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Review

Nonapoptotic caspases in neural development and in anesthesia-induced neurotoxicity

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Apoptosis, classically initiated by caspase pathway activation, plays a prominent role during normal brain development as well as in neurodegeneration. The noncanonical, nonlethal arm of the caspase pathway is evolutionarily conserved and has also been implicated in both processes, yet is relatively understudied. Dysregulated pathway activation during critical periods of neurodevelopment due to environmental neurotoxins or exposure to compounds such as anesthetics can have detrimental consequences for brain maturation and long-term effects on behavior. In this review, we discuss key molecular characteristics and roles of the noncanonical caspase pathway and how its dysregulation may adversely affect brain development. We highlight both genetic and environmental factors that regulate apoptotic and sublethal caspase responses and discuss potential interventions that target the noncanonical caspase pathway for developmental brain injuries.

Highlights

Caspase activity is necessary for normal neuronal development and maturation. Studies are increasingly showing important caspase roles in axonal pathfinding, dendritic arborization, and synaptic plasticity.

There is growing evidence for nonlethal, yet damaging caspase responses to commonly used chemical agents such as ethanol and anesthetics.

Nonlethal, excess caspase activation may result in impaired neuronal arborization and synaptic transmission, leading to long-term behavioral deficits.

Children undergoing surgeries, such as those with congenital heart defects, may be at increased risks for nonapoptotic caspase injuries due to exposure to anesthetics and accompanying genetic susceptibility.

Understanding the molecular and cellular differences between apoptotic and nonlethal caspase activity is crucial for more targeted neuroprotection.

Caspases: more than cell death mediators

Cell death is currently understood to occur in one of the four morphologically defined manners: **apoptosis** (see Glossary), autophagy, necrosis, and entosis [1]. Apoptotic cell death as an intrinsically programmed phenomenon was originally described in the roundworm *Caenorhabditis elegans*. Of the 1090 somatic cells generated during *C. elegans* development, 302 are genetically predetermined to become neuronal cells. Additional 131 cells are produced, however, and these cells undergo programmed cell death with high temporal fidelity during the development of each worm [2]. Unlike in *C. elegans*, mammalian development relies on a more stochastic process of cell elimination [3]. The apoptotic mechanism is orchestrated by **caspases**, cysteine protease enzymes that utilize both other caspase proteins and diverse cellular peptides as their substrates [4].

Caspases are classified into initiator caspases, such as caspase 2, 8, 9, and 10, which are the first to become catalytically active during apoptosis, and effector caspases 3, 6, and 7, which rely on initiator activation and mediate the final steps of programmed cell death [5]. The end result of this sequential protease activity is DNA fragmentation and, ultimately, cell death [4,6]. Apoptosis proceeds either through a death receptor **extrinsic apoptotic pathway** or an intracellular, mitochondria-based intrinsic pathway [7]. The triggers for extrinsic caspase activation involve binding of tumor necrosis factor-α (TNF-α) and Fas to death receptors, while intrinsic, mitochondrial mechanisms are initiated by DNA damage and/or excess reactive oxygen species production [7] (Figure 1).

Over the course of several decades, important neurodevelopmental and injury response roles have been described for the canonical apoptotic pathway [7–9]. More recently, caspase activation has increasingly been associated with both physiological and pathological nonapoptotic outcomes that do not result in neuronal cell death, yet have significant impacts on cell morphology and function. Caspase activation status and catalytic efficiency is known to be influenced by phosphorylation of the enzymes themselves, as well as their target substrates, by kinases [10–12]. Whether

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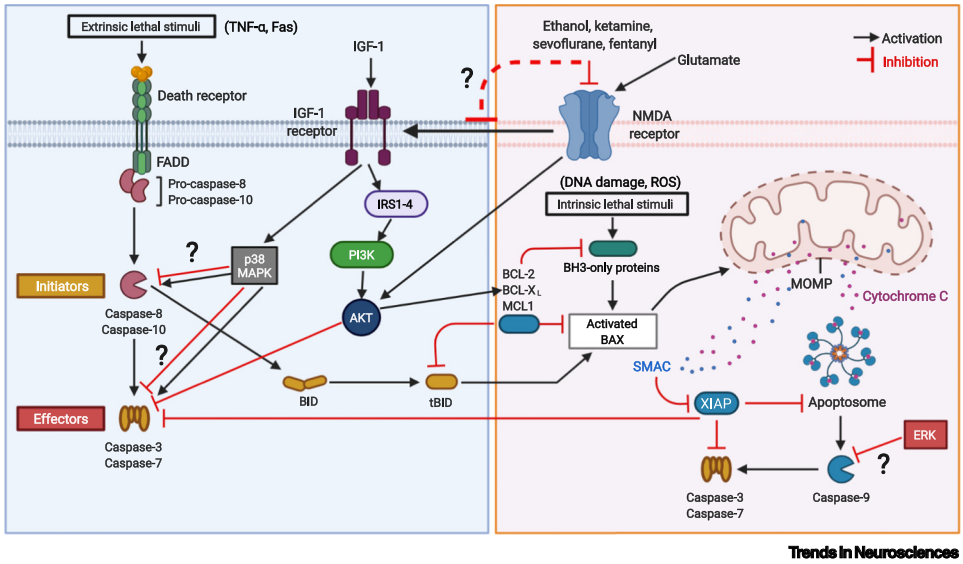

Figure 1. Overview of extrinsic and intrinsic apoptosis and caspase activation modifiers. The flowchart presents a summary of the extrinsic and intrinsic arms of the apoptotic pathway, while highlighting known and hypothetical molecular modifiers of initiator and effector caspase activation. Extrinsic, death receptor-dependent signaling crosstalks with the mitochondrial arm through BCL-2 family proapoptotic proteins such as BID, which when translocated to the mitochondria (translocated BID/tBID) results in activation of the protein BAX, mitochondrial membrane permeabilization, and release of cytochrome C. Cytochrome C forms structures known as apoptosomes, which initiate caspase activation. Effector caspase activation is inhibited by X-linked inhibitor of apoptosis proteins (XIAPs), which are ubiquitin ligases. SMAC acts as a potentiator of apoptosis by inhibiting XIAP function. BCL-2 family antiapoptotic proteins BCL-X_L and MCL1 suppress the intrinsic apoptotic cascade, acting to maintain homeostasis. Other signaling pathways such as PI3K/AKT, p38 MAPK, and ERK act as modulators of caspase signaling through their phosphorylation activity. Chemical agents with both neurophysiological and neurotoxic properties, such as glutamate and ketamine, function as agonists and antagonists of NMDA receptors, which interface with intracellular signaling regulators of caspase activation. Differential recruitment of these signaling pathways and their variable interaction with environmental stimuli may underlie functional differences between apoptotic and nonapoptotic caspase activation. The figure was created with BioRender.com. Abbreviations: AKT, protein kinase B; BAX, BCL2-associated X protein; BCL-2, B-cell lymphoma 2; Bcl-X_L, B-cell lymphoma-extra large; BH3, Bcl-2 homology 3; BID, BH3 interacting-domain death agonist; ERK, extracellular-regulated protein kinase; FADD, Fas-associated death domain-containing protein; FAS, Fas cell surface death ligand; IGF-1, insulin-like growth factor 1; IRS1–4, insulin receptor substrate 1–4; MAPK, mitogen-activated protein kinase; MCL1, myeloid cell leukemia 1; MOMP, mitochondrial outer membrane permeabilization; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SMAC, second mitochondria-derived activator of caspases; TNF-α, tumor necrosis factor-α.

caspase activation and the switch to nonapoptotic signaling predominantly depends on levels of caspase phosphorylation and/or cleavage (Figures 1 and 2) or on modulation by other signaling pathways (Figure 1) is unclear. It also remains unknown how precisely this shift in caspase activity diverts the neuron from proceeding with canonical apoptosis to instead undergoing structural changes (see Outstanding questions).

Neuronal structure is of critical importance to the assembly of functional brain circuits [13]. Disruptions of virtually any of the aspects of neuron structure have been implicated in disorders of neurodevelopment, age-related neurodegeneration, as well as various brain injury scenarios. Caspases are known to have phylogenetically conserved roles in the sculpting of neuronal dendritic arbors through cytoskeletal remodeling and have been demonstrated to contribute to synaptic plasticity [4]. In addition, caspase signaling has been linked to excitotoxic injuries in prior work [4]. Programmed cell death and neurodegeneration has been frequently reported in developmental brain injury scenarios such as anesthesia exposure and alcohol-induced neurotoxicity [9,14,15]. Importantly, the neurotoxic potential of excessive nonapoptotic caspase

Glossary

Apoptosis: a series of intrinsically programmed molecular steps leading to cell death.
Canonical apoptotic signaling: the most well-studied set of reactions leading to cell death, consisting of an extrinsic and intrinsic pathway/arm. Noncanonical apoptotic signaling describes caspase-dependent reactions that do not lead to cell death, yet are important for synaptic pruning, neuritogenesis, and synaptic plasticity.
Caspase: a protease enzyme involved in the execution of apoptosis as well as other nonapoptotic cellular processes.
Extrinsic apoptotic pathway: also termed death receptor pathway, involves binding of an extracellular death ligand (e.g., TNF-α) to a plasma membrane death receptor (e.g., TNF receptor) to initiate apoptosis.
Intrinsic apoptotic pathway: also termed the mitochondrial apoptotic pathway, it is initiated intracellularly, due to DNA damage, ischemia, and oxidative stress.
Sublethal caspase activation: caspase signaling that involves cleaving and activation of effector caspases, without subsequently leading to cell death (as assessed by cell number, nuclear fragmentation via TUNEL staining, and markers of mitochondrial integrity).

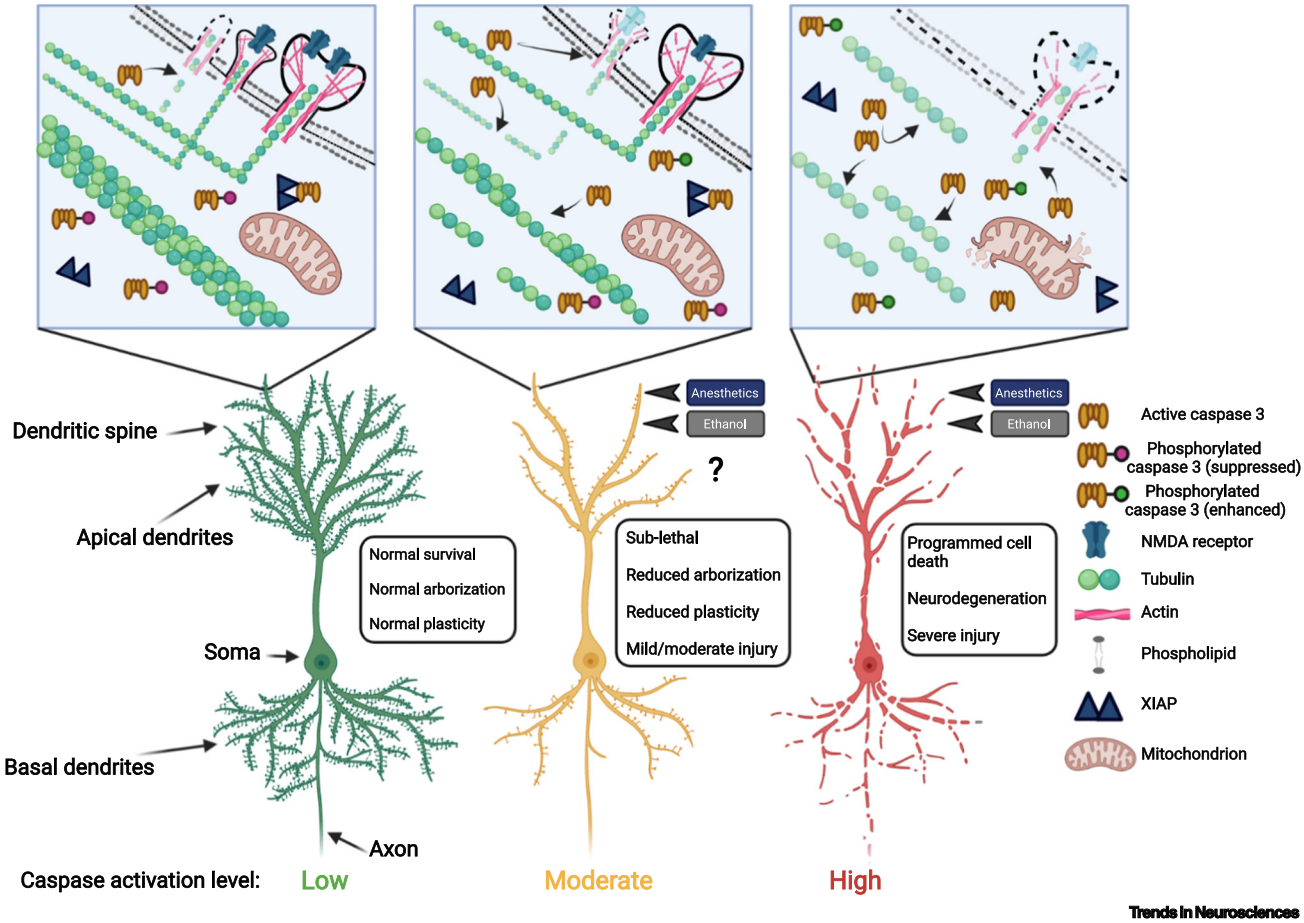

Figure 2. Caspase activation mode and corresponding outcomes in physiological and injury scenarios. The schematic offers a simplified overview of variable, caspase activation level-dependent neurotoxic outcomes (with caspase cleavage and/or phosphorylation levels being key modulators of activation). In pyramidal neurons, dendritic processes contact and receive synaptic inputs from other neurons and enable crosstalk with glial cells. Often, dendrites are covered in dendritic spines, postsynaptic sites of neurotransmission that are dynamically turned over and either stabilized or lost, processes that contribute to synaptic plasticity. These structures are supported and maintained by networks of filament proteins, including actin and tubulin. During normal physiological neuronal development and maturation, a low level of caspase pathway activation is required for dendritic branching and shifts in plasticity required during learning. However, upon exposure to environmental neurotoxins, such as anesthetics and ethanol, enhanced activation of executioner caspases leads to the induction of apoptosis via mitochondria (intrinsic pathway) and extensive cleaving of cytoskeletal proteins. A more complex scenario is increasingly being recognized, whereby a moderate level of caspase activity postexposure may lead to cytoskeletal remodeling, without full cell death. How this level of activation is achieved and modulated is unclear, but may involve variable availability of X-linked inhibitor of apoptosis proteins (XIAPs), genetic susceptibility combined with low-dose neurotoxin exposures, and altered neuronal–glial crosstalk. The figure was created with BioRender.com.

signaling has not been well characterized in the developing brain. Given the capacity for caspase activation by commonly used environmental agents, such as volatile anesthetics, ethanol, and opioids, addressing the possibility of nonapoptotic, sublethal neurotoxicity is particularly pertinent.

In this review, we highlight key aspects of nonapoptotic caspase functions during neuronal development and maturation, as well as unaddressed questions concerning their contribution to pathological processes triggered by developmental brain injury. We then summarize recent findings on how the nonapoptotic caspase pathway contributes to long-term neurological deficits and discuss potential therapeutic avenues.

Diverse roles of the nonapoptotic pathway arm

Caspases during neurogenesis

Besides the canonical apoptotic pathway, caspase activity that triggers nonapoptotic events in the cell is involved in a diverse repertoire of roles, including proper immune function, cell proliferation and differentiation during development, cell fate specification, and cytoskeletal function in both vertebrates and invertebrates [16]. In *Drosophila melanogaster*, the fruit fly ortholog of mammalian initiator caspase 9/2, called *dronc*, restricts the expansion of enteroblast cells, a type of intestinal precursor cell, through a nonlethal regulation of the proliferation-differentiation transition [17,18]. In the *D. melanogaster* CNS, *dronc* plays a similar role, suppressing the production of excess sensory neural precursors in a nonapoptotic manner, thereby contributing to a system-level fine-tuning during cell differentiation [19]. Proper differentiation of mammalian neural stem cells also requires nonapoptotic caspase signaling [20]. For instance, the differentiation of cerebellar granule neurons and Bergmann glia is precisely controlled by nonapoptotic activity of caspase 3 [20–22]. Given the extensive caspase-dependent apoptosis in the rodent cerebellar cortex during the first postnatal week [23,24], a key question that remains to be addressed is how the two disparate functions of caspase 3 are spatiotemporally coordinated. A possible explanation is dosage dependence on caspase 3 cleavage levels (Figures 1 and 2). To test this hypothesis, expression constructs encoding catalytically active caspase 3 subunits under the control of variable strength synthetic promoters may be introduced in different neuronal cell lines [25,26]. This strategy could also be used at different stages of neuronal differentiation and maturation *in vivo*, using tissue- and cell-specific promoters, to clarify if levels of active caspase 3 determine the switch from nonapoptotic to apoptotic pathways.

Caspase-mediated cytoskeletal remodeling

One of the major targets of nonapoptotic caspase signaling is the cytoskeleton. Some of the earliest studies of caspase function, focused on understanding canonical cell death signaling, implicated caspases in the destabilization and cleaving of cytoskeletal proteins such as actin and tau [27,28]. Cytoskeletal remodeling and reorganization underlie cellular morphological changes, such as during cell division, movement, and acquisition of a mature morphology. During neural development, the process of axon guidance is necessary for the establishment of correct early wiring patterns and subsequent synaptic maturation. Inhibition of localized caspase 3 and 8 activity in mouse hippocampal neurons leads to a loss of neurite outgrowth [29]. This effect seems to be dependent on caspase-mediated cleaving of spectrin, a plasma membrane-associated cytoskeletal protein necessary for axonal bending and stretching [30,31]. The aforementioned *D. melanogaster* caspase ortholog *dronc* has been shown to cleave F-actin filaments in the developing salivary glands in nonapoptotic fashion and subsequently prune dendritic arbors in a class of sensory neurons [32]. Similarly, axonal misrouting and impaired synaptic contact establishment occurs in olfactory sensory neurons of mice mutant for caspase 9, an initiator caspase [33]. Furthermore, overexpression of a dominant negative mutant caspase 3 precursor in developing chick embryos, specifically in midbrain presynaptic neurons, led to significantly reduced dendritic complexity, as seen through fewer neuronal branch points and higher order branches [34]. Experiments from these studies show a conserved caspase function in neuronal arbor remodeling across different model species that is strongly dependent on cytoskeletal modulation. Cytoskeletal substrate preference seems to vary to some extent between different caspases, as caspase 3 has been shown to cleave both beta-actin and alpha-tubulin, whereas caspase 6 only targets tubulin [35].

How this substrate specificity is established and regulated in a nonapoptotic context remains an open question. One possibility is the involvement and variable availability of X-linked inhibitor of apoptosis proteins (XIAPs), such as E3 ubiquitin ligase, throughout the neuron [36] (Figures 1

and 2). Given the system-level need for nonapoptotic caspase function in regulating neuronal cytoskeletal architecture, distinguishing apoptotic from sublethal caspase impacts on cytoskeletal proteins at high resolution is an important yet overlooked avenue. Super-resolution microscopy techniques coupled with single-molecule fluorescence tagging of microtubule and actin bundles could provide insights into these distinct caspase-mediated outcomes [37–39].

Caspase signaling in synapses

Apart from being important in the process of neuronal process outgrowth and arborization, caspases have increasingly been linked to synaptic plasticity modulation and normal synaptic physiology (Figure 2). The process of long-term depression (LTD), by which neuronal synapses progressively weaken functionally, accompanied by a reduction in dendritic spine size, depends on a low level of caspase 3/9 activation within dendrites [40]. Curiously, the converse process of long-term potentiation, by which neuronal synapses strengthen over time, seems to be unaffected by suppressing caspase 3/9 activation genetically or pharmacologically. Furthermore, the LTD process dependent on NMDA receptor (NMDAR) function seems to be uniquely dampened by reduced expression of BAD and BAX proteins, both of which are classically associated with promoting intrinsic apoptosis via mitochondria (Figure 1) [41]. More recently, the BAD-BAX-caspase 3 signaling cascade was demonstrated to regulate synaptic vesicle pools by autophagy, with the process being tightly coordinated during associative fear learning in mice [42]. BAX-caspase 3 activation plays a prominent role in the activity-dependent reorganization of corticospinal projections necessary for the acquisition of fine motor task skills [43]. Caspase 3 also directly regulates the levels of AMPA receptor subunit GluA1 in response to chronic stimuli *in vivo* [44]. In zebra finch songbirds, cleaved caspase 3 levels are strongly increased in postsynaptic compartments of the auditory cortex following exposure to novel sounds [45].

Collectively, these findings provide evidence for the involvement of caspase activity during normal physiological processes underlying learning and memory. Better characterization of **sublethal caspase activation** and localization within dendrites and dendritic spines during various learning paradigms will help clarify how caspase activity impacts spine turnover and ion channel expression in experience-dependent plasticity. Are caspases differentially recruited depending on the degree of experience-induced plasticity? Important questions also remain regarding how endogenous caspase activity in these contexts interacts with extrinsic environmental factors known to modify neuronal activity in both physiological and pathological scenarios.

Environmental influences and injury mechanisms

Caspase signaling in excitotoxicity

Intrinsically activated caspases do not operate in isolation, but rather can be influenced by a variety of environmental factors. In neurons, nonapoptotic signaling engagement likely requires a lower activation threshold compared with full programmed cell death. Accordingly, the presence of one or several environmental agents might easily compound the likelihood of pathway activation. Glutamate-induced neurotoxicity, also termed excitotoxicity, is a well-characterized neuronal injury paradigm, particularly in the context of canonical apoptosis [46,47]. Glutamate-induced neurotoxicity occurs due to the presence of supra-physiological levels of the neurotransmitter glutamate, leading to excessive stimulation of ligand-gated ion channel receptors such as NMDA, AMPA, and kainic acid receptors, which culminates in a neurotoxic influx of calcium ions [48,49]. This toxicity is typically manifested through mitochondrial dysfunction, increased generation of reactive oxygen and nitrogen species, a drop in mitochondrial membrane potential, and, ultimately, mitochondrial pore opening followed by apoptosis [49]. The calcium ion influx is known to activate calcium-dependent proteases and phosphatases such as calpains and calcineurin, respectively, which in turn are capable of inducing executioner caspase activity as well, forming a feedback loop [50]. Calpain-10 has been shown to

control actin filament dynamics [51], providing a secondary caspase-independent avenue to excitotoxic dysregulation of the cytoskeleton. More recently, calcineurin signaling downstream of calcium influxes into hippocampal CA1 neurons was found to mediate damage to Ankyrin G, a cytoskeletal protein crucial to the organization of the axon initial segments in said cells [52]. In this study, the injury was ischemic, derived from medial cerebral artery occlusion, which, despite leading to major cytoskeletal reorganization and impaired action potential generation, did not result in significantly increased cell death. Given the known interplay between caspases and calcium-dependent proteases/phosphatases [52–56], and their respective capacities for altering cytoskeletal dynamics, it will be important to dissect their relative contributions to sublethal excitotoxic outcomes in different neuronal populations.

Role of neuronal–glial crosstalk in caspase activation

Neuronal cells do not undergo neurotoxic responses in isolation. The environmental agent causing the insult also acts on glial cells as well as endothelial cells of the vasculature. These non-cell autonomous responses are multi-pronged, with non-neuronal cells experiencing both shared and cell-specific insult effects, which then feed back onto neuronal cells in their proximity. Under physiological conditions, neurons and glial cells engage in extensive crosstalk, due to both physical proximity-based cell–cell contacts, cytoskeletal protrusions known as tunneling nanotubes, extracellular vesicles, as well as paracrine signals [57–59]. In microglial cells, exposure to inflammatory agents such as lipopolysaccharide triggers activation of initiator caspase 8 as well as caspases 3 and 7, which in turn drive the microglia to a proinflammatory phenotype via protein kinase C (PKC)-δ signaling [60]. The caspase activation in this paradigm is not lethal to microglia, yet indirectly leads to neurotoxic outcomes due to microglial activation. In neuronal and astrocyte cocultures, exposure to ketamine, which is known as an NMDAR antagonist, enhances lethal neurotoxicity compared with treatment of neurons alone [61]. Extracellular vesicles, and in particular the long noncoding RNA targeting BDNF contained within them, isolated from the ketamine-treated astrocytes seem to be partially responsible for this effect. Modulatory effects on caspase-dependent apoptosis in neurons by non-neuronal cells may play an even more pertinent role in sublethal caspase activity contexts, with comparatively lower caspase activation thresholds [62].

Anesthetics and ethanol in developmental neurotoxicity

Anesthetics and ethanol are among the best-studied environmental agents implicated in developmental neurotoxicity (Figures 1 and 2 and Box 1) [9,15]. Early work showed that both acute and

Box 1. Clinical findings in anesthesia toxicity and neurodevelopment

Approximately 100 clinical studies have been published evaluating neurodevelopmental outcomes after childhood surgery and anesthesia [110]. Despite concerning evidence from animal studies, clinical results are mixed, likely due to various challenges and limitations inherent to the design of optimal clinical studies (Box 3) [111]. Exposure to general anesthesia is not consistently associated with deficits in academic achievement, general intelligence, or language [88,111,112]. However, a growing number of studies has been reporting associations between general anesthesia exposure during infancy and increased risks for adverse behavioral outcomes, including deficits in executive function, social communication, motor function, and diagnoses of attention deficit hyperactivity disorder (ADHD) [112–114]. These deficits have been reported even after single exposures to anesthesia [115]. Anesthesia length, repeated exposure, dosage regime, and younger age all contribute to enhanced risk for such neurobehavioral impairments [110,112]. Multiple infant anesthetic exposures have been found to more than double the hazard ratio for learning disabilities [116]. The same study found a 30% increase in the hazard ratio for ADHD with a single anesthetic exposure, which, although not statistically significant, is suggestive of the cumulative nature of the damaging effect. It is plausible that lower dose exposures or specific anesthetics in isolation may preferentially trigger nonapoptotic caspase responses, resulting in outcomes different from those of classical apoptosis (see Figure 2 in main text).

The link between anesthesia and canonical caspase-mediated cell death and neurodegeneration in animal models is well established. Whether anesthetics can elicit brain injury via nonapoptotic caspase activation is a pertinent yet largely unexplored avenue of research.

chronic ethanol exposure was associated with caspase 3 activation, neuronal cell death, and neurodegeneration in mouse and rat models, with the response heavily dependent on BAX protein activity [9,63–65]. More recent work intriguingly found that mice lacking primary cilia in forebrain excitatory neurons exhibited sublethal caspase 3 activation after acute ethanol treatment [66]. Dampened PI3K/AKT signaling caused by cilia loss seems to be a key causal factor behind the enhanced caspase response. The same effect on caspase 3 activation was observed with a single dose of ketamine. Curiously, the same ethanol dosage regime used elicited marked neurodegeneration at similar end points in prior studies, with evidence for neuronal cell loss at later times. This discrepancy may be partially explained by the dampening of the caspase 3 response over time, although questions remain over the inconsistency regarding cell loss. In line with prior developmental evidence, the sublethal caspase 3 activation [66] was punctate in presentation and was associated with actin and tubulin degeneration, followed by loss of pyramidal neuron dendritic complexity in ciliopathic mice. Neuronal safeguarding mechanisms, such as PI3K/AKT signaling via primary cilia, may be important modifiers of the degree of caspase activation and determinants of whether a cell commits to apoptosis or undergoes sublethal neurotoxic changes (Figure 1).

Anesthetics as a key risk factor for caspase-induced injury after major neonatal surgeries

Anesthetics in caspase-dependent injury

Anesthetics such as sevoflurane and isoflurane are an indispensable component of complex pediatric surgeries, for instance, corrective heart surgery in patients with congenital heart disease (CHD) (Box 2). Both volatile and injectable anesthetics have been extensively linked to canonical, cell death-associated caspase activity in various animal models of exposure. Originally described in rat models, combinations of midazolam, nitrous oxide, and isoflurane exposure were associated with extensive caspase activation, neurodegenerative changes, and subsequent learning impairments [8,15]. Many of the anesthetics used for major pediatric surgery function as either NMDAR blockers or GABA receptor agonists [8], suggestive of a converging mode of neurotoxicity. Isoflurane and sevoflurane have both been demonstrated to mediate their caspase-dependent neurotoxic effects through disrupting calcium homeostasis and activation of the intrinsic mitochondrial apoptotic cascade [67–70]. In rodent anesthesia models, perinatal exposures to sevoflurane and isoflurane induced long-term behavioral impairments, including memory deficits, increased anxiety, decreased sociability, and impaired motor coordination [14,71]. In infant rhesus macaques, repeated doses, but not a single dose, of isoflurane were associated with deficits

Box 2. Etiologies of neurological deficits observed in congenital heart disease (CHD)

CHD is one of the most common birth defects. Neonatal surgical interventions are critical for patients with complex CHD, leading to significantly reduced case mortality. Given its high prevalence, accounting for around 1% of live births, CHD has huge societal impacts. Despite improvements in mortality prevention, a substantial fraction of CHD patients (estimated at about 1 in 150) go on to exhibit various neurobehavioral abnormalities, including attention deficit, social difficulties, motor issues, and intellectual disability [117]. The Pediatric Heart Network and National Heart, Lung, and Blood Institute working group has reported that one of the most important challenges for neonates undergoing surgery for CHD is long-term sequelae, such as neurodevelopmental deficits [118].

Known and postulated causative factors range from genetic, including *de novo* mutations in chromatin modifier genes [90], to perioperative factors such as hypoxia/ischemia and exposure to anesthetics [119]. Neonates undergoing complex surgeries present a uniquely challenging scenario due to the temporal overlap between surgical factors, such as anesthesia, and ongoing development of several organ systems, including the brain. Past studies indicate that anesthetic use during the neonatal time window is associated with neurotoxicity and an increased risk of behavioral deficits in later life [120]. These include sensory issues, motor deficits, as well as cognitive delays. The neonatal period is characterized by crucial early brain maturation processes, such as early cortical circuit establishment, synaptogenesis, and cerebellar neurogenesis. Disruptions in any of these developmental steps carry a potential risk of physiological dysfunction and resultant behavioral disturbances.

in motor reflexes and increased social anxiety [72]. The dose-dependent effect points to a cumulative model of anesthetic neurotoxicity, with greater exposure frequency leading to greater neuroapoptosis and neurodegenerative changes. Genetic susceptibility or involvement of a second environmental factor (a dual-hit scenario) could therefore plausibly decrease the threshold for caspase activation as well as modify the nature of the response, rerouting it from cell death to structural and physiological remodeling of the cell. Ketamine administration is also commonplace in pediatric cardiac surgery as premedication. Recent reports indicate that multiple courses of ketamine in developing rat models elicit caspase 3-associated neurodegenerative changes in the prefrontal cortex and hippocampus as well as neurobehavioral alterations such as impairments in spatial memory, decreased anxiety, and enhanced novelty-induced behaviors [73–75].

Anesthetic exposure neurotoxicity has also been characterized in the context of age-related cognitive decline and neurodegeneration. Alzheimer's disease has been postulated to result primarily from abnormal accumulation and reduced clearance of β-amyloid (Aβ) peptides in the aging brain [76]. Isoflurane-exposed neonatal mice as well as human neuroglioma cells exhibited increased oligomerization of Aβ42 peptides, which was accompanied by enhanced caspase 3 activation [77] and is known to be upstream of caspase signaling [78], leading to synaptic injuries [79]. Unexpectedly, the use of another anesthetic, propofol, attenuated caspase activation in this paradigm, likely through suppressing Aβ42 oligomerization [77]. The apparently contradicting effects on caspase activation point to the possibility of complex interactions between different anesthetics, when used in combination. The caspase activation signature resulting from their combinatorial use may therefore fall in a sublethal range, where apoptotic and autophagic cell death are avoided, but cytoskeletal and synaptic damage persist.

Potential for sublethal insults

While the majority of anesthetic neurotoxicity research has focused on the canonical, cell death-inducing context, sublethal mechanisms have also been implicated. *In vitro* evidence in neural precursor cells (NPCs) derived from human embryonic stem cells suggests short-term (2 h) exposures to clinically relevant sevoflurane concentrations do not induce cell death, with higher, supraclinical concentrations transiently inhibiting differentiation [80]. In concordance with these findings, earlier research in cultured hippocampal NPCs found that exposures to isoflurane [81] and clinically relevant concentrations of propofol [82,83] did not induce cell death, but did promote neuronal differentiation. Ketamine treatment of *ex vivo* rat cortical NPCs showed an identical trend, with no effect on apoptosis and enhanced neuronal differentiation [84]. These studies collectively suggest a greater resistance to anesthetic-induced cell death in immature neuronal precursors and potentially a more prominent role for nonapoptotic mechanisms in neurogenic regions of the brain during the neonatal time window. In a separate study, isoflurane-treated hippocampal NPCs showed robust increases in cleaved caspase 3 levels without accompanying cell death, as assessed by TUNEL staining [85]. Interestingly, at least part of the mechanistic explanation for the enhanced neuronal differentiation also observed in this study is increased caspase 3 activation, as treatment with Z-DEVD-fmk, a caspase 3 inhibitor, rescued caspase activation and neuronal differentiation levels. If caspase activation in neurogenic regions such as the hippocampus can be sublethally modulated by anesthetics, the neonatal period should be a particularly vulnerable time window, due to extensive neurogenesis taking place.

Detrimental anesthetic effects on dendritic arborization and synaptic ultrastructure have also been demonstrated. Acute exposures of *ex vivo* primary rat cortical neurons for 1 h to sevoflurane or desflurane did not result in overt cell death in the case of sevoflurane; however, both anesthetics led to reduced neurite lengths and dampened spontaneous synaptic currents [86]. Intriguingly, the damaging effects of both anesthetics were mediated through increased

mitochondrial fragmentation, as treatment with an inhibitor of mitochondrial division, Mdivi-1, rescued mitochondrial morphology as well as the synaptic and electrophysiological defects. Mitochondrial fragmentation is a key step during activation of the **intrinsic apoptotic pathway** and is upstream of caspase 3 cleaving and activation (Figure 1). Given the differential level of mitochondrial fragmentation observed between sevoflurane and desflurane [86], which was correlated to cell death, one could speculate that an 'intermediate' level of mitochondrial 'leakiness' leads to a corresponding sublethal caspase activation, which works to suppress neuritogenesis and synaptic maturation while avoiding triggering cell death (as postulated in Figure 2). The findings from this study point to the importance of careful characterization of neuronal phenotypes in anesthetic exposure paradigms where overt neurodegeneration and cell death are not observed. These more subtle phenotypes may be otherwise missed in studies focused on detecting signs of canonical caspase signaling. A major caveat of the aforementioned studies is that they were performed in cultured cells, raising the question of whether these unique, sublethal scenarios can be replicated *in vivo*.

Recently, a large-scale randomized control trial found no difference in neurodevelopmental milestones in 2-year-old children with a follow-up at 5 years, between a group exposed to 1 h of general anesthesia and a control group receiving awake-regional anesthesia [87,88]. While the trial design reflected commonly used regimes during pediatric surgery, it could not assess the effects of repeat exposures nor could it account for potential gene–environment interactions and combinatorial mechanisms (Box 3). Children with CHD often carry polymorphisms in genetic loci that are associated with regulation of apoptosis and cell survival. Among these are the *Akt1* and *Gdf1* genes [89,90], which have known roles in modulating caspase signaling and cell survival [91–94]. This suggests a scenario where genetically susceptible populations, such as certain CHD patients, may be at an increased risk for anesthesia-induced neurotoxicity due to an enhanced likelihood of caspase activation following exposure and caspase-mediated neurodegeneration.

How nonlethal yet damaging caspase responses may be implicated in these cases is unknown and warrants further study. Understanding the switch from cell death-promoting caspase activity to sublethal outcomes would potentially allow for better stratification and more informed treatment of patients suffering from caspase-elicited neurotoxicity.

Therapeutic potential and outlook

Caspase-dependent neurotoxicity is a complex, multistep process involving both autonomous and non-cell autonomous functions and is heavily influenced by timing, dosage, and other underlying

Box 3. Current controversies in anesthesia neurotoxicity

Studies on developmental anesthesia neurotoxicity have mostly focused on canonical, apoptotic caspase signaling and preclinical animal models, primarily rodents and monkeys. Repeated anesthetic exposures in animals lead to robust neurotoxic effects on brain development and maturation [71,74]. A recent clinical trial found no significant effects of a single, 1-h exposure to anesthesia on developmental milestones and neurobehavioral outcomes at 3 and 5 years of age [88]. The difficulty in assessing comprehensive outcomes in humans, as well as the small sample size and fact that trial participants were not screened for genetic status, presents difficulties for fully excluding anesthetics as neurodevelopmental risk factors. In addition, obvious technical and ethical difficulties prohibit distinguishing anesthesia neurotoxicity from the global impact of neonatal surgery such as systemic inflammation. Measuring apoptotic outcomes may also miss more subtle, yet potentially important cellular alterations due to nonlethal caspase-dependent mechanisms.

The cellular and molecular mechanisms behind nonapoptotic neuronal injury and their influence on behavior are largely unexplored, primarily due to historical focus on the canonical caspase pathway. Given the clear importance of this pathway as a source of potential long-term neurological injury, there is an urgent need for in-depth mechanistic characterization in both basic and translational animal models, as well as design of new targeted therapeutics.

covariates. Assuming sublethal neurodegenerative caspase activation is mostly a constrained version of caspase-dependent cell death, the increasing repertoire of caspase inhibitors would provide candidate therapeutics for neuroprotection. Among these, Z-VAD-FMK and Boc-D-FMK show potent anticaspase properties, albeit without inhibiting activity of all caspases and showing non-overlapping specificity [95–97]. Both inhibitors are commercially available; however, they have been shown to induce adverse effects in the past [98,99]. A viable alternative is the pan-caspase inhibitor Q-VD-OPh, with higher efficacy, demonstrable diffusion through the blood–brain barrier, and a better toxicity profile [100,101]. Q-VD-OPh has shown promise in preventing apoptosis in models of perinatal stroke [102], spinal cord injury [103], and Alzheimer's disease [104].

Apart from broad-spectrum caspase enzyme inhibition, therapeutic strategies may be aimed at indirectly dampening caspase activation and subsequent neuronal damage. Mitochondrial fission and fragmentation is a known inducer of intrinsic caspase 3 activation, and inhibitors such as the quinazolinone Mdivi-1 have been shown to protect neuronal stem cells from oxidative damage and apoptosis [105]. The PI3K/AKT pathway has long been known to play a prominent neuroprotective role under various injury paradigms [92,106], partly through restricting caspase activation. Employment of the PI3K/AKT small molecule activator, SC79, may be beneficial in both apoptotic and sublethal injury scenarios, as it has been demonstrated to protect neurons from oxidative stress [107,108].

Given the variety of nonapoptotic roles caspases play during normal neural development and maturation processes, using broad-spectrum caspase inhibitors at critical developmental time points may pose risks. Since many of the sublethal effects of caspase activation take place at lower activation thresholds, it will be important to assess for remnant caspase activity and cytoskeletal integrity in paradigms using caspase inhibitors to suppress cell death. Interference with physiological caspase functioning is of particular concern in scenarios likely requiring continuous inhibitor treatments, such as in progressive neurodegenerative disorders like Alzheimer's, Parkinson's, and Huntington's diseases. Future research on developmental anesthesia neurotoxicity will need to more carefully address the potential sublethal, caspase-dependent adverse outcomes in terms of cell morphology, establishment of functional neural circuitry, and behavior. Assessing caspase activation thresholds in preclinical models alongside thorough titration of therapeutics targeting caspase activation, intracellular signaling such as PI3K/AKT and mitochondrial stability [109] will be necessary to fully characterize and target the sublethal response.

Concluding remarks

Significant progress has been made over the past decades in understanding the molecular mechanisms behind programmed cell death in the nervous system and its effects on cell morphology, physiology, and organismal behavior. Detailed characterization of caspases, their substrates, and the outcomes of their activity has informed the design of new classes of caspase inhibitors with demonstrated efficacy. A primary driving force behind this work was to understand **canonical apoptotic signaling** with the hope of either suppressing it in the context of neurodegenerative insults or promoting it during carcinogenesis. In parallel, the nonlethal mode of caspase activation and signaling has been gaining growing interest. Progress has been made in clarifying the important functions this signaling pathway plays in neuronal dendritic extensions and arborization, experience-induced plasticity, and learning, as well as how it might be co-opted in injury scenarios. Advances in super-resolution imaging, *in vitro* neuronal culture, CRISPR-Cas9 genome editing, and next-generation sequencing methodologies, together with established injury models, are positioning research to dissect the conditions under which sublethal caspase

Outstanding questions

What is the main driver behind the shift from apoptotic to nonapoptotic caspase functions during neuronal development and maturation?

Is the functional divergence in neuronal caspase activity primarily dependent on quantitative, caspase activation threshold-associated changes in signaling, or do other indirect factors play a greater role?

Are neuronal cells more sensitive to nonapoptotic insults at particular times during their morphological and physiological maturation? Are different classes and types of neurons differentially sensitive to caspase signaling?

Are cytoskeletal proteins the primary targets of nonapoptotic caspase activity? How is this substrate specificity governed in neuronal cells? Are different caspases recruited to different substrates in a nonapoptotic context?

To what extent and by what mechanism are environmental neurotoxins implicated in nonlethal caspase signaling? How is the damage constrained and apoptosis avoided?

What role do gene–environment interactions in neurons play in nonapoptotic caspase signaling in physiological and injury contexts? Is there potential for harmful interactions between low-dose anesthetic exposures and genetic susceptibility at the level of nonapoptotic caspases?

If risks for particular pediatric populations (such as those with CHD) are identified, what caspase-targeting therapeutic options are optimal for neuroprotection?

activation takes precedence over caspase-driven apoptosis (see Outstanding questions). These prospective studies will be especially important for deciphering the fine details behind the neurotoxicity of clinically relevant agents such as anesthetics, which remain indispensable for surgical procedures like those in the pediatric CHD population.

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Declaration of interests

The authors declare no competing interests in relation to this work.

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