**Review Article**

**Anesthetic Drug-Induced Neurotoxicity and Compromised Neural Stem Cell Proliferation**


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Abstract

**Introduction.** Because of obvious concerns, it is not possible to thoroughly explore the adverse effects of anesthetics in children. However, the availability of neural stem cell derived models has provided an invaluable tool to examine the etiology of neurotoxicity associated with developmental exposure to general anesthetics. **Stem cells and neurotoxicity.** To evaluate the degree of anesthetic-induced toxicity, cultured neural stem cells were examined, using specific biomarkers, to determine the effects of anesthetics on NSC proliferation/differentiation, the nature of anesthetic-induced toxicity, and possible mechanisms underlying anesthesia toxicity. **Anesthetics and proliferation.** Using recently developed methods, such as dynamic molecular imaging approaches, the relationships between anesthetic-induced NSC damage and alterations in NSC proliferation have been addressed. **Summary.** This review focuses on how anesthetics direct or signal NSCs to undergo apoptosis or necrosis, how neuronal transmitter receptors affect neurotoxicity, and how NSC models may serve as the most expeditious platform for decreasing the uncertainty in extrapolating preclinical data to the human condition.

Keywords neural stem cells; development; proliferation; anesthetics; neural stem cell damage

Core tip Stem-cell-derived models with their capacity to proliferate and differentiate provide advantages for detecting potential anesthetic-induced neurotoxicity. These systems provide reliable and simple in vitro models, that can within a short time frame provide data for evaluating the potential adverse effects of developmental anesthetic exposures and associated cellular mechanisms.

1. Introduction

The ability of cells to maintain a high degree of order in a chaotic universe depends upon the accurate duplication of vast quantities of genetic information carried in chemical form as DNA. This process, called DNA replication, must occur before a cell can produce two genetically identical daughter cells. Neural stem cells (NSCs) are stem cells in the nervous system that can self-renew and give rise to differentiated progenitor cells to generate lineages of neurons as well as glia, such as astrocytes, and oligodendrocytes, a characteristic known as multipotency. Therefore, during development NSCs are the sources of neurons, astrocytes, and oligodendrocytes in the central nervous system (CNS), owing to their multipotency/pluripotency and ability to self-renew. NSCs and neural progenitor cells are present throughout development and persist in the adult nervous system. The proportion of subventricular zone (SVZ) stem cells declines with development, and multipotent stem cells are likely to be present only in regions of ongoing neurogenesis (e.g., anterior SVZ and SVZ underlying hippocampus) in the adult CNS. While the nervous system is one of the earliest organ systems that differentiate from the blastula-stage embryo, and this differentiation can be mimicked in culture, it is important to determine whether stem cells from adult tissues have the same capability and potential as embryonic stem cells.

Various factors contribute to determining the fates of NSCs and affect their transition from maintenance to proliferation to differentiation. Utilization of embryonic NSC-based models should allow for the recapitulation of many aspects of CNS development in vitro, and provide opportunity to identify the roles of specific chemical/cellular factors during developmental processes. Such models should then allow for the evaluation as to whether anesthetics can affect maintenance, proliferation, and/or differentiation.

In the developing brain, the nuclear and mitochondrial genomes incur DNA damage, primarily mediated by free radicals [1,2,3,4]. During embryonic development, accelerated DNA transactions, which support rapid cell proliferation, may even further jeopardize the integrity of DNA [5,6].

It is known that the most commonly used general anesthetics have either NMDA-type glutamate receptor blocking or GABA receptor enhancing properties. A growing body of data indicates that commonly used general anesthetics may cause neuronal apoptotic damage in several major brain regions in animal models including rodents and nonhuman primates, during certain periods of development, particularly the brain growth spurt [7,8,9,10,11,12]. Regarding an association between surgery
early in life and later cognitive problems, it is true that there are no clinical data in humans showing neurotoxicity after pediatric anesthesia exposure (because we are not taking brain biopsies from children undergoing anesthesia). Therefore, it is still controversial about the contribution of anesthetics-mediated neuronal apoptosis (destruction of existing neurons) and impaired neurogenesis (inhibition of generation of new neurons) in the developing brains to the subsequent memory and learning disability.

Recent advances in our understanding of stem cell biology and neuroscience have opened up new avenues of research for detecting anesthetic-induced neurotoxicity, dissecting underlying mechanisms, and developing potential protective/preventative strategies against anesthetic-induced neuronal injury. The application of stem cell models towards understanding issues relevant to developmental neurotoxicology has the potential to advance our understanding of brain-related biological processes, including neuronal plasticity and toxicity [13,14,15,16,17]. This review discusses how stem cells can be used as tools for dissecting out mechanisms underlying anesthetic-induced neural stem cell damage.

2. Stem-cell-derived models and anesthetic-induced toxicity

The nervous system is sensitive to the toxic effects of many chemicals including drugs, environmental agents, and certain naturally occurring substances. Neurotoxicity can result in temporary or permanent damage to the brain or peripheral nervous system and is also a major cause of neurodegenerative diseases. The application of neural stem cell models, coupled with advanced research approaches, will provide valuable platforms from which to study factors that will positively or negatively affect the consequences of pediatric anesthetic exposure.

Many of the anesthetic protocols used in children were developed from those used in adults. However, it is clear that the dynamic developmental physiology characteristic of early life stages is different from those occurring during later developmental stages and children seem to be more vulnerable to the adverse effects of anesthetic exposure [18]. Recently, the availability of stem cells, especially neural stem cells of embryonic origin, with their pluripotency and capacity for proliferation, has provided a valuable tool for examining the developmental effects of anesthetic agents in vitro [19,20]. In fact, neural stem cell models may represent some of the best systems available for evaluating the potential adverse health impacts of pediatric anesthetics. This capability stems from several attributes of neural stem cells including (1) source (neural stem cells can be obtained directly from humans), (2) specificity of cell types (this approach allows for examining the adverse effects of anesthetics on neural stem cells themselves, as well as neurons, astrocytes and oligodendrocytes and the processes involved with differentiation into those cell types), (3) reduced animal use and shortened time frames for study completion, (4) ability to assess regeneration capacity after exposure to toxicants, (5) potential to significantly impact best practices for pediatric anesthetics [21], and (6) the ability to mimic or model particular developmental stages in animals and humans. Embryonic neural stem cells, especially those of human origin, provide great opportunities for identifying potential neurotoxicity associated with exposure to anesthetics such as ketamine. Combining these models with calcium imaging and molecular biological approaches creates great possibilities for elucidating mechanisms underlying the etiology of the neurotoxicity associated with developmental exposures to general anesthetics and may also help identify ameliorative strategies.

Previous work based on mRNA levels showed that NMDA receptor NR1 expression in ketamine-exposed rat pup brains was significantly higher than in controls [12,22,23,24], and subsequent work also showed altered expression levels of the NMDA receptor NR2 family, including NR2A and NR2C [23], after repeated ketamine exposure. This evidence of NMDA receptor upregulation suggests that upon removal of ketamine from the extracellular milieu, the now upregulated NMDA receptor population (a probable compensation for prolonged NMDA receptor blockade by ketamine) will “over” respond to normal levels of extracellular glutamate, resulting in glutamatergic excitotoxicity. Also, utilizing a primary neuronal culture system it was demonstrated that ketamine exposure has a significant impact on subsequent intracellular Ca\(^{2+}\) homoeostasis: the amplitude of calcium influx caused by activating concentrations of NMDA was significantly increased in neurons from ketamine-exposed cultures compared with neurons from control cultures [24]. The NMDA-elicited increases in intracellular Ca\(^{2+}\) were blocked by chelation of extracellular Ca\(^{2+}\) with EGTA, which clearly demonstrates that the NMDA-evoked increases in intracellular calcium originated from an extracellular source, rather than from a depletion or releases of calcium from the endoplasmic reticulum [24]. Since ketamine has well-defined effects on the NMDA receptor at anesthetic concentrations [25], these data provide further support for the hypothesis that continuous blockade of NMDA receptors by anesthetics (e.g., ketamine) causes a compensatory upregulation of NMDA receptors. This upregulation makes neural cells bearing these receptors more vulnerable to the excitotoxic effects of glutamate, because these upregulated NMDA receptors allow for the accumulation of toxic levels of intracellular free calcium [11,26]. The elevated levels of intracellular free calcium that exceed the buffering capacity of mitochondria interfere with electron transport in a manner that results in the increased production of reactive oxygen species (ROS).
In a mechanistic study [24], administration of the anesthetic agent ketamine markedly elevated both nuclear and mitochondrial levels of 8-oxoguanine, a marker of oxidative stress. The concordance between elevated 8-oxoguanine levels, enhanced DNA fragmentation, and increases in the number of cells with DNA strand breaks, following ketamine exposure, suggests key roles for calcium homeostasis and mitochondrial ROS production in inducing neuronal DNA damage and disruption of cell division cycles. Commonly used anesthetics (e.g., propofol, sevoflurane, desflurane, and isoflurane) take their effects by either inhibiting the excitatory neurotransmission mediated by NMDA receptors or enhancing the inhibitory neurotransmission via the excitatory neurotransmission mediated by GABAergic signaling [20, 24, 27, 28]. Therefore, the neurotransmissions conducted by NMDARs and GABA<sub>A</sub>Rs are critical to the normal development of the CNS, and disturbances in glutamatergic or GABAergic signaling would compromise the synaptogenesis, neurogenesis, and neural circuit formation [29, 30].

It is becoming increasingly apparent that mitochondria lie at the center of the cell death regulation process. Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals, are typically produced in mitochondria as electrons leak from the electron transport chain and react with oxygen to form superoxide. ROS generated by mitochondria are not just toxic by-products of respiration, they are also important for cell signaling [31, 32]. In a recent in vitro mechanistic study [24], an increase in the generation of ROS was associated with the increased Ca<sup>2+</sup> influx seen in ketamine-exposed neurons in culture. These ROS appear to originate in mitochondria. Recent evidence suggests that general anesthetics, administered during the peak of synaptogenesis, caused protracted injury to mitochondria including significant enlargement, impairment of their structural integrity, and a decrease in their regional distribution [33]. Along with morphological changes, the general anesthetics also cause functional impairment of immature neuronal mitochondria [34]. Injured mitochondria may be a significant source of reactive oxygen species [24, 34]. Meanwhile, the role of the Bax, proapoptotic gene, in anesthetic-induced apoptosis has been extensively studied [11, 24, 26]. As previously reported, Bax may open pores on the outer mitochondrial membrane, allowing cytochrome c to exit [35]. Cytochrome c, a component of the mitochondrial electron transfer chain, initiates caspase activation when released from mitochondria during apoptosis [36]. Cytosolic cytochrome c could bind to Apaf-1, a cytosolic protein containing a caspase-recruitment domain (CARD). The binding of nucleotide to the Apaf-1/cytochrome c complex triggers its oligomerization to form the apoptosome [37]. The CARDs of Apaf-1 become exposed in the apoptosome, which subsequently recruit multiple procaspase-9 molecules to the complex and facilitate their autoactivation. These executioner caspases subsequently cleave many important intracellular substrates, leading to characteristic morphological changes in apoptosis such as chromatin condensation, nucleosomal DNA fragmentation, nuclear membrane breakdown, externalization of phosphatidylserine, and formation of apoptotic bodies [38].

Taken together, the accumulated data [7, 8, 11, 26, 39] indicate that anesthetic-induced neural damage depends on the amount (dose) given, the duration of exposure, the route of administration, the receptor subtype activated, and the stage of the development at the time of exposure. Undoubtedly, it is the most important that the dosage, duration, and route of administration are well controlled/monitored in clinical practice, pediatric anesthesia, and specific case-studies.

### 3. Anesthetic-induced damage and neural stem cell proliferation

NSCs in the nervous system can self-renew and give rise to differentiated progenitor cells to generate lineages of neurons as well as glia, such as astrocytes and oligodendrocytes. This characteristic is known as multi- or pluripotency. NSCs are present throughout development and persist into adulthood. Multiple classes of NSCs have been identified that differ from each other in their ability to differentiation, their responses to cytokines, and their surface antigen characteristics. NSCs are valuable resources because of their potential to proliferate and differentiate, thus, providing opportunities for applications in neuroscience, in general, and for clinical applications specifically such as in the treatment of neurodegenerative diseases and neurological disorders.

Self-renewal or self-replication is defined as the ability of the stem cell to go through multiple cycles of cell division while maintaining an undifferentiated state (i.e., to generate daughter cells that are identical to the mother cell). The self-replication of NSCs is characterized by two different types of cell division: symmetrical and asymmetrical. Symmetrical cell division refers to the generation of two daughter cells that have the same fate; while asymmetrical cell division refers to the generation of two daughter cells, only one of which remains the same as the mother cell, while the other does not. Typically, symmetrical cell division refers to the generation of two daughter cells, only one of which remains the same as the mother cell, while the other does not. Typically, symmetrical cell division precedes asymmetrical cell renewal, generating enough NSCs to develop the bulk of the CNS. Asymmetrical cell divisions then give rise to more differentiated neural cells, such as progenitor cells, neurons or glia [40].

Enhancement of neurogenesis by pharmaceutical agents would be predicted to improve functional outcome after neuronal damage. Accordingly, there is great interest in using NSCs as screening tools in the early stages of drug development for many neurological disorders. Inhibition of neurogenesis, on the other hand, could be a key pathway for anesthetic-induced neurotoxicity during development [20].
Previously, a variety of cultured cell lines and animal models have been utilized to investigate the neurotoxic effects of anesthetics in studies which have primarily focused on the postnatal period during the brain growth spurt [24, 26, 41, 42]. Since the developmental stage at the time of a chemical/toxicant exposure can critically influence the outcome of neurodevelopment, an embryonic neural stem cell model was utilized in a previous project [20] to study the toxic effects of anesthetics during early developmental stages. This provided an opportunity to allow for the assessment of the effect of the anesthetic propofol on neural stem cell proliferation and differentiation and to monitor associated biochemical outcomes including oxidative DNA damage and apoptotic cascades [20].

Given the importance of NMDA-type glutamate and GABA receptors to the expression of anesthesia-induced neurotoxicity in vivo, it is vital to provide direct evidence that cultured embryonic neural stem cells express functional neurotransmitter receptors of these types. In a recent mechanistic study [20], it was demonstrated that no GABA receptor immunoreactive staining was detected on undifferentiated cultured rat NSCs. And our recent preliminary data showed that there was no receptor-associated intracellular calcium influx detected. However, evidence for the presence of functional NMDA receptors [11, 20, 24] and strong immunoreactive staining for GABA_A receptors [20] was detected on neurons differentiated from the same neural stem cells. These data clearly indicate that during early development embryonic neural stem cells do not express functional neurotransmitter receptors for GABA or NMDA and, thus, any anesthetic-induced neural stem cell damage that occurs in such cells must involve different mechanisms. These might include the effects of anesthetics to increase ROS production resulting in oxidative DNA damage, interruption of neural stem cell proliferation, and associated neural stem cell damage.

To investigate whether enhanced cell damage could be associated with interruptions in cell division cycles, neural stem cell proliferation rates after anesthetic exposures were determined using an EdU (5-ethyl-2′-deoxyuridine) assay [20]. EdU, like 5-bromo-2′-deoxyuridine (BrdU), is a thymidine analog that is incorporated into cells only during the S-phase of cell division and is used as a marker of cellular proliferation. After a 24-hour exposure of neural stem cells to the anesthetic propofol (50 μM), the number of dividing cells was substantially decreased compared with controls suggesting a decrease in the neural stem cell proliferation rate. Coadministration of acetyl-L-carnitine [20], an antioxidant agent, effectively attenuated the decrease in neural cell proliferation produced by propofol (Figure 1).

In living cells, DNA is inherently unstable and its integrity relies on continuous DNA monitoring and repairing activities [43]. During embryonic development, accelerated DNA transactions, which support rapid cell proliferation, may even further jeopardize the integrity of DNA [5, 6, 44]. It has been shown that caspase-3 specifically activates the endonuclease caspase-activated deoxyribonuclease (CAD). CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. It seems that anesthetics can affect neural stem cell proliferation by slowing down or even stopping the cell division cycle, eventually resulting in cell death.

The ameliorating effect of acetyl-L-carnitine on the effects of propofol to inhibit cellular proliferation [20] strongly suggests that propofol can initially cause oxidative mitochondrial damage and dysfunction, subsequently affecting neural stem cell proliferation by slowing down or even stopping cell division and eventually damaging the cells [45, 46, 47].

A growing body of data indicates that molecular imaging with appropriate isotope-labeled biomarkers (radio-tracers) can help to detect and monitor aspects neurotoxicity in animal models [48, 49]. The high-resolution positron emission tomography scanner (microPET) can provide in vivo molecular imaging at a sufficient resolution to resolve both major structures and neuronal activities in the brain. Therefore, imaging technology has great potential for advancing the understanding of brain-related biological processes, including neuronal plasticity, neuronal degeneration/regeneration, and neurotoxicity [48, 49].

In ongoing studies designed to monitor endogenous neural stem cell proliferation in vivo, 3′-deoxy-3′-[18F]-fluoro-L-thymidine ([18F]-FLT), one of the most widely used radiotracers for imaging cell proliferation [50], was utilized after sevoflurane exposure. Sevoflurane, a volatile anesthetic commonly used to anesthetize and sedate pediatric patients, is thought to act as an agonist at GABA_A receptors at anesthesia-relevant concentrations [51]. FLT, a thymidine analog, is taken up by cells and phosphorylated by thymidine kinase (TK) 1, leading to its intracellular trapping. The phosphorylated FLT trapped within cells does not get incorporated into the cellular DNA because it lacks a 3′-hydroxyl. TK 1 is a cytosolic isozyme of TK and its activity closely parallels that of cellular proliferation [52]. Therefore, the retention of [18F]-FLT within the cells serves as an in vivo marker that can be used for the visualization of cell proliferation. In comparison with air-exposed animals, preliminary data indicate that sevoflurane exposure at a clinically-relevant concentration inhibited the uptake of [18F]-FLT in the hippocampus of the developing brain. Here, the inhibitory effect of sevoflurane exposure manifests in an age-dependent manner. These data suggest that by utilization of [18F]-FLT in conjunction with PET imaging, the relationships between anesthetic-induced neurotoxicity and endogenous neural stem cell activity following exposure to general anesthetics can be monitored in vivo. In addition,
critical data can be obtained noninvasively, repeatedly, and quantitatively in the same subjects [53, 54, 55].

There are yet many questions to answer before the findings of pediatric drug-induced neurotoxicity observed in animals can be related to effects in humans. Currently, $[^{18}F]$-FLT is being used in conjunction with PET imaging in rodents to monitor effects on cellular proliferation of exposure to the anesthetic sevoflurane. If such experiments are successful in developing nonhuman primates, then similar studies should be able to help define the pathway to toxicity and allow cross-species comparisons.

4. Summary

Neural stem or progenitor cells are generally uncommitted and so can change their fate after exposure to salient environmental cues. Evidence shows that gene expression and the capacity for self-renewal and differentiation of NSCs are spatially and temporally specified. Stem-cell-derived models with their capacity to proliferate and differentiate provide advantages for detecting potential anesthetic-induced neurotoxicity and underlying mechanisms (Figure 2). These systems provide reliable and simple in vitro models, that can within a short time frame provide data for evaluating the potential adverse effects of developmental anesthetic exposures and associated cellular mechanisms [56]. The use of neural stem cell models, especially those of human origin, holds promise for helping to elucidate relevant mechanisms underlying the etiology of the neurotoxicity associated with developmental exposures to the general anesthetics. Stem-cell-derived models, when combined with advanced research approaches such as calcium and molecular imaging, may also help identify avenues of protection or prevention.

Although stem cells in vitro (e.g., hESC-related neurogenesis models) can mimic the in vivo neuronal development process and provide a simple and unlimited cell source for addressing anesthesia-related issues [57], it is difficult to make an exact comparison between developing brain (3D components) vis-à-vis neural stem cells for anesthetic-induced neurotoxicity. The 2D neural stem
cell cultures, including adult NSC or even iPS-derived NSC models, are lack of 3D structure that more closely resembles actual in vivo microenvironments that include extracellular matrices, adhesion junctions, and so on. Also, it is difficult to determine the percent occurrence of neural stem cells in pediatric versus adult brains.

However, organotypic (3D) culture system provides an invaluable platform for extrapolating preclinical data to human conditions. The main advantage of using organotypic culture model is that this preparation maintains important anatomical relationship and synaptic connectivity, while at the same time preserving the advantages of an in vitro preparation. Comparable and functional high-throughput 3D model platforms and microfluidic cell culture chips have quickly captured the attention of neuroscientists. Studies are continuously revealing new information and it is hoped that therapeutic applications will be developed from both embryonic and adult sources.

Disclaimers
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Conflict of interest
The authors declare that they have no conflict of interest.

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