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Isoflurane exposure leads to apoptosis of neurons and oligodendrocytes in 20- and 40-day old rhesus macaques

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ABSTRACT

Previously we reported that a 5-hour exposure of 6-day-old (P6) rhesus macaques to isoflurane triggers robust neuron and oligodendrocyte apoptosis. In an attempt to further describe the window of vulnerability to anesthetic neurotoxicity, we exposed P20 and P40 rhesus macaques to 5 h of isoflurane anesthesia or no exposure (control animals). Brains were collected 3 h later and examined immunohistochemically to analyze neuronal and glial apoptosis. Brains exposed to isoflurane displayed neuron and oligodendrocyte apoptosis distributed throughout cortex and white matter, respectively. When combining the two age groups (P20 + P40), the animals exposed to isoflurane had 3.6 times as many apoptotic cells as the control animals. In the isoflurane group, approximately 66% of the apoptotic cells were oligodendrocytes and 34% were neurons. In comparison, in our previous studies on *P*6 rhesus macaques, approximately 52% of the dying cells were glia and 48% were neurons. In conclusion, the present data suggest that the window of vulnerability for neurons is beginning to close in the P20 and P40 rhesus macaques, but continuing for oligodendrocytes.

1. Introduction

Worldwide, millions of young children undergo surgery and anesthesia each year. It is becoming increasingly common for pediatric patients of all ages to receive general anesthesia not only for surgical procedures, but also to facilitate long diagnostic procedures and minimally invasive interventions. There is conflicting clinical evidence regarding a potential association between exposure to anesthesia in early childhood and deleterious neurodevelopmental outcomes. While children initially exposed to anesthesia in the first few years of childhood have been shown to have an increased risk of neurodevelopmental deficits in language and cognition, (Ing et al., 2012; Backeljauw et al., 2015) a recent cohort study found that an initial anesthesia exposure after age 3 had no effects on language or cognitive function, but did have deleterious effects on motor function (Ing et al., 2014). Results from two recently published clinical studies suggest that single, short anesthetic episodes may be of less concern (Sun et al., 2016a; Davidson et al., 2016). Today, parents and caregivers often decide to postpone elective pediatric surgery until the child is older with the hope of avoiding any potential neurotoxic anesthetic effects; however, uncertainty remains as to when this window of vulnerability ends.

Substantial animal research suggests that exposure to anesthetics is neurotoxic to the developing brain causing acute apoptosis of neurons/oligodendrocytes and long-term behavior and learning impairment (Jevtovic-Todorovic et al., 2003; Creeley et al., 2014; Brambrink et al., 2012a; Brambrink et al., 2010). The rodent window of vulnerability for neuroapoptosis coincides with the brain growth spurt, a period that encompasses the first two weeks of life in rodents but extends from midgestation to several years after birth in humans. One investigation found that rats exposed to a combination of midazolam, nitrous oxide, and isoflurane on postnatal day 7 (P7) exhibit increased neuroapoptosis, synaptic dysfunction, and long-term cognitive deficits (Jevtovic-Todorovic et al., 2003). In another study, propofol exposure of P5 and P10 rats significantly decreased pyramidal neuronal spine density, whereas similar exposures induced an increase in spine density when administered to P15, 20, or 30 rats (Briner et al., 2011). However, differences in brain maturation rates between rats and humans make translation of data to the clinical setting challenging. Compared to the rodent, the nonhuman primate (NHP) brain more closely parallels human anatomy and

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neurodevelopment. In addition, the NHP model allows one to more closely approximate clinical conditions such as intubation, mechanical ventilation, and constant monitoring of vital signs and thus the ability to maintain physiologic homeostasis. In NHPs, a single study has investigated whether the vulnerability to anesthetic neurotoxicity decreases as animals age (Slikker et al., 2007). As part of a larger study, these authors compared the effects of a 24-hour exposure to ketamine on NHPs at P5 or P35 and found that the frontal cortices of the P5 animals showed apoptosis while those of P35 animals did not (Slikker et al., 2007). Of note, brain development in P5 and P35 monkeys approximates that of 5 and 9 months of age, respectively, in human infants (Dobbing & Sands, 1979; Workman et al., 2013).

We have previously shown that 5 hour isoflurane exposure of the *P*6 neonatal rhesus macaque (*Macaca mulatta*) triggered apoptotic neurodegeneration, and led to apoptosis of oligodendrocytes (Brambrink et al., 2012a; Brambrink et al., 2010). These histopathology findings are similar to those found following P6 NHP exposure to ketamine or propofol (Brambrink et al., 2012b; Creeley et al., 2013). Not only do these anesthesia exposures cause histopathologic changes, but they have also been found to lead to long-lasting cognitive and behavioral deficits in NHPs (Raper et al., 2015; Paule et al., 2011; Coleman et al., 2016). However, the effects of anesthetics on the brains of older NHPs remain unclear. In order to help further describe the window of vulnerability to the neurotoxic effects of anesthetics, the present study was performed to investigate whether the developing brain of nonhuman primates remains vulnerable on postnatal days 20 (P20) or 40 (P40). Of note, the brain development of the P20 rhesus macaque is equivalent to that of a 7-month-old human infant, whereas the P40 approximates that of a 9.5-month-old infant (Workman et al., 2013). Using the same experimental protocols as in our prior studies in P6 animals, we exposed P20 and P40 NHPs to 5 h of isoflurane anesthesia (Brambrink et al., 2012a; Brambrink et al., 2010).

2. Materials and methods

2.1. Animals and experimental procedures

All animal procedures and study protocols were conducted in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of the Oregon National Primate Research Center and Washington University in St. Louis School of Medicine. The subjects for these experiments were 7 female and 5 male infant rhesus macaques that were on average 20 or 40 days old. We included at least one male and one female in each group, and thus did not analyze sex differences. The infant macaques ($n = 3$/group, Table 1) were exposed to either isoflurane for 5 h or no anesthesia.

Infant macaques were taken to an operating room immediately after their dams received intramuscular ketamine sedation (5 to 10 mg/kg; Ketathesia, Butler Schein Animal Health, Dublin, OH). Upon arrival in the operating room, the infant body weight and rectal temperature were recorded. Prior to induction of anesthesia, animals were gently restrained to allow placement of 22-gauge catheter (Introcan Safety, B Braun Medical, Melsungen, Germany) in the saphenous or cephalic vein. Venous blood (0.3 mL) withdrawn from the saphenous vein catheter was used for baseline point-of-care analysis ($CG4^+$ and $EC8^+$ cartridges, iSTAT, Abbott Point of Care, Princeton, NJ). Following blood collection, the catheter was secured in place with tape and maintained throughout the duration of the exposure period in both anesthesia-exposed and control animals. Isoflurane general anesthesia was administered as previously described (Creeley et al., 2014; Brambrink et al., 2012a; Brambrink et al., 2010). Briefly, anesthesia was induced by administering isoflurane via facemask, after which animals were tracheally intubated and mechanically ventilated. Isoflurane (end tidal 1.3–2.5 vol%; Piramal Healthcare, Andhra, India) general anesthesia was regulated to maintain a surgical plane of anesthesia as determined by no movement and not >10% increase in blood pressure or heart rate in response to a mosquito-clamp pinch of the hand or foot (assessed every 30 min). During general anesthesia, the following vital signs were recorded every 15 min: peripheral oxygen saturation, non-invasive blood pressure, heart rate, continuous electrocardiogram, body temperature, end-tidal carbon dioxide, and respiratory rate. Blood gases and metabolic profiles were determined a minimum of every 2 h throughout general anesthesia.

Upon completion of 5 h of isoflurane anesthesia, the isoflurane was discontinued, and animals were extubated when protective reflexes and spontaneous movements returned. The animals were then observed for 3 h in an intensive care unit system (Snyder ICU cage; Snyder MFG, Centennial, CO), which allowed visual monitoring, as well as recording of vital signs, blood gases, and metabolic profiles. Subsequently, the animals received intravenous ketamine (20 mg/kg) and pentobarbital (25 mg/kg). After confirmation of loss of reflexes and deep anesthesia level, the chest was opened, the heart was cannulated, and animals were transcardially perfusion-fixed (4% paraformaldehyde) in preparation for histopathological brain analysis.

Animals that were randomized to the control, no anesthesia group underwent similar handling. The infants were removed from their dams, received an IV catheter, and had baseline vital signs and blood values measured to mimic the stress that animals in the experimental group experienced prior to the induction of anesthesia. Physiologic parameters were collected at two additional time points to mirror isoflurane exposed animals. Eight hours following baseline data collection, final measurements were collected and the awake animals were transferred to pathology for transcardial perfusion as described above.

Table 1
Infant nonhuman primate sex distribution by group.

	Male	Female
P20 Control	$n = 1$	$n = 2$
P20 Isoflurane	$n = 1$	$n = 2$
P40 Control	$n = 2$	$n = 1$
P40 Isoflurane	$n = 1$	$n = 2$

2.2. Histopathology studies

Following in vivo perfusion fixation with 4% paraformaldehyde in phosphate buffer, brains were serially cut into 70 μM sections on a vibratome. Sections were cut coronally in the cerebrum (approximately 800 sections) and sagittally in the cerebellum (approximately 200 sections). Approximately 30 sections per brain (cerebrum + cerebellum) were selected at 2.24 mm intervals and stained using activated caspase 3 (AC3) immunohistochemistry (marker for apoptosis) as previously described (Creeley et al., 2014; Brambrink et al., 2012a; Brambrink et al., 2010).

2.3. Quantification of apoptosis

An experienced neurohistologist blinded to treatment condition counted AC3 positive neurons and oligodendrocytes. Serial brain sections were analyzed via light microscopy with a 10× objective lens and a computer-assisted Microbrightfield Stereo Investigator system (Microbrightfield, Inc., Williston, VT) to record the location and number of dying cells and the dimensions of the field. Location of the cell (white vs. gray matter) and morphological features were used to differentiate AC3 stained neurons from glia. The AC3-positive cell counts were divided by volume in mm^3 to obtain final densities.

2.4. Statistical analysis

Physiological variables are presented as median and range; all other data are presented as means ± standard errors of the mean (SEM). A