105. Tzong KY, Han S, Roh A, Ing C. Epidemiology of pediatric surgical admissions in US children: data from the HCUP kids inpatient database. *J Neurosurg Anesthesiol.* 2012;24(4):391-395.
106. Colon E, Bittner EA, Kussman B, McCann ME, Soriano S, Borsook D. Anesthesia, Brain Changes, and Behavior: Insights from Neural Systems Biology. *Prog Neurobiol.* 2017.

107. DiMaggio C, Sun LS, Ing C, Li G. Pediatric anesthesia and neurodevelopmental impairments: a Bayesian meta-analysis. *J Neurosurg Anesthesiol.* 2012;24(4):376-381.
108. Wang X, Xu Z, Miao CH. Current clinical evidence on the effect of general anesthesia on neurodevelopment in children: an updated systematic review with meta-regression. *PLoS One.* 2014;9(1):e85760.
109. Davidson AJ, Disma N, de Graaff JC, et al. Neurodevelopmental outcome at 2 years of age after general anaesthesia and awake-regional anaesthesia in infancy (GAS): an international multicentre, randomised controlled trial. *Lancet.* 2016;387(10015):239-250.
110. Sun LS, Li G, Miller TL, et al. Association Between a Single General Anesthesia Exposure Before Age 36 Months and Neurocognitive Outcomes in Later Childhood. *Jama.* 2016;315(21):2312-2320.
111. Zhang X, Liu F, Slikker W, Jr., Wang C, Paule MG. Minimally invasive biomarkers of general anesthetic-induced developmental neurotoxicity. *Neurotoxicol Teratol.* 2017;60:95-101.
112. Safe S. Toxicology, structure-function relationship, and human and environmental health impacts of polychlorinated biphenyls: progress and problems. *Environmental health perspectives.* 1993;100:259-268.
113. Thompson MR, Boekelheide K. Multiple environmental chemical exposures to lead, mercury and polychlorinated biphenyls among childbearing-aged women (NHANES 1999-2004): Body burden and risk factors. *Environmental research.* 2013;121:23-30.
114. Thomas K, Xue J, Williams R, Jones P, Whitaker D. *Polychlorinated biphenyls (PCBs) in School Buildings: Sources, Environmental Levels and Exposures.* United States Environmental Protection Agency; 2012.
115. Hu D, Hornbuckle KC. Inadvertent polychlorinated biphenyls in commercial paint pigments. *Environ Sci Technol.* 2010;44(8):2822-2827.
116. Shang H, Li Y, Wang T, et al. The presence of polychlorinated biphenyls in yellow pigment products in China with emphasis on 3,3'-dichlorobiphenyl (PCB 11). *Chemosphere.* 2014;98:44-50.
117. Carpenter DO. Polychlorinated biphenyls (PCBs): routes of exposure and effects on human health. *Reviews on environmental health.* 2006;21(1):1-23.

118. Korrick SA, Sagiv SK. Polychlorinated biphenyls, organochlorine pesticides and neurodevelopment. *Current opinion in pediatrics.* 2008;20(2):198-204.
119. Schantz SL, Widholm JJ, Rice DC. Effects of PCB exposure on neuropsychological function in children. *Environmental health perspectives.* 2003;111(3):357-576.
120. Winneke G. Developmental aspects of environmental neurotoxicology: lessons from lead and polychlorinated biphenyls. *J Neurol Sci.* 2011;308(1-2):9-15.
121. Hany J, Lilienthal H, Sarasin A, et al. Developmental exposure of rats to a reconstituted PCB mixture or aroclor 1254: effects on organ weights, aromatase activity, sex hormone levels, and sweet preference behavior. *Toxicol Appl Pharmacol.* 1999;158(3):231-243.
122. Sable HJ, Powers BE, Wang VC, Widholm JJ, Schantz SL. Alterations in DRH and DRL performance in rats developmentally exposed to an environmental PCB mixture. *Neurotoxicol Teratol.* 2006;28(5):548-556.
123. Schantz SL, Levin ED, Bowman RE, Heironimus MP, Laughlin NK. Effects of perinatal PCB exposure on discrimination-reversal learning in monkeys. *Neurotoxicol Teratol.* 1989;11(3):243-250.
124. Widholm JJ, Clarkson GB, Strupp BJ, Crofton KM, Seegal RF, Schantz SL. Spatial reversal learning in Aroclor 1254-exposed rats: sex-specific deficits in associative ability and inhibitory control. *Toxicol Appl Pharmacol.* 2001;174(2):188-198.
125. Yang D, Kim KH, Phimister A, et al. Developmental exposure to polychlorinated biphenyls interferes with experience-dependent dendritic plasticity and ryanodine receptor expression in weanling rats. *Environmental health perspectives.* 2009;117(3):426-435.
126. Nguon K, Baxter MG, Sajdel-Sulkowska EM. Perinatal exposure to polychlorinated biphenyls differentially affects cerebellar development and motor functions in male and female rat neonates. *Cerebellum (London, England).* 2005;4(2):112-122.
127. Powers BE, Widholm JJ, Lasky RE, Schantz SL. Auditory deficits in rats exposed to an environmental PCB mixture during development. *Toxicological sciences : an official journal of the Society of Toxicology.* 2006;89(2):415-422.

128. Roegge CS, Wang VC, Powers BE, et al. Motor impairment in rats exposed to PCBs and methylmercury during early development. *Toxicological sciences : an official journal of the Society of Toxicology.* 2004;77(2):315-324.
129. Landrigan PJ, Lambertini L, Birnbaum LS. A research strategy to discover the environmental causes of autism and neurodevelopmental disabilities. *Environmental health perspectives.* 2012;120(7):a258-260.
130. Stamou M, Streifel KM, Goines PE, Lein PJ. Neuronal connectivity as a convergent target of gene x environment interactions that confer risk for Autism Spectrum Disorders. *Neurotoxicol Teratol.* 2013;36:3-16.
131. Lyall K, Croen LA, Sjodin A, et al. Polychlorinated Biphenyl and Organochlorine Pesticide Concentrations in Maternal Mid-Pregnancy Serum Samples: Association with Autism Spectrum Disorder and Intellectual Disability. *Environmental health perspectives.* 2016.
132. Rossignol DA, Genuis SJ, Frye RE. Environmental toxicants and autism spectrum disorders: a systematic review. *Transl Psychiatry.* 2014;4:e360.
133. Eubig PA, Aguiar A, Schantz SL. Lead and PCBs as risk factors for attention deficit/hyperactivity disorder. *Environmental health perspectives.* 2010;118(12):1654-1667.
134. Seegal RF. Epidemiological and laboratory evidence of PCB-induced neurotoxicity. *Crit Rev Toxicol.* 1996;26(6):709-737.
135. Gilbert ME, Mundy WR, Crofton KM. Spatial learning and long-term potentiation in the dentate gyrus of the hippocampus in animals developmentally exposed to Aroclor 1254. *Toxicological sciences : an official journal of the Society of Toxicology.* 2000;57(1):102-111.
136. Brouwer A, Longnecker MP, Birnbaum LS, et al. Characterization of potential endocrine-related health effects at low- dose levels of exposure to PCBs. *Environmental health perspectives.* 1999;107 Suppl 4:639-649.
137. Tilson HA, Kodavanti PR. The neurotoxicity of polychlorinated biphenyls. *Neurotoxicology.* 1998;19(4-5):517-525.
138. Voie OA, Fonnum F. Effect of polychlorinated biphenyls on production of reactive oxygen species (ROS) in rat synaptosomes. *Arch Toxicol.* 2000;73(10-11):588-593. 580.htm.
139. Mariussen E, Myhre O, Reistad T, Fonnum F. The polychlorinated biphenyl mixture aroclor 1254 induces death of rat cerebellar granule cells: the

involvement of the N-methyl-D-aspartate receptor and reactive oxygen species. *Toxicol Appl Pharmacol.* 2002;179(3):137-144.

140. Wong PW, Pessah IN. Ortho-substituted polychlorinated biphenyls alter calcium regulation by a ryanodine receptor-mediated mechanism: structural specificity toward skeletal- and cardiac-type microsomal calcium release channels. *Mol Pharmacol.* 1996;49(4):740-751.
141. Howard AS, Fitzpatrick R, Pessah I, Kostyniak P, Lein PJ. Polychlorinated biphenyls induce caspase-dependent cell death in cultured embryonic rat hippocampal but not cortical neurons via activation of the ryanodine receptor. *Toxicol Appl Pharmacol.* 2003;190(1):72-86.
142. Hansen LG. *The Ortho Side of PCBs: Occurrence and Disposition.* Boston: Kluwer Academic Publishers; 1999.
143. Sanchez-Alonso JA, Lopez-Aparicio P, Recio MN, Perez-Albarsanz MA. Polychlorinated biphenyl mixtures (Aroclors) induce apoptosis via Bcl-2, Bax and caspase-3 proteins in neuronal cell cultures. *Toxicol Lett.* 2004;153(3):311-326.
144. Yang D, Lein PJ. Polychlorinated biphenyls increase apoptosis in the developing rat brain. *Curr Neurobiol.* 2010;1(1):70-76.
145. Pessah IN, Cherednichenko G, Lein PJ. Minding the calcium store: Ryanodine receptor activation as a convergent mechanism of PCB toxicity. *Pharmacology & therapeutics.* 2010;125(2):260-285.
146. Berridge MJ. Calcium microdomains: organization and function. *Cell Calcium.* 2006;40(5-6):405-412.
147. Andjelic S, Khanna A, Suthanthiran M, Nikolic-Zugic J. Intracellular Ca2+ elevation and cyclosporin A synergistically induce TGF-beta 1-mediated apoptosis in lymphocytes. *J Immunol.* 1997;158(6):2527-2534.
148. Danieli GA, Rampazzo A. Genetics of arrhythmogenic right ventricular cardiomyopathy. *Curr Opin Cardiol.* 2002;17(3):218-221.
149. Hajnoczky G, Csordas G, Madesh M, Pacher P. Control of apoptosis by IP(3) and ryanodine receptor driven calcium signals. *Cell Calcium.* 2000;28(5-6):349-363.
150. Mariot P, Prevarskaya N, Roudbaraki MM, et al. Evidence of functional ryanodine receptor involved in apoptosis of prostate cancer (LNCaP) cells. *Prostate.* 2000;43(3):205-214.

151. Pan Z, Damron D, Nieminen AL, Bhat MB, Ma J. Depletion of intracellular Ca2+ by caffeine and ryanodine induces apoptosis of chinese hamster ovary cells transfected with ryanodine receptor. *J Biol Chem.* 2000;275(26):19978-19984.
152. Pessah IN, Wong PW. Etiology of PCB Neurotoxicity: From Molecules to Cellular Dysfunction. In: Robertson L, Hansen L, eds. *Progress In Polychlorinated Biphenyl Toxicology*. New York, NY: Academic Press; 2001:179-184.
153. Pessah IN, Hansen LG, Albertson TE, et al. Structure-activity relationship for noncoplanar polychlorinated biphenyl congeners toward the ryanodine receptor-Ca2+ channel complex type 1 (RyR1). *Chem Res Toxicol.* 2006;19(1):92-101.
154. Xu L, Tripathy A, Pasek DA, Meissner G. Potential for pharmacology of ryanodine receptor/calcium release channels. *Annals of the New York Academy of Sciences.* 1998;853:130-148.
155. Pessah IN, Stambuk RA, Casida JE. Ca2+-activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg2+, caffeine, and adenine nucleotides. *Mol Pharmacol.* 1987;31(3):232-238.
156. Kim KH, Bose DD, Ghogha A, et al. Para- and ortho-substitutions are key determinants of polybrominated diphenyl ether activity toward ryanodine receptors and neurotoxicity. *Environmental health perspectives.* 2011;119(4):519-526.
157. Ahn KC, Zhao B, Chen J, et al. In vitro biologic activities of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: receptor-based bioassay screens. *Environmental health perspectives.* 2008;116(9):1203-1210.
158. Papineni RV, O'Connell KM, Zhang H, Dirksen RT, Hamilton SL. Suramin interacts with the calmodulin binding site on the ryanodine receptor, RYR1. *J Biol Chem.* 2002;277(51):49167-49174.
159. Black AM, Pandya S, Clark D, Armstrong EA, Yager JY. Effect of caffeine and morphine on the developing pre-mature brain. *Brain Res.* 2008;1219:136-142.

160. He P, Wang AG, Xia T, et al. Mechanisms underlying the developmental neurotoxic effect of PBDE-47 and the enhanced toxicity associated with its combination with PCB153 in rats. *Neurotoxicology.* 2009;30(6):1088-1095.
161. Szychowski KA, Sitarz AM, Wojtowicz AK. Triclosan induces Fas receptor-dependent apoptosis in mouse neocortical neurons in vitro. *Neuroscience.* 2015;284:192-201.
162. Guo XJ, Fantini J, Roubin R, Marvaldi J, Rougon G. Evaluation of the effect of suramin on neural cell growth and N-CAM expression. *Cancer Res.* 1990;50(16):5164-5170.
163. Sun X, Windebank AJ. Calcium in suramin-induced rat sensory neuron toxicity in vitro. *Brain Res.* 1996;742(1-2):149-156.
164. Dreiem A, Rykken S, Lehmler HJ, Robertson LW, Fonnum F. Hydroxylated polychlorinated biphenyls increase reactive oxygen species formation and induce cell death in cultured cerebellar granule cells. *Toxicol Appl Pharmacol.* 2009;240(2):306-313.
165. Ermak G, Davies KJ. Calcium and oxidative stress: from cell signaling to cell death. *Mol Immunol.* 2002;38(10):713-721.
166. Ravagnan L, Roumier T, Kroemer G. Mitochondria, the killer organelles and their weapons. *J Cell Physiol.* 2002;192(2):131-137.
167. Feng W, Liu G, Allen PD, Pessah IN. Transmembrane redox sensor of ryanodine receptor complex. *J Biol Chem.* 2000;275(46):35902-35907.
168. Pessah IN. Ryanodine receptor acts as a sensor for redox stress. *Pest Manag Sci.* 2001;57(10):941-945.
169. Tofighi R, Johansson C, Goldoni M, et al. Hippocampal neurons exposed to the environmental contaminants methylmercury and polychlorinated biphenyls undergo cell death via parallel activation of calpains and lysosomal proteases. *Neurotoxicity research.* 2011;19(1):183-194.
170. Momeni HR. Role of calpain in apoptosis. *Cell J.* 2011;13(2):65-72.
171. Marger L, Schubert CR, Bertrand D. Zinc: an underappreciated modulatory factor of brain function. *Biochem Pharmacol.* 2014;91(4):426-435.
172. Sensi SL, Paoletti P, Bush AI, Sekler I. Zinc in the physiology and pathology of the CNS. *Nature reviews Neuroscience.* 2009;10(11):780-791.

173. Pochwat B, Nowak G, Szewczyk B. Relationship between Zinc (Zn (2+)) and Glutamate Receptors in the Processes Underlying Neurodegeneration. *Neural Plast.* 2015;2015:591563.
174. Shuttleworth CW, Weiss JH. Zinc: new clues to diverse roles in brain ischemia. *Trends Pharmacol Sci.* 2011;32(8):480-486.
175. Plum LM, Rink L, Haase H. The essential toxin: impact of zinc on human health. *International journal of environmental research and public health.* 2010;7(4):1342-1365.
176. Oteiza PI. Zinc and the modulation of redox homeostasis. *Free radical biology & medicine.* 2012;53(9):1748-1759.
177. Adamo AM, Oteiza PI. Zinc deficiency and neurodevelopment: the case of neurons. *BioFactors (Oxford, England).* 2010;36(2):117-124.
178. Adamo AM, Zago MP, Mackenzie GG, et al. The role of zinc in the modulation of neuronal proliferation and apoptosis. *Neurotoxicity research.* 2010;17(1):1-14.
179. Cho E, Hwang JJ, Han SH, Chung SJ, Koh JY, Lee JY. Endogenous zinc mediates apoptotic programmed cell death in the developing brain. *Neurotoxicity research.* 2010;17(2):156-166.
180. Perry DK, Smyth MJ, Stennicke HR, et al. Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis. *J Biol Chem.* 1997;272(30):18530-18533.
181. Maret W, Jacob C, Vallee BL, Fischer EH. Inhibitory sites in enzymes: zinc removal and reactivation by thionein. *Proc Natl Acad Sci U S A.* 1999;96(5):1936-1940.
182. Taubeneck MW, Daston GP, Rogers JM, Keen CL. Altered maternal zinc metabolism following exposure to diverse developmental toxicants. *Reproductive toxicology (Elmsford, NY).* 1994;8(1):25-40.
183. Koh JY, Choi DW. Zinc toxicity on cultured cortical neurons: involvement of N-methyl-D-aspartate receptors. *Neuroscience.* 1994;60(4):1049-1057.
184. Choi DW, Koh JY. Zinc and brain injury. *Annual review of neuroscience.* 1998;21:347-375.
185. Kim EY, Koh JY, Kim YH, Sohn S, Joe E, Gwag BJ. Zn2+ entry produces oxidative neuronal necrosis in cortical cell cultures. *Eur J Neurosci.* 1999;11(1):327-334.

186. Sensi SL, Yin HZ, Carriedo SG, Rao SS, Weiss JH. Preferential Zn2+ influx through Ca2+-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production. *Proc Natl Acad Sci U S A.* 1999;96(5):2414-2419.
187. Kim YH, Kim EY, Gwag BJ, Sohn S, Koh JY. Zinc-induced cortical neuronal death with features of apoptosis and necrosis: mediation by free radicals. *Neuroscience.* 1999;89(1):175-182.
188. Noh KM, Koh JY. Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2000;20(23):RC111.
189. Brown AM, Kristal BS, Effron MS, et al. Zn2+ inhibits alpha-ketoglutarate-stimulated mitochondrial respiration and the isolated alpha-ketoglutarate dehydrogenase complex. *J Biol Chem.* 2000;275(18):13441-13447.
190. Sheline CT, Behrens MM, Choi DW. Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of NAD(+) and inhibition of glycolysis. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2000;20(9):3139-3146.
191. Jiang D, Sullivan PG, Sensi SL, Steward O, Weiss JH. Zn(2+) induces permeability transition pore opening and release of pro-apoptotic peptides from neuronal mitochondria. *J Biol Chem.* 2001;276(50):47524-47529.
192. McLaughlin B, Pal S, Tran MP, et al. p38 activation is required upstream of potassium current enhancement and caspase cleavage in thiol oxidant-induced neuronal apoptosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2001;21(10):3303-3311.
193. Mukai J, Hachiya T, Shoji-Hoshino S, et al. NADE, a p75NTR-associated cell death executor, is involved in signal transduction mediated by the common neurotrophin receptor p75NTR. *J Biol Chem.* 2000;275(23):17566-17570.
194. Lobner D, Canzoniero LM, Manzerra P, et al. Zinc-induced neuronal death in cortical neurons. *Cell Mol Biol (Noisy-le-grand).* 2000;46(4):797-806.
195. Assaf SY, Chung SH. Release of endogenous Zn2+ from brain tissue during activity. *Nature.* 1984;308(5961):734-736.
196. Aizenman E, Stout AK, Hartnett KA, Dineley KE, McLaughlin B, Reynolds IJ. Induction of neuronal apoptosis by thiol oxidation: putative role of intracellular zinc release. *J Neurochem.* 2000;75(5):1878-1888.

197. Wei G, Hough CJ, Li Y, Sarvey JM. Characterization of extracellular accumulation of Zn2+ during ischemia and reperfusion of hippocampus slices in rat. *Neuroscience.* 2004;125(4):867-877.
198. Song W, Zhang J, Guo J, et al. Role of the dissolved zinc ion and reactive oxygen species in cytotoxicity of ZnO nanoparticles. *Toxicol Lett.* 2010;199(3):389-397.
199. Deng X, Luan Q, Chen W, et al. Nanosized zinc oxide particles induce neural stem cell apoptosis. *Nanotechnology.* 2009;20(11):115101.
200. Caulfield LE, Zavaleta N, Shankar AH, Merialdi M. Potential contribution of maternal zinc supplementation during pregnancy to maternal and child survival. *The American journal of clinical nutrition.* 1998;68(2 Suppl):499s-508s.
201. Goldenberg RL, Tamura T, Neggers Y, et al. The effect of zinc supplementation on pregnancy outcome. *Jama.* 1995;274(6):463-468.
202. Hubbs-Tait L, Kennedy TS, Droke EA, Belanger DM, Parker JR. Zinc, iron, and lead: relations to head start children's cognitive scores and teachers' ratings of behavior. *Journal of the American Dietetic Association.* 2007;107(1):128-133.
203. Swanson CA, King JC. Zinc and pregnancy outcome. *The American journal of clinical nutrition.* 1987;46(5):763-771.
204. Scheplyagina LA. Impact of the mother's zinc deficiency on the woman's and newborn's health status. *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS).* 2005;19(1):29-35.
205. Yu X, Jin L, Zhang X, Yu X. Effects of maternal mild zinc deficiency and zinc supplementation in offspring on spatial memory and hippocampal neuronal ultrastructural changes. *Nutrition (Burbank, Los Angeles County, Calif).* 2013;29(2):457-461.
206. Jankowski MA, Uriu-Hare JY, Rucker RB, Rogers JM, Keen CL. Maternal zinc deficiency, but not copper deficiency or diabetes, results in increased embryonic cell death in the rat: implications for mechanisms underlying abnormal development. *Teratology.* 1995;51(2):85-93.

207. Chowanadisai W, Kelleher SL, Lonnerdal B. Maternal zinc deficiency reduces NMDA receptor expression in neonatal rat brain, which persists into early adulthood. *J Neurochem.* 2005;94(2):510-519.
208. Aimo L, Mackenzie GG, Keenan AH, Oteiza PI. Gestational zinc deficiency affects the regulation of transcription factors AP-1, NF-kappaB and NFAT in fetal brain. *The Journal of nutritional biochemistry.* 2010;21(11):1069-1075.
209. Halas ES, Hunt CD, Eberhardt MJ. Learning and memory disabilities in young adult rats from mildly zinc deficient dams. *Physiology & behavior.* 1986;37(3):451-458.
210. Halas ES, Eberhardt MJ, Diers MA, Sandstead HH. Learning and memory impairment in adult rats due to severe zinc deficiency during lactation. *Physiology & behavior.* 1983;30(3):371-381.
211. Golub MS, Gershwin ME, Vijayan VK. Passive avoidance performance of mice fed marginally or severely zinc deficient diets during post-embryonic brain development. *Physiology & behavior.* 1983;30(3):409-413.
212. Seth R, Corniola RS, Gower-Winter SD, Morgan TJ, Jr., Bishop B, Levenson CW. Zinc deficiency induces apoptosis via mitochondrial p53- and caspase-dependent pathways in human neuronal precursor cells. *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS).* 2015;30:59-65.
213. Corniola RS, Tassabehji NM, Hare J, Sharma G, Levenson CW. Zinc deficiency impairs neuronal precursor cell proliferation and induces apoptosis via p53-mediated mechanisms. *Brain Res.* 2008;1237:52-61.
214. Ra H, Kim HL, Lee HW, Kim YH. Essential role of p53 in TPEN-induced neuronal apoptosis. *FEBS Lett.* 2009;583(9):1516-1520.
215. Clegg MS, Hanna LA, Niles BJ, Momma TY, Keen CL. Zinc deficiency-induced cell death. *IUBMB Life.* 2005;57(10):661-669.
216. Nuttall JR, Oteiza PI. Zinc and the ERK kinases in the developing brain. *Neurotoxicity research.* 2012;21(1):128-141.
217. Mackenzie GG, Zago MP, Erlejman AG, Aimo L, Keen CL, Oteiza PI. alpha-Lipoic acid and N-acetyl cysteine prevent zinc deficiency-induced activation of NF-kappaB and AP-1 transcription factors in human neuroblastoma IMR-32 cells. *Free Radic Res.* 2006;40(1):75-84.

218. Mackenzie GG, Keen CL, Oteiza PI. Microtubules are required for NF-kappaB nuclear translocation in neuroblastoma IMR-32 cells: modulation by zinc. *J Neurochem.* 2006;99(2):402-415.
219. Hanz S, Fainzilber M. Integration of retrograde axonal and nuclear transport mechanisms in neurons: implications for therapeutics. *Neuroscientist.* 2004;10(5):404-408.
220. Duffy JY, Baines D, Overmann GJ, Keen CL, Daston GP. Repeated administration of alpha-hederin results in alterations in maternal zinc status and adverse developmental outcome in the rat. *Teratology.* 1997;56(5):327-334.
221. Carey LC, Coyle P, Philcox JC, Rofe AM. Maternal ethanol exposure is associated with decreased plasma zinc and increased fetal abnormalities in normal but not metallothionein-null mice. *Alcoholism, clinical and experimental research.* 2000;24(2):213-219.
222. Kirsten TB, Chaves-Kirsten GP, Bernardes S, et al. Lipopolysaccharide Exposure Induces Maternal Hypozincemia, and Prenatal Zinc Treatment Prevents Autistic-Like Behaviors and Disturbances in the Striatal Dopaminergic and mTOR Systems of Offspring. *PLoS One.* 2015;10(7):e0134565.
223. Kirsten TB, Queiroz-Hazarbassanov N, Bernardi MM, Felicio LF. Prenatal zinc prevents communication impairments and BDNF disturbance in a rat model of autism induced by prenatal lipopolysaccharide exposure. *Life Sci.* 2015;130:12-17.
224. Nimmervoll B, White R, Yang JW, et al. LPS-induced microglial secretion of TNFalpha increases activity-dependent neuronal apoptosis in the neonatal cerebral cortex. *Cerebral cortex (New York, NY : 1991).* 2013;23(7):1742-1755.
225. Zhang X, Paule MG, Wang C, Slikker W, Jr. Application of microPET imaging approaches in the study of pediatric anesthetic-induced neuronal toxicity. *J Appl Toxicol.* 2013;33(9):861-868.
226. Ali Shah S, Ullah I, Lee HY, Kim MO. Anthocyanins protect against ethanol-induced neuronal apoptosis via GABAB1 receptors intracellular signaling in prenatal rat hippocampal neurons. *Molecular neurobiology.* 2013;48(1):257-269.

227. Shah SA, Yoon GH, Kim MO. Protection of the developing brain with anthocyanins against ethanol-induced oxidative stress and neurodegeneration. *Molecular neurobiology.* 2015;51(3):1278-1291.
228. Reis KP, Heimfarth L, Pierozan P, et al. High postnatal susceptibility of hippocampal cytoskeleton in response to ethanol exposure during pregnancy and lactation. *Alcohol (Fayetteville, NY).* 2015;49(7):665-674.
229. Ullah I, Park HY, Kim MO. Anthocyanins protect against kainic acid-induced excitotoxicity and apoptosis via ROS-activated AMPK pathway in hippocampal neurons. *CNS neuroscience & therapeutics.* 2014;20(4):327-338.
230. Shin EJ, Ko KH, Kim WK, et al. Role of glutathione peroxidase in the ontogeny of hippocampal oxidative stress and kainate seizure sensitivity in the genetically epilepsy-prone rats. *Neurochem Int.* 2008;52(6):1134-1147.
231. Pesic V, Milanovic D, Tanic N, et al. Potential mechanism of cell death in the developing rat brain induced by propofol anesthesia. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience.* 2009;27(3):279-287.
232. Popic J, Pesic V, Milanovic D, et al. Induction of TNF-alpha signaling cascade in neonatal rat brain during propofol anesthesia. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience.* 2015;44:22-32.
233. Shi XY, Wang JW, Cui H, Li BM, Lei GF, Sun RP. Effects of antiepileptic drugs on mRNA levels of BDNF and NT-3 and cell neogenesis in the developing rat brain. *Brain & development.* 2010;32(3):229-235.
234. Lee S, Kim YK, Shin TY, Kim SH. Neurotoxic effects of bisphenol AF on calcium-induced ROS and MAPKs. *Neurotoxicity research.* 2013;23(3):249-259.
235. Liu R, Xing L, Kong D, Jiang J, Shang L, Hao W. Bisphenol A inhibits proliferation and induces apoptosis in micromass cultures of rat embryonic midbrain cells through the JNK, CREB and p53 signaling pathways. *Food Chem Toxicol.* 2013;52:76-82.
236. Ling Z, Gayle DA, Ma SY, et al. In utero bacterial endotoxin exposure causes loss of tyrosine hydroxylase neurons in the postnatal rat midbrain. *Movement disorders : official journal of the Movement Disorder Society.* 2002;17(1):116-124.

Figure Legends

Figure 1. Intrinsic and extrinsic pathways of apoptosis. The intrinsic pathway of apoptosis, illustrated on the left side of the schematic, is mediated by the activation of pro-apoptotic proteins of the Bcl-2 family (e.g., Bid, Bad, PUMA, Noxa, Bmf or Bim) and downregulation of anti-apoptotic Bcl-2 proteins (e.g., Bcl-2, Bcl-XL and Mcl-1). The increased ratio of pro- to anti-apoptotic 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 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 -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 (TNFα, 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 pro-caspase-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 pro-apoptotic proteins Bax and Bak to release cytochrome *c* and other pro-apoptotic 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-κB signaling, which results in rapid transcription of anti-apoptotic genes, including FLIP and IAP1/2. FLIP has been identified in the DISC. Dissociation of Complex 1 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.

Figure 2. Mechanisms of neuronal apoptosis induced by anesthetics with NMDA receptor (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 anti-apoptotic (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 pro-apoptotic proteins Bax and Bad. As illustrated on the right side of the schematic, anesthetics such as ketamine directly antagonize the

NMDA receptor to inhibit Ca^{2+} influx, which downregulates expression of anti-apoptotic and neuroprotective genes. In addition, anesthetics can increase expression of pro-neurotrophins, such as pro-nerve 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).

Figure 3. Mechanisms of PCB-induced neuronal apoptosis. Non-dioxin-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 pro-apoptotic 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 pro-apoptotic proteins (Bax) and downregulate anti-apoptotic proteins (Bcl-2) in neurons. Dioxin-like 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.

Figure 4. Mechanisms by which cellular zinc dyshomeostasis triggers neuronal apoptosis. Neuronal apoptosis can be triggered by shifts in cellular Zn^{2+} towards levels that are either higher (left) or lower (right) than the physiological range. Environmental stressors that increase free Zn^{2+} include: (1) presynaptic kainate receptor agonists (e.g., kainate, domoic acid), which depolarize glutamatergic neurons to release Zn^{2+} sequestered with glutamate in synaptic vesicles; (2) zinc oxide nanoparticles, which release free Zn^{2+}; and (3) elevated levels of reactive oxygen species (ROS), which promote release of Zn^{2+} from metallothionein (MT) proteins. Free Zn^{2+} can move from extracellular to intracellular compartments via the Zn^{2+}-permeable GluR2-lacking AMPA receptors ($AMPAR_{Ca\text{-}Zn}$), voltage-gated Ca^{2+} channels (VGCC) and the transmembrane Na^{+}–Zn^{2+} exchanger. Extracellular Zn^{2+} can also activate the metabotropic Zn^{2+}-sensing receptor (ZnR). High levels of intracellular Zn^{2+} 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 Ca^{2+} 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 Zn^{2+} availability, such as ethanol, can also trigger neuronal apoptosis. Since the NMDAR is inhibited by Zn^{2+}, Zn^{2+} deficiency increases NMDAR activation, which enhances Ca^{2+} influx. Increased intracellular Ca^{2+} activates protein kinase C (PKC) and calmodulin (CaM), which in turn activate NADPH oxidase and NO synthase to increase ROS. While low Zn^{2+} levels promote NF-κB activation, oxidative stress promotes microtubule depolymerization, thereby preventing nuclear translocation of NF-

κB, resulting in decreased expression of pro-survival and anti-apoptotic genes (e.g., cIAP, XIAP). Low intracellular Zn^{2+} levels also activate p38, JNK and p53, which increase transcription of genes encoding pro-apoptotic proteins (e.g., Fas, Bid, Bax, Noxa, PUMA).