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Recent Insights into Molecular Mechanisms of Propofol-Induced Developmental Neurotoxicity: Implications for the Protective Strategies

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Abstract

Mounting evidence has demonstrated that general anesthetics could induce developmental neurotoxicity, including acute widespread neuronal cell death, followed by long-term memory and learning abnormalities. Propofol is a commonly used intravenous anesthetic agent for the induction and maintenance of anesthesia, and procedural and critical care sedation in children. Compared with other anesthetic drugs, little information is available on its potential contributions to neurotoxicity. Growing evidence from multiple experimental models showed a similar neurotoxic effect of propofol as observed in other anesthetic drugs, raising serious concerns

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regarding pediatric propofol anesthesia. The aim of this review is to summarize the current findings of propofol-induced developmental neurotoxicity. We first present the evidence of neurotoxicity from animal models, animal cell culture, and human stem cell-derived neuron culture studies. We then discuss the mechanism of propofol-induced developmental neurotoxicity, such as increased cell death in neurons and oligodendrocytes, dysregulation of neurogenesis, abnormal dendritic development, and decreases in neurotrophic factor expression. Recent findings of complex mechanisms of propofol action, including alterations in microRNAs and mitochondrial fission, are discussed as well. An understanding of the toxic effect of propofol and the underlying mechanisms may help to develop effective novel protective or therapeutic strategies for avoiding the neurotoxicity in the developing human brain.

Keywords

propofol; developmental neurotoxicity; microRNAs; mitochondria

INTRODUCTION

Each year, up to 2% of pregnant women in North America undergo anesthesia during their pregnancy for surgery unrelated to the delivery. In addition, millions of human fetuses, infants, toddlers, and preschool children are exposed to anesthetic drugs every year in the United States and throughout the world due to essential surgical or medical procedures.[1] Since 1979 when the first review article detailed that children had side effects of anesthetic administration, based on several clinical case reports,[2] compelling evidence has emerged linking most anesthetics to adverse consequences on the central nervous system (CNS) in developmental animal models. The toxic effects included acute widespread neuronal cell death followed by long-term memory and learning abnormalities. Several studies have shown that anesthetics induced learning and memory impairment in young animals, but not in adult animals.[3, 4] Importantly, the observed neurotoxicity was specific to the certain developmental period, which is defined as the period of brain growth spurt.[5] This period ranges differently among species. For example, it happens during the first two weeks after birth in rodents. For rhesus monkeys this period ranges from approximately 115-day gestation up to postnatal day (PD) 60, while in humans it starts from about the third trimester of pregnancy and continues until approximately the third year after birth.[6–8] The peak of brain growth spurt occurring at birth in human is centered around one week postnatal in rats. PD2–7 in rats corresponds to the human third trimester.[9] The study by Krzisch et al., showed that propofol anesthesia reduced survival of adult (8- to 10-week-old mice) hippocampal neurons and dendritic maturation.[10] The finding indicates that around PD7 may not be the only vulnerability window to propofol toxicity. The toxic effect of propofol on mature hippocampal neurons will certainly promote future studies to investigate whether these abnormal changes in adult mice can in turn induce learning deficits.

Compared with other anesthetic drugs, less information on propofol neurotoxicity is available. Propofol is highly lipophilic. The intravenous administration of propofol, both for induction and maintenance of sedation throughout treatment, began in 1989.[11] No serious complications were reported during the maintenance or recovery from the anesthesia in the

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patients. In addition, propofol produced fewer side effects (e.g., vomiting or grogginess) compared to other anesthetics. Propofol has since become widely used due to its rapid onset and subsequent washout following the associated medical procedure. Very rapid loss of consciousness after a single dose of propofol and rapid recovery after cessation of administration make propofol an extremely useful agent for inducing and maintaining general anesthesia.[12] Propofol is one of the most commonly used intravenous drugs employed to induce and maintain general anesthesia[i] and it is being used in over 50 countries and in over 75% of all surgeries[ii]. Propofol is also widely used for the induction and maintenance of anesthesia, and procedural and critical care sedation in children. The use of propofol in certain age groups continues to be off-label, as it has been US Food and Drug Administration (FDA) approved for maintenance of anesthesia only in children ≥2 months of age, and for induction of anesthesia in children ≥3 years of age.[96] Because propofol can produce hallucinations and euphoria, propofol is also a drug that is often abused. The number of people abusing propofol, including pregnant women, is increasing.[13] In addition, about 1–2% of pregnant women undergo surgery, unrelated to their delivery, with anesthesia including propofol.[97]

More recently, growing findings showed that propofol could induce developmental neurotoxicity in multiple models (e.g., animal model and cell culture system), raising serious concerns regarding the safety of pediatric propofol anesthesia. Based on current medical advances, pediatric anesthetic exposure is necessary in most cases. Still, substantial consideration of the possibility of human relevance of propofol-induced neurotoxicity observed in animal models is warranted. Since it is also impossible to obtain direct histological evidence of anesthetic neurotoxicity in young patients and their derived neural cell lineages, there have been an increasing number of studies in recent years attempting to develop a stem cell model to examine the toxic effect of general anesthetics on human neurons, and show the toxic effect of these powerful drugs.[14–16] Thus, the aim of this review is to summarize: 1) the current findings of propofol-induced neurotoxicity from animal studies, 2) new stem cell approaches used to study neurotoxicity, and 3) the underlying, complex molecular mechanisms. The potential protective strategies are discussed as well. An understanding of the toxic effect and underlying mechanisms may help to develop more effective novel protective strategies for avoiding the potential neurotoxicity conferred by propofol. This review article cites most of the published studies regarding propofol-induced developmental neurotoxicity. A literature search was conducted using the following electronic search engines: Google (https://www.google.com) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Within each search, the key words of propofol and neurotoxicity were used. The references that are related to the background of the topics and specific discussions shown in the article were cited as well. Possibly relevant references within these articles were also used.

[i]Available at: https://en.wikipedia.org/wiki/Anesthetic. Accessed January 18, 2016
[ii]Available at: http://www.drugaddictiontreatment.com/types-of-addiction/prescription-drug-addiction/propofol-addiction/. Accessed January 18, 2016

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EVIDENCE OF PROPOFOL-INDUCED DEVELOPMENTAL NEUROTOXICITY

For both pre- and postnatal neurotoxicity following propofol exposure in clinical instances, it is difficult to dissect physiological effects specific to propofol, as patients often have underlying alterations due to the medical condition(s) associated with their need to seek treatment. Although this is a challenging area to study, strong evidence from animal models highlights the critical importance of revisiting pediatric propofol use. The remainder of this section first presents the evidence from the well-established animal models of propofol-induced neurotoxicity and then introduces an emerging human stem cell model.

In Vivo Animal Studies

Several studies done in developing rodents, zebrafish, piglets, and primate models showed that propofol alone, or used in combination with other drugs such as sevoflurane (Table 1), could induce neuronal cell death in the brains of these animals and/or lead to learning and memory impairments later in life.[17–22] Animal models provide the advantage of investigation into the *in vivo* cellular basis of alterations following anesthetic exposure, analysis of live animals, and the provision of doses and forms of anesthetics similar to those used routinely in the clinic. Propofol is highly lipophilic and therefore concentrates in lipid-rich tissues such as the brain.[23] The studies in animals[23] and humans[24, 25] indicated that the measured/predicted brain concentration of propofol during maintenance of surgical anesthesia is above 4 μg/ml, and as high as 20 μg/ml.[26, 27] Thus, in the *in vitro* experiments, the doses of propofol used that are within the range of concentrations found in the human brain during surgical anesthesia are referred to as anesthetic doses. The concentrations that are outside this range would be called either sub-anesthetic or supra-anesthetic concentrations. For the *in vivo* animal studies, the dosage required to induce a surgical plane of anesthesia is called anesthetic dose while the dose of propofol which can induce the righting reflex but not loss of response to painful stimuli as assessed by pinprick is called sub-anesthetic dose.

Rodents—The majority of neurotoxicity studies involving propofol have been performed in mice and rats, and have focused on a narrow window of neonatal development. Published literature exists for a number of different doses, exposure frequency, and methods of propofol administration, as the purpose for administration can vary in human medical practice based on severity, duration, and type of treatment. When given a single intraperitoneal propofol injection at doses ranging from sub-anesthetic to high (25 to 300 mg/kg) in 5- to 7-day-old mice, it was determined that the median dose effective for inducing anesthesia was 150 to 200 mg/kg.[28] When these authors measured neuroapoptosis along the same dosage range, the 50 mg/kg sub-anesthetic dose was sufficient to induce significant cell death in the cortex and caudate/putamen, and this effect increased with increasing dose up to 100 mg/kg.[28] The fact that this widespread neuroapoptosis begins to occur at such a low sub-anesthetic dose is an important finding translatable to the clinic.

Several human patient studies have shown that children who received multiple, but not single, anesthetics at a young age have an increased risk of learning disability and attention deficit disorder.[29–31] However, these epidemiological investigations have been retrospective,

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allowing for identification of association but not causality. In addition, many other confounding factors, such as surgical trauma and disease itself, might influence the outcomes of these studies. In order to elucidate the association between multiple anesthetic exposures and cognitive dysfunction, propofol (75 mg/kg) was administered to PD7 rats either as a single dose or in seven doses at 24 h intervals. At PD28 spatial learning, analyzed using Morris water maze, and long-term potentiation in the CA1 (cornus ammonis) region of hippocampus, were significantly reduced in rats that had received seven doses of propofol.[32] Similar observation was also seen in rats following multiple isoflurane exposures. The greater toxic effect of multiple exposures may result from either cumulative toxicity or the sensitization to the effects of subsequent exposure, or a combination of these factors.[33] Although this animal study cannot accurately mimic the clinical situation of multiple anesthetics and surgeries, the data suggest that children receiving multiple exposures to propofol might be at a higher risk.

Rhesus Monkeys—Although the initial rodent studies were extremely important, questions were raised about the translatability of these findings to humans. Brambrink and others opted to use rhesus monkeys to study the effects of anesthetics on the developing brain. This allows for studies in a model more closely related to humans. In addition, the use of larger animals makes it easier to monitor hemodynamic properties of the animals and confounding variables of anesthetic administration such as cardiovascular, respiratory, or metabolic distress. In focusing specifically on apoptosis, PD6 rhesus monkeys exhibited increased neuroapoptosis and oligodendrocyte death following 5 h of 250 to 350 mg/kg/min of propofol infusion. In comparison with monkeys undergoing fetal propofol anesthesia, postnatal propofol exposure appeared to target neurons in the cerebral cortex, while fetal exposure affected more caudal and rostral regions of the brain (e.g., cerebellum, inferior colliculus, caudate, putamen, nucleus accumbens, amygdala, and thalamus) to a greater extent.[20] This study also found that oligodendrocyte death occurred during the time at which myelin generation is beginning to occur.[20] Studies that investigate the time course of developmental events of the CNS are uncovering important clues regarding the brain's susceptibility to toxic effects of propofol. Great headway is undoubtedly being made into the cellular mechanism of apoptosis, and there is continued need for this investigation in order to address potential clinical concerns and preventive/therapeutic possibilities.

In Vitro Cultured Animal Cell Model (Table 2)

In vitro animal cell culture approaches can provide great benefits for the following studies: 1) assessment of propofol toxicity and cell mechanisms without the confounding physiology of intact animal model, 2) looking at neurons independently of other constituents of the brain in order to tease apart the differential effects of propofol depending on cell type, and 3) studying distinct brain regions and different cell types that comprise the nervous system. Based on previous *in vivo* findings that have demonstrated propofol-induced neurodegeneration, Pearn et al. assessed cell morphology and apoptosis by analysis of activated caspase-3 expression in 6- to 10-day-old primary neurons harvested from the neonatal mouse brain.[34] Exposure to propofol for 6 h induced neuroapoptosis as observed in animal brains,[20, 35] and also caused decreased growth of neuron dendritic processes relative to controls.[34]

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In humans, it is also important to consider that synaptogenesis begins in the fetus and continues for the first few years of life, while it mostly occurs prenatally in animal models. In one study, as early as 14 days of gestation in the rats, the fetus was extracted, and neural stem cells (NSCs) were exposed to 10–600 μg/mL of propofol. The results showed that as low as 50μg/mL propofol inhibited cell division and induced oxidative stress. This observation was in contradiction to the results described previously in another study in which NSCs were isolated from hippocampus in PD2 rats and then treated with a lower dose of propofol (2.1 μM/0.37 μg/mL) for 6 h. The results showed that propofol increased neuronal differentiation but was not toxic to NSCs.[36] These studies and somewhat confounding findings collectively represent differential effects of propofol depending on the cell type, developmental age, and brain region. The very long exposure and high concentrations of propofol used in many studies may limit how these data connect to the clinical setting. However, the results of the *in vitro* studies may still be translatable to the human brain, in which the same mechanisms could induce toxicity at different doses due to the increased vulnerability in the whole brain.

Human Stem Cell Model

So far, there is no direct clinical evidence citing anesthetic neurotoxicity in human brain cells. The largest obstacle when studying the neurotoxic effect of anesthetics in pediatric patients is the development of an appropriate human model. Although the results of the epidemiologic studies will be extremely useful, it will be difficult to properly dissect out the effects of anesthetic exposure from the effects of surgery and other potentially confounding variables. The ideal model for these types of studies would be developing neurons from human brains. However, such a model is currently not feasible. Exposing healthy children to anesthetics in order to study these effects, as is done in animal models, would not be ethical. With the development of stem cell technology, we are able to use a model with developing human neurons. The emerging model of human embryonic stem cell (hESC)-derived neurons has allowed us to directly assess the effects of anesthetics on developing human neurons and dissect out the mechanisms by which these anesthetics induce toxicity.

hESCs are derived from the inner cell mass of a blastocyst, can replicate indefinitely, and differentiate into cells from all three germ layers. The proliferation and differentiation potential of hESCs makes them more advantageous than adult stem cells, and allows for mechanistic-based studies that lead to better understanding of development and disease using a human cell line, eliminating potential concerns regarding the relevancy of animal models to humans. Neuronal differentiation could be observed by morphological assessment in the culture after 6 days of culturing the hESC-derived NSCs in neuronal differentiation medium.[14] Two weeks after initiation of differentiation, up to 95% of NSCs differentiated into neurons. These neurons expressed the neuron-specific marker β-tubulin III, the synaptic marker synapsin-1, the postsynaptic protein Homer 1, and the immature neuron marker doublecortin.[14,15,37] In addition, differentiated neurons exhibited functional synapse formation, and responded to neurotransmitter application.[16,38,39] When cells underwent different lengths of exposure to propofol and were subjected to single and multiple exposures, propofol induced cell death in the 2-week-old hESC-derived neurons in a time, dose, and exposure number-dependent manner.[16]

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Ethanol has both NMDA (N-Methyl-D-Aspartate) antagonistic and gamma-aminobutyric acid (GABA)-mimetic properties. Ethanol has long been recognized as neurotoxic to the developing brain. Nash et al. used a similar *in vitro* hESC-based neurogenesis system to study ethanol-induced developmental toxicity. They found that ethanol induced a complex mix of phenotypic changes, including an inappropriate increase in stem cell proliferation and loss of trophic astrocytes.[40] These findings in stem cell-derived human neurons have recapitulated the results reported in animals,[29,41,42] indicating that the human stem cell-derived neural cells represent a promising model to study neurotoxicity, and has opened up avenues of research for advancing the understanding of human brain development, and the issues relevant to anesthetic-induced developmental toxicity in human neuronal lineages under controlled conditions.

One of the major caveats with this *in vitro* stem cell study lies in the relevance of the *in vitro* model to a true *in vivo* system. Although the cultured 2-week-old human stem cell-derived neurons used in the neurotoxicity expressed immature neuron markers, the neuron age-equivalence of *in vitro* model with that of *in vivo* human remains unknown. In addition, there are many cell types present in human brain that interact extensively. Utilizing cultures of pure neurons may not allow for the accurate assessment of the effects of anesthetics on intact brains. These cultured neurons lack stimuli from the complex network of cells that are found *in vivo* and endpoints of interest such as cognitive function cannot be assessed. Thus, establishing an *in vitro* model of co-culturing multiple types of neuronal cells is needed to better mimic the *in vivo* brain environments. It will also be necessary to confirm the *in vitro* findings in animal models. Nevertheless, it is critical to first understand the direct effects of propofol on human neurons, the mechanisms governing propofol-induced neuronal cell death and possible protective approaches before understanding how glial cells may alter these effects and pathways. In summary, development of research models in the area of stem cell biology provides a valuable and promising tool for the investigation of anesthetic-induced developmental neurotoxicity. The findings can potentially be translated to an animal model to further develop protective strategies against anesthetic-induced developmental neuronal cell death and cognitive dysfunction.

MECHANISMS OF PROPOFOL-INDUCED DEVELOPMENTAL NEUROTOXICITY

Despite many findings that propofol induces neurotoxicity in the developing animal brain, the mechanisms by which this toxicity occurs remain largely unknown. Many developmental events (e.g., cell proliferation, neurogenesis, synaptogenesis, and neuron structure remodeling) occur within this period. Thus, neuroapoptosis may not be the only consequence conferred by propofol. Several abnormal changes, such as neurite retraction and dendrite growth reduction, have been demonstrated in few studies.[43] The underlying mechanisms of propofol-induced detrimental effects (such as calcium signaling, mitochondrial fission, neuroinflammation, microRNAs, and neurotrophin expression deregulation) have all been reported.[14,44–46]

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Decreased NSC Survival and Proliferation

NSCs are the precursors of fully-differentiated neural cells, and are present from the early stages of fetal development into adulthood. They are characterized by their ability to self-proliferate, and are multipotent, in that they generate various cell types that constitute the CNS.[47] The advantage of using the cultured NSCs isolated from the prenatal or early postnatal period allow for examining whether or not a broader window for propofol-induced alterations exists. One study extracted NSCs from rat fetuses on gestational day 14 and maintained the cells in culture for 8 days. An anesthetic dose of propofol for 24 h elicited oxidative DNA damage, halted cell proliferation, and adversely affected mitochondrial viability.[48] Antioxidant supplication was sufficient to ameliorate the oxidative stress to a degree. This study demonstrates that propofol toxicity can extend to prenatal development, and also suggests that oxidation may be another emerging mechanism of propofol neurotoxicity. However, it remains unknown how the reduction in NSC number affects the brain development. Another study extracted NSCs from -day-old rats, and maintained the cells in culture for 13 to 14 days before exposure to propofol for 6 to 12 h. Propofol caused an increase in death of NSCs but did not halt NSC division. When NSCs were provided with the necessary factors to differentiate into neurons, the presence of propofol resulted in cell death.[36] This study provides a comprehensive story as to how propofol interacts with NSCs, and also influences differentiation process, which is very useful in better understanding the role of propofol during neurogenesis. Further exploration of the different cellular and molecular characteristics between NSCs and neurons may aid in pinpointing specific targets of propofol.

Altered Neurogenesis

Neurogenesis is defined as neuron generation from NSCs. In humans, neurogenesis begins as early as the first trimester, and appears to be largely complete prior to the age of 10.[49] Studies in rodents indicate that neurogenesis is initiated just prior to the third week of gestation, and is mostly completed by the third week of postnatal life.[50] Although an indefinable number of developmental events occur during the broad period of neurogenesis, attempts have been made to elucidate the specific stage and/or age of cells that are targeted by propofol. In addition to confirming previous findings of propofol-induced neuroapoptosis, Huang et al. showed that a sub-anesthetic dose of propofol exposure in 7- to 9-day-old mice suppressed the proliferation of new neurons.[51] Also, the authors report that morphological maturation of the neurons was delayed. These data suggest that propofol alters multiple cellular processes. A similar study also used thymidine analogs to label proliferating cells in 4-, 8-, and 21-day-old mice, and looked for colocalization between thymidine analog-labeled cells and cells expressing maturation and differentiation markers following propofol exposure. In contrast to the study by Huang et al.,[51] there was no effect of propofol on cell proliferation, though proliferation was assessed 24 h following propofol exposure. Propofol did, however, decrease neuronal differentiation in the 4- and 8-day-old mice.[52] Based on this study and others mentioned previously, it seems that propofol induces changes in neurogenesis and neuronal differentiation.

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Disrupted Dendrite Development

Dendrites are extremely important for cell communication, survival, synaptic plasticity, and impact strength of connection of the neural circuitry. Like other components of neuron development, disruption of dendrite development is believed to be persistent and to impact neural signaling.[53] Several morphological studies have noted altered dendrite growth following propofol exposure. In the earliest stages of dendrite formation, propofol at an anesthetic dose did not result in GABAergic neuron death, but did profoundly decrease the outgrowth of dendritic arbor. Following the supra-anesthetic dose, the reduction in dendrite arbor formation was accompanied by a significant increase in neuroapoptosis. Both anesthetic and supra-anesthetic doses of propofol also decreased dendritic length, but did not decrease the total number of dendrites.[54]

As mentioned previously, dendrite morphology can also affect synapses between cells. Accordingly, Briner et al. simultaneously assessed dendrite and synapse characteristics in the medial prefrontal cortex of the rats. A sub-anesthetic dose of propofol was administered for 6 h. The authors demonstrated that propofol exposure, beginning at 5-, 10-, and 15-day-old, did not affect dendritic arbor development, when measured 5 days post-anesthetic treatment. A decrease in dendritic spine density was observed at only 5- and 10-day-old.[53] In contrast, propofol exposure at 15- and 20-day-old caused increases in dendritic spine density, and exposure at 30, 60, and 90 days had no effect on density. The increase in dendritic spine density following propofol at 15-day-old was accompanied by an overall increase in synapse number. Furthermore, this study suggests that a single administration of propofol is just as sufficient as a 6 h prolonged exposure in inducing these effects listed above.[53] Analysis of the effect of propofol at multiple time points is evidence of the complex interaction between developmental events and anesthetic exposure. However, the effect of these changes of spine density on cognitive function and the mechanism(s) by which propofol differently induces dendrite growth in rats at different ages remains to be determined.

Calcium Overload

Calcium (Ca^{2+}) is a highly important cell signaling molecule, and regulation of its intracellular concentration is crucial to appropriate cell function and survival. Mechanism of propofol anesthetic action involves potentiation of GABA type A ($GABA_A$) receptor function.[43] In the adult rat brain, once the propofol molecule binds to the $GABA_A$ receptor, there is an increased chloride ion influx, causing inhibition by hyperpolarization and achievement of the sedative effect of propofol. In contrast, immature animals show a reversed chloride gradient across the membrane, resulting in the depolarization-induced Ca^{2+} influx and inappropriate cell excitation. In these young mice, Ca^{2+} influx during the critical period of development, in which GABA neurotransmission is excitatory, ranges from the third trimester to 7 days *in vitro*.[55] In humans, this window also starts in the third trimester, but is believed to last until roughly 6-months-of-age.[56]

Since propofol is a known $GABA_A$ agonist, what has been described above is also believed to be applicable for the propofol's effect on Ca^{2+} influx and subsequent apoptosis in the developing CNS. *In vitro* cultured hippocampal neurons exposed to an anesthetic dose of

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propofol for 5 h showed significantly elevated intracellular Ca^{2+} concentration in 4-day-old neurons, but not at PD8. Neuroapoptosis appeared to follow the trend for Ca^{2+} levels, as significant cell death was only reported at 4 days. Interestingly, apoptosis was only exhibited acutely following propofol exposure, as cell death appeared to subside by 48 hours.[57] These data seem to support the existence of a critical developmental window for propofol-induced neurotoxicity similar to findings in other studies. Since Ca^{2+} signaling is the basis of most physiological functions, it seems that future studies should explore dysregulation of signaling cascades that may lead to apoptosis or altered cellular communication within the CNS.

Inflammation

The few studies that exist on neuroinflammation following propofol exposure have yielded mixed results. Many findings have suggested that neuroinflammation is attributed to cognitive decline following surgical procedures, either due to stress or administered agents. For example, exposure to a sub-anesthetic dose of propofol in 8-day-old rats for 6 h revealed that tumor necrosis factor-α (TNF-α) mRNA expression was elevated in the prefrontal cortex but not the hippocampus.[58] However, 20-day-old rats did not show increased expression of these pro-inflammatory cytokines. Additionally, propofol did not affect interleukin-6 (IL-6) or IL-1β expression at either time point tested. mRNA expression of various chemokines was also measured, and ccl23 and ccl13 mRNA were significantly increased relative to controls only at day 10 in both the hippocampus and prefrontal cortex. In all pro-inflammatory molecules that were elevated immediately post-propofol exposure, expression was returned to baseline levels by 24 h.[58] This study highlights the spatial specificity of various cytokines and chemokines induced by propofol, and also the apparent short-term neural effects of propofol. Another study did not find any changes in TNF-α, IL-1, or IL-6 in the plasma to accompany the significant apoptosis in the cortex and hippocampus of 7-day-old mice exposed to propofol for 6 h.[18] The findings from this study may suggest that there is not a strong, if any, relationship between inflammation and neurotoxicity conferred by propofol. Thus, functional roles of inflammation in propofol-induced developmental neurotoxicity await further investigation.

Mitochondrial Fission

Mitochondria are extremely important organelles involved in many cellular processes including energy production, cell signaling, and apoptosis.[59] To maintain proper function, the mitochondria continuously undergo cycles of fusion and fission. Unbalanced fusion/fission may lead to various pathological conditions including neurodegeneration.[60–64] Dynamin-related protein 1 (DRP1) is a key regulator of mitochondrial fission. Phosphorylation of DRP1 by cyclin-dependent kinase 1 (CDK1) at the Serine616 position induces mitochondrial fission.[65–67] DRP1 is primarily distributed in the cytoplasm of a healthy cell, but shuttles between the cytoplasm and the mitochondrial surface.

Twaroski et al. showed that 6 h of propofol exposure at higher concentrations than used clinically, increased hESC-derived neuron death, decreased mitochondrial membrane potential, and led to a detrimental increase in mitochondrial fission. Most control neurons showed elongated, interconnected, and tubular mitochondria, while much shorter and

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smaller mitochondria were prevalent in the propofol-treated culture. In addition, propofol increased the expression of activated DRP1 and CDK1.[68] Inhibition of mitochondrial fission attenuated the propofol-induced neuron death. Nevertheless, the authors have also shown that propofol induced cell death at lower doses after multiple exposures. The increased mitochondrial fission was also observed in ketamine-induced stem cell-derived human neurons.[14] In addition, it has been shown that exposure of neonatal rat pups to general anesthetics (a single injection of midazolam followed by 6 h of nitrous oxide and isoflurane) induced significant increases in mitochondrial DRP1 levels and mitochondrial fission.[69] These findings suggest that the increased mitochondrial fission might be a common signaling pathway of general anesthetic-induced developmental neurotoxicity.

microRNAs

microRNAs are endogenous, small, non-coding RNAs that have been implicated to play important roles in many different disease processes, such as neurodegeneration, by negatively regulating target gene expression.[70] Hundreds of microRNAs have been characterized, many of which are enriched in the brain.[71–73] Alterations of microRNA networks have been shown to cause neurodegenerative disease.[74] As such, miRNAs have become a topic of interest as possible therapeutics and/or therapeutic targets. Recently, several studies demonstrated the important roles of microRNAs in ketamine and propofol-induced developmental neurotoxicity.[75–77] Specifically, the following findings from cell culture models showed that microRNA-21 (miR-21) and microRNA-665 (miR-665) were involved in propofol-induced neuron and astrocyte death, respectively.[37, 77]

miR-21 is a well-established anti-apoptotic factor.[78, 79] Utilizing miR-21 gain- and loss-of-function approaches and the hESC-derived neuron model, Twaroski et al. examined the role of miR-21 in propofol-induced neurotoxicity. miR-21 was artificially upregulated or knocked down in the stem cell-derived neurons using a miR-21 mimic and antagomir, respectively. The results showed that miR-21 overexpression attenuated propofol-induced cell death, while miR-21 knockdown exacerbated these effects.[37] Propofol-induced toxicity in human stem cell-derived developing neurons is likely mediated via miR-21/Sprouty 2/Akt-dependent mechanism.[16] Activated Akt has been reported to promote cell survival in a variety of cell types and prevent apoptosis.[80] Astrocytes represent a major glial cell population in the CNS. They play important roles in brain development, and also maintain neuronal function. miR-665 expression was significantly decreased in the cultured immature rat hippocampal astrocytes. Upregulation of miR-665 suppressed BCL2L1 (BCL2-Like 1, a proapoptotic Bcl-2 protein) and elevated activated caspase-3 expression in the astrocytes, suggesting that miR-665 might be involved in propofol-induced astrocyte death via a caspase-3-mediated mechanism, by negative regulation of BCL2L1.[77]

Dysregulated Neurotrophin Expression

Alterations in the levels of a variety of neurotrophins have been implicated to play a role in anesthetic-induced neurotoxicity in the developing rodent brain. The neurotrophins are a family of secreted proteins that plays important roles in brain development, such as growth, survival, differentiation, and synaptic plasticity of postmitotic neurons. They are comprised of nerve growth factor 9 (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3

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(NT-3), and neurotrophin 4/5 (NT4/5).[81] BDNF is stored as proneurotrophin (proBDNF) within synaptic vesicles and is proteolytically cleaved to mature BDNF (mBDNF) in the synaptic cleft by plasmin, a protease activated by tissue plasminogen activator. mBDNF triggers prosurvival signaling, while proBDNF binds to the p75 neurotrophin receptor ($p75^{NTR}$) and activates RhoA, which regulates actin cytoskeleton polymerization prior to cell apoptosis. Pearn et al. extracted neurons from 1- to 2-day-old normal and $p75^{NTR}$ knockout mice, and exposed the neurons for 6 h to an anesthetic dose of propofol at 5 to 7 days *in vitro*. Propofol induced neuroapoptosis through a proBDNF/$p75^{NTR}$ pathway. Knockout of $p75^{NTR}$ or administration of TAT-Pep5 (A specific $p75^{NTR}$ inhibitor) attenuated propofol-induced neuroapoptosis.[34]

Neurotrophins bind the tyrosine kinase (Trk) receptors such as TrkA, TrkB, and/or TrkC. The binding of neurotrophins to Trk receptors leads to the activation of signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[82–84] It has been shown that a sub-anesthetic dose (25 mg/kg) of propofol exposure to PD7 rats induced 1) downregulation of NGF expression in the cortex and thalamus at defined time points between 1 and 24 h after propofol treatment, and 2) a decrease of activated Akt.[85] Consistent with the study utilizing a single administration of propofol, multiple injections in the mice also affected neurotrophin (e.g., BDNF and NT-3) mRNA levels and altered the Akt signaling pathway.[86] Surprisingly, these authors found that propofol treatment caused transient enhancement of motor function 1 day post-administration, but found no difference in memory between groups.[86] Collectively, these data suggest that the propofol-mediated neurotoxic effect is mediated, at least in part, by neurotrophic downregulation.

Collectively, propofol-induced cognitive dysfunction might be related to complicated cellular and molecular mechanisms as described above such as apoptosis, interference with brain developmental events (e.g., cell proliferation, neurogenesis, and synaptogenesis), and neuron structural changes. Contribution of propofol-induced neuronal changes to long-term cognitive impairment is a critical topic for future research. In addition, the time sequences in which molecular mechanisms that might be responsible, such as calcium signaling, ROS production, mitochondrial fission, neuroinflammation, microRNAs, and neurotrophin expression deregulation, are not clear. So far, functional roles of downregulated miR-21 and DRP1-mediated mitochondrial fission have only been reported in propofol-mediated neuron death. Whether miR-21 and mitochondrial fission are associated with other general anesthetic-induced developmental neurotoxicity remains to be determined.

Most general anesthetics function through NMDAR and/or GABAAR. Several anesthetics (e.g., isoflurane, ketamine, and propofol) have been shown to cause similar cellular and molecular mechanisms (e.g., synaptic changes, inflammation, free oxygen radicals, and/or calcium overload) in developmental neurotoxicity as observed during propofol treatment. However, so far there is no convincing experimental evidence showing which molecular perturbation leads to a detrimental anesthetic effect. The cause-effect relationship among various neurotoxic mechanisms is largely unknown. Thus, a future study will focus on the dissection of drug-specific mechanisms and the order of the abnormal events that take place during anesthetic exposure.

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PROTECTIVE STRATEGIES

If propofol does indeed lead to cellular damage and cognitive dysfunction in the developing human brain, it is critical to identify therapeutic interventions to combat these effects, as anesthetic use is necessary based on the current state of medicine. Based on the studies presented above, some protective approaches have been used to counteract propofol toxicity in either *in vitro* cell culture or intact animal models by introducing exogenous agents or supplemented molecules and/or factors that have been shown to be downregulated by propofol (Table 3). Several major protective strategies that have been reported are summarized as follows: 1) Scavenging ROS production: Acetyl-L-carnitine is a supplemental antioxidant. Following propofol exposure at a sub-anesthetic dose, acetyl-L-carnitine attenuated oxidative damage in NSCs,[48] suggesting a feasible clinical intervention. Future studies are also necessary to elucidate the protective effect of acetyl-L-carnitine and other ROS scavengers (e.g., Trolox and EUK-134) against the propofol-induced various detrimental effects (e.g., neuroapoptosis); 2) Lithium: It was found that lithium supplementation prevented propofol-induced cell death.[87] However, many hurdles must be overcome in order to determine if lithium is a safe treatment option; 3) Erythropoietin: Erythropoietin has been shown to reduce neurodegeneration in neonatal rats in a dose-dependent manner;[88] 4) Manipulation of the expression of target microRNA expression: As described previously, propofol decreased miR-21 expression in hESC-derived neurons. When miR-21 was overexpressed, neuronal death was attenuated and returned to control levels.[37] Since miR-21 is endogenous, this neuroprotective method represents a way to directly combat the actions of propofol by restoring a target of propofol to normal levels; and 5) Inhibition of mitochondrial fission: Propofol increased mitochondrial fission in stem cell-derived human neurons, and mdivi-1 (a mitochondrial fission inhibitor) attenuated this toxicity.[68] Some drug-mediated neuroprotective effects as described above (e.g., mitochondrial fission blocker mdivi-1 and ROS scavenger Acetyl-L-carnitine) were only reported to be associated with changes in cultured neuronal lineages, including NSCs and stem cell-derived neurons. Whether these neuroprotective agents observed in the *in vitro* culture model can prevent propofol-induced long-term cognitive dysfunction in animal models remains to be determined.

The neuroprotective action of miR-21 and mdivi-1 were only observed in propofol-treated cultured neurons.[37,68] There have also been studies on neuroprotection following exposure to anesthetic agents other than propofol. ROS scavengers, lithium, and erythropoietin have been associated with neuroprotective effects against other general anesthetics. For instance, ROS scavengers elicited neuroprotection following ketamine or an anesthetic cocktail (midazolam, isoflurane, and nitrous oxide).[14,89] Lithium could counteract both ketamine- and propofol-mediated neuroapoptosis in PD5 mice.[87] Lithium also inhibited sevoflurane-induced cognitive impairment in mice.[90] Erythropoietin was found to protect against ketamine-induced apoptosis in cultured rat cortical neurons through the Akt cell survival pathway.[91] A single administration of erythropoietin immediately after postnatal exposure to sevoflurane reduced both early activation of neuroapoptosis and late onset of neurologic disorders in rats.[92] Although these neuroprotective strategies extend across multiple anesthetics of varying exposures and different experimental models, similarities in the

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players and pathways involved in anesthetic-induced neurotoxicity may be beneficial in approaching treatment, should a similar effect be observed in children. Additionally, these studies may foster a better understanding of the mechanism(s) of brain injury following anesthetic exposure.

CONCLUSION AND FUTURE PERSPECTIVE

In summary, this review focused on the collection of studies related to propofol exposure in developing animals and cultured cells. The fact that propofol exposure induces significant neuroapoptosis, and influences NSC proliferation, neurogenesis, dendrite development, and cognitive function in developing animal and human stem cell models is extremely compelling. In addition, the studies summarized above have demonstrated candidate pathways involved in cell survival, morphology, brain development, and functional outcomes, and will be highly useful in the future of this field. Specifically, the presented evidence will promote discovery and development of more rational protective strategies and identification of new members of pathways involved in the detrimental changes in the developing brain elicited by propofol.

Future studies should attempt to consider the stages and events of neural development, how propofol differentially acts at each of these stages and implicates functional significance at the level of whole-animal physiology. It also seems appropriate to apply the evidence from studies on anesthetic exposure in general to the experiments specifically related to propofol-induced neurotoxicity, as there is much overlap in experimental findings. Moving forward, what is perhaps most crucial is that scientists relate the extensive findings from animal models back to the human population. Without a doubt there are many obstacles and limitations in approaching this task, evidenced by the lack of human data on neurotoxicity as a direct result of anesthetics. In addition to the animal models, breakthroughs utilizing human stem cells may aid in answering questions with regards to children, but do not directly confirm the human relevance. New advancements in imaging and CNS biomarkers should also be employed in future investigations.

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Anesth Analg. Author manuscript; available in PMC 2017 November 01.

Table 1

Experimental evidence of propofol-induced developmental neurotoxicity in animal models

Propofol Dose	Time of Neurotoxicity Analyzed	Animals and Age	Neurotoxicity Region	Major Findings	Reference
Single exposure					
150 mg/kg injection	6 h and 32 days following exposure	C57BL6 mice (PD7)	Cortex and hippocampal CA1	Increased caspase 3; no changes on TNFα, IL-1β, IL-6	Yang et al.[18] 2014
100 mg/kg injection	6 h following exposure	BALBc mice (PD5)	Hippocampal dentate gyrus, CA3, CA1	Increased caspase 3; $p75^{NTR}$ activation	Pearn et al.[34] 2012
25 mg/kg injection	8 and 16 h following exposure	Wistar rats (PD14)	Cortex and thalamus	Increased fluoro-Jade stained neurons; altered p-Akt, p-ErK, BDNF, NGF	Popic et al. [19]2012
0.4 mg/kg/min for 2 h	6 h following exposure or PD10 and 28	Sprague Dawley rats (Gestational day 18)	Hippocampal CA1 and CA3	Increased caspase 3; impaired learning and memory on PD28	Xiong et al.[35] 2014
3 mg/kg followed by 350–450 mcg/kg/min for 5 h	3 h following exposure	Rhesus macaque (Gestational age120 days and PD6)	Subcortical and caudal regions	Increased caspase 3 in neurons and oligodendrocytes; no caspase-3 positive astrocytes	Creeley et al.[20] 2013
100 mg/kg followed by 5 subsequent injections at 50 mg/kg at a rate of one injection per hour	6 h following exposure	C57BL6 mice (PD56 to PD 70)	Adult-born 11 days or 17 days old hippocampal neurons	Impaired the survival and maturation of adult-born hippocampal neurons in a developmental stage-dependent manner in mice	Krzisch et al.[10] 2013
Multiple exposures					
40 mg/kg then 20 mg/kg/h for 4 h (5 injections for total 6 h)	0 and 18 h following exposure	Wistar rats (PD10)	Hippocampus and cortex	Increased TNFα, CCL2/3 mRNA and decreased IL-6 mRNA	Kargaran et al.[58] 2014
30 mg/kg every 90 min (3 injections)	6, 12, 24 h following exposure	Wistar rats (PD6)	Cortex and thalamus	Increased caspase 3; decreased BDNF and NT-3	Karen et al.[86] 2013
40 mg/kg then 20 mg/kg/h for 4 h (5 injections for total 6 h)	24 h following exposure	Wistar rats (PD5-10)	Prefrontal cortex	Decreased dendritic spine density	Briner et al.[53] 2011

Legend: PD: postnatal day; BDNF: brain-derived neurotrophin factor; NGF: nerve growth factor; TNFα: tumor necrosis factor α; CCL2/3: chemokine (C-C Motif) ligand 2/3; IL-1β: interleukin-1β; IL-6: Interleukin-6; CA1 and CA3: region I and III of hippocampus proper; $p75^{NTR}$ neurotrophin receptor p75; Erk: extracellular signal-regulated kinases

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Table 2

Preclinical evidence of propofol-induced neurotoxicity in cultured cells

Propofol Dose	Exposure Duration	Time of Neurotoxicity analyzed	Cell Sources	Cell Age	Major Findings	Reference
10 μg/mL (56.10 μM)	6 h	18 h following exposure	Human stem cell-derived neurons	2 weeks after differentiation	Increased TUNEL-positive neuron death; decreased miR-21 and activated Akt; increased Spouty 2	Twaroski et al.[37] 2014
8.91 μg/mL (50 μM)	24 h	End of treatment	Neural stem cells from gestational day 14 rat fetuses	DIV8	Increased cell death	Liu et al.[48] 2014
0.02–17.83 μg/mL (0.1–1000 μM)	3 h	End of treatment	Hippocampus from Sprague Dawley embryos	DIV7	Increased caspase 3-positive apoptotic neurons; decreased Bcl-2 and NFκB p65	Zhong et al.[93] 2014
0.53 μg/mL (3 μM)	6 h	2 h following exposure	Whole brain from BALBc mice	DIV5-7	Increased caspase 3 and Drebin; p75 neurotrophin receptor activation	Pearn et al.[34] 2012
1.27 and 12.73 μg/mL (7.1 and 71.4 μM)	6 and 12 h	End of treatment	Hippocampal precursor cells from Sprague Dawley neonatal rats	DIV13-14	Increased LDH release	Sall et al.[36] 2012
0.89 and 8.91 μg/mL (5 and 50 μM)	5 h	1 h following exposure	Hippocampi from Sprague Dawley neonatal rats	DIV4	Increased caspase 3,7-positive neurons; increased intracellular free Ca^{2+}	Kahraman et al.[94] 2008
50 μg/mL (280 μM)	4 h	End of treatment	Subventricular zone from Sprague Dawley neonatal rats	DIV1-2	Decreased dendritic growth	Vutskits et al.[26] 2005

Legend: DIV: days in vitro; TUNEL:Terminal deoxynucleotidyl transferase dUTP nick end labeling; LDH: lactate dehydrogenase; Bcl-2: B-cell lymphoma 2; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cell.

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Table 3

Protective strategies of propofol-induced neurotoxicity

Neuroprotective Agents	Propofol Dose	Animal or Cell Sources	Protective Outcomes	Reference
Lithium	1 × 50 mg/kg IP for 2 h	C57/Bl6 mice (PD5)	Decreased caspase-3 positive neurons in cortex and caudate/putamen	Straiko et al.[87] 2009
Acetyl-l-carnitine	50 μM for 24 h	Cultured neural stem cells isolated from gestational day 14 rat fetuses and on the eighth day in culture	Increased neuron stem cell proliferation; reduced oxidative stress; decreased neuron death	Liu et al.[48] 2014
Pre-miR-21	20 μg/mL for 6 h	14-day-old human stem cell-derived neurons	Decreased TUNEL-positive neurons; downregulated Sprouty 2	Twaroski et al.[37] 2014
Dexmedetomidine	8.0 mg/kg IV plus 1.2 ± 0.2 mg/kg/min for 1 h	Sprague-Dawley rats (embryonic day 20)	Decreased caspase-3 activation; improved neurocognitive deficit	Li et al.[95]2015
Erythropoietin	3 × 30 mg/kg IP at 0, 90 and 180 min	Rats (PD6)	Low-dose, but not high-dose rEPO decreased neuronal degeneration scores	Zacharias et al.[88] 2010
Mdivi-1	20 μg/mL for 6 h	14-day-old cultured human stem cell-derived neurons	Decreased TUNEL-positive neurons and mitochondrial fission	Twaroski et al.[68] 2015

Legend: rEPO: recombinant Erythropoietin; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; PD: postnatal day; IP: intraperitoneal injection; IV: intravenous injection.