

Ketamine-induced Neuroapoptosis in the Fetal and Neonatal Rhesus Macaque Brain

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ABSTRACT

Background: Exposure of rhesus macaque fetuses for 24 h or neonates for 9 h to ketamine anesthesia causes neuroapoptosis in the developing brain. The current study clarifies the minimum exposure required for and the extent and spatial distribution of ketamine-induced neuroapoptosis in rhesus fetuses and neonates.

Method: Ketamine was administered by IV infusion for 5 h to postnatal day 6 rhesus neonates or to pregnant rhesus females at 120 days' gestation (full term = 165 days). Three hours later, fetuses were delivered by cesarean section, and the fetal and neonatal brains were studied for evidence of apoptotic neurodegeneration, as determined by activated caspase-3 staining.

Results: Both the fetal (n = 3) and neonatal (n = 4) ketamine-exposed brains had a significant increase in apoptotic profiles compared with drug-naive controls (fetal n = 4; neonatal n = 5). Loss of neurons attributable to ketamine exposure was 2.2 times greater in fetuses than in neonates. The pattern of neurodegeneration in fetuses was different from that in neonates, and all subjects exposed at either age had a pattern characteristic for that age.

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What We Already Know about This Topic

- Previous work has shown that sustained exposure to ketamine (e.g., 9–24 h) produces neurodegeneration in the developing rhesus macaque brain.

What This Article Tells Us That Is New

- Shorter exposure intervals (e.g., 5 h) also produce neurodegeneration. Patterns of neuroapoptosis are different in fetal versus neonatal rhesus macaque brain, with the number of damaged cells being greater in the fetal brain.

Conclusion: The developing rhesus macaque brain is sensitive to the apoptogenic action of ketamine at both a fetal and neonatal age, and exposure duration of 5 h is sufficient to induce a significant neuroapoptosis response at either age. The pattern of neurodegeneration induced by ketamine in fetuses was different from that in neonates, and loss of neurons attributable to ketamine exposure was 2.2 times greater in the fetal than neonatal brains.

IN recent years, several classes of drugs, including those that block *N*-methyl-D-aspartate (NMDA) glutamate receptors, those that activate γ -aminobutyric acid receptor type A (GABA_A), and ethanol (which has both NMDA antagonist and GABA-mimetic properties), have been shown to trigger widespread apoptotic death of neurons in the developing animal brain.^{1–5} Vulnerability to the apoptogenic mechanism reaches its peak during the developmental period of rapid synaptogenesis,^{1,2} also known as the brain growth spurt period, which in rodents occurs primarily during the first 2 weeks after birth but in humans extends from approximately midgestation to several years after birth.⁶

Drug-induced developmental neuroapoptosis has been shown at both light and electron microscopic levels to have classic morphologic characteristics of apoptosis.^{1,3,5,7,8} The cell death process involves translocation of Bax protein to mitochondrial membranes and extra-mitochondrial leakage of cytochrome c, followed by activation of caspase-3.^{9–11} Because commitment to cell death occurs before the caspase-3 activation step,¹¹ activated caspase-3 (AC3) immunohistochemistry reliably identifies cells that have progressed beyond the point of cell death commitment. Therefore, AC-3 immunohistochemistry has been used extensively for

marking dying neurons in recent studies focusing on drug-induced developmental neuroapoptosis.^{3,8-27}

Neuroapoptosis induced in the developing brain by NMDA antagonist and GABA-mimetic drugs potentially is relevant in a public health context because there are many agents that the developing human brain may be exposed to that have NMDA antagonist or GABA-mimetic properties, including drugs that are sometimes abused by pregnant mothers (ethanol, phencyclidine, ketamine, nitrous oxide, barbiturates, and benzodiazepine²⁸⁻³⁰‡‡) and many drugs used worldwide in obstetric and pediatric medicine as anticonvulsants, sedatives or anesthetics. Of particular concern is mounting evidence from animal studies 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 brain^{3,13-18,22,23,26} and that exposure of the developing rodent brain to these agents can result in long-term neurobehavioral impairment.^{5,26,31-34} In addition, there is evidence potentially linking anesthesia exposure in infancy with long-term neurobehavioral deficits in both human³⁵⁻³⁹ and non-human⁴⁰ primates.

The important question of whether anesthetic drugs can trigger neuroapoptosis in the developing nonhuman primate (NHP) brain was first addressed by Slikker and colleagues, who reported^{20,21} that IV infusion of ketamine triggered neuroapoptosis in the rhesus macaque brain at postnatal day 5 if the infusion was continued for 9 or 24 h but not if it was stopped at 3 h. In addition, it was shown that a 24-h ketamine infusion triggered neuroapoptosis in the gestational day 120 (G120) fetal rhesus macaque brain,²⁰ but briefer periods of fetal exposure were not studied. More recently, we have shown²⁷ that a neuroapoptosis response is induced in the 6-day-old rhesus macaque brain by 5-h exposure to the potent volatile anesthetic isoflurane. To further clarify the clinical relevance of ketamine's apoptogenic potential, we administered ketamine by IV infusion for 5 h to pregnant (G120) or postnatal day 6 rhesus macaques and examined the fetal or neonatal brains 3 h later for histologic evidence of apoptotic neurons.

Materials and Methods

Animals and Experimental Procedures

All animal procedures were approved by the Oregon National Primate Research Center (Beaverton, Oregon) and Washington University Medical School Institutional Animal Care and Use Committees (St. Louis, Missouri) and were conducted in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

‡‡ NSDUH Report. Substance use among women during pregnancy and following childbirth. Substance Abuse and Mental Health Services Administration, Office of Applied Studies. Rockville, Maryland, 2009. Available at: <http://oas.samhsa.gov/2k9/135/PregWoSubUse.htm>. Accessed July 20, 2011.

Two studies were performed, one pertaining to fetal and the other to neonatal subjects.

Fetuses. To produce time-mated pregnant dams, cycling females were paired for 4 days with fertile males beginning on day 10 ± 2 of the menstrual cycle (first day of menses = day 1; gestation length was counted from the first day of pairing, first day = day 0). Pregnancy was confirmed by blood progesterone concentrations of greater than 1 ng/ml at 29 ± 2 and 42 ± 3 days after the menses before mating. Blood samples were collected from conscious animals using minimal restraint. The pregnant dams were not exposed to any sedative agents throughout the pregnancy. Pregnant female macaques at G120 were exposed for 5 h to no anesthesia ($n = 4$) or to an IV infusion of ketamine ($n = 3$). Full-term gestation for these macaques is 165 days.

At the initiation of the experiment, the pregnant females were gently restrained according to standard methods (in the same apparatus used for blood sampling, as described, so the animals are well accustomed to the procedure) to facilitate placement of an IV catheter for administration of ketamine. Anesthesia was induced by a bolus of ketamine (10 mg/kg), which was followed by a continuous ketamine infusion at $10-85 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ for 5 h, augmented by additional IV boluses as needed to maintain an intermediate surgical plane of anesthesia, defined as no movement and not more than 10% increase in heart rate or blood pressure in response to a profound mosquito-clamp pinch at hand and foot (checked every 30 min). Animals assigned to ketamine anesthesia were intubated tracheally (conventional direct laryngoscopy, 4.0 ID endotracheal tube; Mallinckrodt, Hazelwood, MO), mechanically ventilated using a conventional anesthesia machine ventilator (Hallowell EMC, Pittsfield, MA) to control oxygenation and gas exchange, and maintained using extended physiologic monitoring (continuous end-tidal carbon dioxide, FiO_2 , electrocardiogram, peripheral oxygen saturation, noninvasive blood pressure [every 15 min], body temperature [esophageal temperature probe, Smiths Medical ASD, SurgiVet Monitor; Smith Medical, Waukesha, WI], blood gases, metabolic profile including pH, base excess, blood urea nitrogen, hematocrit, hemoglobin, sodium, chloride, potassium, and serum glucose and lactate concentrations [every 2 h; i-STAT, Princeton, NJ]). IV fluids (lactated Ringer's solution) and glucose were substituted as needed, and physiologic temperature was maintained using a warming device and forced warm air (Bair Hugger; Arizant Healthcare Inc., Eden Prairie, MN). Ultrasonography was performed hourly to monitor fetal heart rate and health of the fetus. After 5 h, the ketamine infusion was stopped and the animals were observed for 3 h. The trachea was extubated when appropriate, and the animals were then returned to climatized cages and offered biscuits and water as tolerated for the remainder of the observation period. At 8 h after time 0, ketamine anesthesia was induced again (10 mg/kg IV, followed by a continuous infusion at $50-85 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$), and a cesarean section was performed to deliver the

fetus, which was euthanized for tissue collection (high-dose phenobarbital followed by transcardial perfusion according to National Institutes of Health guidelines). After recovery from anesthesia, the dam was given appropriate analgesic and was returned to her home cage. Dams that were randomized to receive no anesthesia (control group) were measured at baseline, received an IV injection of saline by the same method used in the ketamine-treated animals, and were returned to their cages, where they had access to light food and water *ad libitum*. Eight hours later, a cesarean section was performed to remove the fetus for transcardial perfusion under deep anesthesia as described, and the dam was allowed to recover according to the paradigm described.

Neonates. Neonates were delivered naturally from dams that had been group housed or were specifically paired with males as part of the time-mated breeding program. Pregnancy was determined by ultrasound under light sedation of the dam using ketamine (5–10 mg/kg) during the first or early second trimester. On postnatal day 6, neonatal rhesus macaques were exposed for 5 h to no anesthesia (n = 5) or to IV ketamine (n = 4). Neonates were gently hand restrained; after IV access was established and blood drawn for baseline measurements, the animals assigned to receive general anesthesia were given ketamine (20 mg/kg bolus IV) followed by a continuous IV infusion (20–50 mg · kg⁻¹ · h⁻¹ for 5 h) and additional IV boluses for maintenance of an intermediate surgical plane of anesthesia, defined as no movement and not more than 10% increase in heart rate or blood pressure in response to a profound mosquito-clamp pinch at hand and foot (checked every 30 min). The animals were tracheally intubated (semirigid fiber-optic endoscope; Karl Storz America, El Segundo, CA; 2.0 ID endotracheal tube; Mallinckrodt), mechanically ventilated (small animal ventilator; Harvard Apparatus, Holliston, MA), and maintained using extended physiologic monitoring (continuous end-tidal carbon dioxide, F_{IO}₂ [Capnomac; Datex Ohmeda, Madison, WI], electrocardiogram, peripheral oxygen saturation, noninvasive blood pressure [every 15 min; SurgiVet Advisor V9200; Smith Medical], rectal temperature [hand-held rectal thermometer, Vicks, V965F; KAZ Inc., Proctor and Gamble, Cincinnati, OH]), blood gases, metabolic profile, including pH, base excess, blood urea nitrogen, hematocrit, hemoglobin, sodium, chloride, potassium, and serum glucose and lactate concentrations [every 2 h; i-STAT]). After 5 h, the ketamine infusion was stopped and the animals were observed for 3 h. The trachea was extubated when appropriate, and the animals were then kept in an infant monkey incubator (Snyder ICU cage; Snyder Mfg., Centennial, CO) and were provided formula as tolerated. At the end of the 3-h observation period (8 h after time 0), and after final measurements, the animals received ketamine (20 mg/kg IV), followed by high-dose phenobarbital to secure a deep level of anesthesia, and were then transcardially perfusion-fixed to prepare the brain for histopathologic analysis. Animals that were randomized to the control group (no anesthe-

sia) were measured at baseline, received an IV injection of saline to mimic the stress to which the experimental animals were exposed before being anesthetized, and were then returned to their mothers until final measurements and euthanasia 8 h later.

Rationale for the Ketamine Protocol Used. The current study pertaining to ketamine is part of a larger research program aimed at comparing the potential toxic impact of several anesthetic drugs on the developing NHP brain. To make such a comparison, each agent must be administered in a manner that provides the same level of anesthesia for the same duration of time. Thus, the 5-h ketamine perfusion protocol used in this study was chosen to match a specific depth and duration of anesthesia that is used for each anesthetic drug being tested. The authors recognize that ketamine is not commonly used as a stand-alone drug in either pediatric or obstetric anesthesia to maintain patients in a fully anesthetized state for an extended period of hours, and this is a caveat that must be taken into consideration in interpreting the human significance of the findings.

Histopathologic Methods

For histopathologic analysis, the perfusion-fixed neonatal and fetal brains were cut on a vibratome into 70- μ M serial coronal sections across the entire rostrocaudal extent of the forebrain and midbrain and into serial sagittal sections across the brainstem and cerebellum. Sections were selected at 2-mm intervals and stained for detection and quantification of apoptotic profiles by AC3 immunohistochemistry, as described previously.^{3,8–27} Before the AC3 stain was applied, 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.

It can be considered a limitation of the current study that only a single posttreatment survival interval was used and only a single method (AC3 staining) was used to detect apoptotic profiles. We chose the AC3 staining procedure for several reasons: (1) It has been shown in numerous previous studies^{3,8–26} to mark faithfully cellular profiles that are undergoing apoptotic degeneration after exposure to various apoptogenic drugs, including ketamine^{13,15,16,23}; (2) It has been demonstrated that cellular profiles marked by the AC3 stain are also marked by the cupric silver stain, which selectively stains cells that are dead or dying^{1–5,12}; (3) It has been confirmed by electron microscopy that these cells display all of the classic ultrastructural characteristics of cells undergoing cell death by apoptosis^{1–3,7,8,12}; and (4) It has been found^{9,27} that the AC3 stain is valuable, not only for identifying cells that are undergoing apoptosis, but also for revealing whether the cell is in an early or advanced stage of degeneration and for revealing what type of cell is undergoing degeneration. The AC3 stain can provide this infor-

Table 1. Physiologic Variables of Pregnant Females (G120) during Ketamine Anesthesia

—	Anesthesia Time		
	0.5 h	2.5 h	4.5 h
Maternal			
Temp (°C)	37.6 (37.3–37.9)	37.7 (37.2–38.2)	37.8 (37.5–38.5)
MAP (mmHg)	94 (87–101)	68 (59–74)	61 (51–70)
HR (/min)	148 (139–161)	115 (101–132)	128 (106–144)
Fetal HR (/min)	178 (168–190)	160 (138–185)	157 (133–190)
Maternal			
ETCO ₂ (mmHg)	35 (33–29)	31 (27–37)	30 (24–37)
pH (arterial)	7.44 (7.38–7.49)	7.43 (7.40–7.46)	7.41 (7.40–7.44)
PACO ₂ (mmHg)	37 (35–39)	34 (31–38)	33 (32–34)
PAO ₂ (mmHg)	289 (284–294)	178 (122–259)	223 (209–249)
Hb (mg/dl)	13 (11–14)	12 (10–13)	12 (11–12)
Glucose (mM)	64 (59–73)	70 (47–92)	71 (56–91)
Lactate (mM)	0.9 (0.7–1.2)	0.5 (1.1–4.9)	0.7 (0.6–0.9)

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

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).

mation because, in the early stages of apoptotic degeneration, brain cells generate copious amounts of AC3 protein, which fills the cell body and its processes, so that the full microanatomy of the cell can be visualized by immunohistochemical staining with AC3 antibody in this

early stage. In the ensuing several hours, the processes shrivel and become fragmented, the cell body becomes condensed or rounded up into a spherical shape, and these changes signify that the cell is progressing from early to advanced stages of cell death.²⁷

Table 2. Physiologic Variables of Dams and Fetuses Immediately before and after Cesarean Section

Group	Dams after Induction of Anesthesia and before Cesarean Section		Fetus Immediately after Cesarean Section (Umbilical Artery)
	Temp (°C)	Control (n = 4)	37.7 (37.3–38.2)
	Ketamine (n = 3)	38.8 (38.5–39.2)	
MAP (mmHg)	Control (n = 4)	54 (42–68)	
	Ketamine (n = 3)	70 (57–84)	
HR (/min)	Control (n = 4)	171 (145–191)	
	Ketamine (n = 3)	155 (133–68)	
Fetal HR (/min)	Control (n = 4)	171 (165–183)	
	Ketamine (n = 3)	175 (140–213)	
ETCO ₂ (mmHg)	Control (n = 4)	34 (26–41)	
	Ketamine (n = 3)	28 (23–34)	
pH (venous)	Control (n = 4)	7.27 (7.20–7.35)	7.23 (7.20–7.25)
	Ketamine (n = 3)	7.39 (7.38–7.40)	7.17 (7.08–7.26)
PvCO ₂ (mmHg)	Control (n = 4)	43 (38–48)	58 (53–62)
	Ketamine (n = 3)	35 (34–36)	65 (49–81)
			—
PvO ₂ (mmHg)	Control (n = 4)	56 (48–63)	16 (10–26)
	Ketamine (n = 3)	43 (38–47)	12 (11–13)
			—
Hb (mg/dl)	Control (n = 4)	14 (12–15)	13 (12–16)
	Ketamine (n = 3)	13 (12–14)	15 (14–15)
			—
Glucose (mM)	Control (n = 4)	83 (61–96)	56 (34–66)
	Ketamine (n = 3)	53 (46–65)	50 (34–66)
Lactate (mM)	Control (n = 4)	6.7 (3.5–9.2)	4.0 (2.8–5.5)
	Ketamine (n = 3)	0.8 (0.7–0.8)	4.5 (2.9–6.1)

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

ETCO₂ = end-tidal carbon dioxide; Hb = hemoglobin; HR = heart rate; MAP = mean arterial pressure (noninvasive); PvCO₂ = venous carbon dioxide tension; PvO₂ = venous oxygen tension; Temp = temperature (rectal).

Quantification of Apoptotic Cells

AC3-positive cellular profiles were counted by an investigator who was blinded to the treatment condition. Each stained section was comprehensively scanned by light microscopy using a $\times 10$ objective lens and a computer-assisted stereoinvestigator system (Microbrightfield Inc., Williston, VT) with an electronically driven automatic stage to plot the number and location of each AC3-stained neuronal profile in each section. The total area scanned and the total number of stained neurons per section and per brain were computer-recorded. The total area was converted to volume by multiplying by the thickness of the section ($70 \mu\text{M}$), and the total number of neurons was divided by the total volume of tissue within which counts were made to yield a density count (number of stained profiles per mm^3) for each brain. Density counts were converted to total numbers of apoptotic neurons in a given brain by calculating the mean volume of tissue per counted section and multiplying by the number of serial sections cut from the entire brain. The density of apoptotic neurons (number per mm^3) times the total brain volume yielded an accurate estimate of the total number of apoptotic neurons per brain.

Statistical Evaluation

There are important ethical and logistical considerations regarding NHP experimentation. It is important to include as few animals as are needed to answer research questions with as much precision as possible. Previous experiments in NHP 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 five per group.^{13,14,16,20–22,23–25,27} For statistical analysis, an unpaired Student *t* test with Welch correction, where appropriate, was used, a two-sided *P* value < 0.05 was judged significant, and the 95% CI for the mean difference provided a measure of precision. Statistical analysis was performed with Analyze-It[®] Statistical Software (Leeds, United Kingdom) for Microsoft Excel[®] (Redmond, WA).

Results

Fetuses

The pregnant dams were assigned on postconception day 120 ± 2 to receive general anesthesia (ketamine group; body weight range 6.15–7.45 kg; $n = 3$) or not (control group; 5.40–7.45 kg; $n = 4$). Induction (10 mg/kg, IV) and maintenance of ketamine anesthesia was generally well tolerated, and in all animals, the trachea was intubated for airway control. The animals retained adequate spontaneous ventilation and were connected to the breathing circuit of a conventional anesthesia machine to control oxygenation and gas exchange. Blood gases, acid-base status, laboratory variables, glucose and lactate concentration, as well as hemodynamic variable

and body temperature were controlled within species-specific physiologic limits throughout the experimental period. Fetal heart rates remained within physiologic limits and varied in concert with the maternal heart rate. The animals were extubated without problems within approximately 90 min after the ketamine infusion was stopped, and the dams remained somnolent and were kept in a climatized cage under direct observation until the scheduled cesarean section. The cesarean sections were conducted using ketamine general anesthesia and standard intraoperative monitoring according to established protocols at the Oregon National Primate Research Center surgical center. The animals tolerated the procedure well and retained stable vital signs, blood gases, and acid-base levels within physiologic limits (table 1). 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 transcardial perfusion fixation as described in Materials and Methods. Fetal body weight (ketamine group 215–237 g; control group 129–252 g) and other standard body dimensions were age appropriate for the macaque G120 time point.

During the first 90 min, ketamine doses of $1.10\text{--}1.35 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ were necessary to achieve the desired anesthetic depth (no movement and less than 10% increase in heart rate or blood pressure in response to profound mosquito-clamp pinch). The total doses of ketamine per animal were 260.4–432.6 mg/kg during the 5-h anesthesia period ($48.1\text{--}86.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). One dam showed clinical signs of seizure activity (eye blinking, bilateral upper extremity

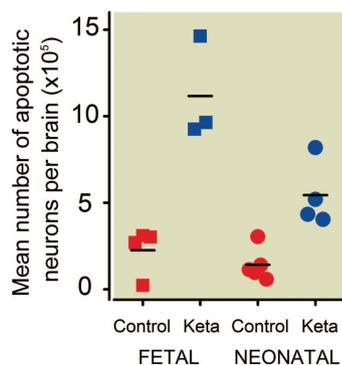


Fig. 1. Neuroapoptosis, as detected by activated caspase-3 (AC3) positivity, induced in the fetal and neonatal monkey brain by ketamine exposure for 5 h. The mean number of AC3-positive neuronal profiles in the ketamine-exposed fetal brains was 4.9-fold higher than in the drug-naïve fetal control brains. The difference between the means for ketamine-exposed versus control brains was 8.92×10^5 (95% confidence interval [CI], 13.18×10^5 to 4.65×10^5 , $P = 0.003$). The mean number of AC3-positive neuronal profiles in the ketamine-exposed neonatal brains was 3.83-fold higher than in the drug-naïve neonatal control brains. The difference in the mean number of apoptotic neurons between the ketamine and control groups was 4.02×10^5 (95% CI, 6.29×10^5 to 1.74×10^5 , $P = 0.004$).

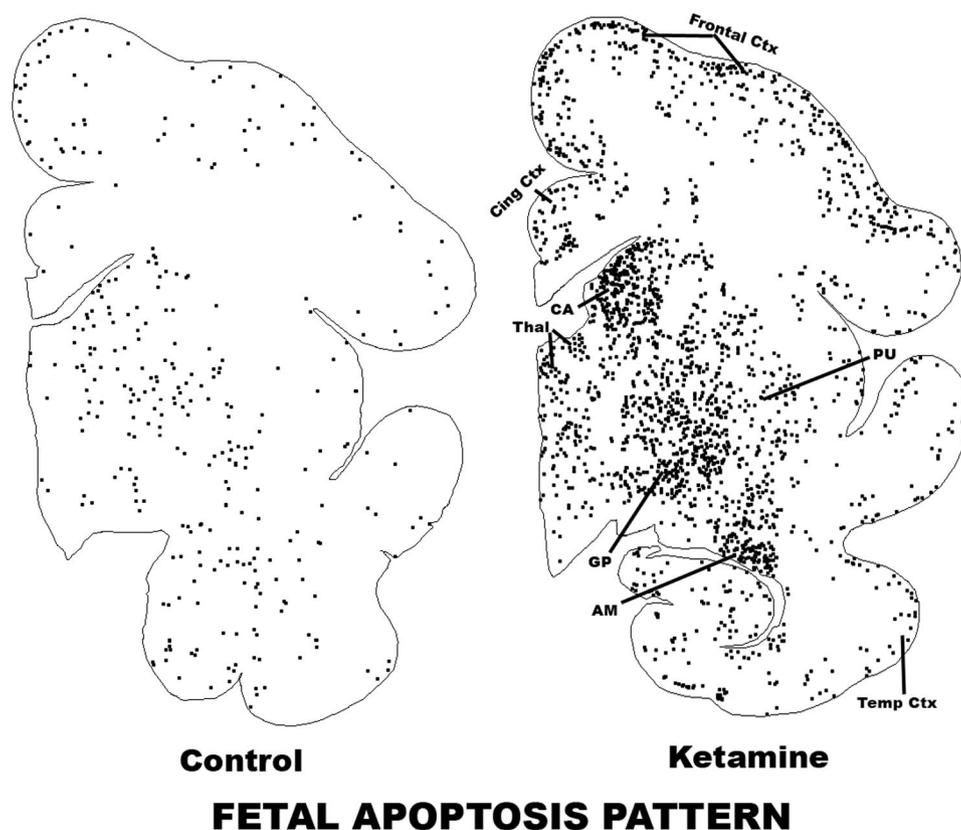


Fig. 2. Computer plot depicting the pattern of neuroapoptosis in ketamine-exposed *versus* control fetal macaque brains. These sections display several gray matter zones, including frontal, cingulate (Cing) and temporal (Temp) cortices, thalamus (Thal), caudate nucleus (CA), putamen (PU), globus pallidus (GP), and amygdala (AM). An outline of each section was sketched into the computer, and the location of each activated caspase-3-stained neuron was marked by a *black dot*. The pattern of neuronal staining in the control brain caused by natural neuroapoptosis is similar to the pattern in the ketamine-exposed brain, but stained neuronal profiles in the former are sparse and in the latter are abundant and heavily concentrated in regions such as the frontal cortex, thalamus, caudate nucleus, globus pallidus, and amygdala.

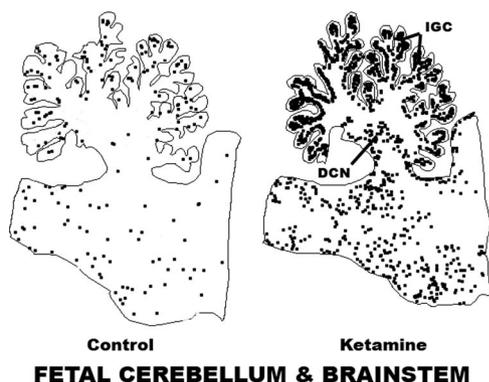
jerks) at 85 min of ketamine anesthesia. This animal received 2.5 mg IV diazepam, which immediately stopped the clinical seizure-like activity. The subsequent clinical course of this animal was uneventful.

Animals assigned to the control group had unremarkable baseline measurements and underwent cesarean section 5 h later according to the same protocol and monitoring. At time of delivery, the measured physiologic variables (fetal heart rate, metabolic status, and blood gases of dam and fetus) were within physiologic limits and comparable with those in the ketamine group. One dam in the control group showed an anion gap-positive metabolic acidosis with increased lactate levels. Additional laboratory evaluation of this animal revealed mild aspartate aminotransferase elevation and low globulin levels. Differential diagnoses include subclinical chronic liver or renal disease, chronic infection, or a tumor disease. At the time of the experiment, the animal was clinically unremarkable as was the delivered fetus. The brain of this fetus had a low apoptotic profile count similar to the other brains in the control group.

Quantitative evaluation of AC3-stained brain sections from the fetal brains (G120) revealed that the mean (\pm SD)

number of AC3-positive neuronal profiles per brain in the three ketamine-exposed fetal brains was $11.17 \times 10^5 \pm 2.99 \times 10^5$, and in the four control fetal brains was $2.26 \times 10^5 \pm 1.37 \times 10^5$ (fig. 1), which amounts to a 4.9-fold increase in the number of apoptotic neurons in the ketamine-exposed fetal brains compared with the drug-naïve controls. The difference between the means for ketamine-exposed *versus* control brains was 8.92×10^5 (95% CI, 13.18×10^5 to 4.65×10^5 , $P = 0.003$).

The pattern of augmented neuroapoptosis in the ketamine-exposed fetal brains was widespread. Brain regions preferentially affected were the cerebellum, brainstem, inferior and superior colliculi, several thalamic nuclei, caudate nucleus, putamen, globus pallidus, nucleus accumbens, several neuronal groups in the hypothalamus and basal forebrain, and all divisions of the neocortex and several limbic cortical regions, with the notable exception of the hippocampus, which showed very little effect. The most severely affected regions were the cerebellum, caudate nucleus, putamen, and nucleus accumbens. The pattern of neuroapoptosis induced by ketamine in the fetal brain is illustrated in figures 2 and 3. The histologic appearance of neurons undergo-



FETAL CEREBELLUM & BRAINSTEM

Fig. 3. Computer plot showing the pattern of neuroapoptosis in sagittal sections of the cerebellum and brainstem of ketamine-exposed *versus* control fetal macaque brains. The apoptotic profiles in the cerebellar folia are in the internal granule cell (IGC) zone and those at the base of the cerebellum are in the region of the deep cerebellar nuclei (DCN).

ing apoptosis in the NHP fetal brain is illustrated in figures 4 and 5.

Neonates

On the day of the experiment, the infant macaques (ketamine group [n = 4], mean age 5.7 days, 449–680 g body weight; control group [n = 5], mean age 5.6 days, body weight 430–546 g) were assigned to receive general anesthesia or not. IV induction (20 mg/kg) and maintenance of ketamine anesthesia were well tolerated by all infant animals. At adequate anesthetic depth, the trachea was intubated in all animals and the lungs were mechanically ventilated, which allowed control of blood gases and acid-base status within physiologic limits throughout the 5 h of general anesthesia. Active warming and fluid and glucose application maintained basic homeostasis, documented by continuous intraoperative monitoring of hemodynamic variables and point-of-care laboratory results throughout the entire experimental period (table 3). Within 45 min after cessation of the ketamine infusion, the infant macaques were weaned from the ventilator, extubated without complications, and maintained in an animal incubator at physiologic condition. Animals remained drowsy and were observed directly for 3 h until transcardial perfusion.

In the first 90 min, $0.52\text{--}1.28\text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ketamine was applied to the neonatal macaques to achieve the desired anesthetic depth (no movement and less than 10% increase in heart rate or blood pressure in response to profound mosquito-clamp pinch). The total doses of ketamine per infant macaque were 92–280 mg/kg during the 5-h anesthesia ($18.40\text{--}56.0\text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). There were no adverse effects of ketamine anesthesia observed in the neonatal cohort.

Quantitative evaluation of AC3-stained sections from the postnatal day 6 neonatal brains revealed that the mean (\pm SD) number of apoptotic neuronal profiles per brain in the four ketamine-exposed neonatal brains was $5.44 \times 10^5 \pm$

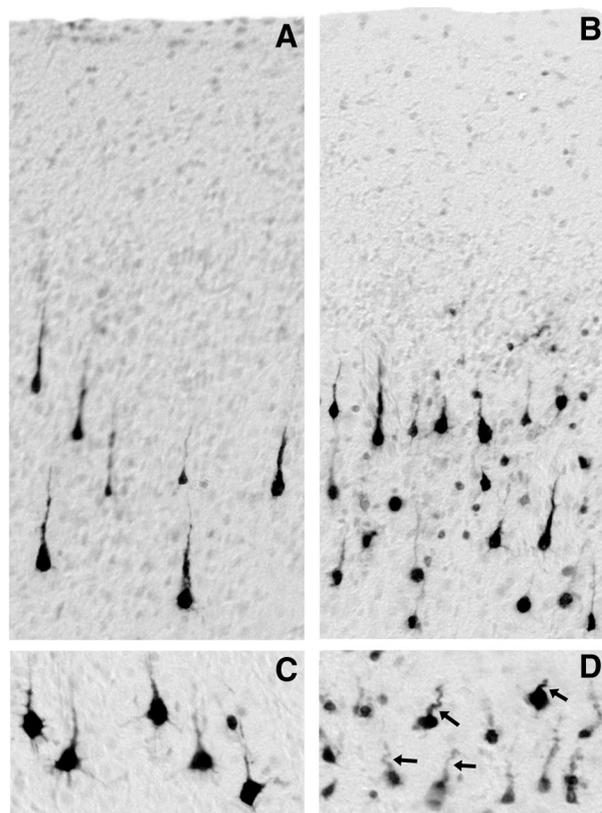


Fig. 4. All panels are from fetal brains harvested 3 h after a 5-h exposure to ketamine. They display the histologic appearance of activated caspase-3 (AC3)-positive neuronal profiles in layers III (A) and V (C) of the frontal cortex, layer III of the temporal cortex (B), and the pyramidal cell layer in the subiculum (D). These panels depict neurons in several stages of degeneration. Subicular neurons are in a late stage of degeneration because they are very sensitive and tend to degenerate early; this is revealed by faint and incomplete staining and by the corkscrew deformity of their apical dendrites (arrows, D). Affected neurons in layer III of the temporal cortex are heterogeneous; some degenerate early and some late. The early-dying cells have degenerated beyond recognition and present as faintly stained small round dots (B). Neurons in layer V of the frontal cortex (C) are relatively resistant and have just begun to degenerate. Therefore, they are robustly AC3-positive and show very few signs of structural deformity.

1.89×10^5 and in the five control neonatal brains was $1.42 \times 10^5 \pm 0.95 \times 10^5$ (fig. 1), which equals a 3.83-fold increase in the number of apoptotic neurons in the ketamine-exposed brains compared with the brains from drug-naïve controls. The difference in the mean number of apoptotic neurons between the ketamine and control groups was 4.02×10^5 (95% CI, 6.29×10^5 to 1.74×10^5 , $P = 0.004$).

Ketamine caused a less widespread pattern and less dense concentration of neuroapoptosis in the neonatal brains than in the fetal brains. A major difference between the fetal and neonatal response to ketamine was that many of the caudal and subcortical brain regions that were affected preferentially in the fetal brains, including the cerebellum and brainstem,

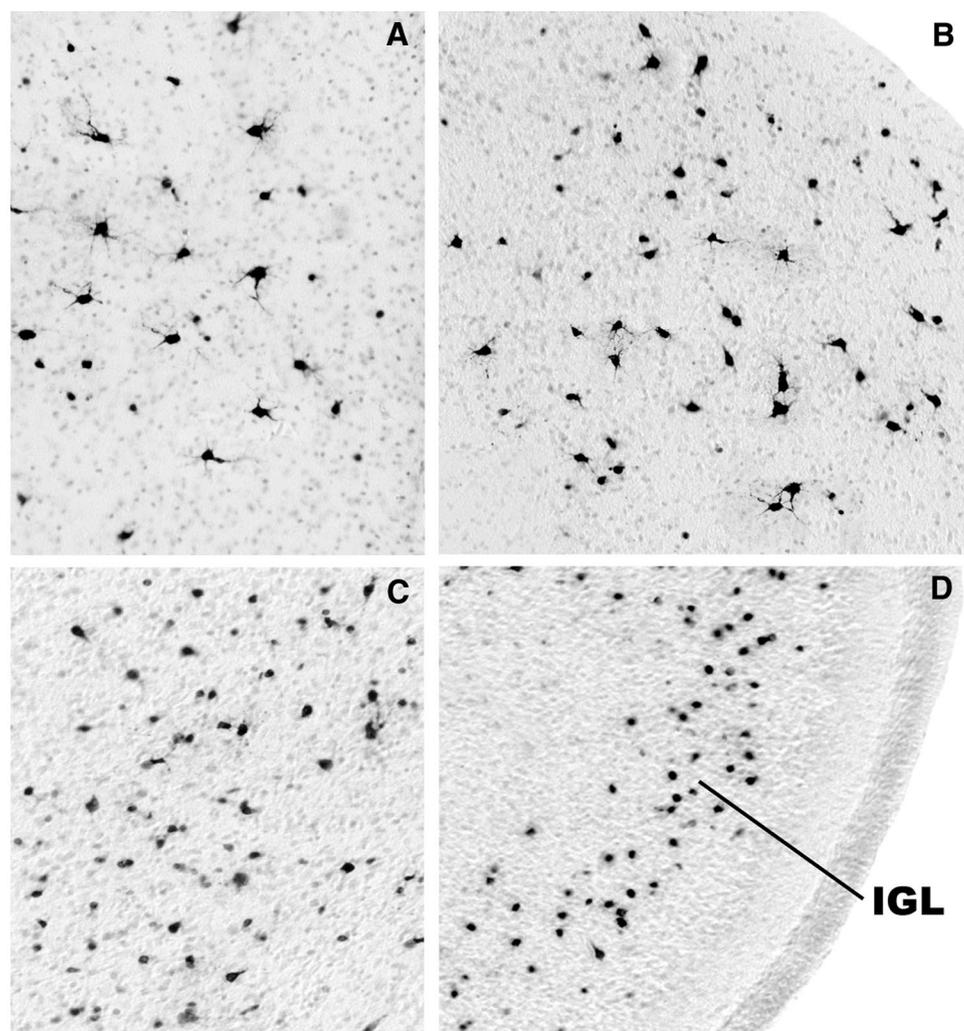


Fig. 5. The histologic appearance of activated caspase-3–positive neuronal profiles in the lateral septum (A), anterodorsal thalamic nucleus (B), caudate nucleus (C), and cerebellum (D) of fetal brains harvested 3 h after a 5-h exposure to ketamine. Most of the affected neurons in the septum (A) and thalamus (B) are large multipolar neurons that are relatively resistant and thus are in a relatively early stage of degeneration. The affected neurons in the caudate nucleus (C) are morphologically heterogeneous. A small cell type degenerates rapidly and presents as a faintly stained, small, condensed, shrunken structure amid other larger cells that degenerate on a slightly later time schedule. The most sensitive cell population in the cerebellum (D) is one that is located in the inner portion of the inner granule cell layer (IGL). These cells degenerate rapidly after exposure of either infant rodents or fetal monkeys to alcohol or anesthetic drugs. As explained in previous publications,^{8,39} it is not clear whether they are granule cells or other cell types that are migrating through the granule cell layer.

showed little or no neuroapoptotic response in the neonatal brains. A major exception to this rule was that the strongest neuroapoptotic response to ketamine in the neonatal brain was in the basal ganglia (caudate nucleus, putamen, nucleus accumbens, globus pallidus) and several thalamic areas (fig. 6). These same regions, although not necessarily the same neuronal populations within each region, also had a high neuroapoptotic count after ketamine exposure of fetal brains. The neuroapoptotic response in the cerebral cortex of ketamine-exposed neonates was distributed evenly across cortical regions and was increased only moderately compared with controls in most cortical regions. This distribution is different from that recently described in neonatal macaque brains exposed to isoflurane.²⁷ The apoptotic response after

isoflurane exposure was most prominent in several divisions of the neocortex, especially the temporal cortex (layers II and IV) and the primary visual cortex (layers II and V). The striking difference in pattern and density of neuroapoptosis in the primary visual cortex after isoflurane exposure *versus* ketamine exposure is illustrated in figure 7.

The rate of natural neuroapoptosis and of ketamine-induced neuroapoptosis in the fetal *versus* neonatal period is illustrated in figure 8. The rate of neuroapoptosis declines between the fetal and neonatal period for both phenomena, and the decline is more rapid for ketamine-induced than for natural neuroapoptosis. However, because the magnitude of the drug-induced phenomenon is so much higher, the two curves perhaps will approach zero at roughly the same time in

Table 3. Physiologic Variables of Neonatal Animals (Postnatal Day 6) during Ketamine Anesthesia and Recovery

	Group	Time				
		Baseline	Anesthesia			3 h Recovery
			0.5 h	2.5 h	4.5 h	
Temp (°C)	Control (n = 5)	37.7 (37.3–38.2)	—	—	—	37.8 (37.7–37.9)
	Ketamine (n = 4)	36.5 (35.9–37.1)	36.8 (36.8–36.8)	38.0 (37.7–38.4)	38.3 (37.9–39.3)	38.0 (37.7–38.4)
MAP (mmHg)	Control (n = 5)*	—	—	—	—	—
	Ketamine (n = 4)	*	65 (60–76)	50 (41–59)	43 (35–50)	51 (44–60)
HR (/min)	Control (n = 5)*	—	—	—	—	—
	Ketamine (n = 4)	*	187 (147–210)	166 (128–218)	163 (122–210)	196 (160–222)
ETCO ₂ (mmHg)	Control (n = 5)*	—	—	—	—	—
	Ketamine (n = 4)	—	26 (23–28)	29 (28–30)	28 (28–28)	—
pH (venous)	Control (n = 5)	7.30 (7.27–7.34)	—	—	—	7.31 (7.23–7.36)
	Ketamine (n = 4)	7.29 (7.24–7.34)	7.43 (7.39–7.49)	7.33 (7.22–7.41)	7.34 (7.29–7.37)	7.28 (7.12–7.39)
PvCO ₂ (mmHg)	Control (n = 5)	43 (39–56)	—	—	—	40 (37–45)
	Ketamine (n = 4)	39 (37–42)	28 (24–36)	33 (29–37)	36 (30–43)	40 (35–48)
PvO ₂ (mmHg)	Control (n = 5)	25 (20–34)	—	—	—	33 (28–40)
	Ketamine (n = 4)	28 (26–31)	37 (30–41)	35 (21–41)	33 (28–40)	28 (25–31)
Hb (mg/dl)	Control (n = 5)	18 (16–19)	—	—	—	15 (14–17)
	Ketamine (n = 4)	14 (12–15)	12 (10–15)	11 (9–13)	11 (10–12)	11 (10–13)
Glucose (mM)	Control (n = 5)	92 (55–159)	—	—	—	74 (51–95)
	Ketamine (n = 4)	66 (52–73)	114 (69–233)	131 (105–166)	90 (60–155)	56 (46–67)
Lactate (mM)	Control (n = 5)	3.9 (1.9–5.3)	—	—	—	3.1 (1.8–5.5)
	Ketamine (n = 4)	6.1 (3.3–9.5)	2.69 (1.6–4.4)	3.2 (1.1–4.9)	2.1 (1.6–2.8)	3.0 (1.2–4.4)

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

* Variables not obtained.

Baseline = animal awake, no sedation, hand restrained, IV access established; 3-h recovery = animal awake, residual mild sedation, hand restrained, IV access in place; ETco₂ = end-tidal carbon dioxide; Hb = hemoglobin; HR = heart rate; MAP = mean arterial pressure (noninvasive); Pvco₂ = venous carbon dioxide tension; Pvo₂ = venous oxygen tension; Temp = temperature (rectal).

the late neonatal or early juvenile period. The dashed lines with arrows represent the amount of neuroapoptosis that can be attributed to ketamine treatment at each age (8.92×10^5 for fetuses and 4.02×10^5 for neonates). The value for fetuses is 2.22 times greater than the value for neonates.

Dose Effects

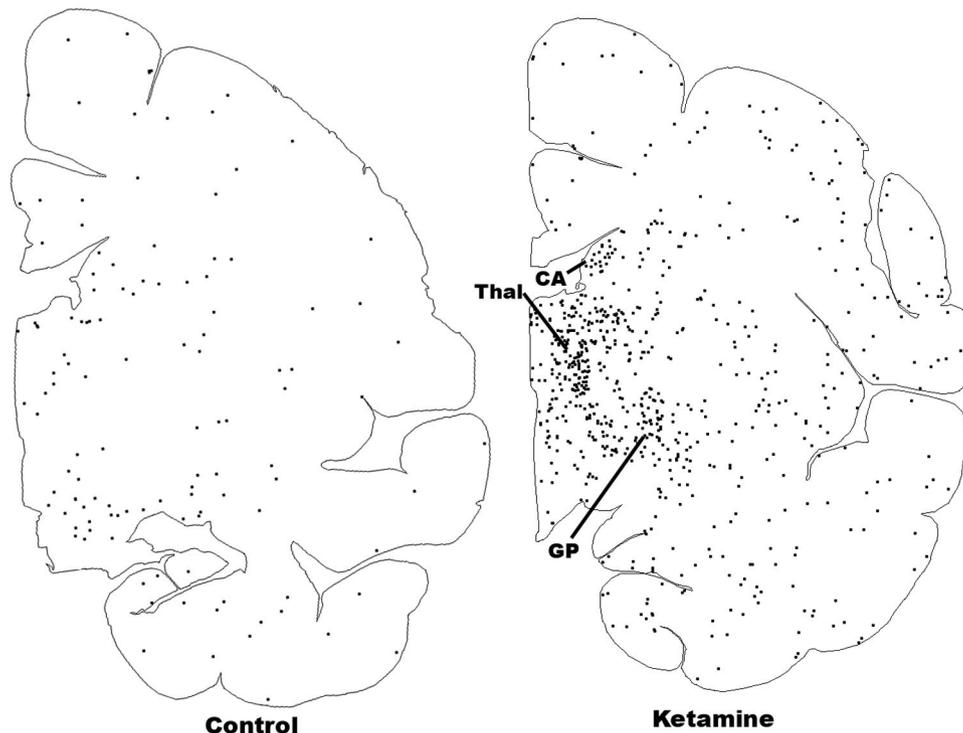
Ketamine anesthesia was titrated clinically to achieve a moderate anesthesia plane (see Materials and Methods), and the total doses of ketamine per kilogram body weight varied between individuals and between the fetal and neonatal cohorts. Although individual biologic variability and the small number of animals preclude reaching firm conclusions regarding linearity, a linear dose-response relationship is suggested by the fact that at each age, the lowest dose was associated with the lowest apoptosis count and higher doses were associated with correspondingly higher counts, with the exception of one neonate that received a high dose and had a lower than would have been predicted neuroapoptotic score (table 4).

Discussion

It has been estimated that the minimum ketamine exposure duration for inducing neuroapoptosis in the developing NHP brain lies somewhere between 3 and 9 h²⁰. The current findings clarify that a 5-h ketamine infusion is sufficient to

induce a significant neuroapoptotic response in either the fetal or neonatal NHP brain. A comparison of the brain growth spurt curve for the rhesus macaque with that for the human⁶ suggests that the rhesus brain at G120 may be at a developmental stage approximately equivalent to the brain of a mid- to late-third-trimester human fetus (or prematurely born infant), and the postnatal day 6 rhesus may be equivalent to that of a 4–6-month-old human infant. Therefore, the period in human development to which the current data are potentially most relevant would be an 8-month interval from the middle of the third trimester of gestation to approximately 6 months after birth.

The total number of neurons that showed AC3 positivity as a result of ketamine exposure was more than two times greater when exposure occurred in the fetal period. This suggests that human risk for ketamine-induced neuroapoptosis may be greatest at some point in the third trimester of prenatal development, which corresponds in general to the range of brain ages for human infants born prematurely. Such infants often are exposed to anesthetic drugs for procedural sedation and/or surgery. Because a G120 rhesus brain equates closely to a late, but not early, third trimester human brain, this points to the need for NHP studies examining ketamine neurotoxicity at additional gestational ages corresponding to a human brain age earlier in the third trimester. Similarly, whereas all of the recent human epidemiologic



NEONATAL APOPTOSIS PATTERN

Fig. 6. Computer plot revealing the pattern of neuroapoptosis in ketamine-exposed *versus* control neonatal macaque brains. These sections are cut through the forebrain at the same level as the fetal sections in figure 3. Although, the density of apoptotic neural profiles is decreased in the neonatal compared with the fetal brain, the pattern at both ages features a high density of apoptotic profiles in the basal ganglia, including the caudate nucleus (CA), globus pallidus (GP), and adjacent thalamus (Thal) relative to other regions of the neonatal brain.

studies pertaining to developmental anesthesia neurotoxicity^{35–39} have focused on full-term infants and children, the focus of future human research should be expanded to include third trimester fetuses and prematurely born infants.

Our data indicating that ketamine, an NMDA antagonist, may be more damaging to the fetal than neonatal NHP brain, has implications in both a clinical anesthesia and a drug abuse context. Maternal abuse of drugs is the most common cause of preventable developmental brain damage in modern society. Alcohol, the most widely and frequently abused drug in human experience, which has damaged more fetal brains (fetal alcohol spectrum disorder) than any drug in human history, is also an NMDA antagonist, as is phencyclidine (1-(1-phenylcyclohexyl)-piperidine [Angel Dust]), a dissociative anesthetic and notorious psychotomimetic drug of abuse. ‡‡ In recent years, ketamine has become a popular drug of abuse in the United States and other regions of the world.^{28–30} Our findings identify the third trimester of pregnancy as a developmental period when neurons may be at peak sensitivity to the apoptogenic action of NMDA antagonist drugs. This information is relevant to the use of ketamine to anesthetize pregnant women or premature infants^{41–43} and also to the maternal abuse of ketamine, phencyclidine, or alcohol during this period of heightened fetal sensitivity.

A comparison of the fetal and neonatal patterns of damage induced in the macaque brain by ketamine suggests that in the fetal period, caudal and some subcortical brain regions transition through their window of vulnerability, so that a number of these regions are vulnerable in the fetal but not neonatal period. A similar finding was reported recently⁴⁴ for fetal macaques exposed to alcohol at G120 *versus* at G155 (1 week before term). However, it is noteworthy that several subcortical brain regions, especially the basal ganglia and thalamus, were severely affected in both the fetal and neonatal period. This observation has potentially important implications, in that a deficit in neuronal mass of the basal ganglia has been emphasized in several reports as a prominent finding in children who were exposed *in utero* to alcohol,^{45,46} and the same finding was reported recently in children exposed *in utero* to antiepileptic drugs.⁴⁷ Alcohol and antiepileptic drugs have apoptogenic properties similar to those of ketamine.^{2–4,13,44}

The pattern of degeneration induced by ketamine in the fetal NHP brain differed substantially from the pattern induced by ketamine in the neonatal brain. This signifies that if anesthesia-induced neuroapoptosis in the primate brain gives rise to lasting neurocognitive disabilities, the nature of the disabilities will vary as a function of the time of exposure. This will complicate the task of establishing correlations be-

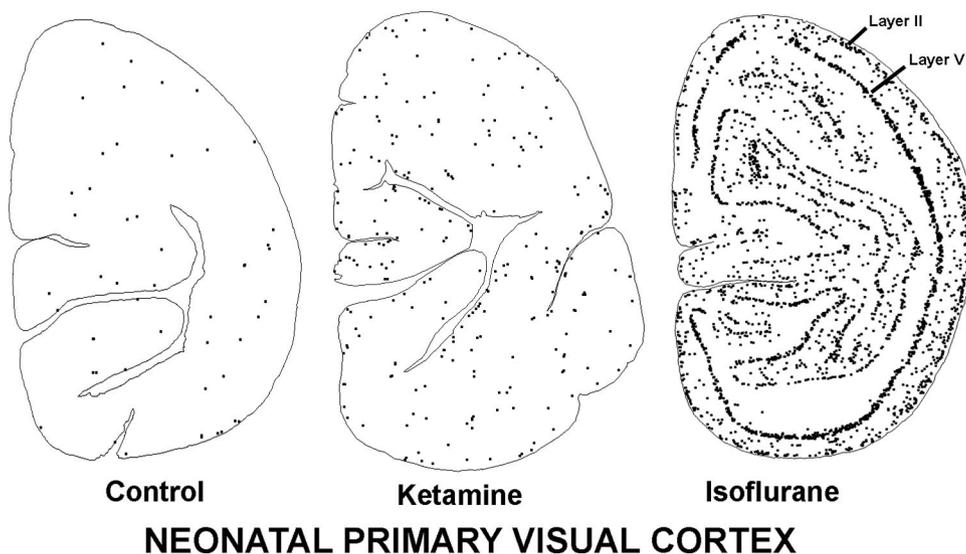


Fig. 7. Apoptosis induced by ketamine *versus* isoflurane in the neonatal primary visual cortex. The ketamine illustration is from the current study, and the isoflurane illustration is reproduced from the authors' previous publication pertaining to isoflurane-induced neuroapoptosis.²⁷ The control brain has a sparse and randomly scattered pattern of neuroapoptosis. The ketamine-exposed visual cortex has a moderately increased number of neural apoptotic profiles, and they are distributed in a less random pattern. The isoflurane-exposed visual cortex is heavily studded with apoptotic neural profiles, and they are organized in a distinctive laminar pattern corresponding to the location of neurons in layers II and V.

tween anesthesia exposure of the developing human brain with subsequent neurobehavioral disturbances, in that the disturbances will display wide-ranging heterogeneity correlated not with anesthesia *per se*, but with age at the time of exposure. In addition, the duration of exposure and type of anesthetic drug and many other interacting variables will complicate the analysis.

Our findings document important differences in the patterns of neuroapoptosis induced in the neonatal NHP brain

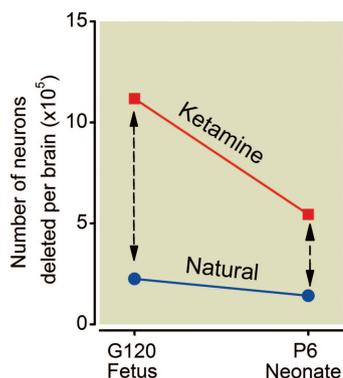


Fig. 8. The impact of ketamine treatment on neurons in the fetal *versus* neonatal brain is depicted in terms of the mean number of neurons affected in the brain by ketamine at each age. For comparison, the mean number undergoing natural neuroapoptosis in the control brains is also illustrated. The rate of neuroapoptosis induced by ketamine declines more sharply between the fetal and neonatal period than the rate for natural neuroapoptosis. The *dashed lines with arrows* represent the amount of apoptosis that can be attributed to ketamine treatment at each age. The number of neurons affected by ketamine exposure in the fetal period is 2.2 times greater than that in the neonatal period.

by ketamine, as observed in the current study, *versus* isoflurane, as observed in a previous study.²⁷ The most prominent difference is that the isoflurane pattern was heavily concentrated in the temporal and occipital lobes, where it featured a laminar pattern of degeneration affecting many neurons in layers II and IV of the temporal cortex and layers II and V of the primary visual cortex. The ketamine-exposed neonatal brains displayed a pattern more evenly and lightly distributed over various divisions of the cerebral cortex, and none of the ketamine-exposed brains showed a laminar pattern of dense degeneration selectively affecting large numbers of neurons in specific layers of the temporal and primary visual cortices.

Our findings corroborate those of Slikker *et al.* regarding NHP susceptibility to ketamine's apoptogenic action, but

Table 4. Fetal and Neonatal Dose and Response Data

Animal Identification	Dose (mg/kg)	NA Density (Profiles/mm ³)
FE 17	260	35.54
FE 15	339	36.86
FE 16	433	65.97
Neo 13	92	5.35
Neo 14	160	10.24
Neo 16	260	14.00
Neo 15	280	7.60

All neuroapoptosis (NA) values for both fetuses and neonates are consistent with a linear dose-response relationship, except for Neonate 15, whose NA score is lower than would be theoretically predicted. Ketamine doses indicated for each fetus are expressed in mg/kg of the body weight of the respective dam; doses for each neonate are expressed in mg/kg body weight of the respective neonate.

FE = fetus; NA = neuroapoptotic; Neo = neonate.

some of our observations are different from theirs. They reported that in both neonatal and fetal rhesus macaques, ketamine triggered neuroapoptosis in only one brain region, the frontal cortex.²⁰ Our findings document a more widespread pattern of neuroapoptosis in both the fetal and neonatal macaque brain after 5-h exposure to ketamine. We believe these divergent results can best be explained by differences in experimental protocol. For example, our methods for sampling and counting apoptotic profiles are different from theirs, and their experiments, in which ketamine was found to induce neuroapoptosis, are based on a prolonged time frame and ours on a much shorter time frame. They maintained the ketamine infusion for either 24 or 9 h and allowed an additional 6-h survival time before obtaining the brains for histologic analysis. Thus, the total time for their experiments was either 30 or 15 h. We terminated drug exposure at 5 h and allowed an additional 3 h survival, for a total duration of 8 h. We geared our experiments to a shorter time frame because we have observed in numerous previous studies in rodents^{3,5,9,11,12-14,16,19} and in NHPs exposed to alcohol⁴⁴ that evidence for neuroapoptosis (caspase 3 activation) is detectable in many brain regions within approximately 3–5 h after initiation of drug exposure. To reliably detect this rapid apoptosis response, we have found AC3 immunohistochemistry to be the best method. However, once the apoptosis cascade becomes evident by AC3 staining, it transpires rapidly to end-stage cell death. Within a time frame of 5–6 h, the apoptotic profiles begin to lose immunoreactivity, undergo degradation into small fragments, and are phagocytized.^{3,11} Thus, if brains are examined at 15 or 30 h after initiation of anesthesia, it is possible to detect late-dying cells, but the most sensitive cells (those that died early) may not be detected.

Paule *et al.*⁴⁰ have reported relatively robust and apparently permanent neurocognitive deficits in rhesus macaques after neonatal exposure to ketamine for 24 h. The human relevance of this finding is difficult to assess because of the long exposure time. Our demonstration that, within a more clinically relevant exposure time, both ketamine and isoflurane induce a significant neuroapoptosis response in the developing monkey brain clearly indicates the need for well-designed research to clarify whether these more clinically relevant anesthesia exposure conditions are associated with long-term neurobehavioral disturbances.

In conclusion, our data demonstrate that a 5-h exposure to ketamine, an anesthetic drug with NMDA antagonist properties, is sufficient to induce a significant neuroapoptosis reaction in the developing NHP brain at either a fetal or neonatal age. The apoptotic response at the fetal age (early third trimester) was 2.2 times greater than at the neonatal age, which signifies that the third trimester of pregnancy may be a period when the primate brain is particularly sensitive to the neuroapoptogenic action of NMDA antagonist drugs. This is potentially important in a public health context because at this developmental age, human fetuses and prema-

ture infants are exposed to NMDA antagonist drugs (ketamine, nitrous oxide), either alone or together with other neuroactive agents, for sedative, analgesic, or anesthetic purposes, and fetuses also may be exposed to NMDA antagonists (ketamine, phencyclidine, alcohol) by mothers who abuse these drugs for recreational purposes.

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