Published in final edited form as:

Anesthesiology. 2014 March ; 120(3): 626–638. doi:10.1097/ALN.0000000000000037.

Isoflurane-induced Apoptosis of Neurons and Oligodendrocytes in the Fetal Rhesus Macaque Brain

Catherine E. Creeley, Ph.D., Krikor T. Dikranian, M.D., Ph.D., Gregory A. Dissen, Ph.D., Stephen A. Back, M.D., Ph.D., John W. Olney, M.D., and Ansgar M. Brambrink, M.D., Ph.D.

Department of Psychiatry (C.E.C., J.W.O.) and Department of Anatomy and Neurobiology (K.T.D.), Washington University School of Medicine, St. Louis, Missouri, and the Division of Neuroscience (G.A.D.) and Department of Anesthesiology and Perioperative Medicine (A.M.B.), Oregon National Primate Research Center, Portland, Oregon, and the Department of Pediatrics (S.A.B.), Oregon Health and Science University, Portland, Oregon

Abstract

Background—The authors have previously shown that exposure of the neonatal nonhuman primate (NHP) brain to isoflurane for 5 h causes widespread acute apoptotic degeneration of neurons and oligodendrocyte. The current study explored the potential apoptogenic action of isoflurane in the fetal NHP brain.

Methods—Fetal rhesus macaques at gestational age of 120 days (G120) were exposed *in utero* for 5 h to isoflurane anesthesia (n = 5) or to no anesthesia (control condition; n = 4), and all regions of the brain were systematically evaluated 3 h later for evidence of apoptotic degeneration of neurons or glia.

Results—Exposure of the G120 fetal NHP brain to isoflurane caused a significant increase in apoptosis of neurons and of oligodendrocytes at a stage when oligodendrocytes were just beginning to myelinate axons. The neuroapoptosis response was most prominent in the cerebellum, caudate, putamen, amygdala, and several cerebrocortical regions. Oligodendrocyte apoptosis was diffusely distributed over many white matter regions. The total number of apoptotic profiles (neurons + oligodendrocytes) in the isoflurane-exposed brains was increased 4.1-fold, compared with the brains from drug-naïve controls. The total number of oligodendrocytes deleted by isoflurane was higher than the number of neurons deleted.

Conclusions—Isoflurane anesthesia for 5 h causes death of neurons and oligodendrocytes in the G120 fetal NHP brain. In the fetal brain, as the authors previously found in the neonatal NHP brain, oligodendrocytes become vulnerable when they are just achieving myelination competence. The neurotoxic potential of isoflurane increases between the third trimester (G120) and the neonatal period in the NHP brain.

There is mounting evidence that anesthetic and anticonvulsant drugs and alcohol cause widespread apoptotic neurodegeneration in the developing brains of several animal species,^{1–5} including nonhuman primates (NHPs),^{6–12} and that neonatal exposure to these agents is associated with long-term neurobehavioral disturbances in both rodents^{3,13–17} and NHPs.¹⁸ More importantly, there is recent evidence from several independent research groups suggesting that exposure of human infants to brief anesthesia is associated with

Copyright © 2013, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins.

Address correspondence to Dr. Brambrink: Department of Anesthesiology and Perioperative Medicine, Oregon Health and Science University, UHN-2, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239-3098. brambrin@ohsu.edu.

The first two authors contributed equally to this article.

The authors declare no competing interests.

increased risk for neurobehavioral impairment.^{19–25} Further complicating the picture is evidence that exposure of infant rhesus macaques to isoflurane causes apoptosis not only of neurons, but also of glial cells. Our recent findings^{10,11} indicate that in the neonatal NHP brain the vulnerable glial cell type is in the oligodendrocyte lineage, and oligodendrocytes become vulnerable at a time when they are just beginning to myelinate axons that interconnect neurons throughout the central nervous system. In the isoflurane-exposed neonatal NHP brains, apoptotic oligodendrocytes were distributed diffusely throughout many white matter (WM) regions, and the total number of dying oligodendrocytes in WM was approximately equal to the total number of dying neurons in gray matter.

Isoflurane and related halogenated ethers are frequently used in pediatric and obstetric anesthesia. Thus, it is not uncommon for a human neonate or premature infant to be exposed to these agents. At this stage, the neurodevelopmental age of a human infant is equivalent to that of a third-trimester NHP fetus. Nothing is known regarding the vulnerability of the fetal primate brain to the apoptogenic action of isoflurane on either neurons or glia. Therefore, we administered isoflurane to pregnant rhesus macaques for 5 h at a gestational age of 120 days (G120; approximately mid-third trimester), and delivered the fetus by cesarean section 3 h later for histopathological evaluation of the brains. Here we will describe the effects of isoflurane on both neurons and glia in the G120 NHP brain, and will compare these effects in the fetal brain with those we have described previously in the isoflurane-exposed neonatal NHP brain.

Materials and Methods

Animals and Experimental Procedures

The study protocol and all related procedures were conducted with the approval of the Animal Care and Use Committees of the Oregon National Primate Research Center at Oregon Health and Science University and Washington University Medical School, and were in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The methods used to provide general anesthesia for the study animals were similar to those previously reported^{9,10} for anesthesia exposure of NHP fetuses, and were in keeping with standards for human pediatric anesthesia currently endorsed by the American Society of Anesthesiologists and similar professional societies.

In brief, the subjects were time-mated pregnant rhesus macaques at a gestational age of 120 days (G120; full term for these macaques = 165 days), and were exposed for 5 h to isoflurane (n = 5; range, G118–G122) or no anesthesia (n = 4; range, G117–G124). Animals received an IV catheter followed by propofol (2 mg/kg IV bolus), placed on standard American Society for Anesthesiology monitoring, and were tracheally intubated for airway protection and to allow mechanical ventilation and precise application of isoflurane (direct laryngoscopy, 4.0 ID endotracheal tube [Mallinckrodt, Hazelwood, MO]; veterinarian ventilator; Hallowell EMC [Pittsfield, MA]; end-tidal 1.0–1.5 volume-%; Capnomac [Datex Ohmeda, Madison, WI]). The volatile anesthetic was titrated according to a predefined clinical endpoint that represents an intermediate surgical plane of anesthesia (deep nail-bed stimulation at hand and foot [mosquito-clamp pinch] results in no motor response and only a mild sympathetic response evident by an increase of 10% or less in heart rate or blood pressure; monitored every 30 min). This type of response to the standardized deep nail-bed stimulation is typical for a level of anesthesia that would allow skin incision and the initiation of a moderate surgical intervention. Anesthetic management was aimed at maintaining homeostasis. Extended physiologic monitoring included end-tidal gas measurements, electrocardiogram findings, blood pressure and peripheral oxygen saturation, esophageal temperature, as well as blood gases and metabolic status.^{8–10} IV fluids and

glucose were provided and animals were warmed as needed. Fetal heart rate was determined every hour by using ultrasonography.^{9,10}

At 5 h after time zero, isoflurane was discontinued, the animals were extubated after 10–15 min, and returned to their cages when appropriate for the remainder of the 3-h observation period. At 8 h after time zero, general anesthesia was induced again after the above strategy, followed by immediate cesarean section under aseptic conditions and following state-of-the-art standard operating procedures established at Oregon National Primate Research Center (skin incision to delivery <5 min). Samples from umbilical venous and arterial blood were used to determine fetal and placenta well-being, and after brief morphologic measurements (body weight; dimensions of hand, foot, biparietal diameter, crown-to-rump; <1 min) the fetus received IV phenobarbital (umbilical vein) to induce deep barbiturate coma and allow *in vivo* transcatheter perfusion fixation according to National Institutes of Health guidelines in order to prepare the brain for histopathological analysis.

After separation from the fetus, the dam was managed according to Oregon National Primate Research Center standard operating procedures (balanced anesthesia including opioid analgesics until completion of operative procedure; appropriate postoperative pain medication; return to home cage).

Dams randomized to the control group (no anesthesia; n = 4) were handled exactly the same way as described above for the isoflurane-treated animals, but instead of being administered general anesthesia these animals received IV saline. They were then returned to their cage with access to food and water *ad libitum* for the remainder of the experimental period. At 8 h after time zero, the fetus was removed *via* cesarean section under general anesthesia. Further handling of the fetus including *in vivo* brain perfusion was conducted as described above, and the dam was further managed according to the above paradigm.

Histopathology Methods

The histopathologic evaluation was performed using similar strategies as reported previously,^{10,11} and additional analytic means were employed. In brief, after *in vivo* perfusion fixation (4% paraformaldehyde) and serial cutting of the entire brains (70 μ M sections), four series of adjacent sections (2-mm intervals; across the entire brain) were individually stained to determine the effects of isoflurane anesthesia on both neurons and glia in the fetal NHP brain. In particular, activated caspase-3 (AC3) nonfluorescent immunohistochemistry (first series) was used as a marker for cellular apoptosis,^{8–13} in order to track the morphological changes (progression from early to late stages) and to allow counting and mapping the location of both apoptotic neurons and glia after isoflurane exposure. DeOlmos cupric silver staining (second series) was used as a marker for all cells that were dying or were already dead. Fractin immunohistochemistry (third series) was used as a marker of caspase-mediated proteolysis in brain tissue,^{26,27} and myelin basic protein (MBP) immunohistochemistry (fourth series) was used to visualize premyelinating or myelinating oligodendrocytes.^{10,28} In addition, immunofluorescent double staining experiments were conducted to evaluate type and maturational state of cells that undergo apoptosis after isoflurane exposure by using AC3 (marker for apoptosis), glial fibrillary acidic protein (marker for astrocytes), Iba1 (marker for microglia and macrophages), platelet-derived growth factor receptor- α (marker for oligodendrocyte progenitors), MBP (marker for premyelinating and myelinating oligodendrocytes), CC-1 (marker for mature oligodendrocytes), and NeuN (marker for mature neurons). All immunohistochemical double stainings were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize changes in nuclear chromatin pattern indicative of apoptotic cell death.

On the basis of our recent findings^{10,11} that glial apoptosis in the neonatal NHP brain impinges on oligodendrocytes at a time when they are beginning to achieve myelination potential, we conducted a pilot experiment aimed at gaining insight into the myelination status of the G120 fetal NHP brain. Traditionally, luxol fast blue has been used to assess myelination, but recent research²⁹ indicates that immunohistochemical staining with antibodies to MBP provides a sensitive measure of the time of onset of myelination in various central nervous system regions. MBP staining detects oligodendrocytes that are terminally differentiated at either the premyelinating or myelinating state.^{10,28} Therefore, using MBP immunohistochemistry we stained sections at several rostrocaudal levels from the frontal pole to the cervical spinal cord to define the extent of myelination. We undertook a detailed analysis of three levels: prefrontal regions rostral to the septum; a midrostrocaudal level that included the internal capsule, centrum semiovale and the globus pallidus; and a caudal level of the brain, which included the brain stem and cerebellum.

Specific staining protocols^{10,11} were as follows: Our DeOlmos cupric silver method and nonfluorescent staining methods for AC3 have been described previously.^{4,8-12} For MBP (1:100; MAB 395; EMD Millipore, Billerica, MA), fractin (1:400; AB3150; EMD Millipore), Iba1 (1:500; 019-18741; Wako Chemicals, Richmond, VA), and CC-1 (1:200; OP80; Calbiochem, San Diego, CA) immunostaining methods, we used the Vectastain Elite ABC kit with Vector VIP as chromogen (Vector Laboratories, Inc., Burlingame, CA).^{4,8-12} Immunofluorescent detection of caspase-mediated cell death employed an AC3 rabbit primary polyclonal antibody (9661B; 1:500; Cell Signaling Technology, Inc., Danvers, MA). Floating sections were incubated overnight at room temperature. After the sections were rinsed in phosphate-buffered saline (3 × 5 min) they were incubated for 2 h at room temperature with fluorescent goat antirabbit Alexa Fluor 555 (1:1,000, Invitrogen™, Life Technologies™, Grand Island, NY), rinsed in phosphate-buffered saline, and coverslipped with Vectashield mounting medium. Double staining for AC3 with MBP (1:100, MAB 395; EMD Millipore), NeuN (1:200, MAB 377; EMD Millipore), and glial fibrillary acidic protein (1:500; Mab360; EMD Millipore) was accomplished using complementary fluorescent secondary antibodies (1:1,000, Alexa Fluor 488). Double staining for AC3 with platelet-derived growth factor receptor- α employed a goat anti-platelet-derived growth factor receptor- α antibody (1:6; AF-307-NA; R&D Systems, Inc., Minneapolis, MN) followed by complementary fluorescent secondary antibodies (1:100 Alexa Fluor 488 and Alexa Fluor 555; Invitrogen™). Double staining for Fractin (1:400; AB3150; EMD Millipore) and MBP (1:100; MAB 395; EMD Millipore) was achieved using complementary fluorescent (1:1,000; Alexa Fluor 488 and Alexa Fluor 555; Invitrogen™) secondary antibodies. Before CC-1 staining, sections were exposed to antigen retrieval treatment (10 mM Trizma-Base, 1 mM EDTA, pH 9.0; 90°C water bath for 10 min). All immunofluorescent colabeling experiments included DAPI in the mounting medium (Vectashield; Vector Laboratories) to clarify whether cellular profiles staining positive for apoptosis markers also showed nuclear chromatin pattern indicative of apoptotic cell death (condensation and clumping into one or more large spherical or crescent-shaped bodies). Regions of interest were viewed and digital images were obtained by using a Leica DFC310 Microsystems fluorescence microscope (North Central Instruments, Inc., Plymouth, MN) operated by Leica Application Suite software (Leica Microsystems, Inc., Buffalo Grove, IL).

Quantification of Apoptosis

The quantitative analysis of cellular apoptosis used the same approach as reported previously,⁸⁻¹¹ and was conducted by an investigator blinded to the treatment conditions. In brief, brain sections were selected in an unbiased manner at 2-mm intervals from serial sections cut across the entire brain and analyzed by using a computerized light microscopy system (×10; Microbrightfield Inc., Williston, VT). On each section, each AC3-positive

profile was identified and, on the basis of location and morphological appearance, precisely plotted in the scanned area as either a red dot (neuron) or white dot (glia) using the computer interface. The computerized information (brain area scanned; number of sections per brain, number of plotted neurons or glia) allowed estimation of the total number, as well as the density (per mm³), of apoptotic profiles for the entire brain or any given region within the brain. In addition, computer plots showing the regional distribution of stained profiles were generated and plots for each region of each brain for each treatment condition were compared. The final AC3 counts were then used to compare the magnitude of apoptotic degeneration of neurons and glia in the control *versus* isoflurane-exposed brains.

Statistical Analysis

Data are presented as mean $\pm$ SD. Student *t* test with Welch correction, where appropriate, was used. A two-sided *P* value less than 0.05 was judged significant and the 95% CI for the mean difference provided a measure of precision. Statistical analysis was performed with Prism Software (GraphPad Software, Inc., La Jolla, CA).

Results

On postconception day 120 ± 1 (mean $\pm$ SEM), dams were randomized to receive general anesthesia (isoflurane group; body weight range, 6.8–10.6 kg; *n* = 5) or no anesthesia (control group; 6.9–10.6 kg; *n* = 4). Their baseline physiology was within normal limits at the time of induction of anesthesia (IV propofol). A conventional anesthesia machine allowed the precise application of the volatile anesthetic isoflurane that was titrated to a defined plane of anesthesia and monitored breath-by-breath by using an anesthetic gas monitor (see Materials and Methods for details). Standard methods for intraoperative anesthesia management published by the American Society for Anesthesiology resulted in species-specific physiologic conditions throughout the experimental period, documented by hemodynamic variables, body temperature, blood gases, acid–base status, laboratory variables, glucose and lactate level (table 1). Fetal heart rates during the anesthetic remained within physiologic limits and varied in concert with the maternal heart rate (table 1). Recovery from anesthesia was fast and all animals were extubated without problems within 15 min after the administration of isoflurane was stopped. Subsequently, the dams recovered quickly to baseline alertness and were kept in a climatized cage under direct observation until scheduled cesarean section. All dams tolerated the surgical procedure well and retained stable vital signs, blood gases, and acid–base levels within physiologic limits (table 2). The time from induction of general anesthesia to clamping of the umbilical cord of the fetuses was less than 10 min in all cases, and fetal heart rate monitoring before surgical incision and umbilical arterial and venous blood samples confirmed fetal well-being until the cord was clamped (table 2), which was followed immediately by transcatheter perfusion fixation as described in Materials and Methods. Fetal body weight (isoflurane group 207–280 g; control group 129–252 g) as well as other standard body dimensions were age appropriate for the macaque G120 time point. Animals assigned to the control group had unremarkable baseline measurements and underwent cesarean section 8 h later according to the same protocol and monitoring. Immediately before and during cesarean section the measured physiologic variables (including maternal hemodynamics, fetal heart rate, temperature, as well as metabolic status and blood gases of dam and fetus) were within physiologic limits and comparable to those in the isoflurane group (table 2).

Patterns of Cell Death in Gray Matter and White Matter

The regional distribution of cells undergoing acute apoptotic degeneration, as assessed by AC3 staining, in the G120 NHP fetal brain at a representative midrostrocaudal level, or at the level of the brain stem and cerebellum, after exposure to isoflurane or no anesthesia is

illustrated in figure 1. Neuronal degeneration at the midrostrocaudal level in the isoflurane-exposed brains was prominent in several regions, including the caudate nucleus, putamen, nucleus accumbens, amygdala, and several divisions (frontal, parietal, temporal) of the neocortex. At the cerebellar–brain stem level, the highest density of degenerating neurons was in the internal granule cell layer of the cerebellum and in the inferior colliculus. Degenerating glial profiles were distributed diffusely throughout the WM at all levels of the neuraxis, and the distribution pattern conformed to the location of specific WM tracts and pathways at each level. The pattern of degeneration in control brains was similar to the pattern in isoflurane-exposed brains but the magnitude of degeneration was much greater in the latter (see Quantitative Evaluation below).

Neuronal Response to Isoflurane Exposure

In the cerebral cortex, the majority of AC3-positive neuronal profiles were large cells with prominent apical dendrites and were distributed in layers two to four (fig. 2A). Immunofluorescent double staining demonstrated that NeuN consistently colocalized with AC3 in these cells, confirming their neuronal identity, and DAPI counterstaining revealed changes in the pattern of nuclear chromatin, indicative of apoptotic cell death (fig. 2, B and C).

In the caudate and putamen there was a high density of AC3-positive cells, which did not colabel with other markers for either neurons or glia. These AC3-positive cells were found in dense clusters throughout the caudate nucleus and putamen. The majority had medium sized, oval-shaped cell bodies, but a small subset of these often included one or two large multipolar profiles (fig. 2D). We have previously described and illustrated both, the medium-sized and large profiles in caudate/putamen undergoing apoptotic cell death after exposure of the NHP fetal brain to alcohol¹² or after exposure of the infant rodent brain to alcohol or anesthetic drugs.^{3,4}

In the cerebellum a large number of AC3-positive profiles were found in the inner portion of the internal granule cell layer (fig. 2E) that did not stain for NeuN. This is the same cell population that we have previously described as being highly sensitive to alcohol-induced degeneration in the fetal NHP brain¹² and infant mouse brain.³⁰ At an earlier age in both mouse³⁰ and NHP brain¹² we have found that these vulnerable cells are located in the medullary zone and have not yet entered the granule cell zone, signifying that they are in a migratory status. Others have described cells that have this same migratory trajectory as basket or Golgi inter-neurons that migrate from the medullary zone through the internal granule cell layer to reach their final destination in the vicinity of the Purkinje cell layer.³¹

Glial Response to Isoflurane Exposure

Following up on our preliminary observation that sections from isoflurane-exposed fetal NHP brains (fig. 3) have abundant AC3-positive profiles distributed widely throughout WM regions (fig. 3, A and D), we stained sections adjacent to the AC3-stained sections with the DeOlmos cupric silver method (marker for dying cells), and observed that approximately the same number of cells in the same WM locations were silver positive (fig. 3, B and E), which provides confirmatory evidence that these WM cells are dead or in the process of dying.

Next, we performed immunohistochemical staining of adjacent sections with antibodies to fractin, which reportedly is a marker for caspase-mediated proteolysis in either neurons²⁷ or glia.²⁶ We carefully screened cerebrocortical and other gray matter regions where adjacent sections stained with AC3 revealed extensive neuronal apoptotic cell death, and found no evidence for fractin staining of these degenerating neurons. By contrast, in WM regions, fractin reproducibly stained a large population of cells that appeared, by numbers and

location, to be the same population stained by AC3. Moreover, the morphological appearance of profiles stained by fractin (fig. 3, C and F) closely resembled the profiles stained by AC3 and silver (compare fig. 3, D–F), which supports the interpretation that all three cell death markers are selectively staining the same population of dying WM cells. However, in the isoflurane-exposed G120 fetal NHP brain, where there is abundant apoptotic cell death of both, neurons and glia, antifractin antibodies detected only the apoptotic process in glia.

Because many of the cells in the WM belong either to the astrocyte, microglial/macrophage, or oligodendrocyte lineages, we performed an immunofluorescence double-label experiment in which AC3-stained sections were also stained for markers specific to these cell types. We found that the AC3-positive profiles did not colabel for glial fibrillary acidic protein (a marker for astrocytes; fig. 3G). We also conducted double staining with AC3 and Iba1 (marker for microglia/macrophages) or platelet-derived growth factor receptor- α (marker for early oligodendrocyte progenitors). Consistent with our previous findings in the neonatal NHP brain,¹¹ we found no evidence in the fetal brain for colabeling of AC3 with either of these markers (data not shown), signifying that isoflurane toxicity does not target microglia, oligodendrocyte progenitors, or astrocytes in the NHP brain at these times of development.

Collectively, the abovementioned findings support the hypothesis that in the fetal brain of the NHP the WM cells vulnerable to anesthesia are oligodendrocytes, which are beyond the progenitor stage of maturation. These findings suggested the related hypothesis that the topography of fetal glial apoptosis induced by isoflurane is related to the relative distribution of premyelinating and myelinating oligodendrocytes across the neuraxis. To test this, we stained for MBP to visualize the oligodendrocytes at three distinct rostrocaudal levels: a rostral level where little or no myelination was seen, a midrostrocaudal level where there was abundant early myelination, and a caudal level where WM tracts were densely myelinated.

WM Findings at a Rostral Level

MBP staining in control frontal cortex at or rostral to the level of the septum revealed no evidence for myelination, but at this level there were numerous MBP-positive premyelinating oligodendrocytes. In control brains these oligodendrocytes, had a dense arbor of MBP-labeled processes (fig. 4A). The morphological appearance of some of these oligodendrocytes, especially in the isoflurane-exposed brains, suggested that they may be degenerating. The signs of early degeneration were subtle and difficult to distinguish from the normal condition; the plasma membrane became blurred and indistinct and the processes became beaded with small blobs of MBP-positive cytoplasm. In later stages, the degenerative condition was more obvious (fig. 4B) and closely resembled the dying profiles when stained with AC3, silver, or fractin (compare fig. 4B with fig. 3, B, D, and F). Immunofluorescent double staining with MBP and AC3 revealed that many cells in the rostral WM colabel for MBP and AC3. An example of one such cell in very early stages of degeneration is depicted in figure 4, C–E. MBP positivity (fig. 4, C and E) identifies this cell as premyelinating oligodendrocyte (no other cell in the brain stains positive for MBP), and AC3 positivity (fig. 4, D and E) reveals that it is dying by apoptosis. DAPI counterstaining (fig. 4E, inset) demonstrates that this dying cell had multiple balls of condensed nuclear chromatin, consistent with apoptosis.

WM Findings at a Midrostrocaudal Level

At a midrostrocaudal level, MBP staining in control brains revealed that several WM tracts were undergoing early to moderately advanced myelination (fig. 5A). In these regions, MBP-positive oligodendrocytes were plentiful, and some were engaged in myelinating

axons, whereas others were premyelinating (fig. 5, B and C). Typically, regions where early myelination was occurring featured many small foci of axons that became visible in increments as MBP-positive oligodendrocyte processes developed sheaths around them (fig. 5, C and D). In some regions the myelination process was more advanced, and the foci of myelination were more confluent. Oligodendrocytes that were actively myelinating axons, had a more simplified arbor of processes, each of which appeared to contact and simultaneously myelinate multiple axons (fig. 5D). Some of these oligodendrocytes, especially in the isoflurane-exposed brains, showed signs of advanced degeneration. Interestingly, the degenerative process, as revealed by MBP staining, presented in two different patterns, depending on whether the oligodendrocyte was actively myelinating when it committed to cell death. The first pattern presented as a halo of MBP-positive debris in starburst configuration surrounding a condensed cell body (as shown in fig. 4B), consistent with a commitment to cell death that occurred in a premyelinating oligodendrocyte. The second pattern consisted of MBP-positive debris distributed in a linear pattern, consistent with degeneration of a myelinating oligodendrocyte and its associated myelin sheaths (fig. 5E).

Immunofluorescent double staining with MBP and AC3 at this midrostrocaudal level revealed premyelinating and myelinating oligodendrocytes that colabeled for MBP and AC3. An example of a myelinating oligodendrocyte that colabeled for AC3 and MBP, and was in an early stage of degeneration, as evidenced by its relatively intact morphological appearance, is depicted in figure 6, A–C. The MBP staining (fig. 6, A and C) identified myelinating oligodendrocytes in which AC3 was diffusely distributed within the apparent arbor of processes and myelin sheaths (fig. 6, B and C). DAPI counterstaining (fig. 6C) demonstrates multiple small balls of condensed nuclear chromatin in close apposition to the nuclear-limiting membrane, which confirms the diagnosis of early-stage apoptotic cell death.

WM Findings at a Caudal Level

At a caudal cerebellar/brain stem level, WM tracts were heavily myelinated and MBP staining provided an excellent means of visualizing these myelinated pathways, but MBP did not visualize any oligodendrocyte cell bodies in these densely myelinated pathways. Fractin/MBP double staining of these densely myelinated pathways in isoflurane-exposed brains revealed many degenerating cell bodies within the WM tracts. In figures 7, A and B, this phenomenon is illustrated in the cerebellar peduncle, a very densely myelinated pathway. Staining of adjacent sections with AC3 (fig. 7C) or the silver method (fig. 7D) confirmed that large numbers of cells with oligodendrocyte morphology were undergoing apoptotic cell death in the cerebellar peduncle after isoflurane exposure. By their morphology, these degenerating cells appear to be myelinating oligodendrocytes, but proving their identity is problematic. They do not stain positive for MBP, presumably because they have transferred all their MBP content to the myelin sheaths and then have ceased storing MBP in their cell bodies. By maturational status, they can be considered mature oligodendrocytes, but it is difficult to visualize them with markers for mature oligodendrocytes, because when they are degenerating they rapidly lose immunoreactivity for these markers. For example, we found previously in the neonatal NHP brain¹³ that when these cells are undergoing isoflurane-induced apoptosis, they stain intensely for AC3, but faintly or not at all for Olig 2 or CC-1, which are currently recognized as markers of mature oligodendrocytes.^{11,32} Consistent with our previous observations in neonatal WM, in the current study, we found rare examples of faint CC-1 colabeling of dying oligodendrocytes that were intensely positive for AC3 and presented with a similar morphology as those in figure 7, C and D (fig. 8, A–C), but in the majority of cases, CC-1 colabeling was absent.

Quantitative Evaluation

Quantitative evaluation of AC3-stained sections from the brains exposed to isoflurane ($n = 5$) revealed that the mean ($\pm$ SD) number of all AC3-positive profiles (neurons + glia) per brain was $18.9 \times 10^5 \pm 5.5 \times 10^5$, and in the four control brains was $4.6 \times 10^5 \pm 2.7 \times 10^5$ (fig. 9). Hence, there was a 4.1-fold increase in the number of apoptotic profiles in the isoflurane-exposed brains compared with the brains from drug-naïve controls. The difference in the mean number of apoptotic profiles between the isoflurane and control groups was 14.3×10^5 (95% CI, 21.4×10^5 to 7.2×10^5 ; $P = 0.002$).

When the initial counts were performed, based on location (white vs. gray matter) and morphological appearance, we assigned all AC3-stained profiles to either a neuronal or glial category. In the isoflurane-treated group, the mean number of AC3-positive neuronal profiles was 7.8×10^5 and the mean number of AC3-positive glial profiles was 11.1×10^5 , which yields a ratio of degenerating neurons to glia of 41:59. Subtracting the total mean number of apoptotic profiles per control brain from the total mean number per isoflurane-exposed brain yields the total mean number of dying cells that can be attributed to isoflurane exposure. That number for the fetal brain is 14.3×10^5 .

Discussion

Our findings demonstrate that *in utero* exposure of the early third-trimester NHP fetus to isoflurane anesthesia for 5 h is sufficient to cause a robust apoptotic cell death response affecting both neurons and glia. Isoflurane-induced apoptosis of neurons and glia has been described previously in the neonatal NHP brain,^{8,11} but this is the first report describing susceptibility of the NHP fetus to this toxic action of isoflurane. The glial cell type that is selectively affected in both the neonatal and fetal brain is in the oligodendrocyte lineage, and onset of vulnerability occurs at a maturational stage when the oligodendrocyte is just beginning to myelinate axons. Neither oligodendrocyte progenitors nor other glial cell types were affected. The overall number of apoptotic profiles (neurons + oligodendrocytes) in the isoflurane-exposed brains was 4.1 times greater than in the drug-naïve control brains. Of the total number of cells deleted from the isoflurane-exposed brains, 41% were neurons and 59% were oligodendrocytes.

The pattern of neuronal degeneration induced by isoflurane in the fetal NHP brain is different from the pattern we have previously described in the neonatal NHP brain.⁸ In that study, a laminar pattern of neuronal degeneration was strikingly evident in specific layers and regions of the cerebral cortex (layers 2 and 4 of the auditory and somatosensory association cortices in the temporal and parietal lobes, and layers 2 and 5 of the primary visual cortex in the occipital lobe). Other cortical and subcortical regions in the neonate were affected, but not as severely. In addition, there was a tendency for rostral brain regions to be more affected and caudal regions, for example the cerebellum, to be spared. By contrast, in the current study of the fetal brain, isoflurane induced dense degeneration in caudal brain regions, including especially the cerebellum and inferior colliculus. The fetal brains also displayed more dense neuronal degeneration than the neonatal brains in rostral subcortical regions, including the basal ganglia (caudate, putamen, nucleus accumbens), amygdala, and in several nuclei of the thalamus. Neuronal degeneration was distributed evenly and with mild to moderate severity across all divisions of the neocortex of the isoflurane-exposed fetal brains, and the pattern was not as densely concentrated in specific layers as was found in the neonatal brains.

The pattern of oligodendrocyte degeneration coincided with the location of axonal pathways throughout the fetal brain. Oligodendrocyte vulnerability became evident at a maturational stage when oligodendrocytes began accumulating MBP in their cytoplasm and plasma

membranes before myelination. These premyelinating and myelinating oligodendrocytes appeared to be similarly vulnerable to the toxic action of isoflurane. Persistence of vulnerability in regions with more advanced myelination is further corroborated by our previous finding¹¹ of a robust oligodendrocyte toxic response in the neonatal NHP brain, which is uniformly more heavily myelinated than the G120 fetal brain.

In addition to differences in injury patterns, the total number of cells (neurons and oligodendrocytes) that die in response to isoflurane exposure is different between fetal and neonatal NHP brain. Although in the current study fetal NHP brains on average lost 14.3×10^5 cells after 5-h isoflurane anesthesia, approximately 2.5-times more cells (36.9×10^5) were deleted from neonatal NHP brains after isoflurane exposure for the same duration (previous study⁸ involving infant macaques at postnatal day 6). This indicates that the toxic potential of isoflurane for the developing NHP brain increases between the G120 fetal period and the P6 neonatal period, when isoflurane is delivered for the same duration, and titrated to a comparable surgical plane.

Evidence that the regional pattern and quantity of cell loss varies as a function of age at time of drug exposure, suggests that potential long-term behavioral consequences may also vary as a function of age at time of exposure. Thus, in human studies, if age at time of exposure varies widely, so may the type and severity of neurobehavioral outcomes vary widely, and this is a potential confound in interpreting such studies. Evidence that the toxic response to isoflurane of both neurons and oligodendrocytes in the NHP brain is relatively robust at the fetal age of G120, and is similarly robust at the neonatal age of P6 (human equivalent period would be from mid-third trimester to 6 months after birth),^{8,33} suggests the need for further studies to determine at which developmental age the window of vulnerability opens and at what age it closes for both the neuroapoptosis and oligoapoptosis phenomena.

In several recent human studies,^{19–25} a significant association has been reported between exposure to anesthesia in infancy and subsequent neurobehavioral disturbances. Although some authors^{23,24} have reported that increased risk is associated only with multiple anesthesia exposures, other studies support the interpretation that a single brief exposure may be sufficient,^{19,20,22,25} and some studies^{21,22} have reported that increased risk for neurobehavioral disturbances correlates positively with increased duration of exposure. Although many full-term human infants who require anesthesia are not exposed multiple times or for long durations, this is not true for infants, especially premature infants, who require prolonged inpatient intensive care. Premature human infants are in the same neurodevelopmental age range as the fetal NHP subjects in the current study, and they are often exposed to moderate anesthesia (procedural sedation) intermittently or sometimes continuously for a period of several days, and may also receive deep anesthesia for surgery. The potential of prolonged anesthesia exposure to cause long-term neurobehavioral disturbances is unknown (because it has not been studied). However, data have recently become available for other apoptogenic agents, such as antiepileptic drugs, to which human fetuses and infants are sometimes exposed chronically. The antiepileptic drug, valproate, is particularly potent in triggering neuroapoptosis in the developing rodent brain,² and recent studies document that daily exposure of human fetuses to valproate during the third trimester of pregnancy is associated with a 9-point deficit in intelligence.³⁴ In addition, fetuses exposed to other antiepileptic drugs (carbamazepine, lamotrigine, phenytoin) had impaired verbal abilities at age of 4.5 yr.³⁵ It is well recognized that a high percentage of premature infants experience behavioral delays and neurocognitive impairment,³⁶ but anesthetic drugs have received limited attention as potential contributory factors. Our findings suggest the need for well-designed studies to shed further light on this important issue.

A potential explanation for the relatively potent apoptogenic action of isoflurane would be that it has both γ -aminobutyric acid_A agonist and *N*-methyl-D-aspartate receptor antagonist properties.³⁷ This interpretation is consistent with evidence from rodent studies that anesthesia protocols comprising a combination of these two classes of drugs cause more severe neuroapoptosis and/or neurobehavioral disturbances.^{3,15} Alcohol, which has both *N*-methyl-D-aspartate receptor antagonist and γ -aminobutyric acid_A agonist properties, causes a particularly severe neuroapoptosis reaction in both the infant rodent^{1,4} and fetal NHP brain,¹² and alcohol is well known to have deleterious effects on the fetal human brain and have long-term adverse neurobehavioral consequences.³⁸ Also relevant is evidence that alcohol, like isoflurane, causes both neuroapoptosis¹² and oligoapoptosis,³⁹ and that both alcohol¹² and isoflurane exposure (as shown in this study) of NHP fetuses causes severe damage in the NHP basal ganglia, which coincides with evidence that loss of neuronal mass in the basal ganglia is a consistent finding in patients diagnosed with fetal alcohol spectrum disorder.³⁸ Similarly, recent neuroimaging evidence⁴⁰ documents loss of neuronal mass in the basal ganglia of human patients exposed during the third trimester of pregnancy to antiepileptic drugs that have apoptogenic properties resembling those of alcohol and isoflurane.² Another relevant correlation is that all our studies focusing on neuroapoptosis in the early neonatal period (rodents) or fetal period (NHPs), regardless whether the apoptogenic agent is alcohol, ketamine, propofol, or isoflurane, have documented dense degeneration of a specific population of neurons in the cerebellum.^{9–12,30} It is not known what the neurodevelopmental significance may be, but the fact that all such agents have robust toxic impact on this specific population of immature neurons during a specific developmental time window supports the concept that maturation-dependent mechanisms define both the temporal and regional patterns of susceptibility of neurons and glia to apoptosis during fetal and neonatal brain development. Future research is needed to define the extent to which the mechanisms of action of these different agents overlap.

Whether deletion of oligodendrocytes from the developing brain when they are just beginning to myelinate axons has lasting deleterious effects remains to be determined. Immunohistochemical staining with antibodies to MBP allowed the visualization of oligodendrocytes as they were undergoing acute degeneration, and also helped to detect that disintegration of newly formed myelin sheaths accompanies the degenerative reaction, but it will require a more detailed study with specialized methods, including electron microscopy, to clarify whether, or to what extent, axonal injury may occur. The fact that premature infants are often exposed multiple times or for prolonged periods to anesthetic drugs is potentially problematic in that this could entail cumulative oligodendrocyte loss, and perhaps progressive axonal pathology which, together with cumulative loss of neurons, would increase the chances for longterm neurobehavioral disturbances. Only well-designed long-term survival studies in translationally relevant models will be able to identify the functional consequences of this combined neuronal–glial injury.

Acknowledgments

Support was provided by the Intellectual and Developmental Disabilities Research Center at Washington University through the grant No. P30 HD062171 from the from the National Institutes of Health, Bethesda, Maryland, and by grant No. HD052664 and HD37100 from the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, Bethesda, Maryland, and by grant No. OD011092 from the National Institutes of Health, Bethesda, Maryland, for the operation of the Oregon National Primate Research Center.

References

1. Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovskaya V, Hörster F, Tenkova T, Dikranian K, Olney JW. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science*. 2000; 287:1056–60. [PubMed: 10669420]

2. Bittigau P, Sifringer M, Genz K, Reith E, Pospischil D, Govindarajulu S, Dzietko M, Pesditschek S, Mai I, Dikranian K, Olney JW, Ikonomidou C. Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proc Natl Acad Sci U S A*. 2002; 99:15089–94. [PubMed: 12417760]
3. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci*. 2003; 23:876–82. [PubMed: 12574416]
4. Olney JW, Tenkova T, Dikranian K, Qin YQ, Labruyere J, Ikonomidou C. Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. *Brain Res Dev Brain Res*. 2002; 133:115–26.
5. Rizzi S, Carter LB, Ori C, Jevtovic-Todorovic V. Clinical anesthesia causes permanent damage to the fetal guinea pig brain. *Brain Pathol*. 2008; 18:198–10. [PubMed: 18241241]
6. Slikker W Jr, Zou X, Hotchkiss CE, Divine RL, Sadovova N, Twaddle NC, Doerge DR, Scallet AC, Patterson TA, Hanig JP, Paule MG, Wang C. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. *Toxicol Sci*. 2007; 98:145–58. [PubMed: 17426105]
7. Zou X, Liu F, Zhang X, Patterson TA, Callicott R, Liu S, Hanig JP, Paule MG, Slikker W Jr, Wang C. Inhalation anesthetic-induced neuronal damage in the developing rhesus monkey. *Neurotoxicol Teratol*. 2011; 33:592–7. [PubMed: 21708249]
8. Brambrink AM, Evers AS, Avidan MS, Farber NB, Smith DJ, Zhang X, Dissen GA, Creeley CE, Olney JW. Isoflurane-induced neuroapoptosis in the neonatal rhesus macaque brain. *Anesthesiology*. 2010; 112:834–41. [PubMed: 20234312]
9. Brambrink AM, Evers AS, Avidan MS, Farber NB, Smith DJ, Martin LD, Dissen GA, Creeley CE, Olney JW. Ketamine-induced neuroapoptosis in the fetal and neonatal rhesus macaque brain. *Anesthesiology*. 2012; 116:372–84. [PubMed: 2222480]
10. Creeley CE, Dikranian KT, Evers A, Dissen GA, Olney JW, Brambrink AM. Propofol-induced apoptosis of neurons and oligodendrocytes in the fetal and the neonatal Rhesus macaque brain. *Br J Anaesth*. 2013; 110(suppl 1):i29–38. [PubMed: 23722059]
11. Brambrink AM, Back SA, Riddle A, Gong X, Moravec MD, Dissen GA, Creeley CE, Dikranian KT, Olney JW. Isoflurane-induced apoptosis of oligodendrocytes in the neonatal primate brain. *Ann Neurol*. 2012; 72:525–35. [PubMed: 23109147]
12. Farber NB, Creeley CE, Olney JW. Alcohol-induced neuroapoptosis in the fetal macaque brain. *Neurobiol Dis*. 2010; 40:200–6. [PubMed: 20580929]
13. Wozniak DF, Hartman RE, Boyle MP, Vogt SK, Brooks AR, Tenkova T, Young C, Olney JW, Muglia LJ. Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. *Neurobiol Dis*. 2004; 17:403–14. [PubMed: 15571976]
14. Satomoto M, Satoh Y, Terui K, Miyao H, Takishima K, Ito M, Imaki J. Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice. *Anesthesiology*. 2009; 110:628–37. [PubMed: 19212262]
15. Fredriksson A, Pontén E, Gordh T, Eriksson P. Neonatal exposure to a combination of *N*-methyl-D-aspartate and gamma-aminobutyric acid type A receptor anesthetic agents potentiates apoptotic neurodegeneration and persistent behavioral deficits. *Anesthesiology*. 2007; 107:427–36. [PubMed: 17721245]
16. Stratmann G, Sall JW, May LD, Bell JS, Magnusson KR, Rau V, Visrodia KH, Alvi RS, Ku B, Lee MT, Dai R. Isoflurane differentially affects neurogenesis and long-term neurocognitive function in 60-day-old and 7-day-old rats. *Anesthesiology*. 2009; 110:834–48. [PubMed: 19293705]
17. Sanders RD, Xu J, Shu Y, Januszewski A, Halder S, Fidalgo A, Sun P, Hossain M, Ma D, Maze M. Dexmedetomidine attenuates isoflurane-induced neurocognitive impairment in neonatal rats. *Anesthesiology*. 2009; 110:1077–85. [PubMed: 19352168]
18. Paule MG, Li M, Allen RR, Liu F, Zou X, Hotchkiss C, Hanig JP, Patterson TA, Slikker W Jr, Wang C. Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys. *Neurotoxicol Teratol*. 2011; 33:220–30. [PubMed: 21241795]

19. DiMaggio C, Sun LS, Kakavouli A, Byrne MW, Li G. A retrospective cohort study of the association of anesthesia and hernia repair surgery with behavioral and developmental disorders in young children. *J Neurosurg Anesthesiol.* 2009; 21:286–91. [PubMed: 19955889]
20. DiMaggio C, Sun LS, Li G. Early childhood exposure to anesthesia and risk of developmental and behavioral disorders in a sibling birth cohort. *Anesth Analg.* 2011; 113:1143–51. [PubMed: 21415431]
21. Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL, Warner DO. Early exposure to anesthesia and learning disabilities in a population-based birth cohort. *Anesthesiology.* 2009; 110:796–4. [PubMed: 19293700]
22. Block RI, Thomas JJ, Bayman EO, Choi JY, Kimble KK, Todd MM. Are anesthesia and surgery during infancy associated with altered academic performance during childhood? *Anesthesiology.* 2012; 117:494–3. [PubMed: 22801049]
23. Flick RP, Katusic SK, Colligan RC, Wilder RT, Voigt RG, Olson MD, Sprung J, Weaver AL, Schroeder DR, Warner DO. Cognitive and behavioral outcomes after early exposure to anesthesia and surgery. *Pediatrics.* 2011; 128:e1053–61. [PubMed: 21969289]
24. Sprung J, Flick RP, Katusic SK, Colligan RC, Barbaresi WJ, Bojani K, Welch TL, Olson MD, Hanson AC, Schroeder DR, Wilder RT, Warner DO. Attention-deficit/hyperactivity disorder after early exposure to procedures requiring general anesthesia. *Mayo Clin Proc.* 2012; 87:120–9. [PubMed: 22305025]
25. Ing C, DiMaggio C, Whitehouse A, Hegarty MK, Brady J, von Ungern-Sternberg BS, Davidson A, Wood AJ, Li G, Sun LS. Long-term differences in language and cognitive function after childhood exposure to anesthesia. *Pediatrics.* 2012; 130:e476–85. [PubMed: 22908104]
26. Schulz R, Vogel T, Mashima T, Tsuruo T, Kriegstein K. Involvement of Fractin in TGF-beta-induced apoptosis in oligodendroglial progenitor cells. *Glia.* 2009; 57:1619–29. [PubMed: 19330858]
27. Rossiter JP, Anderson LL, Yang F, Cole GM. Caspase-3 activation and caspase-like proteolytic activity in human perinatal hypoxic-ischemic brain injury. *Acta Neuropathol.* 2002; 103:66–3. [PubMed: 11841033]
28. Baumann N, Pham-Dinh D. Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev.* 2001; 81:871–27. [PubMed: 11274346]
29. Back SA, Luo NL, Borenstein NS, Volpe JJ, Kinney HC. Arrested oligodendrocyte lineage progression during human cerebral white matter development. Dissociation between the timing of progenitor differentiation and myelinogenesis. *J Neuropathol Exp Neurol.* 2002; 61:197–11. [PubMed: 11853021]
30. Dikranian K, Qin YQ, Labruyere J, Nemmers B, Olney JW. Ethanol-induced neuroapoptosis in the developing rodent cerebellum and related brain stem structures. *Brain Res Dev Brain Res.* 2005; 155:1–13.
31. Zhang L, Goldman JE. Generation of cerebellar interneurons from dividing progenitors in white matter. *Neuron.* 1996; 16:47–4. [PubMed: 8562089]
32. Kitada M, Rowitch DH. Transcription factor co-expression patterns indicate heterogeneity of oligodendroglial subpopulations in adult spinal cord. *Glia.* 2006; 54:35–6. [PubMed: 16673374]
33. Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum Dev.* 1979; 3:79–3. [PubMed: 118862]
34. Meador KJ, Baker GA, Browning N, Clayton-Smith J, Combs-Cantrell DT, Cohen M, Kalayjian LA, Kanner A, Liporace JD, Pennell PB, Privitera M, Loring DW. NEAD Study Group. Cognitive function at 3 years of age after fetal exposure to antiepileptic drugs. *N Engl J Med.* 2009; 360:1597–605. [PubMed: 19369666]
35. Meador KJ, Baker GA, Browning N, Cohen MJ, Bromley RL, Clayton-Smith J, Kalayjian LA, Kanner A, Liporace JD, Pennell PB, Privitera M, Loring DW. NEAD Study Group. Effects of fetal antiepileptic drug exposure: Outcomes at age 4. 5 years. *Neurology.* 2012; 78:1207–14. [PubMed: 22491865]
36. Løhaugen GC, Gramstad A, Evensen KA, Martinussen M, Lindqvist S, Indredavik M, Vik T, Brubakk AM, Skranes J. Cognitive profile in young adults born preterm at very low birthweight. *Dev Med Child Neurol.* 2010; 52:1133–8. [PubMed: 21175467]

37. Shelton KL, Nicholson KL. GABA(A) positive modulator and NMDA antagonist-like discriminative stimulus effects of isoflurane vapor in mice. *Psychopharmacology (Berl)*. 2010; 212:559–69. [PubMed: 20697696]
38. Riley EP, McGee CL. Fetal alcohol spectrum disorders: An overview with emphasis on changes in brain and behavior. *Exp Biol Med (Maywood)*. 2005; 230:357–65. [PubMed: 15956765]
39. Creeley CE, Dikranian KT, Johnson SA, Farber NB, Olney JW. Alcohol-induced apoptosis of oligodendrocytes in the fetal macaque brain. *Acta Neuropathologica Communications*. 2013; 1:23. [PubMed: 24252271]
40. Ikonomidou C, Scheer I, Wilhelm T, Juengling FD, Titze K, Stöver B, Lehmkuhl U, Koch S, Kassubek J. Brain morphology alterations in the basal ganglia and the hypothalamus following prenatal exposure to antiepileptic drugs. *Eur J Paediatr Neurol*. 2007; 11:297–1. [PubMed: 17418601]

What We Already Know about This Topic

- Exposure of neonatal nonhuman primates to isoflurane can cause widespread apoptosis in neurons and glia, but its effects in fetal animals was unknown

What This Article Tells Us That Is New

- Exposure of third-trimester fetal macaque monkeys to isoflurane *in utero* caused widespread apoptosis of neurons and of oligodendroglia that are critical for myelination
- Compared with its neurotoxicity in neonatal macaques, isoflurane produced quantitatively less apoptosis with a more diffuse distribution in the fetus, consistent with developmental changes in toxic potential

Fig. 1.

Distribution of apoptotic cell death in gray matter and white matter of fetal nonhuman primate brain. This figure presents computer plots displaying the location of activated caspase-3 (AC3)-positive profiles in sections at a midrostrocaudal (A and B) or cerebellar/brain stem (C and D) level of a fetal control brain (A and C), or a fetal brain exposed to isoflurane (ISO; B and D). *Red dots* = neuronal profiles; *white dots* = glial (oligodendrocyte) profiles. The distribution pattern is similar in the control and isoflurane-exposed brains, but the density of stained profiles is much greater in the isoflurane-exposed sections. Neuronal populations preferentially affected include those in the frontal (FC) and temporal (TC) cortices, the caudate nucleus (CN), putamen (Pu), amygdala (Am), and in all folia of the cerebellum. Vulnerable oligodendrocytes were distributed diffusely throughout white matter regions in a pattern corresponding to the location of major axonal pathways, such as the corona radiata (CR), centrum semiovale (CSO), internal capsule (IC), anterior commissure (AC), optic chiasm (OC), and cerebellar peduncle (CP).

Fig. 2.

Neurons undergoing cell death after isoflurane exposure. *A* is a bright field image displaying activated caspase-3 (AC3)-positive large neurons with prominent apical dendrites in layers 2, 3, and 4 of the fetal frontal cortex in various stages of degeneration. *B* is an immunofluorescent image showing an encircled cellular profile in the frontal cortex that stains positive for NeuN (neuronal marker), and *C* demonstrates that this neuron colabels for AC3 (apoptosis marker). Counter-staining with 4',6-diamidino-2-phenylindole (blue; *C*) reveals condensed chromatin balls in the nucleus (sign of apoptotic cell death). *D* and *E* are bright field images displaying AC3-positive profiles in the caudate nucleus and cerebellum, respectively. In the caudate (*D*) the dying cells appear in clusters and most are small and round or spindle-shaped, but a few are larger multipolar profiles. In the cerebellum, most AC3-positive cells are small and round or spindle-shaped, and are located in the internal granule cell (GC) layer. The bar represents 50 μ M in *A*, 40 μ M in *D* and *E*, and 10 μ M in *B* and *C*. PC = Purkinje cell layer.

Fig. 3.

Glial cells undergoing cell death after isoflurane exposure. *A* depicts the typical appearance of glial cells stained with antibodies to activated caspase-3 (AC3) in the frontal cortical white matter of an isoflurane-exposed fetal nonhuman primate brain. *B* and *C* are from adjacent sections of frontal white matter of the same brain showing that silver and fractin staining detects dying cells in the same location and in approximately the same numbers, and in each case the dying cells have a similar morphological appearance. The boxed regions in *A–C* are shown at higher magnification in *D–F*. Immunofluorescent double staining performed on an adjacent section with antibodies to glial fibrillary acidic protein (GFAP; *green*) and (AC3; *red*) shows that the dying AC3-positive white matter cells do not colabel for GFAP, therefore are not astrocytes (*G*). Counterstaining with 4',6-diamidino-2-phenylindole (*blue*) reveals balls of condensed chromatin in the nucleus of the AC3-positive cell, which confirms the apoptosis diagnosis. The bar in *A* represents 50 μM in *A–C*, and 12 μM in *D–F*, and 10 μM in *G*.

Fig. 4.

Premyelinating oligodendrocytes in the rostral forebrain. *A* shows the typical appearance of a myelin basic protein (MBP)-positive premyelinating oligodendrocyte in the frontal cortical white matter of a control brain at a rostral level where myelination has not yet begun. The premyelinating oligodendrocyte has a diffuse arbor of processes surrounding the cell body, which are diffusely labeled with MBP. *B* is from the same white matter region of an isoflurane-exposed brain and depicts the appearance of an MBP-stained degenerating oligodendrocyte. The radial processes are diffusely fragmented and condensed and form a halo surrounding the shrunken cell body in a starburst configuration that resembles the appearance of degenerating white matter cells stained by activated caspase-3 (AC3), fractin, or silver (compare fig. 4B with fig. 3, A–F). *C–E* are from the same region of an isoflurane-exposed brain and display a premyelinating oligodendrocyte that stains positive for both MBP (*C* and *E*) and AC3 (*D* and *E*), signifying that it is undergoing cell death. This cell is in an early stage of degeneration, as supported by the fact that AC3 staining (*D*) reveals an array of relatively intact processes. The *inset* in *E* shows that the nucleus of this cell has an abnormal chromatin pattern (condensed spherical balls; 4',6-diamidino-2-phenylindole-positive, *blue*) indicative of apoptosis. The *bar* in *A* represents 10 μ M in all panels.

Fig. 5.

Myelinating oligodendrocytes at a midrostrocaudal level: All panels are stained immunochemically with antibodies to myelin basic protein (MBP). *A* presents an overview showing different stages of myelination at a midrostrocaudal level of a control brain. In the cerebrocortical mantle, and in the transition zone between gray and white matter, there is no myelination. But in the subjacent corona radiata (CR) oligodendrocytes are beginning to myelinate axons. (*B*) A magnified view of the boxed region in *A*, displays an area where myelin formation is in its early stages, and *C* is a magnified view of the boxed region in *B*, illustrating in detail the fundamentals of early myelination. The process begins (*C*) by a single oligodendrocyte contacting multiple axons, which are visualized as focal segmental myelination. Segments of myelinated axons are seen in isolated foci of myelination, whereas in regions of more confluent myelination, the segments of myelinated axons are longer and more continuous. Primary oligodendrocytes, in addition to myelinating their own patch, extend long processes (*arrow*) to other nearby patches, which perhaps facilitates integration of the patches. Regions of white matter showing significant myelination in *A* are CR, centrum semiovale (CSO), internal capsule (IC), globus pallidus (GP), and the optic tract (OT). Very little myelination is seen in other regions such as the caudate nucleus (CN), putamen (Pu), corpus callosum (CC), and thalamus (Th). *D* illustrates at higher magnification that as premyelinating OLs initiate myelination, they tend to lose their highly arborized appearance, and the processes contact multiple axons (*arrow*) that are intensely MBP-positive. *E* displays an MBP-positive oligodendrocyte in an advanced stage of degeneration. Note that the degeneration products are organized in a linear pattern consistent with degeneration of the myelin sheaths as well as the oligodendrocyte soma. The *bar* in *A* represents 500 μM in *A*, 50 μM in *B*, 12 μM in *C*, 5 μM in *D*, and 4 μM in *E*.

Fig. 6.

Immunofluorescent myelin basic protein (MBP)/activated caspase-3 (AC3) double labeling of a myelinating oligodendrocyte. *A* illustrates an oligodendrocyte that is intensely MBP-positive (*green*) and has processes that are in contact with multiple MBP-positive myelin sheaths. *B* shows that this same cell is intensely AC3-positive (*red*). Its cell body and processes, even the distal tips that are in continuity with myelin sheaths, are labeled with AC3. The merged image in *C* confirms that *A* and *B* are the same cell, and the inset reveals a 4',6-diamidino-2-phenylindole pattern of nuclear chromatin clumping indicative of apoptotic cell death. The *bar* in *A* represents 15 μ M for all panels.

Fig. 7.

Oligodendrocyte apoptosis in a heavily myelinated pathway. *A* is a bright field image of the cerebellum of an isoflurane-exposed fetus, which has been stained with antibodies to myelin basic protein (MBP). This overview documents that at this caudal region of the G120 nonhuman primate brain white matter tracts are already heavily myelinated. The *boxed region* outlines an area in the center of the cerebellar peduncle, the major white matter tract serving the cerebellum. *B–D* are from the boxed region of adjacent sections stained with antibodies to fractin and MBP (*B*), activated caspase-3 (*C*), or with the silver method (*D*). (*B*) A fluorescent image double stained with antibodies to MBP (*green*) and fractin (*red*) reveals many dying cells that are positive for fractin (marker of apoptotic cells), but there are no MBP-positive cell bodies, even though the myelinated axons are intensely MBP positive. This is characteristic of all densely myelinated pathways; oligodendrocytes in these pathways do not stain positive for MBP. *C* and *D* confirm with two additional markers of cell death (activated caspase-3 and silver, respectively) that fractin is reliably detecting cell death in this heavily myelinated pathway. The *bar* in *A* represents 500 μM in *A* and 50 μM in *B–D*.

Fig. 8.

CC-1 staining in a myelinated pathway. *A* shows that in myelinated areas, there is an abundance of cells that stain positive for CC-1 (*green*). *B* depicts the same scene stained for activated caspase-3 (AC3; *red*), and *C* is a merged image revealing that the AC3-positive cell colabels with a CC-1-positive profile (*circled profile* in *A*). CC-1 positivity of this cell is relatively faint and appears to be confined to the nucleus. The majority of other AC3-positive cells in this region did not display even a faint CC-1 signal. The *bar* in *B* represents 15 μ M for all panels.

Fig. 9. Total mean number of apoptotic profiles (neurons + glia) per brain of G120 fetal rhesus macaques exposed to isoflurane (ISO; n = 5) or to no drug (control; n = 4). AC3+ = activated caspase 3-positive.

Table 1

Physiologic Variables of Pregnant Females (G120) during Isoflurane Anesthesia

	Anesthesia Time		
	1.0 h	2.5 h	4.5 h
Maternal			
Temp, °C	36.7 (34.8–37.0)	37.8 (35.5–37.9)	37.7 (37.0–38.4)
MAP, mmHg	57 (48–68)	57 (49–64)	55 (46–65)
HR, min ⁻¹	138 (122–157)	139 (128–144)	142 (138–150)
Fetal HR, min ⁻¹	150 (140–155)	150 (130–160)	154 (138–175)
Maternal			
EtCO ₂ , mmHg	30 (24–37)	32 (24–39)	33 (23–40)
pH, arterial	7.45 (7.44–7.54)	7.48 (7.44–7.50)	7.48 (7.44–7.54)
PaCO ₂ , mmHg	34 (31–37)	32 (26–33)	31 (21–33)
PaO ₂ , mmHg	350 (102–598)*	151 (106–195)	125 (81–169)
Hb, mg/dl	11 (10–13)	10 (10–12)	11 (8–12)
Glucose, mM	53 (46–80)	53 (42–64)	49 (40–62)
Lactate, mM	1.2 (0.8–1.5)	0.9 (0.5–2.9)	1.2 (0.5–4.5)

Data are presented as median (range: min – max).

* At this time point, two animals were still ventilated with FIO₂ = 1.0

EtCO₂ = end-tidal carbon dioxide; fetal HR = ultrasound-guided measurements of fetal heart rate (six measurements/time point/animal); Hb = hemoglobin; HR = heart rate; MAP = mean arterial pressure (noninvasive); PaCO₂ = arterial carbon dioxide tension; PaO₂ = arterial oxygen tension (FIO₂ = 0.4); Temp = temperature (rectal).

Table 2

Physiologic Variables of Dams and Fetuses Immediately before and after C/S

	Group	Dams after Induction of Anesthesia and before C/S	Fetus Immediately before Clamping of Umbilical Cord (Umbilical Artery Sample)
Temp, °C	Control	37.7 (37.3–38.2)	
	Isoflurane	37.3 (35.7–37.6)	
MAP, mmHg	Control	54 (42–68)	
	Isoflurane	57 (56–68)	
HR, min ⁻¹	Control	171 (145–191)	
	Isoflurane	163 (145–182)	
Fetal HR, min ⁻¹	Control	171 (165–183)	
	Isoflurane	161 (130–191)	
EtCO ₂ , mmHg	Control	34 (26–41)	
	Isoflurane	36 (31–37)	
pH, arterial	Control	7.27 (7.20–7.35)	7.23 (7.20–7.25)
	Isoflurane	7.34 (7.33–7.38)	7.26 (7.19–7.32)
PaCO ₂ , mmHg	Control	43 (38–48)	58 (53–62)
	Isoflurane	36 (31–40)	48 (43–69)
PaO ₂ , mmHg	Control	140 (111–168)	16 (10–26)
	Isoflurane	296 (219–373)*	8 (5–19)
Hb, mg/dl	Control	14 (12–15)	13 (12–16)
	Isoflurane	12 (10–14)	12 (10–13)
Glucose, mM	Control	83 (61–96)	56 (34–66)
	Isoflurane	64 (50–65)	41 (27–48)
Lactate, mM	Control	6.7 (3.5–9.2) [†]	4.0 (2.8–5.5) [†]
	Isoflurane	2.3 (1.8–4.5)	2.1 (1.6–4.2)

Data are presented as median (range: min – max).

* FIO₂ = 1.0.[†] Increased lactate blood level in the nonmedicated control animals likely reflect higher stress levels during transition from the home cage environment before induction of anesthesia for C/S.C/S = cesarean section; EtCO₂ = end-tidal carbon dioxide; Hb = hemoglobin; HR = heart rate; MAP = mean arterial pressure (noninvasive); PvCO₂ = venous carbon dioxide tension; PvO₂ = venous oxygen tension (FIO₂ = 0.4); Temp = temperature (rectal).