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Application of Nonhuman Primate Models in the Studies of Pediatric Anesthesia Neurotoxicity

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Numerous animal models have been used to study developmental neurotoxicity associated with short-term or prolonged exposure of common general anesthetics at clinically relevant concentrations. Pediatric anesthesia models using the nonhuman primate (NHP) may more accurately reflect the human condition because of their phylogenetic similarity to humans with regard to reproduction, development, neuroanatomy, and cognition. Although they are not as widely used as other animal models, the contribution of NHP models in the study of anesthetic-induced developmental neurotoxicity has been essential. In this review, we discuss how neonatal NHP animals have been used for modeling pediatric anesthetic exposure; how NHPs have addressed key data gaps and application of the NHP model for the studies of general anesthetic-induced developmental neurotoxicity. The appropriate application and evaluation of the NHP model in the study of general anesthetic-induced developmental neurotoxicity have played a key role in enhancing the understanding and awareness of the potential neurotoxicity associated with pediatric general anesthetics. (Anesth Analg 2022;134:1203–14)

GLOSSARY

[^{18}F]-FDG = [^{18}F]-labeled fluorodeoxyglucose; **[^{18}F]-FEPPA** = [^{18}F]-labeled fluoroethoxybenzyl-N-(4-phenoxypyridin-3-yl) acetamide; **4-HNE** = 4-hydroxynonenal; **BBB** = blood-brain barrier; **CNS** = central nervous system; **CO_2** = carbon dioxide; **CSF** = cerebral spinal fluid; **DEGs** = differentially expressed genes; **FDA** = US Food and Drug Administration; **GA** = general anesthetics; **GABA** = gamma-aminobutyric acid; **GD** = gestational day; **MicroPET/CT** = micro positron emission tomography/computed tomography; **N_2O** = nitrous oxide; **NCTR OTB** = National Center for Toxicological Research Operant Test Battery; **NHP** = nonhuman primate; **NMDA** = N-methyl-D-aspartate; **NR1** = the NR1 subunit of the glutamate/NMDA receptor; **NSC** = neural stem cell; **PE** = phosphatidylethanolamine; **PET** = positron emission tomography; **PG** = phosphatidylglycerol; **PND** = postnatal day; **PS** = phosphatidylserine; **ROIs** = regions of interest; **Spo_2** = peripheral oxygen saturation; **TSPO** = translocator protein

General anesthetics (GAs) primarily exert their effects on patients by either suppressing excitatory or potentiating inhibitory synaptic transmission.[1–5] In the past 3 decades, it has been confirmed with a large body of rat,[6–9] mouse,[10–13] and nonhuman primate (NHP)[14–17] studies that prolonged exposure to commonly used GA agents at clinically relevant concentrations during the brain growth spurt period can induce neurotoxicity in the immature brain. The evidence of developmental neurotoxicity from animal experiments has given rise to public health concerns over the safety of pediatric general anesthesia.[18] Food and Drug Administration (FDA) released an announcement (www.fda.gov/Drugs/DrugSafety/ucm532356.htm) warning about the potential adverse effects to brain development caused by prolonged (ie, >3 hours) or multiple GA exposure(s) for surgeries or procedures in children younger than 3 years or in pregnant women during their third trimester. Meanwhile, both retrospective and prospective studies on human patients have been performed.[19–23] Results indicate that a single bout of brief general anesthesia in children younger than 3 years of age is not likely associated with an increased incidence of negative neurodevelopmental outcomes later in life. Due to confounding factors that compromise the neurodevelopment of children undergoing surgery, the relationship between lengthy or frequent

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pediatric general anesthesia procedures and neurodevelopmental impairment in pediatric patients remains controversial.[23,24]

With progress in anesthesia support and intraoperative monitoring, more surgical interventions are now available for pediatric patients and even the intrauterine fetus. These are done under various regimens of anesthesia or sedation,[25–28] leading to more frequent and possibly prolonged exposure of the developing brain to GAs. Moreover, some pediatric patients with life-threatening conditions are subject to complex surgical treatments and prolonged anesthetic exposure during the first 3 years of life.[24,29–34] The NHP model has been crucial in describing the risks associated with early life anesthesia in some of the most vulnerable patients.

The results of GA-induced developmental neurotoxicity obtained from animal experiments cannot be expected to perfectly predict effects on human patients because of the differences in species and the settings of animal GA exposures. Nonetheless, using animal models to study GA-induced developmental neurotoxicity is irreplaceable due to the ethical considerations of pediatric clinical studies. Despite the hurdles of translating insights of GA-induced neurotoxicity acquired in animal studies to the management of pediatric general anesthesia, animal models remain a potent tool in exploring GA-induced adverse effects on neurodevelopment in humans.

The animal models used to examine the neurotoxic effects of GA on neurodevelopment are diverse, including, but not limited to, the nematode (*C. elegans*),[35] zebra fish,[36] rodent,[7,13,37,38] NHP,[16,39,40] etc.[41,42] Although various animal models have been utilized in the reported research, the animals' age when being exposed to anesthetics has been strictly defined to coincide with key periods in brain development, most frequently synaptogenesis. This is a critical stage of brain development when synapsis formation is exuberant and neural cells are sensitive to neurotoxic agents.[9,16,43,44] In studies using neonatal rats, the amount of GA-induced neuronal cell death in both the cerebral cortices and thalamus nuclei increased significantly in comparison with control animals from postnatal days (PNDs) 3 to 14, with the greatest increment in the number of apoptotic neurons on PND 7. This indicates that the severity of GA-induced apoptotic neurodegeneration could be correlated with rapidity of synaptogenesis.[9,44] Although rodent models are widely used in preclinical anesthesia research, the use of the rat for pediatric anesthetic studies is practically problematic because of anesthesia performance, monitoring physical robustness, and biofluid collection (such as blood and cerebral spinal fluid [CSF]).

In the rhesus macaque, the peak of synaptogenesis occurs from gestational day (GD) 122 to PND 5 and is greatly reduced by PND 35.[45–48] Among the animal models used to determine the neurotoxic potential of GAs and other drugs or substances, eg, methylphenidate, nicotine, cocaine, marijuana, etc, NHP models are considered more predictive because of similarities with humans in genetics, reproduction, development, neuroanatomical organization, and cognitive capacities.[49–54] For the GA studies, the specific advantages of the NHP models include easy management of the anesthesia procedure, monitoring physical wellbeing during exposure, and availability (repeated) of blood and/or CSF sample collection. No other commonly used animal model has a functional fetal placental unit, a propensity for single birth, and a fetal-to-maternal weight ratio comparable to that of humans.[55,56] In the following sections, we will discuss the use of the NHP model in studying GA-induced neurotoxicity, including the setting of GA exposure, animal monitoring during GA exposure, and the indices of developmental neurotoxicity.

THE DEPTH OF ANESTHESIA

GA agents exert pervasive effects on the central nervous system (CNS) and other body systems in a dose-dependent manner.[57] Optimal dosing of GA agents is critical during experimental anesthetic exposure to simulate general anesthesia and to produce adequate analgesia without deranging the subjects' physiological status. In animal studies on anesthesia-induced developmental neurotoxicity,[14–16,37,58–60] a light anesthesia plane, where animals lacked voluntary movement to physical stimuli, has been attained and maintained steadily by inhalational or intravenous anesthetic agents after induction to prevent overdosing of the animals. In addition to testing animals' withdrawal movement to physical stimuli, more reactions to physical stimuli were tested in NHP models, including the presence of a palpebral reflex,[16] increase of heart rate, or changes in blood pressure on pinch of paws.[14,16,60] These studies indicated that accurate titration and maintenance of the anesthetic plane (live monitoring of heart rate and blood pressure) can be ensured in neonatal NHP models. It is crucial to have the correct levels of anesthesia to assess anesthetic-induced neural damage when predicting the risk posed by these agents/procedures to humans.

MONITORING AND MAINTAINING OF PHYSIOLOGICAL STATUS

Circulatory and respiratory inhibitions are common predictable adverse effects of general anesthesia using either inhalational or intravenous anesthetic agents.[61–63] Small animal models exposed to anesthetic agents need to have vital signs monitored and their normal physiological status maintained.[64] In animal models for anesthesia-induced developmental neurotoxicity, a

range of measures have been designated to intensively monitor physiological parameters, including vital signs, blood oxygenation levels, and homeostasis, to assess if any alterations, including hypoxia or hypotension, occur during anesthetic exposure.[6,13,37]

During anesthetic exposures, vital signs of neonatal monkeys can be continuously monitored including heart rate, respiratory rate, blood pressure, and rectal temperature.[16,60,65] The homeostatic status in the exposed neonatal monkeys can be evaluated by continuously monitoring physiological parameters, including hemoglobin oxygen saturation, end-expiration of carbon dioxide (CO_2) concentration, and blood glucose levels using venous blood gas analysis at intervals of 1 to 2 hours during anesthetic exposure.[14–16,39,60,65] Noninvasive pulse oximetry that measures hemoglobin oxygenation level (Spo_2) using the pulse-dependent photoplethysmographic signal[66] has been used to monitor arterial blood oxygen saturation. These noninvasive measures in anesthetic-treated neonatal monkeys were recorded every 15 minutes; the systolic, diastolic, and arterial mean pressures were measured every 30 minutes in animals showing no major physiological disturbance but that did display signs of neurotoxicity.[16,60,65] The presence of hypercarbia, respiratory acidosis, hypotension, and hypoglycemia in GA-treated neonatal monkeys was also monitored. Neonatal monkeys were supported with either 5% dextrose administrated enterally (via a gastric tube in treated animal and a feeding bottle in control animal)[16] or lactated Ringers solution given intravenously.[67] To prevent hypoventilation, tracheal intubation and mechanical ventilation were applied to neonatal monkeys after induction.[14,65,67,68] The ability to maintain physiological parameters provides additional confidence that observed changes are the result of anesthesia-related neurotoxicity and not the consequence of nonspecific effects.

Interestingly, due to GA-associated suppression of cardiopulmonary functions, it is suspected that anesthetic-induced apoptotic neurodegeneration in the immature brain may be caused by hypoxia-/ischemia-induced excitotoxicity.[69–72] To distinguish potential contribution of hypoxia/ischemia from GA-induced neurotoxicity to acute apoptotic neurodegeneration after prolonged exposure to anesthetic agents, it is important to recognize the difference between hypoxia-ischemia-induced cell death and anesthetic-induced acute apoptotic neurodegeneration.[73–75] The acute cytopathological alterations induced by hypoxia-ischemia is excitotoxic in nature, characterized by "edematous degeneration of neuronal dendrites and cell bodies" as verified by electron microscopy.[73] These cytopathological changes evolved rapidly, in a few hours, resulting in end-stage necrosis. In the case of GA-induced developmental neurotoxicity, the ensuing apoptotic cell death occurred in cortical neurons in superficial layers, displaying nuclear condensation and nucleus membrane fragmentation in ultrastructure assessments.[16,37,73] These findings suggest a potential dissociation in the histological markers associated with anesthesia-related neurotoxicity with those best attributed to hypoxia/ischemia.

GA-INDUCED NEUROTOXICITY IS DEPENDENT ON EXPOSURE DURATION

Many preclinical studies using either in vitro or in vivo models have demonstrated that GAs, which are either N-methyl-D-aspartate (NMDA)-type receptor antagonists (eg, ketamine and nitrous oxide [N_2O]) or gamma-aminobutyric acid (GABA) receptor agonists (eg, sevoflurane and propofol), induced neuronal cell death in a dose or duration of exposure-dependent manner.[76,77] The NHP models were used in only a few studies.[14,16,39,60,65,68,78–80] However, the contributions of these experiments are important in terms of translational validity due to the similarities of NHPs to humans in development, neuroanatomy, and recognitive functions.[81] In the studies on ketamine-induced neurotoxicity in neonatal rhesus monkeys, it was shown that prolonged exposure to ketamine (9 or 24 hours) induced a significant increase of neuronal cell death as compared to control animals in layers II and III of the frontal cortex, indicated by caspase-3 immunostaining, Fluoro-Jade C, and silver stains. Ketamine exposure for a shorter duration, ie, 3 hours, did not elicit similar apoptotic neurodegeneration.[16,80] In another independent study,[82] a ketamine exposure of 5 hours to neonatal rhesus monkeys (PND 6) and fetuses (at GD 120) was sufficient to elicit a significant increase of apoptotic neuronal cell death, with a more severe apoptotic profile in fetuses than neonates. In isoflurane anesthesia maintained at the surgical plane in PND 5 rhesus macaques, apoptotic neuronal death in the cerebral cortex was increased 13-fold compared with control monkeys following the 5-hour exposure.[65] These experiments in NHP models were performed with close monitoring of similar physiological parameters as used in pediatric anesthesia. With physiological parameters maintained in a normal range in the treated neonatal monkeys, the apoptotic neuronal cell death accrued in an exposure duration-dependent manner. These data indicate that the neurotoxic effects following GA exposure in the NHP model should be attributed to mechanisms other than hypoxia/ischemia. In addition, repeated 4-hour exposures of neonatal rhesus monkeys (PNDs 6–10, 14, and 28) to sevoflurane at clinically relevant concentrations resulted in increased emotional reactivities to the stress of a human intruder compared with the control animals. These data may be interpreted as long-term adverse neurodevelopmental consequence

of repeated anesthesia.[78] These experiments using NHP models have demonstrated their essential value in preclinical investigation of anesthesia-induced developmental neurotoxicity.

PEDIATRIC ANESTHETIC-INDUCED NEUROTOXICITY IS DEPENDENT ON DEVELOPMENTAL AGE

Exposure of the developing mammal to NMDA receptor agonists[83] or antagonists[84] perturbs the endogenous NMDA receptor system and results in enhanced neuronal cell death.[85] The early life stage/growth spurt is a period with intense synaptic remodeling and myelin formation. It has been hypothesized that excessive suppression and/or stimulation of neuronal activity during synaptogenesis activates an internal signal for a developing neuron to enter a programmed cell death. Previous studies[16,80] demonstrated that ketamine (noncompetitive NMDA receptor antagonist) exposure for 24 hours significantly elevated neuronal cell death in the cerebral cortex, as manifested by enhanced caspase-3 expression, increased Fluoro-Jade-C staining, and silver-impregnated neuronal cells observed in PND 5 neonatal monkeys and GD 122 fetuses (early developmental stage) but not in PND 35 monkeys. Consistently, a recent study[86] indicated that prolonged exposure to sevoflurane anesthesia (for 8 hours) had no effect on brain damage and/or neural inflammatory responses in the adult monkey brain. These studies revealed that the relatively mature and/or adult monkey brain are very resistant to the neurotoxic effects of general anesthesia in comparison to the developing monkey brain. These data also suggested that cortical neuronal cell death following prolonged anesthetic exposure was attributed to brain susceptibility to anesthetic exposure during development, rather than suppressed cardiac and respiratory functions during exposure. In addition, these data indicated that the physiological stimulation of NMDA receptors is necessary for neuronal synaptogenesis, differentiation, and survival during development. Disturbing NMDA receptor activation decreases synaptogenesis and cell-cell interactions.[87,88]

PEDIATRIC ANESTHETIC-INDUCED BEHAVIORAL DEFICITS IN NHP MODELS

Preclinical studies indicated that in early life, prolonged or repeated exposure to GAs induced acute widespread apoptotic neurodegeneration in the cerebral cortex and subcortical structures, resulting in delayed, long-lasting behavioral impairments involving memory and learning functions.[12,37,59,89] In addition to hippocampus-dependent memory and learning functions, other components of psychological processes, including motivation and inhibition, sequence learning, and counting, which are based on the circuits connecting the prefrontal cortex and the striatum, have been evaluated in animal models of neonatal anesthesia.[15,78,79,90,91] Due to the similarities in behavioral complexity and psychological capacities between NHPs and humans,[92] the NHP models for GA-induced cognitive impairment are valuable to translational studies on anesthetic-induced developmental neurotoxicity. The National Center for Toxicological Research Operant Test Battery (NCTR OTB), an array of operant behavioral tasks, has been designed to test cognitive function following exposure to toxicants or drugs in monkeys[53,93,94] and, with adaption, in children.[95,96] Following 24-hour ketamine anesthesia on PND 5 or 6, ketamine-treated monkeys attained significantly lower scores than the control animals in OTB tests with the same training, indicating significant impairment in color and position discrimination, motivation, sequence learning, and concept formation.[15] In another experiment with rhesus monkeys in which subjects were exposed to 1% isoflurane plus 70% N_2O for 8 hours, the response rate (ie, the rate of lever pressing for reinforcers) recorded in those anesthesia-exposed monkeys was significantly lower than that in control animals, indicating reduced motivation in response and reinforcement learning.[79] Being relevant to human in social interaction, basic emotions, and behavioral complexity, NHPs have also been utilized in models for assessment of socioemotional maturation and motor skills development after anesthesia exposures.[14,78,97–99] It was shown that socially reared rhesus monkeys that had been exposed to isoflurane (0.7%–1.5%, 5 hours per session) repeatedly (at PNDs 6, 9, and 12) had motor reflex deficits and higher anxiety at 1 year of age.[14] Similarly, multiple exposures of neonatal rhesus monkeys (first session on PNDs 6–10 and 2 additional sessions on PNDs 14 and 28) to sevoflurane (maintained at 2.5%, 4 hours) led to increased anxiety behavior when confronted by an intruder.[78] Among the various animal models, the NHP model appears more relevant in translational studies on the cognitive impairments related to pediatric anesthesia.

APPLICATION OF MINIMALLY INVASIVE AND TRANSLATABLE APPROACHES TO DETECT ANESTHETIC-INDUCED NEURONAL DEATH IN NHP MODELS

Numerous studies suggest prolonged exposure to anesthetic(s) during the late gestational period, and infancy is associated with an increased risk of neuronal damage (including neural apoptosis) and neurocognitive impairment.[6,16,17,80,85,100,101]

There are a variety of similarities, as well as some very important differences, in modeling and understanding the molecular mechanisms underlying the pathogenesis of disorders/diseases in the developing

primate brain compared to the situation in rodents. Because the brain growth spurt in both human and NHPs extends over a longer period than in rodents, matching the timing of a developmental event in humans and NHPs is less problematic than correlating the same periods between primates and rodents. It is generally believed that the NHP fetus and the human fetus have a more similar degree of maturation at birth compared to rodents. It has also been demonstrated that the starting age for quantitative imaging of neuronal activity is much earlier in primates (eg, newborn or PND 5/6 monkeys) than that in rodents (eg, PND 21 rats). Therefore, application of NHP models in identifying anesthetic-induced developmental neurotoxicity by molecular imaging technology has been repeatably reported.[102–104]

Among the imaging techniques, positron emission tomography (PET) with radiolabeled (eg, ^{11}C or ^{18}F) tracers can dynamically visualize complex interactions between physiological targets and ligands while providing information about the time of on-set, characterization, and quantification of biological processes.[105–109] However, it should be mentioned that not all tracers used for detecting neuronal damage/cell death are equally/efficiently utilized in both rodents and NHPs,[103] due to their maturation levels and the nature of architecture of blood-brain barrier (BBB) during the development. Here, based on our experience, [^{18}F]-labeled fluorodeoxyglucose ([^{18}F]-FDG) and [^{18}F]-labeled fluoroethoxybenzyl-N-(4-phenoxypyridin-3-yl) acetamide ([^{18}F]-FEPPA)[102,110,111] may be used to successfully monitor anesthetic-induced neural damage in the developing monkey brain.

[^{18}F]-FEPPA is a ligand for the translocator protein (TSPO). In the CNS, TSPOs are expressed primarily on activated microglia and can be used as biomarkers of neural damage and inflammation.[112–114] Thus, anesthetic-induced alterations/adverse effects could be evidenced by increased radiolabeled uptake (tracer accumulation) in specific regions of interest (ROIs). For example, our previous studies demonstrated that prolonged exposure (9 hours) of neonatal monkeys (PND 5/6) to a clinically relevant concentration (2.5%) of sevoflurane (a volatile anesthetic agent commonly used in anesthesia and sedation in pediatric patients) results in significant neuroinflammation as indicated by increased accumulation of [^{18}F]-FEPPA in the frontal cortex 24 hours and 7 days after anesthetic exposure.[102]

Taken together, monitoring changes in the uptake of specific radiotracers, eg, [^{18}F]-FEPPA that highlights adverse events such as neurotoxicity and gliosis, anesthetic-induced neurodegeneration can be repeatedly assessed in in vivo NHP models.[102,115] Additionally,[103,116] the severity of anesthetic-induced brain damage and the duration of the damage, as well as potential protection, can systematically be evaluated in developing primate models.[102]

GENETIC (DNA MICROARRAY) AND LIPIDOMIC PROFILING (IDENTIFYING BIOMARKERS) IN NHP MODELS

The NHP, eg, rhesus monkeys, being 93% genetically similar to humans[117] can, in many instances, more accurately predict how pathological conditions arise in humans, and the vulnerability of the primate brain to toxicants/chemicals is closely related to the maturity of brain development.[16]

To dissect underlying mechanisms associated with anesthetic- (eg, sevoflurane) induced neurotoxicity, DNA microarray technology was used in NHP models to check, analyze, and interpret the gene-environment interaction and/or gene expression profile on the brain tissues from specific ROIs, such as frontal cortex.[40,118,119] DNA microarray technology simultaneously detects perturbations of tens of thousands of genes in 1 single experiment. Previously, we demonstrated that 576 genes of 43,663 probes were identified as differentially expressed genes (DEGs) in PND 5/6 monkeys following their prolonged exposure (eg, 9 hours) to sevoflurane at a clinically relevant concentration (2.5%).[40] These DEGs were closely associated with multiple significant terms or pathways including developmental process, abnormal morphology of CNS, and neuronal and synaptic transmissions, as well as associated with cell death and survival and cell cycling pathways.[40]

The most important information obtained from the microarray analysis is that a considerable number of DEGs after sevoflurane exposure were associated with networks of lipid metabolism.[40] Lipid peroxidation is one of the most commonly reported indices of oxidative stress and has been implicated as a contributing factor in a range of degenerative diseases/disorders including anesthetic-induced neurotoxicity.[120] Altered lipid composition has been found at the earliest diagnostic stages of degenerative diseases,[121–131] suggesting that aberrant lipid metabolism may be one of the determinants of neuronal damage. Even small perturbations in the nervous system can be reflected in changes in lipid content, composition, or both. It should be mentioned that GAs are highly lipid soluble and may have some impact on lipid profile because of this property. Meanwhile, in contrast to neonatal rodent models, repeated biofluid (blood and/or CSF sample) collections are practically accessible in neonatal NHP. Therefore, evaluating whether and/or how anesthetic agents might affect lipids, the most abundant component in the brain (other than water) would greatly aid in determining the underlying mechanisms of anesthesia-induced neurotoxicity. The discovery of plasma (blood) and/or CSF

lipid profiles as biomarkers for the early detection of anesthetic-induced neurotoxicity, especially during the most sensitive developmental brain growth spurt period, would provide new opportunities for developing procedures with fewer or no neurotoxic effects.

Data from mass spectral analyses indicated that broad classes of lipids changed after sevoflurane (2.5%; 9 hours) exposure. The membrane phospholipid contents, such as phosphatidylethanolamine (PE),[132,133] phosphatidylserine (PS),[134–136] and phosphatidylglycerol (PG),[137] were significantly decreased in brains of sevoflurane-exposed neonatal monkeys.[40] The levels of 4-hydroxynonenal (4-HNE) were markedly increased by sevoflurane. 4-HNE is an aldehydic product of membrane lipid peroxidation that negatively affects brain mitochondrial complexes II and III and is associated with mediating oxidative stress-induced neuronal apoptosis.[138,139] Since PE and PS are critical apoptotic components located on mitochondrial and cytoplasmic membranes, and PG is exclusively present in mitochondrial membranes,[125,127–129,131,137,140–142] these findings suggest that mitochondria are affected early in the course of sevoflurane-induced neurotoxicity.

Meanwhile, to verify whether the altered lipids can serve as effective biomarkers for anesthetic-induced neurotoxicity, blood samples were collected from anesthetized (by sevoflurane) and control animals at 2-hour intervals during the experiment and analyzed for potential lipid changes.[40] Consistent with brain (tissue) lipid data, the mass level of PE in blood plasma of the neonatal monkeys was substantially decreased, and 4-HNE levels were significantly increased after sevoflurane exposure compared with controls.[120] These data clearly indicate that the changes observed in blood largely reflect lipid alterations in the brain of the same neonatal monkeys.

It is known that membrane lipids (eg, those on mitochondrial membrane) play critical roles in cell signaling and membrane trafficking/permeability, and alteration of membrane lipids is an important component of anesthetic-induced neurotoxicity following prolonged exposure (ie, 9 hours). These findings from both brain tissues and blood plasma suggest that prolonged exposure of the developing primate brain to anesthetics results in disruption of lipid classes and phospholipid integrity and negatively alters ion channel proteins and the function of innumerable proteins integral to membranes. Thus, dynamically monitoring the alteration of lipid composition/integrity in the blood plasma during anesthesia may serve as sensitive biomarkers and aid in determining the presence and severity, as well as the underlying mechanisms of anesthesia-induced neurotoxicity.

Biomarkers that are related to chemical or other toxic exposures and/or their effects on biological systems offer tremendous potential for the early detection and management of many different diseases and adverse health conditions.[40] It is well known that CSF is intimately involved with the cellular functions of the CNS and that lipid levels in the CSF best reflect the cellular lipid metabolic and catabolic activities of the CNS. Since CSF composition is dictated by brain metabolite production rates,[143] examination of CSF lipid metabolites, as biomarkers of CNS function and damage, may assist in understanding the biochemical changes that occur in the brain during anesthesia. Therefore, changes in lipid profiles in brain tissues and blood plasma may be carefully analyzed to determine whether these alterations match CSF lipid fingerprints and correlate with anesthetic-induced neuronal damage.

Biomarkers that are present in easily accessible biofluids, such as blood, urine, and CSF, enable the quick assessment of the presence, progression, and treatment response of various diseases and toxicities. It should be emphasized that not all preclinical animal models are suitable for biomarker identification. In the PND 7 rat model, the size of the pups severely limits the amount of blood and CSF that can be repeatedly collected for the lipidomic analyses (biomarker identification). Among the animal (preclinical) models used to assess anesthetic-induced neurotoxicity during the development, NHPs are evolutionally closer to humans than other animals (species), and their comparatively large size offers numerous practical advantages for biomarker detection.[16,40,80]

IN VITRO MEASUREMENTS OF ANESTHETIC-INDUCED NEUROTOXICITY

To examine the neurotoxicity induced by anesthetic agents, the NCTR pediatric anesthetic research team initially established primary cell culture systems using brain tissue (frontal cortex) from PND 3 monkeys.[17,85] It was demonstrated that addition of 10-μM ketamine results in an approximately 58% loss of viability at 6 hours and approximately 75% at 24 hours. These experiments also demonstrate that ketamine-induced neuronal cell death in monkey frontal cortical cultures appears to be both apoptotic and necrotic in nature.[17,85] Ketamine produces an apparent increase in the NMDA receptor NR1 subunit expression. Coadministration of NR1 antisense oligodeoxynucleotides specifically prevents synthesis of NMDA receptor NR1 protein and subsequently blocks the neuronal loss induced by ketamine.[17,85]

The application of these advanced approaches and the monkey primary culture model allowed for the investigation of cellular mechanisms that may be associated with anesthetic-induced cell death. These systematic in vitro studies have provided critical information for understanding that general

pediatric anesthetics cause neuronal cell death by a mechanism involving compensatory upregulation of NMDA receptors. Anesthetic- (eg, ketamine) induced neuronal cell death is associated with an increased Ca^{2+} influx/calcium overload, which is thought to be caused by glutamatergic stimulation of upregulated NMDA receptors.[17,85]

LIMITATIONS AND FUTURE DIRECTIONS OF NHP-RELATED STUDIES

Limitations

Most preclinical studies of neurotoxicity rely on animals including NHP models. Although NHPs models are the closest animal models to humans regarding genetics, physiology, and behavior, NHP models have their limitations. Given the complexity of translational research and the fact that any animal model cannot totally reproduce the human condition, such as important pharmacokinetic differences between humans and NHPs, there are many challenges and opportunities when NHPs are used for translation.[144] Meanwhile, the use of NHPs versus other models such as rodents is complicated by the many human-like characteristics of NHPs, leading to questions from researchers, stakeholders, and the public about their use for research.[144]

It is suspected that long duration exposure of infants to commonly used anesthetics/analgesics may lead to impaired cognitive development.[101] However, it remains uncertain if the neurodevelopmental deficits are caused by general anesthesia or the surgical procedure/underlying condition that requires the use of general anesthesia.[145,146] There is increasing realization that variation in genetics or other factors such as the social interactions of individual rhesus or cynomolgus macaques can influence experimental results. Also, it is impractical (slow and expensive) for comparing/conducting multiple drugs/dosing regimens or high-throughput methods, because of the expense associated with each animal in an NHP-based study and the potential difficulty of obtaining adequate numbers of females, neonates, or juveniles.

Future Directions

It is difficult to study critical dose-response and time-course data on anesthetic-/analgesic-induced neural damage in humans. With the ability to better interrogate specific mechanisms and the opportunity to apply emerging technologies that cannot easily occur in vivo, in vitro models[6,17,85,147] have been used that can overcome limitations and provide additional data. Particularly, the availability of neural stem cell (NSC) models from NHPs with their capacity to reproduce the most critical developmental processes, including proliferation and differentiation, may serve as effective alternatives when evaluating anesthesia-related neurotoxicity. To develop new and improved testing methods that can better predict human responses to pharmaceuticals and/or environmental chemicals, it is proposed that the nonhuman NHP NSC culture systems may facilitate mechanistic studies including anesthetic neurotoxicity that are difficult in animal models (eg, limited availability of neonatal monkeys), but are becoming increasingly necessary to understand and prevent the damaging effects of perinatal anesthetic exposure. Recently, the NCTR Pediatric Anesthetic Research Team successfully established an NHP NSC model from the embryos of rhesus monkeys. In this preliminary study, cortical and hippocampal NSCs were successfully harvested from GD 80 fetal monkey brain, and NHP NSCs were established that can mimic in vivo cellular development. This NSC model is an unlimited resource for obtaining differentiated neurons, astrocytes, and oligodendrocytes with high yields. Therefore, cultured NSCs and/or cells differentiated from them can be exposed to GAs at selected concentrations (including lower and higher doses versus threshold) and for different durations (including short-term and long-term exposures). These regimens include establishing the limitations of the assay and thresholds of response that can be used to predict potential in vivo toxicity. Thus, the availability of NHP primary NSC culture models will bring biology that closely mimics the human condition to the laboratory. Application of these in vitro NHP models will not only minimize/reduce animal use in developmental neurotoxicity research but also provide a more human-relevant model of scientific and regulatory interest to allow for increased exploration of cellular and molecular mechanisms underlying anesthetic-induced neurotoxicity.

Another important/critical and consistent element in the field of anesthesia research is to do preclinical studies that use clinically relevant doses of anesthetics, eg, ketamine, to mimic the clinical situation, in the presence of a surgical stress in neonatal animals. Data in support of a correlation between surgery and subsequent physiological changes have accumulated, and it remains uncertain if neurodevelopmental deficits are directly caused by general anesthesia.[145,146] Because it is difficult to disentangle the effect of anesthesia per se from the effects of surgery or preexisting pathologies that necessitate surgery, it is essential to continue studies in neonatal NHPs to obtain valuable information on potential neuroprotective effects of anesthetics (such as ketamine) and/or ketamine-induced neural degeneration. Importantly, the size and physiological similarity of NHPs to humans allow for an opportunity to test this theory.

Table. Preclinical (Pediatric) Anesthetic Studies in NHP Models/NCTR

Drug	Author	Title
Ketamine	Wang et al (2006)[17]	Blockade of N-methyl-D-aspartate receptors by ketamine produces loss of postnatal day 3 monkey frontal cortical neurons in culture
Ketamine	Slikker et al (2007)[16]	Ketamine-induced neuronal cell death in the perinatal rhesus monkey
Ketamine	Zou et al (2009)[80]	Prolonged exposure to ketamine increases neurodegeneration in the developing monkey brain
Isoflurane and N_2O	Zou et al (2011)[60]	Inhalation anesthetic-induced neuronal damage in the developing rhesus monkey
Ketamine	Paule et al (2011)[15]	Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys
Isoflurane and N_2O	Zhang et al (2012)[115]	MicroPET/CT imaging of [^{18}F]-FEPPA in the nonhuman primate: a potential biomarker of pathogenic processes associated with anesthetic-induced neurotoxicity
	Talpos et al (2019)[79]	Early life exposure to extended general anesthesia with isoflurane and nitrous oxide reduces responsivity on a cognitive test battery in the nonhuman primate
Isoflurane and N_2O	Liu et al (2012)[119]	Assessment of potential neuronal toxicity of inhaled anesthetics in the developing nonhuman primate
Sevoflurane	Liu et al (2015)[40]	Potential adverse effects of prolonged sevoflurane exposure on developing monkey brain: from abnormal lipid metabolism to neuronal damage
Sevoflurane	Wang et al (2018)[148]	Lipidomics reveals a systemic energy deficient state that precedes neurotoxicity in neonatal monkeys after sevoflurane exposure

Abbreviations: [^{18}F]-FEPPA, [^{18}F]-labeled fluoroethoxybenzyl-N-(4-phenoxypyridin-3-yl) acetamide; MicroPET/CT, micro positron emission tomography/computed tomography; NCTR, National Center for Toxicological Research; NHP, nonhuman primate.

SUMMARY

During the last 15 years, anesthesia-/analgesia-related neurotoxicity has been systematically studied (Table) at the NCTR using NHP models (eg, neonatal rhesus monkeys). It is important to mention that all animal procedures performed by the NCTR Pediatric Anesthetic Research Team were approved by the NCTR Institutional Animal Care and Use Committee and conducted in full accordance with the PHS Policy on Humane Care and Use of Laboratory Animals. All monkeys were housed in the FDA's NCTR NHP facility. Animal procedures were designed to minimize the number of animals required and reduce any pain or distress associated with the experimental procedures.

In general, as a complementary system, when combined with biochemical, pathological, pharmacokinetic, behavioral, genetic, and molecular imaging approaches, the NHP can be an efficient, mechanism-focused tool for studying the adverse effects associated with anesthetic exposures. It has played a crucial role in clarify the risks associated with early life anesthesia that would not be possible in rodent studies or in most clinical trials. Moreover, ongoing research with the developing NHP might also facilitate biomarker identification for early detection of anesthetic-induced neurotoxicity and provide a system to bridge the gap between preclinical and clinical research. If so, the NHP will have helped to describe and minimize the risk associated with perinatal anesthesia.

DISCLOSURES

Name: Cheng Wang, MD, PhD.
Contribution: This author helped with experimental design, conducted part of the experiments (mentioned in the manuscript), drafted, and revised the manuscript.
Name: Shuliang Liu, PhD.
Contribution: This author helped conduct part of the experiments (mentioned in the manuscript) and draft the sections of Introduction and Monitoring and Maintaining of Physiological Status.
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