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Preclinical Research Into the Effects of Anesthetics on the Developing Brain: Promises and Pitfalls

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Abstract

Every year millions of children are treated with anesthetics and sedatives to alleviate pain and distress during invasive procedures. Accumulating evidence suggests the possibility for deleterious effects on the developing brain. This has led to significant concerns among pediatric anesthesiologists and to the formation of the Pediatric Anesthesia NeuroDevelopmental Assessment (PANDA) group and its biannual symposium. Not surprisingly, the majority of the data in this field have thus far been derived through laboratory research. Accordingly, this review summarizes the current state of animal research in this field, introduces some of the findings presented at the PANDA symposium, and addresses some of the difficulties in translating these findings to pediatric anesthesia practice, as discussed during the symposium. The symposium participants' consensus was that significant preclinical and clinical research efforts are still needed to investigate this important concern for child health.

Keywords

anesthesia; sedation; animal research; in vitro; in vivo; brain; neonate; infant; pediatric; neuroapoptosis; neurotoxicity

Developmental disabilities affect a substantial number of children, with estimates for prevalence ranging between 13% and 17%.^{1,2} Chronic intrauterine exposure to environmental chemicals has been recognized as a significant hazard for neurotoxicity.² More recently, early postnatal exposure to general anesthetics and sedatives has been found to be deleterious to the developing animal brain. Some of the detrimental effects observed after prolonged anesthetic exposure in immature animals include enhanced neuronal apoptosis, alterations in dendritic architecture, impairment in neurogenesis and gliogenesis,

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decreases in neurotrophic factors, destabilization of the cytoskeleton, and impairment of neurological function (reviewed in detail elsewhere).^{3,4} Since millions of children are exposed to general anesthetics and sedatives every year,⁵ serious concerns have been raised regarding the safety of this practice. For obvious ethical reasons, this phenomenon cannot be easily studied in humans. Exposing infants to anesthesia without indication or randomizing children to anesthesia or no anesthesia during painful interventions is impossible. Accordingly, animal research remains crucial to improving our understanding of the effects of anesthetics and sedatives on the developing brain to elucidate the mechanism of anesthetic neurotoxicity, to discover potentially noninjurious compounds or mitigating strategies, and to finally make recommendations for clinical practice. This report will therefore present some of the questions raised by pediatric practitioners regarding animal research into anesthetic neurotoxicity during the 2012 Pediatric Anesthesia NeuroDevelopmental Assessment (PANDA) Symposium sponsored by the Columbia University in New York. Moreover, we will summarize recent findings from animal research presented at the meeting and discuss the advantages and disadvantages of the animal model systems used in the investigation of anesthesia-induced, developmental neurotoxicity.

DISCUSSION ABOUT THE ROLE OF ANIMAL RESEARCH IN ANESTHESIA NEUROTOXICITY

Pediatric practitioners' interest in the effects of anesthetics in the developing brain has been progressively growing since the 1990s. Groundbreaking rodent studies demonstrating increased neuronal cell death after ketamine exposure in very young animals raised concerns about pediatric sedation and anesthesia practices.⁶ Subsequently, extensive research efforts have been carried out to elucidate the effects of anesthetics in the developing nonhuman brain and to assess neurological outcomes after exposure to anesthetics and sedatives in very young children. The 2012 PANDA symposium provided the unique opportunity for a direct dialogue between basic scientists, clinical researchers, and pediatric practitioners. These discussions led to several points raised by the symposium participants on the important role of animal research in the study of this clinically important phenomenon.

Several questions raised during the symposium will require further animal studies to build upon the present knowledge that in vivo and in vitro work have already provided. For instance, the mechanism of neurotoxicity secondary to anesthetic exposure has been a major area of focus. The emphasis of a substantial number of initial studies has been on apoptotic cell death as the main characteristic of anesthetic toxicity. In addition to cell death, recent in vivo and in vitro studies involving dissociated cell cultures and brain slice cultures have pointed to other structural and functional abnormalities, which will be discussed below. It is currently unclear whether all the toxic effects observed in the developing animal brain are part of 1 spectrum of injury that eventually results in neuronal apoptosis or whether they are entirely distinct phenomena. Clarifying these uncertainties will certainly improve our understanding of the mechanism of pediatric anesthetic neurotoxicity. Along those lines, participants in the symposium expressed substantial optimism that future development of

appropriate animal models will also aid in elucidating candidate pathways for therapeutic interventions in clinical trials.

It was also noted during discussions at the symposium that preclinical studies offer the best opportunity for rigorous pharmacological investigations of the effects of anesthetic dose, duration, and repetitive exposures on the developing brain, which are required before accepting a causative relationship between exposure and neurological impairment. It is still unclear whether brief exposures are noninjurious and whether a threshold dose, duration, or frequency exists beyond which neurotoxicity is inevitable. Despite substantial progress in the field, the consensus was that additional small rodent studies were needed to answer this question in principle; however, concerns were raised, which will be addressed later in this review, that more sophisticated models, such as primates, may be required to translate the small rodent findings to humans.

A closely related, but equally unanswered, question raised by the PANDA panel was the comparative toxic potency of anesthetics and sedatives and whether certain agents, such as opiates or α_2 -adrenergic receptor agonists, may be substantially less harmful to the developing brain. Moreover, further investigation was encouraged of adjunct treatments to reduce the effects of potentially toxic anesthetics that are required to provide adequate depth of anesthesia during surgical procedures, for which animal models are ideally positioned. There currently exists very little work on the effects of adjuncts, such as low doses of opioids, sedatives, or adjuncts on the development of neurological effects of general anesthetics.

Another key issue raised by discussants at the symposium was whether anesthetic exposure affected all brain cells in all brain regions equally or whether some were more vulnerable than others. Answering this question will be critical in predicting a behavioral phenotype and potential cognitive deficits, thereby helping to translate findings from animal studies to humans. Furthermore, because maturation of different brain regions occurs along dissimilar timelines, novel discoveries in this field of research may help better predict distinct windows of vulnerability for different brain regions. However, although animal studies cannot definitively answer these questions for clinical practice, they may nonetheless suggest neuropsychological domains to be tested in humans to detect more subtle putative effects of anesthetic toxicity.

Finally, there was some discussion about whether animal models can help determine whether toxic effects of anesthetics might be mitigated or reversed after the exposure. If substantiated, postexposure interventions may become a priority if exposure to deleterious agents has already occurred or cannot be entirely avoided due to the lack of alternatives.

IN VIVO ANIMAL MODELS

Harmful effects from prolonged exposure to anesthetics or sedatives have been observed during in vivo experiments in a wide variety of animal species, ranging from nematodes, chickens, mice, rats, guinea pigs to piglets and nonhuman primates (reviewed in detail by Loepke and Davidson⁷). The most widely studied deleterious phenomenon after exposure in immature animals is apoptotic or programmed cell death. Apoptosis is a physiological,

energy-consuming process that eliminates approximately 50% to 70% of neurons during normal brain development.^{8,9} Prolonged exposure to anesthetics or sedatives, such as chloral hydrate, clonazepam, diazepam, midazolam, nitrous oxide, desflurane, enflurane, halothane, isoflurane, sevoflurane, ketamine, pento-barbital, phenobarbital, propofol, and xenon can briefly, but dramatically, increase the number of neurons undergoing apoptotic cell death, compared with unexposed animals. Although this phenomenon has been confirmed in vitro as well, eliminating some physiological confounders, it currently remains unknown whether anesthesia-induced neuroapoptosis only accelerates physiological programmed cell death in cells determined to die at a later time point or whether it eliminates cells not destined to die, as in pathologic apoptosis. Conversely, repetitive pain in the neonatal period has also been shown to alter behavior and cognitive function in adulthood, to decrease pain thresholds, and to increase vulnerability to stress and anxiety disorders or to chronic pain syndromes later in life.^{10,11}

Importantly, animal studies have identified a narrow window of susceptibility for neuronal cell death induced by compounds acting as NMDA antagonists and/or GABA agonists, such as ketamine, isoflurane, or ethanol. Current thinking is that apoptosis is only triggered in newborn rodents before 14 days of age or in monkeys younger than 35 days of age,^{6,12-14} suggesting an innate vulnerability of the developing brain. However, preliminary data presented at the symposium by A.W.L. suggest that certain brain regions may be affected by the phenomenon even beyond the first 2 weeks of life. The effects of early life exposure to anesthetics on the other predominant brain cell types, astrocytes and oligodendrocytes, currently remain unclear. At the symposium, John W. Olney, MD presented recently published data obtained in 5-day-old rhesus monkeys demonstrating that immature oligodendrocytes undergo apoptotic cell death after isoflurane exposure early in life.¹⁵ These cells become vulnerable to isoflurane-induced apoptosis at a maturational stage when they are just beginning to myelinate axons. The magnitude of the oligoapoptosis effect was approximately equal to the magnitude of the neuroapoptotic effect in these neonatal monkey brains.¹⁵ In another symposium presentation by Philip G. Morgan, MD, apoptotic cell death and age during exposure was examined in the nematode *Caenorhabditis elegans*. The mechanism of this cell death process is not entirely clear but may be related to decreases in brain-derived neurotrophic factor.^{16,17} Moreover, anesthetic exposure can lead to actin depolymerization in neurons and astrocytes, cytoskeletal destabilization, and impairment of astrocyte morphologic differentiation and maturation, which may precipitate in brain cell death.^{18,19} These effects were part of the symposium presentation by Piyush Patel, MD, FRCPC.

Anesthetics have also been found to affect dendritic and synaptic brain architecture. Anesthetics have been found to either increase or to a decrease dendritic arborization and synaptic density, depending on the age of exposure, and therefore the developmental state of the brain regions being examined.^{20,21}

Studies presented by Greg Stratmann, MD, PhD and Mark Baxter, PhD suggest that aside from these structural abnormalities, subsequent neurological function can also be affected after neonatal anesthetic exposure. Several studies have observed neurological dysfunction, especially in hippocampal-based memory tasks, in adult animals exposed as neonates,

whereas other functional domains remained unimpaired.²²⁻²⁴ Maternal behavior and grooming represents a critical factor in subsequent performance of rodent pups in learning and memory tasks,²⁵ which may affect neurological outcome in studies of anesthetic exposure.²⁶ The relationship of the various structural abnormalities and functional impairment is currently not entirely clear, as neonatal neuronal cell death may not be inevitably linked to subsequent neurocognitive impairment.^{23,26}

The obvious advantage of using live animals in anesthetic neurotoxicity research is that experiments are performed in intact organisms with preserved biological systems, testing the effects of interventions on all biological functions. However, even though some of the primate studies use sophisticated medical techniques, including endotracheal intubation and mechanical ventilation, considerable differences remain between all in vivo animal studies and human pediatric anesthesia. To evaluate whether findings from animal models can be generalized to clinical practice, it is critical to examine how well animal studies represent the human clinical scenario and to acknowledge their limitations. To date, no animal model has been developed that covers all aspects of human physiology and pathophysiology during surgical procedures. The task of modeling potential vulnerabilities of the developing human brain in animal models is complicated by the fact that human central nervous system development is much more protracted than that of any of the model species, including nonhuman primates, and human neurocognitive performance is also much more complex compared with lower animals. To demonstrate toxic effects, animal studies have been criticized for using exposure times that are longer than the average clinical procedure and doses for injectable anesthetics that are often 10 to 100 times higher than routine pediatric anesthetic doses. However, pharmacokinetic and pharmacodynamics data in model species are lacking, and animals have demonstrated increased anesthetic requirements on a weight-based comparison with humans. To account for some of the known species differences, allometric scaling estimates animal drug doses on a body surface area basis to be approximately 3-, 6-, or 12-fold higher for monkeys, rats, or mice, respectively, compared with drug doses used in human medicine.^{27,28} Although scaled drug doses are still lower than those used in anesthetic neurotoxicity studies, they are more analogous to clinical practice than weight-based doses.

Concentrations of inhaled anesthetics used during animal research into anesthetic neurotoxicity are much closer to human clinical concentrations and have demonstrated an age-related variation in potency, similar to that observed during clinical anesthesia. However, the anesthetics' dramatic effects on acid-base homeostasis in some animal studies complicate dose-finding studies.²⁹⁻³¹ Hypercarbia, metabolic acidosis, hypoglycemia, and even significant mortality have been observed during anesthetic exposure in small rodents.^{23,26,32} Endotracheal intubation and mechanical ventilation do not seem to completely obviate these abnormalities,³² questioning the direct human applicability of prolonged anesthetic exposures in small rodents.

Environmental enrichment can dramatically increase neurogenesis in rodents and has shown to dramatically obviate some the neurological impairment observed after neonatal anesthetic exposure.³³⁻³⁵ Children face daily cognitive challenges in their "enriched" environment,

which may affect their postexposure neurogenesis compared with animals raised in bare laboratory housing.

Another dissimilarity between in vivo animal studies and clinical anesthesia practice involves the significant comorbidities, stress, and painful stimulation linked to the administration of anesthetics and analgesics in clinical pediatric medicine but not in the majority of neurotoxicity animal studies. Some animal research incorporating concomitant painful stimulation during administration of anesthetic and analgesic drugs have found anesthetic neuroprotection of the deleterious effects of unopposed pain^{10,36} and diminution of the anesthetics' neurotoxic effects,^{10,37} whereas others have not.³⁵

Brain architecture and development are vastly different between human and all model species. Overall brain size and neuronal numbers are substantially greater in humans than in most animals. Primate brains have gyri and sulci, whereas rodent brains have a lissencephalic, smooth surface. Brain development occurs over a much longer period of time in humans than in any of the animal species and whereas considerable brain development takes place postnatally in rodents, most critical steps in humans occur in utero.³⁸ At birth, the immature brain's size is one third of the adult brain, doubles in size within the first year of life, and reaches 90% of its eventual size by 6 years of age.³⁹ This dramatic growth spurt coincides with a remarkable overabundance of neurons and neuronal connections. Less than half of the neurons generated during development survive into adulthood.^{8,9} Superfluous neurons that lose in the competition for a limited amount of trophic factors are removed by programmed cell death. In addition to an overabundance in the total number of neurons generated during early development, the mammalian brain also forms an excess of neuronal connections, or synapses, during that period. Dependent on brain region, synaptic density reaches its maximum in infants and young toddlers between 3 and 15 months of age and will be progressively reduced by about half into adulthood.⁴⁰ Accordingly, the first several years after birth represent a critical period of development for many brain regions, and recent findings in animals suggest that exposure to anesthetics or sedatives may interfere with proper neuronal development, brain architecture, and subsequent function. Because the anesthetic effects in animals seem to be highly targeted to a particular age, it is critical to identify the corresponding maturational stage of the human brain. Contemporary comparison studies using computational models have approximated the 7-day-old rodent, a commonly used model for neuroapoptosis, to be comparable in brain maturity to a 20- to 22-week-gestation human fetus.⁴¹⁻⁴³ Similarly, brain development in the 5-day-old rhesus monkey, according to these models, approximates a human fetus during the 31st week of gestation. It therefore remains unknown whether these models have direct applicability to clinical pediatric anesthesia.

IN VITRO ANIMAL MODELS

In vitro studies present a different set of advantages and limitations, which allow them to be complementary to whole animal in vivo models. In vitro models that have been used in the literature and were discussed at the PANDA symposium include dissociated cell culture and brain slice cultures. The principal advantage of these systems over in vivo work is the focus on cellular mechanisms, a centrally important point for devising mitigating strategies in

neurotoxicity research. From the earliest published use of tissue culture in neuroscience, the proof of the neuron doctrine by Ross Harrison in 1910,⁴⁴ to the most current work on brain development, in vitro studies have been crucial in identifying mechanism. Attendees at the PANDA symposium who use these methods pointed out that they are more economical, rapid, and flexible compared with in vivo models and better adapt to screening approaches, particularly those involving rigorous investigations of pharmacology.

Current cell culture models used in anesthetic neurotoxicity consist of immortalized cell lines and primary cells. Experimental protocols to assay apoptotic cell death are typically designed for immortalized cell lines, due to their relative ease of transfection with exogenous genetic material relative to primary cells. Although no data were presented at the PANDA symposium using cell lines, it was noted that immortalized cell lines with neuronal features may serve as good models to further explore neuronal apoptosis, as the pathways involved are likely to be similar to true neurons. For example, cell lines with neuronal properties such as the pheochromocytoma-derived PC12 line and the neuroblastoma-derived SH-SY5Y line have been used to explore the mechanisms by which proapoptotic pathways are activated by isoflurane,⁴⁵ differentially activated by isoflurane and sevoflurane,⁴⁶ and inhibited by propofol.⁴⁷ The use of cell lines has been particularly common in research of anesthetic neurotoxicity of mature neurons in the context of Alzheimer disease,^{48,49} raising expectations that pathways identified in these studies may have relevance to developing neurons as well. However, cell lines alone cannot be used to address many of the anesthetic effects observed using in vivo models, as isolated cells do not elaborate functional axons, dendrites, and synapses, which are the key elements of neurons that arise during development.

Dissociated cultures of primary cells are likely to be of use in investigating mechanisms of pediatric anesthetic neurotoxicity, as they have been fundamental to the study of developmental neuroscience, beginning with pioneering studies of Banker and Cowan⁵⁰ on hippocampal neurons in culture. Protocols for primary cultures of late embryonic rodent hippocampal and cortical neurons are readily available and can be used to produce high-quality, consistent supplies of neurons that recapitulate the developmental events observed in vivo.⁵¹ These cultures can be used for live or fixed imaging, electrophysiology, molecular biology, or biochemistry. Advantages over in vivo preparations include the synchrony with which developmental events occur and the ease with which individual neurons can be monitored by imaging or electro-physiology techniques, as well as being relatively easy to acquire and inexpensive to perform.

There are several notable examples of how primary cultures can be used in the published neurotoxicity literature and in preliminary reports presented at the PANDA symposium. Piyush Patel, MD presented previously published data in mixed cortical and hippocampal dissociated cultures showing that isoflurane can disrupt the actin cytoskeleton in developing neurons through effects on RhoA.¹⁹ Cell culture models are ideal for the study of the cytoskeleton, which typically requires high-resolution single-cell imaging. Key processes in neuron development, including synaptogenesis as well as axon and dendrite development, are all highly dependent on the actin cytoskeleton,⁵² and actin regulation has been identified as a potential target of anesthetics.⁵³ One of the earliest and most convincing demonstrations

of the effects of anesthetics on actin in the nervous system was performed in dissociated hippocampal cultures. In this study, live imaging techniques revealed that volatile anesthetics block actin-dependent morphologic changes in dendritic spines.⁵⁴ In this regard, Dr. Patel presented unpublished data demonstrating effects of propofol on the cytoskeleton; these data indicate that propofol might produce injury by destabilizing the cytoskeleton in neurons.

Neurites can also be easily studied in dissociated primary neuron culture, as individual axons and dendrites can easily be resolved by microscopy. C.D.M. presented data derived from dissociated cortical neuron cultures showing that isoflurane and propofol interfere with axon specification and the acquisition of neuronal polarity, some of which is published in an accompanying article.⁵⁵ Furthermore, C.D.M. showed that anesthetics with activity at GABA_A receptors interfere with growth cone responses to axon guidance cues, potentially interfering with the establishment of neuronal connectivity.

Dissociated cell culture models are not limited to the widely used methods of preparing postmitotic cortical and hippocampal neurons. Neuronal progenitors can be isolated and cultured, and using this technique, Crosby and colleagues showed that isoflurane reduces the proliferative capacity of neuronal stem cells without causing substantial cell death in this cell population.⁵⁶ Furthermore, glial cells are critical to brain development and are another possible target for anesthetic agents that can be investigated using cell culture. Cultures of immature primary astrocytes can be prepared and studied in vitro with the same advantages of primary neuronal cultures. This system has been used to show that isoflurane also interferes with astrocyte development through effects on actin stress fibers.¹⁸

The principal limitations of dissociated cultures are the lack of context and patterned activity, and although it remains the optimal system for reductionist experiments to explore mechanism, it is not the only system that can be used for in vitro neurotoxicity research. Organotypic slice cultures can be prepared from both cortex and hippocampus,⁵⁷ representing a useful compromise between dissociated cultures and in vivo experiments. It was pointed out at the PANDA symposium that slice culture is considerably easier and more cost effective than whole-animal experiments for rigorous pharmacological experimentation that establish concentration-response, time course, and reversibility. Furthermore, slice cultures do not suffer from physiological derangements, such as hypotension, hypothermia, and hypoventilation, which can all confound in vivo studies of anesthetic agents. Unlike cell cultures, slice culture does preserve connectivity and can be used to investigate circuit formation. For example, C.D.M. presented unpublished data at the symposium from organotypic cortical slice cultures, which demonstrates that anesthetics can interfere with axon guidance mediated by the repulsive guidance cue Semaphorin 3A, which led to further mechanistic investigations in dissociated cell culture that are described above. Although both synaptogenesis and apoptosis can be studied in dissociated cell culture, the more complete environment of the slice culture setting enhances the applicability of findings in this model system to the intact organism.^{19,58,59} Although brain slice cultures cannot entirely substitute for whole-animal studies, they serve as a valuable intermediate allowing the investigator to hone in on the effects under study, which in turn can make subsequent in vivo work more effective and efficient.

CONCLUSIONS

In summary, both in vitro and in vivo animal research are critical for trying to elucidate the mechanism and cellular selectivity of the effects of anesthetic exposure in the developing brain. Animal models are crucial in comparison studies into the toxic potency of different anesthetic compounds. Moreover, they will be central in helping to devise mitigating strategies during and after anesthetic exposure. Several important barriers exist in directly translating animal research to human clinical practice, as outlined above. Accordingly, whether pediatric exposure to anesthetics does interfere with subsequent brain function can only be fully answered by prospective human trials. However, the consensus at the PANDA symposium was that additional animal research is urgently needed using in vivo rodent and primate models to guide clinical studies and that in vitro studies will be crucial in directing and refining in vivo work, thereby improving our understanding of the mechanism of anesthetic neurotoxicity and its human applicability.

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