Isoflurane-induced Neuroapoptosis in the Neonatal Rhesus Macaque Brain

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ABSTRACT

Background: Brief isoflurane anesthesia induces neuroapoptosis in the developing rodent brain, but susceptibility of non-human primates to the apoptogenic action of isoflurane has not been studied. Therefore, we exposed postnatal day 6 (P6) rhesus macaques to a surgical plane of isoflurane anesthesia for 5 h, and studied the brains 3 h later for histopathologic changes.

Method: With the same intensity of physiologic monitoring typical for human neonatal anesthesia, five P6 rhesus macaques were exposed for 5 h to isoflurane maintained between 0.7 and 1.5 end-tidal Vol% (endotracheally intubated and mechanically ventilated) and five controls were exposed for 5 h to room air without further intervention. Three hours later, the brains were harvested and serially sectioned across the entire forebrain and midbrain, and stained immunohistochemically with antibodies to activated caspase-3 for detection and quantification of apoptotic neurons.

Results: Quantitative evaluation of brain sections revealed a median of 32.5 (range, 18.0–48.2) apoptotic cells/mm³ of brain tissue in the isoflurane group and only 2.5 (range, 1.1–5.2) in the control group (difference significant at P = 0.008). Apoptotic neuronal profiles were largely confined to the cerebral cortex. In the control brains, they were sparse and randomly distributed, whereas in the isoflurane brains they were abundant and preferentially concentrated in specific cortical layers and regions.

Conclusion: The developing non-human primate brain is sensitive to the apoptogenic action of isoflurane and displays a 13-fold increase in neuroapoptosis after 5 h exposure to a surgical plane of isoflurane anesthesia.

What We Already Know about This Topic

- Prolonged ketamine infusion causes programmed cell death (apoptosis) in brains of neonatal non-human primates
- Brief anesthesia also causes apoptosis in neonatal rodents

What This Article Tells Us That Is New

- Five hours of isoflurane anesthesia in 6-day-old non-human primates caused a large increase in neuronal apoptosis in several brain regions 3 h later, although the effect of this on cognitive development is not known.

ACUTE exposure of infant rats or mice to several classes of drugs, including those that block N-methyl-D-aspartate glutamate receptors and those that activate γ-aminobutyric acid-A receptors and ethanol (which has both N-methyl-D-aspartate antagonist and γ-aminobutyric acid-mimetic properties), triggers widespread apoptotic death of neurons in the developing brain.1–5 The window of peak vulnerability to the apoptogenic mechanism coincides with the developmental period of rapid synaptogenesis,1,2 also known as the brain growth spurt period, which in mice and rats occurs primarily during the first 2 weeks after birth, but in humans extends from about mid-gestation to several years after birth.6

The cell death process triggered by these drugs has been demonstrated at both light and electron microscopic levels to have classic morphologic characteristics of apoptosis,1,3,5,7,8 a form of cell death in which the cell actively mediates its own demise. Studies undertaken to examine gene-regulated biochemical pathways revealed that the cell death process in-isoflurane-induced neuroapoptosis in the Neonatal Rhesus Macaque Brain
duced by these drugs involves translocation of Bax protein to mitochondrial membranes where it disrupts membrane permeability, allowing extramitochondrial leakage of cytochrome c, followed by a sequence of changes culminating in activation of caspase-3. Commitment to cell death occurs before the caspase-3 activation step; therefore, immunohistochemical detection of neurons positive for activated caspase-3 (AC3) provides a reliable means of mapping and quantifying dying cells that have already progressed beyond the point of cell death commitment. Accordingly, AC3 immunohistochemistry has been used extensively for marking dying neurons in recent studies focusing on drug-induced developmental neuroapoptosis.

The observation that N-methyl-d-aspartate antagonist and γ-aminobutyric acid-mimetic drugs induce widespread neuronal degeneration in the developing brain has relevance in a public health context, because there are many agents in the human environment that have N-methyl-d-aspartate antagonist or γ-aminobutyric acid-mimetic properties, including drugs that are sometimes abused by pregnant mothers (ethanol, phencyclidine, ketamine, nitrous oxide, barbiturates, and benzodiazepines), and many drugs used worldwide in obstetric and pediatric medicine as anticonvulsants, sedatives, or anesthetics. Particularly, concerning is mounting evidence that even single or brief exposure to clinically relevant doses of commonly used anesthetics (ketamine, midazolam, propofol, isoflurane, sevoflurane, and chloral hydrate) may trigger a significant neuroapoptosis response in the developing rodent brain.

It has also been reported that exposure of the developing rodent brain to these agents can result in long-term neurobehavioral impairments. In addition, there is preliminary evidence potentially linking anesthesia exposure in infancy with long-term neurobehavioral deficits in both humans and non-human primates.

Research aimed at clarifying whether anesthetic drugs can trigger neuroapoptosis in the developing non-human primate brain is limited to studies in which it was reported that intravenous infusion of ketamine triggered neuroapoptosis in the postnatal day 5 (P5) rhesus macaque brain if the infusion was continued for 9 or 24 h, but not if it was stopped at 3 h. Potent volatile anesthetic agents, such as isoflurane, are frequently used to administer general anesthesia to pregnant women and to human neonates and infants requiring surgery. Therefore, we undertook the current study in which P6 rhesus macaque neonates were exposed to a surgical plane of isoflurane anesthesia for 5 h, and the brains were processed 3 h later for subsequent histopathologic evaluation.

Materials and Methods

Animals and Experimental Procedures

All animal procedures were approved by the Oregon National Primate Research Center (Beaverton, OR) and Washington University Medical School (St. Louis, MO) Institutional Animal Care and Use Committees and were conducted in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The subjects for these experiments were infant rhesus macaques that were exposed for 5 h either to room air (n = 5) or to isoflurane (end tidal 0.7–1.5 vol%; n = 5). Sex distribution in both the control and isoflurane groups was three

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females and two males. The mean age of the control and isoflurane animals was 5.6 and 6.0 days old, respectively. Criteria for maintaining a surgical plane of anesthesia were no movement and not more than 10% increase in heart rate or blood pressure in response to a profound moskito-clamp pinch at hand and foot (checked every 30 min). After mask induction (isoflurane), animals were tracheally intubated (semirigid fiberoptic endoscope; Karl Storz America, El Segundo, CA; 2.0 ID endotracheal tube; Mallinckrodt, Hazelwood, MO), mechanically ventilated (small animal ventilator; Harvard Apparatus, Holliston, MA), and maintained using full physiologic monitoring (continuous end-tidal carbon dioxide, end-tidal isoflurane, FiO₂ [Capnomac; Datex Ohmeda, Madison, WI], electrocardiogram, peripheral oxygen saturation, noninvasive blood pressure [every 15 min], rectal temperature [Surgivet Advisor V9200; Smith Medical, Wankesha, WI], blood gases, metabolic profile including pH, base excess, blood urea nitrogen, hematocrit, hemoglobin, Na, Cl, K, and serum glucose and lactate levels [every 2 h; i-STAT, Princeton, NJ]). After extubation of the trachea, animals were kept for 3 h in an infant monkey incubator (Snyder ICU cage; Snyder MFG, Centennial, CO) and were formula fed as tolerated. After the 3-h observation period, the animals received high-dose phenobarbital and were transcardially perfusion fixed to prepare the brain for histopathologic analysis.

**Histopathology Methods**

For histopathologic analysis, brains were cut serially into 70-μm coronal sections across the entire rostro-caudal extent of the forebrain and midbrain (~800 sections per brain). Sections were selected at 2.24-mm intervals (~25 sections per brain) and stained for detection of apoptotic profiles by AC3 immunohistochemistry, as described previously.3,8–25 Before applying the AC3 stain, sections were processed for antigen retrieval to reduce background nonspecific staining and maximize the AC3-specific signal. For antigen retrieval, sections were immersed in a citrate buffer, pH 6.0, and subjected to heat in a pressure cooker for 10 min.

We chose the AC3 staining procedure for several reasons: (1) it has been shown in numerous previous studies3,8–25 to faithfully mark neurons that are undergoing apoptotic degeneration after exposure to various apoptogenic drugs, including isoflurane15,16,22; (2) it has been demonstrated that cellular profiles marked by the AC3 stain are also marked by the cupric silver stain that selectively marks neurons that are dead or dying1–5,12; (3) it has been confirmed by electron microscopy that these cells display all of the classic ultrastructural characteristics of neurons undergoing apoptotic cell death.1–3,7,8,12 In addition, it has been found9 that the AC3 stain is valuable not only for identifying neurons that are undergoing apoptosis but also for revealing whether the cell is in an early or advanced stage of degener-
Physiologic Variables in the Experimental Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Temp, °C</th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
<th>ETCO₂, %</th>
<th>pH (venous)</th>
<th>P CO₂, mmHg</th>
<th>Hb, mg/dl</th>
<th>Glucose, mm</th>
<th>Lactate, mm</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>0.5 h</td>
<td>2.5 h</td>
<td>4.5 h*</td>
<td>3-h Recovery</td>
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<td>Control</td>
<td>37.7 (37.3–38.2)</td>
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<td>Isoflurane</td>
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<td>37.3 (35.5–38.2)</td>
<td>37.9 (37.6–38.6)</td>
<td>37.9 (37.3–38.6)</td>
<td>38.3 (37.7–38.3)</td>
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<td>Isoflurane</td>
<td>—‡</td>
<td>176 (150–193)</td>
<td>185 (146–201)</td>
<td>169 (149–217)</td>
<td>204 (160–233)</td>
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<tr>
<td>Isoflurane</td>
<td>—‡</td>
<td>27 (22–34)</td>
<td>27 (26–31)</td>
<td>28 (22–33)</td>
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<td>7.30 (7.27–7.34)</td>
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<td>7.31 (7.25–7.37)</td>
<td>7.34 (7.33–7.47)</td>
<td>7.37 (7.35–7.39)</td>
<td>7.38 (7.34–7.43)</td>
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<td>43 (39–56)</td>
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<td>18 (16–19)</td>
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<td>Control</td>
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<td>15 (12–17)</td>
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<td>Isoflurane</td>
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<td>12 (9–17)</td>
<td>10 (7–11)</td>
<td>9 (9–11)</td>
<td>14 (14–17)</td>
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<td>Control</td>
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<td>77 (55–159)</td>
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<td>Isoflurane</td>
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<td>75 (58–97)</td>
<td>103 (78–112)</td>
<td>82 (63–177)</td>
<td>63 (56–73)</td>
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<td>Control</td>
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<td>—</td>
<td>4.5 (1.9–5.3)</td>
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<td>Isoflurane</td>
<td>—‡</td>
<td>5.1 (2.6–10.1)</td>
<td>3.6 (2.4–4.8)</td>
<td>3.5 (2.6–4.8)</td>
<td>3.4 (3.1–5.2)</td>
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* Data are presented as median (range).
† Variables not obtained.
‡ Data represent n = 4, because of a spurious sample from Iso-1. **P < 0.05 vs. control.

Statistical Evaluation

There are important ethical and logistical considerations regarding non-human primate experimentation. It is important to include as few animals as needed to answer research questions with as much precision as possible. As there are no published data indicating the amount of potential neuroapoptosis after exposure of the neonatal macaque to isoflurane, it was difficult to estimate how many animals might be needed for this study. However, previous experiments in non-human primates and rodents have shown that, when neonatal animals are exposed to drugs that consistently and potently induce neuroapoptosis, the apoptosis is extensive and can be demonstrated compellingly with a small number of animals, typically between three and seven animals per group. Therefore, we decided a priori to use five experimental and five control animals, present the raw data for each group graphically, with medians and full ranges for density counts of apoptotic neurons per cubic millimeter of brain tissue, and compare median difference in density counts between the isoflurane and control groups using the Mann–Whitney U statistical test. A two-sided P value less than 0.05 would be judged significant, and the 95% confidence interval for the median difference would provide a measure of precision. Statistical analysis was performed with Analyze-It® Statistical Software (Leeds, United Kingdom) for Microsoft Excel® (Redmond, WA).

Table 1. Physiologic Variables in the Experimental Groups

<table>
<thead>
<tr>
<th>Group (n = 5)</th>
<th>Time</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>Temp, °C</td>
<td>37.7 (37.3–38.2)</td>
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<tr>
<td>MAP, mmHg</td>
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<td>ETCO₂, %</td>
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<td>pH (venous)</td>
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<td>Hb, mg/dl</td>
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<td>Glucose, mm</td>
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<td>Lactate, mm</td>
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</table>

Data are presented as median (range).
* Data represent n = 4, because of a spurious sample from Iso-1.
† P < 0.05 vs. control.
‡ Variables not obtained.

EtCO₂ = end-tidal carbon dioxide; Hb = hemoglobin; HR = heart rate; MAP = mean arterial pressure (noninvasive); P CO₂ = venous carbon dioxide tension; PVO₂ = venous oxygen tension; Temp = temperature (rectal).
Results

All animals randomly assigned to the experimental protocol survived induction and maintenance of anesthesia. Tracheal intubation and mechanical ventilation allowed the controlled administration of the volatile anesthetic at the desired level, and maintenance of vital signs, blood gases, and metabolic values within normal limits (see figs. 1–3, Supplemental Digital Content 1, which contains graphs showing mean arterial pressure, serum glucose, and hemoglobin levels, http://links.lww.com/ALN/A573; also see table 1, Supplemental Digital Content 2, which is a table listing physiologic data, http://links.lww.com/ALN/A574). Continuous fluid and glucose application as well as active warming ensured homeostasis throughout the entire experimental period. Recovery was fast after cessation of isoflurane delivery and animals were extubated without complications and maintained in an animal incubator at physiologic conditions under direct visual observation. All infants tolerated formula milk within the first 2 h after the end of the anesthetic.

Quantitative evaluation of AC3-stained brain sections across the entire extent of the forebrain and midbrain revealed that the median density (range) of AC3-positive neuronal cells per cubic millimeter of brain tissue in the five isoflurane-exposed brains was 32.5 (18.0–48.2) and in the five control brains was only 2.5 (1.1–5.2) (fig. 1). This translates to a 13-fold increase in density of apoptotic cells in the brains of the animals that were exposed to isoflurane. The difference in the median density of apoptotic cells per cubic millimeter of brain tissue between the isoflurane and control groups was 30 (95% confidence interval, 15.5–45.7, \( P = 0.008 \)).

The apoptotic neurons were located primarily in various divisions of the cerebral cortex. In the controls, they were sparse and randomly distributed, whereas in the isoflurane-exposed brains they were more abundant and tended to be densely concentrated in specific cortical regions or layers. In the isoflurane-exposed brains, the primary visual cortex, known as the striate cortex because of its conspicuous laminar organization, had an exceedingly high density of AC3 positive profiles, which were concentrated primarily in layers II and V (figs. 2 and 3). The next most severely affected regions were the temporal and somatosensory cortices (figs. 4 and 5). All cortical regions of the isoflurane-exposed brains were more severely affected than any cortical region in the control brains. The frontal cortex, which is reportedly more sensitive than other brain regions to ketamine-induced neuroapoptosis in P5 rhesus macaques,19,20 was more severely affected in the isoflurane brains than in the concurrent controls but was relatively spared compared with other cortical regions of the isoflurane-exposed brains (fig. 4).

Morphologic analysis of the stained profiles of individual cells revealed that both early and advanced stages of degeneration could be detected (figs. 3, 5, and 6), but the majority of AC3-positive profiles were in a relatively early stage. The reaction was not confined to a specific cell type, but rather affected several different cell types, including large multipolar neurons (layers V and VI), pyramidal neurons both large and small (layers IV and V), and interneurons, especially those concentrated in layer II of various divisions of the cerebral cortex (figs. 3 and 5). An important distinction between the appearance of stained cells in the control brains versus the isoflurane-exposed brains was that the majority of stained profiles in the control brains were in a late stage (rounded-up cell body with few or no intact processes), and the majority in the isoflurane brains were in an earlier stage. This reflects the fact that the latter are responding within an acute time frame to a recent drug challenge, whereas the former have begun degenerating spontaneously at various more remote intervals in the absence of any drug challenge.

Discussion

We have demonstrated that exposing P6 infant rhesus macaques to a moderate surgical plane of anesthesia for 5 h,
while maintaining physiologic stability, is associated with a 13-fold increase in the rate of neuroapoptosis compared with nonanesthetized controls. The increased neurodegeneration was demonstrable in widespread distribution across all divisions of the cerebral cortex. The reaction was most severe in the primary visual cortex, least severe in the frontal cortex, and of intermediate severity in the remaining cortical regions. A wide variety of neuronal cell types were affected, and while the majority were in an early stage of degeneration when examined 3 h after cessation of anesthesia, some cell populations showed morphologic evidence of being in various stages of advanced degeneration. This signifies, consistent with observations in rodent studies,3,9,34 that some neuronal populations are more sensitive to the toxic stimulus and, therefore, begin dying earlier.

Because rhesus macaques are neurodevelopmentally precocious compared with humans, a P6 rhesus infant is approximately equivalent to a 6-month-old human infant (fig. 7), or a P8 to P12 infant rodent.6 In rodents during the early postnatal period (P1–P3), subcortical neurons are vulnerable and cerebrocortical neurons are spared, then there is a period (P4–P7) when both cortical and subcortical neurons are vulnerable, and finally a period (P8–P12) when cerebrocortical neurons remain vulnerable while subcortical vulnerability is waning.1,2 During this latter period, the cortical neurons most severely affected are in layers II, IV, and V. The pattern of neurodegeneration observed in the isoflurane-exposed P6 rhesus macaque was limited primarily to various divisions of the cerebral cortex and preferentially affected layer II interneurons and pyramidal neurons in layers IV and V. Thus, the
Degenerative reaction to isoflurane in the P6 rhesus macaque is consistent with the findings in P8 to P12 infant rodents, both with respect to brain regions and cell types that are preferentially involved. This suggests the type of reaction that might occur in the human infant brain if humans are susceptible to this toxic mechanism. Whether humans are susceptible is not a question that can be answered definitively by research on non-human species.

Slikker et al.\textsuperscript{19,20} reported that an intravenous infusion of ketamine induces a neuroapoptosis reaction in the P5 rhesus macaque brain if the infusion is continued for 9 or 24 h, but not if it is stopped at 3 h. These authors confined their quantitative evaluation to a single brain region, the frontal cortex, after making the preliminary qualitative observation that this region of the ketamine-exposed brains was rich in degenerating profiles compared with several subcortical regions (striatum, thalamus, hippocampus, and amygdala). Our findings are in agreement with theirs, regarding susceptibility of the neonatal macaque brain to anesthesia-induced neuroapoptosis and with respect to cerebrocortical neurons being more sensitive than subcortical neurons at this developmental age. Regarding the potential implication of their findings that it may require at least 9 h of anesthesia exposure to induce neuroapoptosis, our findings clarify, consistent with observations in rodents,\textsuperscript{13,14,16} that a 5-h exposure period is sufficient and that the apoptotic response can be detected as early as 3 h after a 5-h exposure period. Regarding their finding that 3 h of ketamine anesthesia was not sufficient to induce neuroapoptosis in the neonatal non-human primate brain, this important issue warrants further investigation, in which their observation is based on quantitative data gathered only from a single brain region (frontal cortex) and from a small number (n = 3) of experimental subjects. In light of our observation in P6 rhesus macaques that the frontal cortex, although susceptible to isoflurane-induced neuroapoptosis at this developmental age, was less sensitive than other regions of the cerebral cortex, in future research examining this issue it will be important to include all regions of the cerebral cortex in the quantitative evaluation.

In summary, our findings indicate that exposure of the P6 rhesus macaque brain for 5 h to isoflurane, under clinically relevant conditions, induces widespread neuroapoptosis affecting all divisions of the cerebral cortex. Our studies leave unanswered whether shorter durations of isoflurane anesthesia would also trigger acute apoptotic neurodegeneration and whether either brief or prolonged exposure of the infant non-human primate brain to isoflurane can cause long-term neurocognitive deficits. Research addressing these important questions is urgently needed.

References


Fig. 7. Timing of the brain growth spurt period in human versus non-human primates. The curves represent the changing rates of brain growth for humans and rhesus monkeys in the perinatal period. In both species, the rate of brain growth increases rapidly to a peak level then gradually descends to a lower level sustained into adolescence. Note that the timing of the growth spurt in relation to birth occurs earlier in monkeys than in humans, and it tapers down to a low level at an earlier age. Thus, in the monkey infant at postnatal day 6, the brain growth rate has decreased to about 25% of its peak value, whereas in the human infant at postnatal day 6, the brain growth rate is just reaching its peak and does not taper down to the 25% level until the age of 6 months (see dashed line). Therefore, in terms of comparative brain growth spurt timing, the 6-day-old monkey infant is the equivalent of a 6-month-old human infant. Another relevant comparison is that the brain weight of the infant rhesus monkey at birth is 76% of its adult brain weight, whereas the brain weight of the newborn human is only 27% of its adult brain weight.\textsuperscript{6} Adapted from Early Hum Dev 1979; 3:79–83.\textsuperscript{6}