

## RESEARCH ARTICLE

# Clinical Anesthesia Causes Permanent Damage to the Fetal Guinea Pig Brain

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## INTRODUCTION

General anesthetics are unique pharmacological agents widely used in modern anesthesiology because of their potent inhibition of neuronal activity, which is clinically manifested as loss of consciousness and/or insensitivity to painful stimuli. Over the last 60 years, great advances in anesthesiology have made complex surgical interventions possible even in premature infants and fetuses *in utero*. Although exposure of the immature central nervous system to general anesthetics is considered to be safe, recent reports of *in vivo* (21, 22, 24, 61, 62) and *in vitro* (57) studies indicate that common general anesthetics may be damaging to developing neurons. For instance, several classes of intravenous anesthetics, such as barbiturates (3, 22, 42), benzodiazepines (3, 22, 42) and ketamine (21, 50, 53), have been shown to cause severe and widespread neuronal degeneration in the immature mammalian brain. In addition, we recently showed that exposure of immature rats at the peak of their brain development (ie, synaptogenesis) to the volatile anesthetic isoflurane, either alone or in combination with midazolam, an intravenous benzodiazepine, and nitrous oxide (N<sub>2</sub>O), an inhalational anesthetic, for 6 h triggered widespread neurodegeneration that was apoptotic in nature (24, 61). Moreover, when rats exposed to these anesthetics in infancy were tested as juveniles and adults, they were found to have persistent learning and memory deficits (24).

## Abstract

Exposure of the immature brain to general anesthesia is common. The safety of this practice has recently been challenged in view of evidence that general anesthetics can damage developing mammalian neurons. Initial reports on immature rats raised criticism regarding the possibly unique vulnerability of this species, short duration of their brain development and a lack of close monitoring of nutritional and cardiopulmonary homeostasis during anesthesia. Therefore, we studied the neurotoxic effects of anesthesia in guinea pigs, whose brain development is longer and is mostly a prenatal phenomenon, so that anesthesia-induced neurotoxicity studies of the fetal brain can be performed by anesthetizing pregnant female pigs. Because of their large size, these animals made invasive monitoring of maternal and, indirectly, fetal well-being technically feasible. Despite adequate maintenance of maternal homeostasis, a single short maternal exposure to isoflurane, whether alone or with nitrous oxide and/or midazolam at the peak of fetal synaptogenesis, induced severe neuroapoptosis in the fetal guinea pig brain. As detected early in post-natal life, this resulted in the loss of many neurons from vulnerable brain regions, demonstrating that anesthesia-induced neuroapoptosis can cause permanent brain damage.

Thus, it appears that the use of common general anesthetics in clinically relevant doses may not be as innocuous to the developing mammalian brain (reviewed by 37) as was previously thought. However, several issues regarding the technical approach used in initial studies, particularly the duration of animals' exposure to anesthesia and the maintenance of their nutritional and cardiopulmonary homeostasis while under anesthesia, have raised severe criticism. For example, the neurotoxic anesthesia protocols were regarded as being prolonged (average 6 h), taking proportionally substantial time during the 2 to 3 weeks of rat and mouse brain development. As the majority of exposures to pediatric anesthesia are relatively short, studies comparing the susceptibility of species with different durations of brain development to anesthesia protocols of similar or shorter duration are crucial for appropriate risk assessment.

As noted, another important criticism regards the nutritional and cardiovascular status of immature animals while under anesthesia. In clinical settings, all vital functions and metabolic parameters of pediatric patients under anesthesia are closely monitored and tightly controlled. This was technically not feasible when infant rats and mice were studied, giving rise to the possibility that inadequacy of tissue perfusion, ventilation or oxygenation, as well as severe hypoglycemia (2, 44) during anesthesia could be, at least in part, responsible for anesthesia-induced neurotoxicity. This notion

was reinforced by the recent finding that mouse pups anesthetized with isoflurane developed severe hypoglycemia (29), which is known to jeopardize brain development and may cause learning disabilities (8, 33, 34).

Thus, our initial work on rats and mice, as well as that done in other laboratories, could not address two important issues: (i) can short exposure to anesthesia trigger apoptotic neurodegeneration in species with longer brain development? (ii) does close control over cardiorespiratory parameters (eg, blood pressure, heart rate, tissue oxygenation, and ventilation) and metabolic homeostasis (eg, blood glucose, bicarbonate levels and arterial blood pH) patterned after the actual clinical anesthesia setting have any effect on the severity of anesthesia-induced developmental neuroapoptosis? It is technically impossible to monitor all of these parameters in immature rats because of their small size. Therefore, to address these important questions, we designed a study with guinea pigs, in which brain development lasts about 10 weeks, or approximately five times longer than in rats (9) and is mostly a prenatal phenomenon which allows tight control over vital functions via continuous monitoring of the pregnant mother during anesthesia. As anesthetics readily cross the utero-placental barrier, our experimental design is based on the fact that fetal well-being is highly dependent on maternal well-being (16, 28, 39, 40, 48, 58). Thus, maintenance of maternal vital functions during anesthesia is an attainable way to assure fetal homeostasis.

Another question that needed to be addressed was whether exposure to anesthesia at the peak of brain development leads to neuronal loss in the most vulnerable brain regions. Although it is a useful marker for detecting developmental apoptosis, caspase activation is a rapidly occurring and transient process having somewhat different timing in different neurons within the same brain region, making it impossible to cumulatively assess neuronal damage in all vulnerable neurons at one time point. Therefore, we designed this study to assess overall neuronal loss many days after anesthesia treatment (first post-natal week), by which time dying neurons were phagocytized and the neuronal debris had been removed.

The main hypothesis that guided this study was that, despite the assurance of adequate maternal nutritional and cardiorespiratory stability during a short exposure to clinically relevant anesthesia, the fetal guinea pig brain would suffer significant and widespread anesthesia-induced apoptotic neurodegeneration. This, we hypothesized, would ultimately lead to neuronal loss in vulnerable brain regions.

## EXPERIMENTAL METHODS

### Animals

Pregnant Hartley guinea pigs were used for all experiments. We chose to use guinea pigs rather than rats, which have been used in earlier studies, because their brain growth is five times longer than that in rats and occurs predominantly prenatally. Thus, anesthesia-induced neurotoxicity studies of the fetal brain can be performed by anesthetizing pregnant mothers. Because of the relatively large size of guinea pigs, invasive monitoring of maternal and indirectly fetal vital signs are technically feasible.

The duration of the guinea pig gestation is 59–72 days (41). We selected three groups of guinea pigs based on the stage of the fetal

brain growth spurt: 20–25 days of pregnancy (early stage) (average maternal body weight,  $973 \pm 46$  g); 35–40 days (peak stage) (average body weight,  $1108 \pm 25$  g), and over 50 days (late stage) (average body weight,  $1324 \pm 55$  g) (9). These guinea pigs were randomly assigned to one of three groups: the experimental group, in which animals were given continuous anesthesia (total number of animals was 17, bearing a total of 55 fetuses), a sham control group that was given continuous fentanyl infusion (total number of animals was 6, bearing a total of 16 fetuses), or the “true” control group, given no anesthesia for 4 h (total number of animals was 4, bearing a total of 11 fetuses). All possible efforts were made to minimize both the number of pregnant animals used and any pain they might experience. All experiments were approved by The Animal Use and Care Committee of the University of Virginia Health System and were conducted in accordance with the US Public Health Service’s Policy on Human Care and Use of Laboratory Animals.

### Anesthesia

Nitrous oxide ( $N_2O$ ) and oxygen (GTS, Allentown, PA, USA) were delivered using a calibrated flowmeter. Isoflurane (Abbott Laboratories, Chicago, IL, USA) was administered using an agent-specific vaporizer that delivered a set percentage of this anesthetic. Midazolam (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was dissolved in 10% dimethyl sulfoxide (DMSO) immediately before administration. For control experiments, 10% DMSO was used as a vehicle. Fentanyl, an intravenous narcotic, and rocuronium, an intravenous muscle relaxant, were purchased, respectively, from Abbott Laboratories and Baxter Pharmaceutical Solutions, LLC, Bloomington, IN, and administered using a multi-channel infusion pump (Harvard Apparatus, PHD 2000).

### Anesthesia treatments

Before the initiation of the assigned protocols, the pregnant guinea pigs were first put to sleep using isoflurane in oxygen (3-L flow) delivered via mouth cone, after which atropine (0.05 mg/kg, s.c.) was administered. Once asleep, the animals were orotracheally intubated with a polyethylene tube (outside diameter, 2.5 mm, inside diameter 2.1 mm). Artificial ventilation was initiated with oxygen (40-vol%), nitrogen (57–58-vol%), and isoflurane as needed (2–3-vol%), under conditions of 6–8 mL/kg tidal volume, and 23–40 strokes per minute, delivered by a SAR-830 ventilator (CWE Inc., Ardmore, PA, USA). The exhaled gases (eg, oxygen, carbon dioxide, nitrous oxide and isoflurane) were continuously analyzed using an infrared analyzer (Datex Ultima, Helsinki, Finland). Postinduction of general anesthesia, one polyurethane catheter was inserted into the left carotid artery for continuous monitoring of blood pressure, heart rate and arterial blood gas; a second polyurethane catheter (PE50) was introduced into the right jugular vein for continuous infusion of intravenous fluid (Lactate Ringer solution at 4 mL/kg/h). To facilitate artificial ventilation, the pregnant animals were paralyzed by continuous intravenous infusion of the muscle relaxant rocuronium (15 nmol/kg/minute) (12), initiated after placement of an intravenous line and discontinued 30 minutes before termination of the anesthesia protocol.

After the placement of invasive lines, the pregnant animals were allowed 15–20 minutes to stabilize, at which point induction

of an anesthesia protocol was initiated. The minute ventilation was adjusted to maintain stable physiological parameters, which were continually monitored. The experimental group of pregnant guinea pigs was divided into four subgroups; each subgroup was randomly assigned to receive one of the following clinically relevant anesthesia protocols: isoflurane alone (0.55-vol%, ISO), isoflurane (0.55-vol%) + N<sub>2</sub>O (75-vol%), isoflurane (0.55-vol%) + midazolam (1 mg/kg, i.m.), or isoflurane (0.55-vol%) + nitrous oxide (75-vol%) + midazolam (1 mg/kg, i.m.) (I + N + M) for 4 h. The inhaled oxygen concentration was approximately 24-vol%. The use of nitrous oxide (75-vol%) and midazolam (1 mg/kg, i.m.), either individually or in combination, was not possible due to ethical considerations. At the required doses, these agents do not provide adequate depth of anesthesia which is crucial to the comfort of intubated and mechanically ventilated guinea pigs. Sham control animals were exposed to the same procedures as were experimental animals, but fentanyl alone was used for the maintenance of anesthesia. Fentanyl was administered after placement of the intravenous line, first as a bolus (30 µg/kg, i.v. given slowly over 15 minutes), then by 4 h of continuous infusion (15 µg/kg/h) (46). "True" control animals were neither exposed to any anesthesia, nor to invasive monitoring so that the histopathological properties of the fetal control brains could be directly compared with those of age-matched experimental and sham control fetal brains.

At the termination of any anesthesia protocol, the pregnant animals were kept normothermic and were closely inspected. As soon as they resumed a regular breathing pattern, airway reflexes and purposeful movements, the invasive lines were removed and the trachea was extubated. Soon after anesthesia, pregnant guinea pigs were capable of feeding well and did not require supplemental hydration.

### Monitoring of maternal vital functions

The adequacy of ventilation and oxygenation was continuously monitored by analyzing the composition of maternal exhaled gases (end tidal oxygen and carbon dioxide, measured, respectively, in mmHg—EtO<sub>2</sub> and EtCO<sub>2</sub>) collected from the exhalation limb of the anesthesia breathing circuit and by analyzing the composition of blood gases in the arterial blood obtained from the maternal left carotid artery (pH, partial pressure of carbon dioxide in mmHg—pCO<sub>2</sub>, partial pressure of oxygen in mmHg—pO<sub>2</sub>, bicarbonate concentration in mmol/L, oxygen saturation, SaO<sub>2</sub> in %) (Nova Biomedical, Waltham, MA, USA). Maternal arterial blood pressure was continuously monitored using a blood pressure transducer (23Db transducer referred to the mid-thoracic level) connected to a cannula (a polyethylene catheter-PE-50) inserted into the left carotid artery, which also allowed the sampling of arterial blood as needed. Systolic, diastolic and mean arterial pressures were obtained using custom software in Pascal computer language and an Intel digital computer. Maternal heart rate was derived from the pressure signal. Maternal animal core temperature was continuously monitored with a rectal temperature probe and maintained using heating pads. Maternal blood glucose levels were measured, using a glucometer (Accu-check Advantage) at regular intervals during anesthesia (Nova Biomedical), hourly postanesthesia for 2 h, and immediately before the removal of fetuses.

### Histopathological studies

The fetuses and infants were quickly weighed, deeply anesthetized and perfused with 4% paraformaldehyde in cacodylate buffer (0.1%), pH 7.4, for histopathology studies of the brain at different intervals after maternal anesthesia. The effects of anesthesia were tested at two time points, based on which histological analysis was performed. For caspase-3 and -9 immunochemical staining (ICC), an excellent method for marking neurons that are in an early stage of apoptosis (17, 61), the interval was 2 h; for NeuN and Nissl staining, useful for assessing the overall neuronal density in any given brain region, the interval was 35–40 days after anesthesia (during the first week of post-natal life). Whereas caspase-3 ICC was used for all quantification analyses, caspase-9 ICC was used for selected cases involving the most vulnerable fetal brains [35–40 days of gestational age (GA)] exposed to either "true" or sham control conditions or to a triple anesthesia combination.

For activated caspase-9 and caspase-3 staining, 50-µm-thick vibratome sections of fetal brains were washed in 0.01 M phosphate-buffered saline (PBS), quenched for 10 minutes in a solution of methanol containing 3% hydrogen peroxide, then incubated for 1 h in blocking solution (2% BSA/0.2% milk/0.1% triton X-100 in PBS). This was followed by incubation overnight in caspase-specific antibodies—either rabbit anti-active caspase-9, diluted 1:2000 or anti-active caspase-3, diluted 1:1000 in blocking solution (D175, Cell Signaling Technology, Beverly, MA, USA). After incubation with D175 primary antibody, the sections were incubated for 1 h in secondary antibody (goat anti-rabbit 1:200 in blocking solution), then reacted in the dark with ABC reagents (standard Vectastain ABC Elite Kit, Vector Labs, Burlingame, CA, USA). The sections were then preincubated for 10 minutes in a filtered mixture containing 6 mL of 0.1 M Tris buffer, 2 mg 3, 3'-diaminobenzidine (DAB), and 400 mg imidazole, then for 15 minutes in 6 mL of the same DAB/imidazole/Tris mixture containing 3-µL H<sub>2</sub>O<sub>2</sub>.

To study the neuronal densities in selected brain regions at completion of brain development, infant brains (n = 21) were perfused and fixed with 4% paraformaldehyde in cacodylate buffer (0.1%), pH 7.4. 35–40 days after anesthesia treatment; that is, during the first week of post-natal life. We used Nissl and NeuN staining, well-established techniques for labeling neurons. The most susceptible brain regions, and therefore the ones of most interest, were, among the cortical areas, the retrosplenial, parietal, cingulate, occipital and piriform regions, and among the subcortical areas, the amygdala, subiculum, hippocampus and anterior thalamus (35). For Nissl staining, the fixed brains were blocked in paraffin, cut into 7-µm sections, and stained. For NeuN staining, neurons were immunolabeled using a monoclonal antibody against neuron-specific nuclear protein (NeuN; 1:100; Chemicon).

### Quantitative histology

To determine the density of caspase-3 and Nissl-stained neurons in a given brain region, we used the optical disector and fractionator method. A counting frame (0.05 × 0.05 mm, disector height, 0.05 mm for caspase-stained; 0.05 × 0.05 mm, disector height, 0.007 mm for Nissl-stained) and a high numerical aperture objective lens were used to visualize neurons. Once a brain region of interest was identified, three random areas within that region were

subsampled using Stereo Investigator 6.02.2 computer software, which enabled us to count all neuronal profiles of interest with equal probability of their being included in the sample and not counted repeatedly. Unbiased subsampling of each area in each brain region was performed by randomly selecting 10–12 viewing fields in each area. The counting frame was positioned for counting at different focal levels in three different coronal sections (Stereo Investigator System, MicroBright Field, Inc, Williston, VT, USA) (24, 59, 61, 62). The numerical density of caspase-3 or Nissl-stained neurons in any given region was expressed as the number of cells/mm<sup>3</sup>. By using the optical disector and fractionator method, we were able to estimate neuronal densities in each brain region of interest without determining their precise anatomical boundaries (59). The counting was done by an investigator who was blind to the experimental conditions.

### Statistical analyses

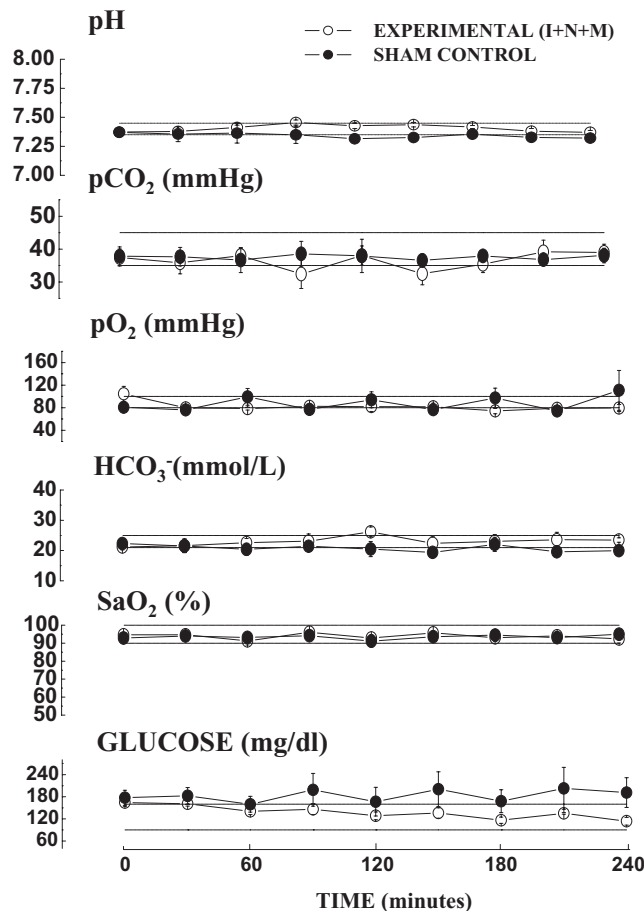
Anesthesia-induced pathomorphological changes (the stereological cell count data) were assessed using ANOVA models that included a between-subject variable such as treatment group and fetal age (20–25, 35–40 and over 50 days *in utero*). The within-subject variable was brain regions. When ANOVAs with repeated measures were conducted, the Bonferroni correction or Tukey's test was used to help maintain prescribed alpha levels (eg, 0.05).

## RESULTS

The adequacy of maternal oxygenation or ventilation, tissue perfusion and nutritional stability (Figure 1), as well as the stability of maternal vital signs (Figure 2), were regularly monitored and recorded every 30 and 15 minutes, respectively. We noted no significant differences between the experimental and sham pregnant guinea pigs in any physiological parameters at any point during the 4 h of anesthesia. The tight control is confirmed by the fact that recorded values were not significantly different from normal physiological ranges for guinea pigs (indicated with dotted lines in each graph) (5). Although blood glucose levels declined slightly in the experimental animals during the second half of exposure to anesthesia, neither the difference compared with sham controls nor variations in glucose stability were significant when compared with the normal physiological range (Figure 1). This finding suggests that anesthesia *per se* did not result in either severe hypo- or hyperglycemia. Figures 1 and 2 illustrate the results obtained from the experimental group that was exposed to a triple anesthesia combination (isoflurane, nitrous oxide and midazolam-I + N + M, which was the most neurotoxic). Similar findings were obtained when isoflurane, alone or in combination with midazolam or nitrous oxide, was administered (data not shown).

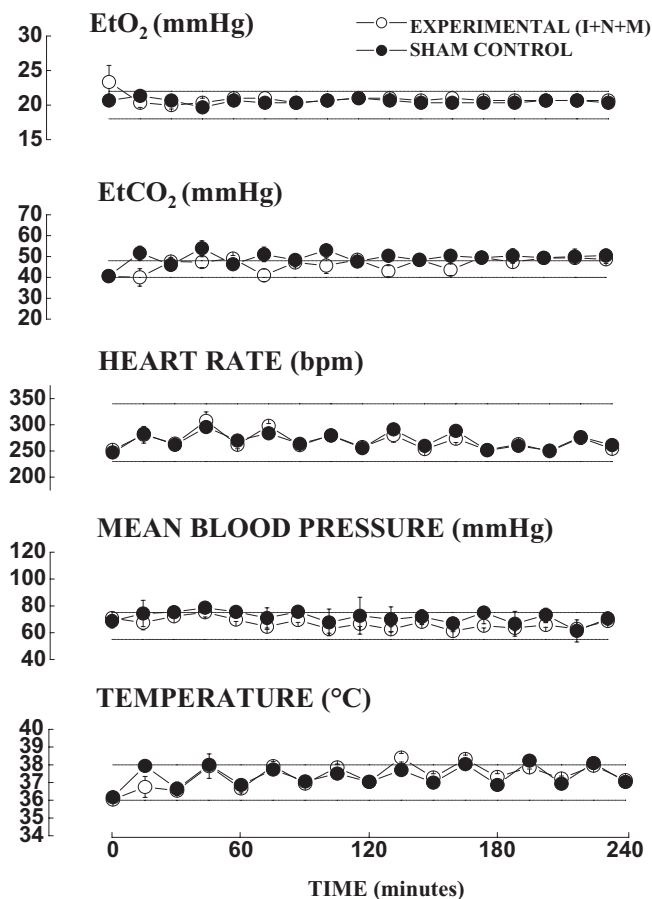
To study the regional distribution and to compare the severity of anesthesia-induced fetal neuroapoptosis caused by a variety of anesthesia protocols administered to the pregnant female pigs, we examined an extensive list of brain regions stained with caspase-3 in fetal guinea pigs at the peak of their brain development (35–40 days GA) (Figures 3 and 4, Tables 1 and 2).

While studying the developing fetal cortex, we observed that the neurons in the mid-third zone (cortical layers IV and V) were consistently labeled with activated caspase-3, whereas other cortical layers were minimally affected (cingulate cortical layer II) or



**Figure 1.** Anesthesia does not cause significant cardiorespiratory or metabolic disturbances in pregnant guinea pigs as determined by analysis of arterial blood gases. Conducting these analyses every 30 minutes during exposure to anesthesia revealed no significant differences in any of the measured parameters in the experimental animals [exposed to isoflurane (0.55-vol%) + N<sub>2</sub>O (75-vol%) + midazolam (1 mg/kg, i.m.)—(I + N + M)] (n = 3 pregnant guinea pigs) compared with the sham animals (exposed to a continuous fentanyl infusion, 15 µg/kg/h) (n = 3 pregnant guinea pigs). The physiological range for guinea pigs for each parameter is indicated by a dotted line.

were not affected by anesthesia treatments. Therefore, we focused our density studies on the mid zone because it appeared to be most sensitive. As shown in Table 1, the density of caspase-3-positive neurons in the “true” controls varied somewhat in different cortical regions (eg, retrosplenial, parietal, cingulate, occipital and piriform cortices), although the number of neurons undergoing apoptosis was small. A low concentration of ISO (0.55-vol%) induced a statistically significant increase in neuroapoptosis, on the average, three- to sixfold, in all cortical regions when compared with “true” controls (\**P* < 0.001). When either N<sub>2</sub>O (75-vol%) or midazolam (1 mg/kg, i.m.) was combined with isoflurane (0.55-vol%) (ISO + N<sub>2</sub>O and ISO + midazolam, respectively), we found further worsening of apoptotic damage compared with that in response to isoflurane alone. When compared with “true” controls, the degree



**Figure 2.** Anesthesia does not cause significant disturbances in the vital signs of pregnant guinea pigs. Close monitoring of vital signs was performed continuously and recorded every 15 minutes during exposure to anesthesia. No significant differences in any of the measured parameters in the experimental animals [exposed to isoflurane (0.55-vol%) + N<sub>2</sub>O (75-vol%) + midazolam (1 mg/kg, i.m.)—(I + N + M)] (n = 3 pregnant guinea pigs) compared with the sham animals (n = 3 pregnant guinea pigs). Physiological range for guinea pigs for each parameter is indicated by a dotted line.

of neuroapoptosis with double anesthesia combinations was, on the average, 7- to 16-fold higher ( $*P < 0.001$ ).

As Figure 3 shows, the effect of the triple anesthesia combination containing isoflurane (0.55-vol%), nitrous oxide (75-vol%), and midazolam (1 mg/kg, i.m.) [Experimental (I + N + M)] was extremely severe. For example, compared with “true” and sham controls, the increases in apoptotic damage were, on the average, 17- to 23-fold ( $*P < 0.001$ ) and 6- to 10-fold ( $**P < 0.01$ ), respectively, in all cortical regions of interest. Although a somewhat higher degree of apoptosis was detected in sham controls than in “true” controls, the difference was not statistically significant.

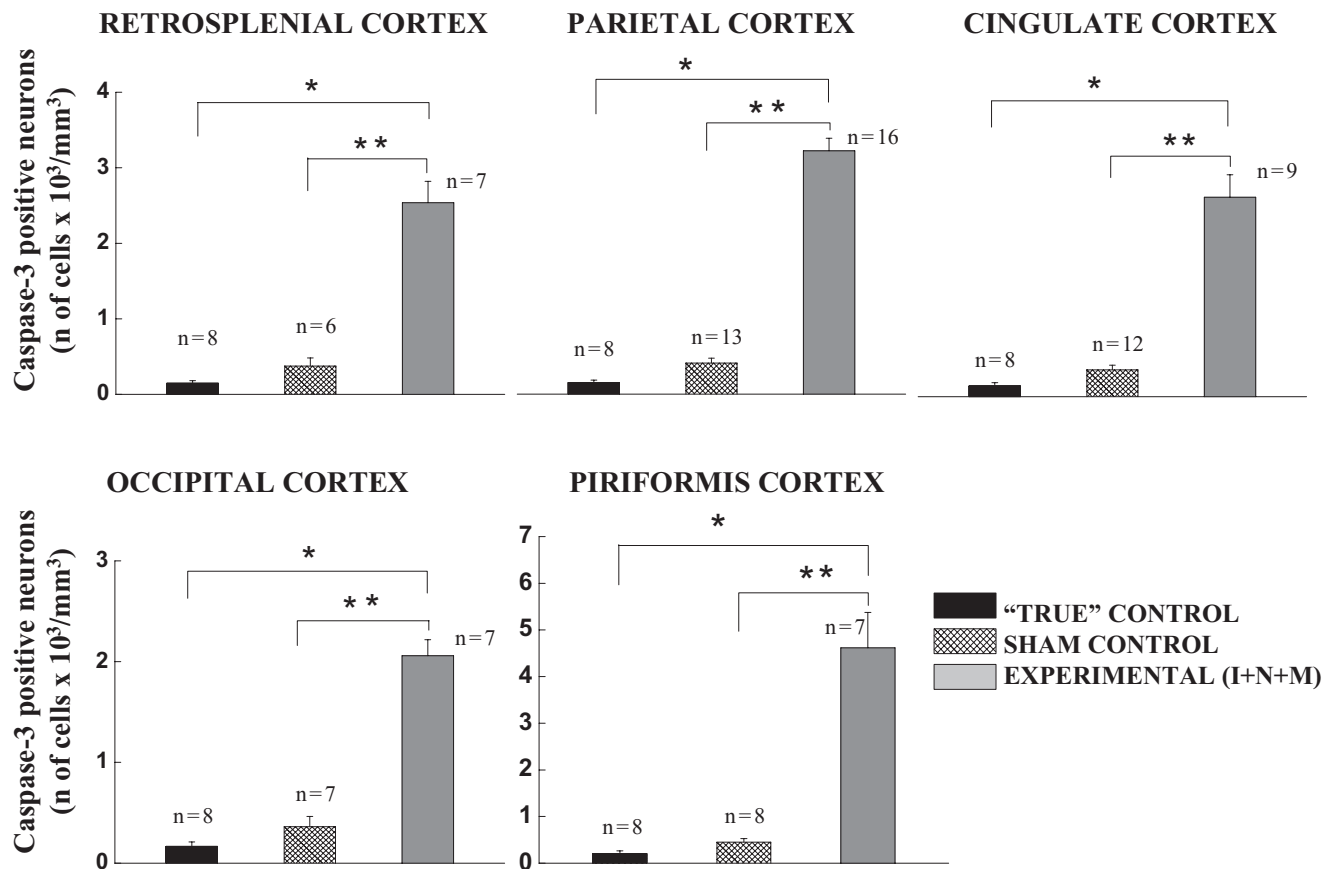
The studies of subcortical brain regions focused on the amygdala, subiculum, hippocampus and anterior thalamus because they were most vulnerable (Table 2, Figure 4). As indicated in Table 2, the “true” controls exhibited a low degree of developmental neuroapoptosis as measured by a low density of caspase-3-positive neurons. However, exposure to isoflurane

alone resulted in a significant increase in neuroapoptosis in the fetal amygdala (except in the nucleus centralis), subiculum and hippocampus (except in the CA1 region) compared with the “true” controls ( $*P < 0.001$ ) (approximately 3- to 11-fold higher density of caspase-3-stained neurons in isoflurane-treated fetuses). Despite an approximately twofold higher density of caspase-3-positive neurons in anterior thalamic nuclei (eg, nucleus lateralis, nucleus medialis, nucleus ventralis, and nucleus reticularis) the difference was not statistically significant when compared with the “true” controls. When either N<sub>2</sub>O (75-vol%) or midazolam (1 mg/kg, i.m.) was combined with isoflurane (0.55-vol%), we found an overall worsening of apoptotic damage compared with that in response to isoflurane alone (except in the amygdaloid nuclei medialis and basalis medialis). When compared with the “true” controls, the degree of neuroapoptosis with double anesthesia combinations was, on the average, 4- to 25-fold higher ( $*P < 0.001$ ).

As shown in Figure 4, we detected a somewhat higher degree of apoptosis in sham than in “true” controls, although the difference was not statistically significant. However, when the triple anesthesia combination containing isoflurane (0.55-vol%), N<sub>2</sub>O (75-vol%), and midazolam (1 mg/kg, i.m.) [Experimental (I + N + M)] was tested, the neuroapoptosis was extremely severe overall. For example, compared with “true” and sham controls, the increases in apoptotic damage were, on the average, 4- to 31-fold ( $*P < 0.001$ ) and 3- to 10-fold ( $**P < 0.01$ ), respectively, in all subcortical regions of interest. Interestingly, in certain subcortical regions (eg, nucleus lateralis, ventralis and reticularis thalami; amygdaloid nucleus basalis medialis and the CA3 region of the hippocampus) double anesthesia combinations (Table 2) were as damaging or somewhat more damaging than was the triple combination (Figure 4), although the differences were not statistically significant.

When the vulnerability of the same brain regions were examined in fetal guinea pigs in the early stages of brain development (20–25 days GA), we found that a triple anesthesia cocktail was, again, most damaging compared with the “true” controls (an approximately 6- to 20-fold increase in apoptosis) ( $P < 0.05$ ), whereas isoflurane alone was not significantly more neurotoxic than the “true” controls (data not shown). The studies of fetal guinea pig brains in the late stages of development (50+ days) revealed a complete lack of sensitivity to the triple anesthesia cocktail; that is, we were unable to detect any signs of caspase-3 activation in any vulnerable brain regions (n = 5 fetuses) (data not shown).

Figure 5 illustrates caspase-3 and caspase-9 stages of apoptotic neurodegeneration observed in the mid-third zone (cortical layers IV and V) of the parietal cortex of fetal brains (35–40 GA) exposed to the triple anesthesia combination, the most damaging anesthesia protocol. Early stage caspase-9 activation a precursor step in caspase-3 activation (32, 61, 63), is shown in the upper portion of Figure 5. Practically undetectable caspase-9 labeling was noted in “true” controls and only *very* minimal labeling was noted in sham controls, whereas there was substantial caspase-9 activation detected in the experimental cortex. Subsequent-stage caspase-3 labeling, a final step leading to DNA fragmentation and cell death, is illustrated in the lower portion of Figure 5. A substantial increase in caspase-3 staining was noted in the experimental cortex compared with “true” and sham controls. Note similar laminar distribution of staining in both stages of apoptotic neurodegeneration,



**Figure 3.** Anesthesia induces significant caspase-3 activation in fetal guinea pig brains in several cortical brain regions. The sensitivity of cerebral cortices in fetal guinea pig brains (35–40 days gestational age) to general anesthesia is quantified as the number of caspase-3-positive cells/mm<sup>3</sup> in the mid-zone of the cortex (layers IV and V). The density of caspase-3-positive neurons in the “true” controls was minimal in all cortical regions (eg, retrosplenial, parietal, cingulate, occipital and piriform cortices). Although a somewhat higher degree of caspase-3 label-

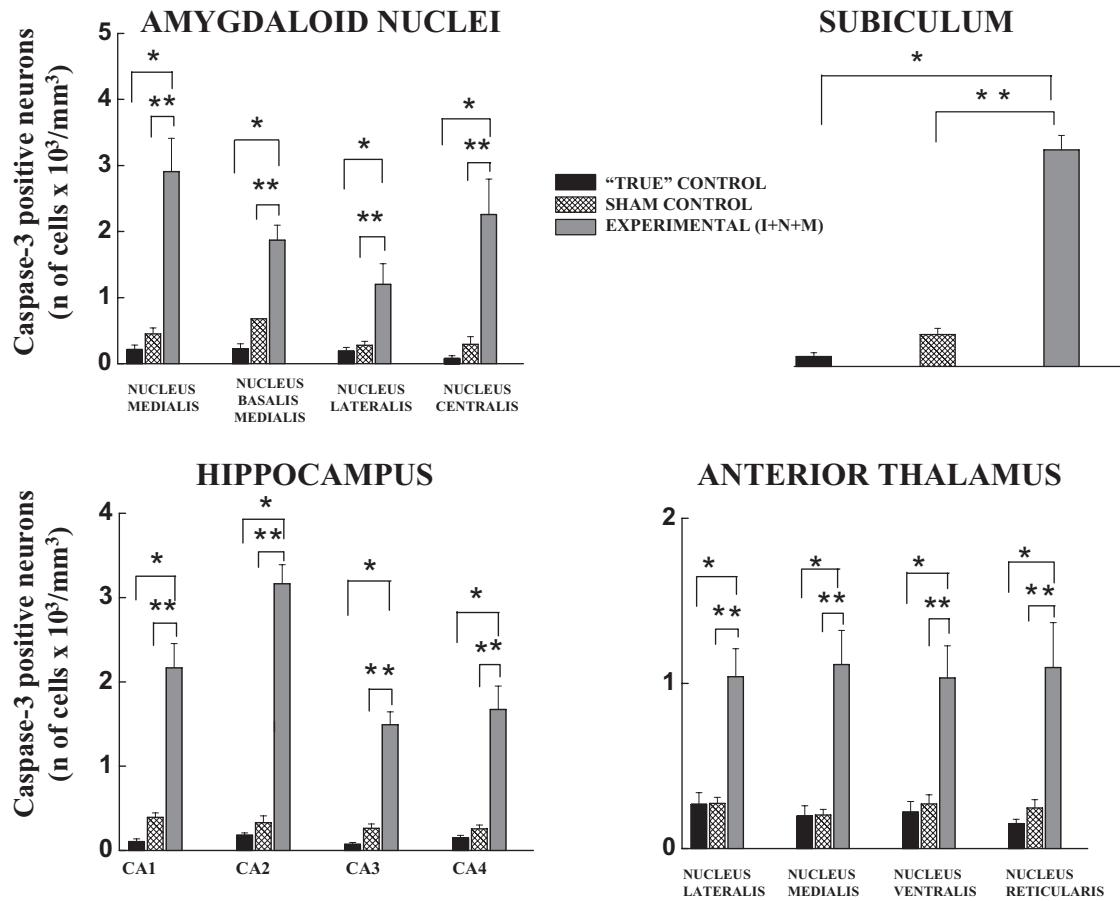
ing was detected in the sham controls (exposed to a continuous fentanyl infusion, 15 µg/kg/h), the difference was not statistically significant when compared with the “true” controls. Triple anesthesia containing isoflurane (0.55-vol%), N<sub>2</sub>O (75 vol-%), and midazolam [1 mg/kg, i.m. (I + N + M)] induced a significant increase in caspase-3 labeling compared with the “true” and sham controls (\**P* < 0.001, and \*\**P* < 0.01, respectively). (The number of fetuses per each data point is indicated in the graph.)

suggesting that caspase-3 activation is, at least in part, mediated via the activation of caspase-9.

Because caspase-3 activation occurs when the neurons are committed to die, we set out to determine the severity of anesthesia-induced overall neuronal loss in the most vulnerable brain regions once the dying neurons were phagocytized and the neuronal debris had been removed. Hence, we examined the most vulnerable regions in young guinea pig brain many days after anesthesia treatment (during the first week of post-natal life) using Nissl staining. Because the triple combination of anesthetics given at the peak of the growth spurt of the guinea pig brain (35–40 days GA) was most damaging in the majority of vulnerable brain regions, we compared this experimental group with the “true” and sham controls treated at the same age. Figure 6 illustrates the neuronal densities in the mid-zone (cortical layers IV and V) of the most vulnerable cortical regions (eg, retrosplenial, parietal, cingulate, occipital and piriform cortices) of the control (“true” and sham) and experimental animals. We found a significant difference in anesthesia-induced neuronal loss in the experimental animals vs. the “true” and sham

controls (\**P* < 0.01, and \*\**P* < 0.05, respectively). The overall anesthesia-induced neuronal loss in the experimental animals varied from 40% to 50% compared with the controls. A comparison of the “true” and sham controls revealed similar neuronal densities in all cortical regions.

The neuronal densities in vulnerable subcortical brain regions (eg, amygdala, subiculum, anterior thalamus and hippocampus) of the two control groups (“true” and sham) and the experimental (I + N + M) animals are evaluated in Figure 7. In general, we found a significant neuronal loss in the experimental animals when compared with the two control groups (\**P* < 0.01, and \*\**P* < 0.05, respectively); the overall anesthesia-induced neuronal loss in the experimental group varied from 30% to 50% compared with the control groups. When the two control groups were compared, we found that the neuronal densities in all subcortical brain regions were similar. The representative microphotographs of hippocampal CA1 region (left lower panels) show a substantial decrease in neuronal densities in an experimental animal compared to a “true” control.



**Figure 4.** Anesthesia induces significant caspase-3 activation in fetal guinea pig brains in several subcortical brain regions. The sensitivity of amygdala, subiculum, hippocampus and anterior thalamus in fetal guinea pig brains (35–40 days gestational age) to general anesthesia is quantified as the number of caspase-3-positive cells/mm<sup>3</sup> in specific nuclei or regions, as indicated in each panel. Both control groups exhibit a low degree of developmental neuroapoptosis, with somewhat higher, although not significant, caspase-3 staining in the sham controls

(exposed to a continuous infusion of fentanyl, 15 µg/kg/h) compared with the “true” controls. The triple anesthesia combination containing isoflurane (0.55-vol%), N<sub>2</sub>O (75-vol%) and midazolam (1 mg/kg, i.m.) (I + N + M) induced a significant increase in caspase-3 labeling compared with the “true” and sham controls (\**P* < 0.001, and \*\**P* < 0.01, respectively) (number of fetuses per each data point: 5–7 for amygdaloid nuclei, 5–10 for subiculum, 5–12 for hippocampus and 5–11 for anterior thalamus).

**DISCUSSION**

In this study, we show that the commonly used general anesthetic isoflurane, alone or in combination with either nitrous oxide or midazolam or both, induces severe and widespread apoptotic

neurodegeneration in fetal guinea pigs at the peak of the brain growth spurt. This damage ultimately leads to a loss of a significant number of neurons in vulnerable regions of the brain, suggesting that anesthesia-induced neuroapoptosis can result in permanent brain damage. The intravenous narcotic fentanyl did not

**Table 1.** Cortical brain regions.

	Caspase-3 positive neurons number of cells/ mm <sup>3</sup> (fold increase from “true” control)			
	True (n = 8)	ISO (n = 4)	ISO + N <sub>2</sub> O (n = 6)	ISO + midazolam (n = 3)
Retrosplenial cortex	149 ± 32	899 ± 112* (6)	1194 ± 150* (8)	1577 ± 511* (10.6)
Parietal cortex	144 ± 33	697 ± 99* (4.8)	1656 ± 252* (11.5)	1007 ± 59* (7)
Cingulate cortex	138 ± 43	433 ± 61* (3.1)	1352 ± 296* (9.8)	1247 ± 134* (9)
Occipital cortex	167 ± 45	590 ± 25* (3.5)	1286 ± 257* (7.7)	1258 ± 541* (7.5)
Piriformis cortex	203 ± 59	1236 ± 148* (6.1)	2117 ± 159* (10.4)	3177 ± 233* (15.7)

\**P* < 0.001 compared to “true” controls (see text).

**Table 2.** Subcortical brain regions.

		Caspase-3 positive neurons number of cells/mm <sup>3</sup> (fold increase from "true" control)			
		True (n = 5–7)	ISO (n = 4)	ISO + N <sub>2</sub> O (n = 6)	ISO + midazolam (n = 3)
Amygdaloid nuclei	Nucleus medialis	213 ± 69	1017 ± 151* (4.8)	730 ± 124* (3.4)	1806 ± 455* (8.5)
	Nucleus basalis medialis	221 ± 80	966 ± 108* (4.4)	831 ± 72*	3160 ± 858* (14.3)
	Nucleus lateralis	187 ± 58	523 ± 169* (2.8)	980 ± 341* (5.2)	728 ± 169* (3.9)
	Nucleus centralis	72 ± 48	285 ± 149 (4)	726 ± 203 (10.1)	1399 ± 28* (19.4)
Subiculum		158 ± 65	1162 ± 319* (7.4)	1343 ± 132* (8.5)	2502 ± 356* (15.9)
Hippocampus	CA1	104 ± 33	388 ± 103 (3.7)	529 ± 149 (5.1)	1501 ± 172* (14.4)
	CA2	179 ± 29	1267 ± 107* (7.1)	2376 ± 205* (13.3)	1874 ± 98* (10.5)
	CA3	72 ± 23	788 ± 105* (11)	1829 ± 113* (25.4)	1472 ± 39* (20.4)
	CA4	145 ± 33	590 ± 155* (4.1)	789 ± 116* (5.4)	645 ± 98* (4.4)
Anterior thalamus	Nucleus lateralis	269 ± 71	349 ± 78 (1.3)	952 ± 233* (3.5)	1434 ± 265* (5.3)
	Nucleus medialis	195 ± 63	515 ± 139 (2.6)	787 ± 138* (4)	868 ± 104* (4.5)
	Nucleus ventralis	219 ± 66	369 ± 106 (1.7)	869 ± 191 (4)	1597 ± 461* (7.3)
	Nucleus reticularis	151 ± 26	402 ± 116 (2.7)	662 ± 165 (4)	1090 ± 202 (7.2)

\* $P < 0.001$  compared to "true" controls (see text).

significantly increase apoptotic damage in vulnerable fetal brain regions and consequently did not induce a significant decrease in neuronal densities later on during development.

The quantification analyses of anesthesia-induced developmental neuroapoptosis presented here are based on the activation of caspase-3 because it represents the final and converging step in apoptotic activation, the point at which a neuron is committed to die. The fact that activated caspase-9 labeling in fetal brains exposed to a severely neurotoxic triple anesthesia combination shows a similar pattern of distribution suggests that caspase-3 activation is mediated, at least in part, via the activation of caspase-9. In the future, detailed mechanistic studies should address the involvement and relative importance of different apoptotic cascades in anesthesia-induced apoptotic neurodegeneration in fetal guinea pig brain.

The brain's growth spurt consists of several stages that are precisely timed and vary in duration among species. In humans, its duration is fairly long, spanning both the *in utero* (last trimester of pregnancy) and post-natal (first couple of years) periods. In guinea pigs and rats, its duration is much shorter (10 and 2 weeks, respectively) and takes place either mostly *in utero* (in guinea pigs) or mostly after birth (in rats) (9). In guinea pigs, the species of interest for this study, synaptogenesis, an important stage of the brain's growth spurt, includes the period of intense formation of synapses, which starts midway during *in utero* life and culminates by day 50 (25, 27, 31). Studies of not only the number of synaptic junctions but synaptic maturity (eg, the number of dense projections per junction, the length of the junction and the narrowing of the synaptic cleft) have determined that at the end of the gestational period (59–72 days) (41) the guinea pig brain is almost fully developed (25); at birth, the guinea pig is essentially fully functional and its eyes are open (31).

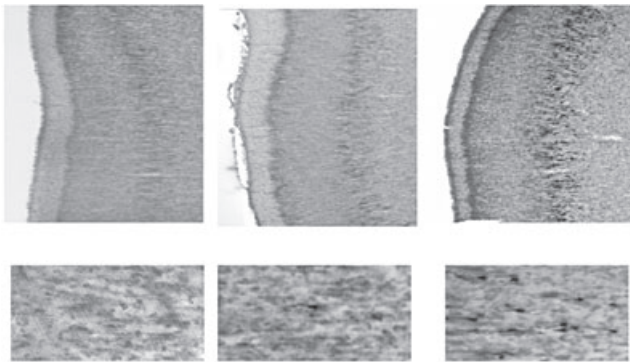
To appropriately evaluate the risks posed by commonly used anesthetics in human anesthesia, it is important to understand whether a species with a longer brain growth spurt is less vulnerable to brief exposure to anesthesia. Our study indicates that, at the peak of brain growth, only 4 h of anesthesia, which represents a brief time during the guinea pig brain development (on the average, 70 days), caused significant and widespread neuroapoptotic

damage in the fetal brain. This damage is comparable in intensity to the damage to the immature rat brain after 6 h of anesthesia (24, 61, 62) despite the fact that the duration of brain development in guinea pigs is approximately five times longer than it is in rats.

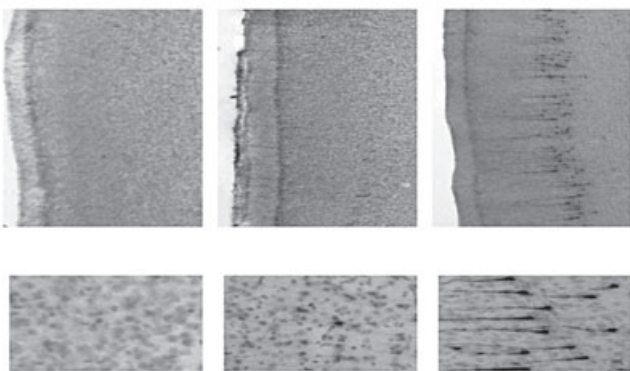
However, according to the present study and a previously published report (61), it appears that the timing of exposure to anesthesia is more important than its duration; that is, the same anesthesia protocol administered when development of the immature brain is at its peak causes significantly more damage than if the insult occurs later in development. The reason is that the neuronal tasks at the peak of brain development are multifaceted and include several important elements (eg, migration, formation of synapses, differentiation, maturation). These elements are precisely timed and require highly coordinated neuronal communications (see 63 for a review), factors that make the immature neurons especially vulnerable if the circumstances of their normal functioning have been altered by an external insult such as exposure to anesthesia.

In daily practice, the tight control of vital signs and functions during anesthesia is crucially important to assure adequate homeostasis. By anesthetizing pregnant guinea pigs, we were able to mimic the clinically relevant anesthesia setup and to monitor the adequacy of maternal ventilation, oxygenation, and tissue perfusion at any point in time, which was technically impossible with immature rats because of their small size. We found that despite strict control of all maternal vital parameters, the amount of fetal neuronal damage that occurred in the experimental animals was many-fold greater than was the case in either the sham or "true" controls. It is important to note that in designing these experiments, because direct monitoring of fetal ventilation, oxygenation and perfusion in guinea pigs was not technically feasible, we relied on the common assumption that careful monitoring and tight control over maternal vital functions and well-being during anesthesia assured adequate well-being of a fetus. This assumption has been tested in the practice of obstetric anesthesia and in several animal studies. The results have shown that any abnormalities in maternal cardiopulmonary functions could have a negative impact on the fetus (1, 4, 54) and that therapeutic interventions aimed at correcting maternal vital functioning could be beneficial for the fetus (11, 16). Because all anesthetics, including the ones used in our study,

## CASPASE-9 STAINING



## CASPASE-3 STAINING



“TRUE” CONTROL SHAM CONTROL EXPERIMENTAL (I+N+M)

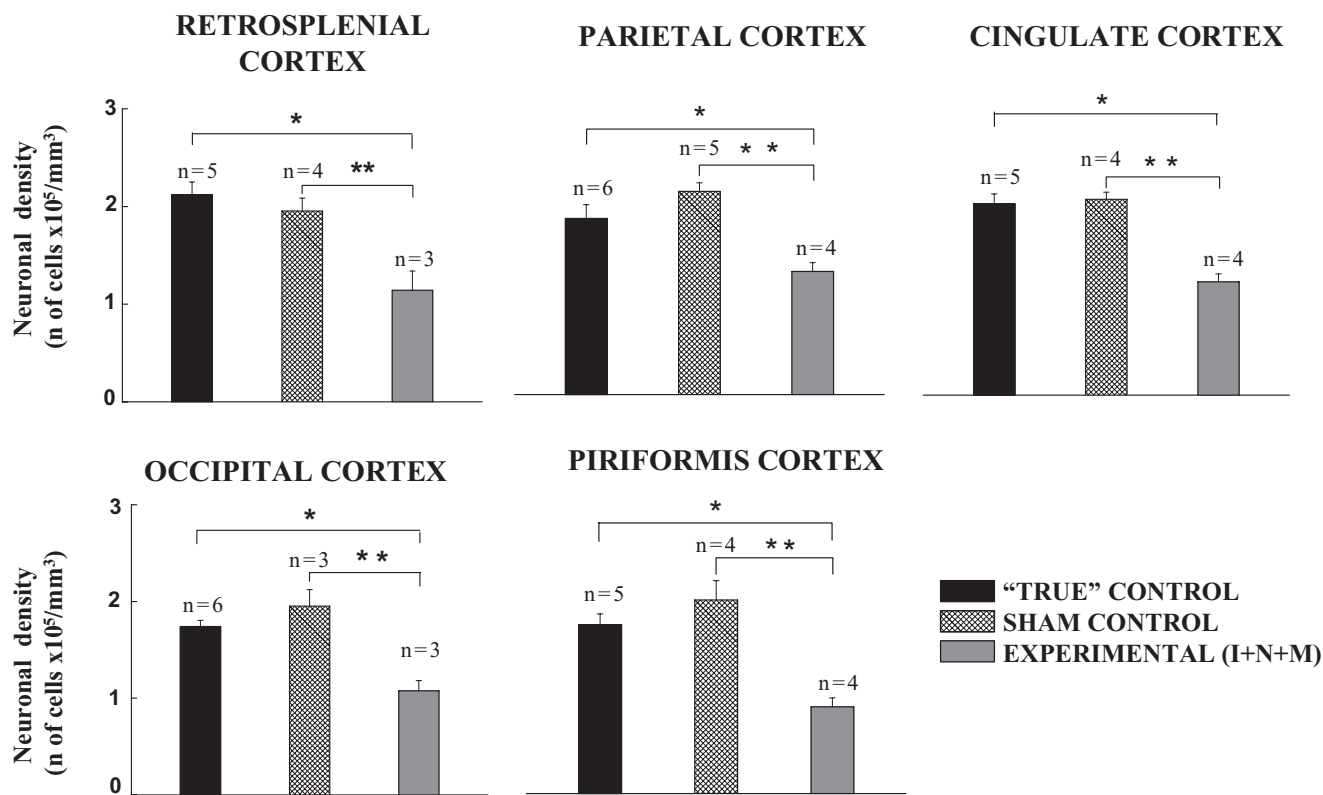
**Figure 5.** Various stages of anesthesia-induced apoptotic neurodegeneration in the mid-zone of parietal cortex (cortical layers IV and V). Exposure of fetal guinea pigs at the peak of their brain development (35–40 days gestational age) to the triple anesthesia combination (isoflurane, 0.55-vol%, + N<sub>2</sub>O, 75-vol%, + midazolam, 1 mg/kg, i.m.) (Experimental—I + N + M) induced significant caspase-9 activation, a precursor step in caspase-3 activation. Practically undetectable and only a very minimal caspase-9 labeling was noted in the “true” (no anesthesia) and sham controls, (exposed to a continuous infusion of fentanyl, 15 µg/kg/h), respectively. Significant caspase-3 labeling was noted in the experimental cortex compared with that of the two control groups. Note the similar laminar distribution of staining in both stages of apoptotic neurodegeneration (caspase-9 microphotographs: magnification 10× in upper panels; 40× in lower panels) (caspase-3 microphotographs: magnification 10× in upper panels; 60× lower panels).

easily cross the utero-placental barrier (10, 18, 30, 36, 60) without significantly affecting *in utero* placental blood flow (6, 11, 43), we believe that pregnant guinea pigs are a good model for assessing fetal anesthesia-induced neurotoxicity. Because the apoptotic neurodegeneration observed in the experimental fetal brains was more severe than that observed in the sham and “true” control fetal brains, despite adequate maintenance of maternal vital functions, we conclude that general anesthesia *per se* is the main culprit, not a hypoxia/hypercarbia or metabolic imbalance (7).

Although fentanyl’s potential to induce neuroapoptosis during the brain growth spurt has not been studied, several reports suggest that chronic administration of other narcotics (eg, morphine, buprenorphine) very early during development (in neurogenesis) as well as later (in adulthood) can modulate a variety of apoptotic markers (eg, c-jun, caspase-3) (13, 19). This results in obvious changes in neuronal morphology, such as reduced dendritic arborization and density of dendritic spines (49, 52) that, in turn, may lead to abnormalities in the formation of neuronal circuitries. Our findings with continuous infusion of fentanyl suggest that brief exposure to this intravenous narcotic, unlike brief exposure to volatile anesthetic isoflurane, does not induce significant neuroapoptosis or significant neuronal deletion when compared with the controls. However, establishing whether prolonged exposure to fentanyl (eg, for days and weeks) during critical stages of the brain growth spurt could induce severe apoptotic neurodegeneration would be important, especially because continuous administration of fentanyl is a part of the standard sedation protocol in many neonatal and pediatric intensive care units.

The development of neuronal circuitries in the mammalian brain is complex, and although certain stages are activity-independent (56), formation of the majority of neuronal synapses is highly dependent on constant neuronal signaling. Thus, refinement and fine-tuning of synaptic connections is practically impossible when this signaling is severely inhibited (26). Based on our findings, it seems that combining different general anesthetics to achieve the desired depth of anesthesia increases the vulnerability of the developing brain to neuroapoptotic damage. For instance, although a low, clinically relevant, concentration of isoflurane alone (at 0.55-vol%, which is only 0.5 MAC—the minimum alveolar anesthetic concentration that prevents purposeful movement to supramaximal noxious stimulation in 50% guinea pigs) (51) was pro-apoptotic at the peak of brain growth spurt, the severity of neuroapoptosis was significantly augmented by the addition of very low concentrations of nitrous oxide (at 75-vol% which is only 0.375–0.5 MAC for guinea pigs) (55) and a low dose midazolam (commonly used in sedation protocols) (45). It remains to be determined whether the higher toxicity potential of anesthesia cocktails compared with a single anesthetic is caused by a more profound generalized inhibition of neuronal activity caused by an anesthetic combination or caused by, at least in part, a combined and synchronized modulatory effect(s) on two of the most important neuro-transmitter systems during the development—glutamate (nitrous oxide-induced inhibition of N-methyl-D-aspartate system) (23) and  $\gamma$ -amino-butyric acid-GABA (isoflurane/midazolam-induced over-stimulation of GABAergic system) (14).

The development and function of NMDA and GABA<sub>A</sub> receptor systems in fetal guinea pigs could be modulated by maternal exposure to environmental challenges. For example, maternal hypoxia has been shown to cause selective and differential modification of recognition, co-activation and modulation the NMDA receptor ion channel complex in fetal guinea pig cortices. This resulted in increased affinity and basal activation, ultimately leading to hyperstimulation of the NMDA receptor ion channel complex and a possible increase in fetal susceptibility to hypoxia (38). Maternal exposure to ethanol resulted in alterations in fetal hippocampal GABA<sub>A</sub> receptor expression, and pharmacological properties, thus contributing to hippocampal-related behavioral abnormalities and



**Figure 6.** Anesthesia induces significant neuronal loss in vulnerable cortical brain regions. Neuronal density counts were obtained using Nissl-stained slides. Fully developed guinea pig brains (first week of post-natal life) of the “true” controls were compared with the experimental animals that had been exposed to the triple anesthesia (isoflurane, 0.55-vol%, + N<sub>2</sub>O, 75-vol%, + midazolam, 1 mg/kg, i.m.) or the sham procedure (continuous fentanyl infusion, 15 µg/kg/h) when they were at the

peak of their development (35–40 days gestational age). The sham and “true” controls maintain similar neuronal densities, whereas the experimental animals show a significant decrease in neuronal densities in all vulnerable cortical brain regions compared with the “true” and sham control groups (\* $P < 0.01$ , and \*\* $P < 0.05$ , respectively). (The number of fetuses per each data point is indicated in the graph.)

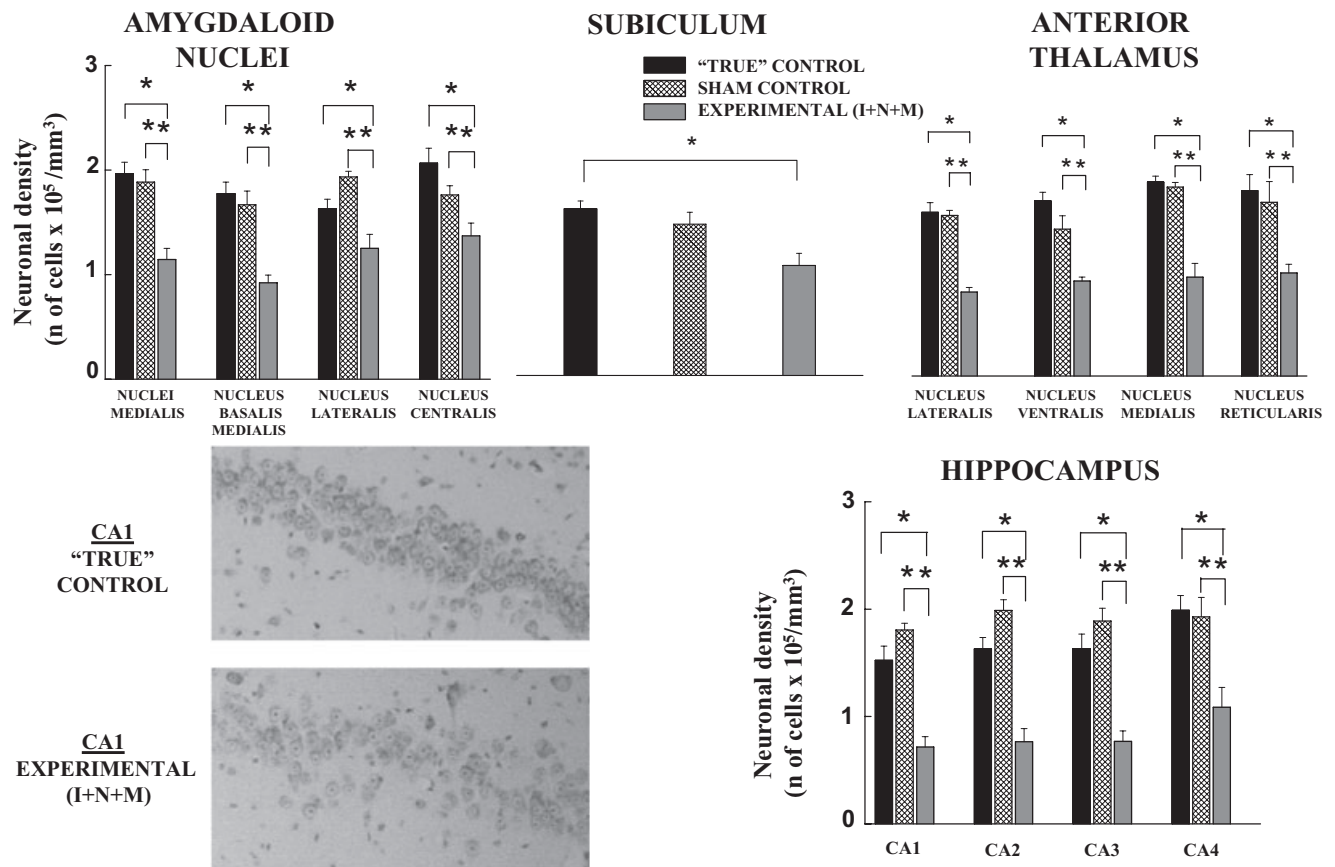
cognitive deficits in guinea pig offspring (20). Although detailed studies of the function and subunit composition of these two receptor systems in all brain regions of fetal guinea pigs are not available, it is reasonable to propose that the brain-region-specific differences in vulnerability of the fetal guinea pig brain to a variety of anesthetic protocols could be caused by unique receptor/ion channel composition and interplay at each point during fetal brain development.

Several pathways of anesthesia-induced apoptotic cell death have been described, including mitochondria-dependent and -independent (death receptor-dependent) (61, 62), as well as neurotrophic factor-dependent (32), suggesting that anesthesia-induced developmental neuroapoptosis is a complex phenomenon. However, it has been established that caspase-3 activation is the final step at which a developing neuron is committed to die, demonstrating that immunocytochemical analysis using activated caspase-3 is a reliable method for mapping neurons dying by apoptosis (21, 22, 24, 42, 61, 62).

Physiological “pruning” of the redundant neurons is commonly observed in the developing mammalian brain. It is estimated that less than 1% of the total neuronal population (with some regional

variations) succumb to apoptosis (21, 22). Thus, only a small percentage of the neuronal population is expected not to survive during the normal brain growth spurt. Our findings with “true” control animals support this notion. However, based on our evaluation of the experimental animals, it appears that general anesthesia increases neuronal apoptosis many times over, leading to an alarming increase in neuronal “pruning” (as much as 50% in most vulnerable brain regions). Similar severity of damage has been described in the hippocampus (approximately 30% neuronal loss in the CA1 region) of young post-natal guinea pigs chronically exposed to ethanol *in utero* (15) which also resulted in impaired cognitive functioning (47). Although the functional importance of anesthesia-induced neuronal loss in guinea pigs remains to be determined, our studies of rats suggest that early exposure to general anesthesia during the brain growth spurt causes permanent impairment of learning and memory capabilities (24).

In conclusion, our study demonstrates that a single short maternal exposure to commonly used general anesthesia induces significant neuroapoptotic degeneration in developing fetal guinea pig brains. This leads to a loss of developing neurons in the most vulnerable brain regions.



**Figure 7.** Anesthesia induces significant neuronal loss in vulnerable subcortical brain regions. Neuronal density counts were obtained using Nissl-stained slides. Fully developed guinea pig brains (first week of post-natal life) in the "true" control group were compared with the experimental animals that had been exposed to triple anesthesia (isoflurane, 0.55-vol%, + N<sub>2</sub>O, 75-vol%, + midazolam, 1 mg/kg, i.m.) or the sham procedure (continuous infusion of fentanyl, 15 µg/kg/h) when they were at the peak of their development (35–40 days gestational age).

Note that the "true" and sham groups maintain similar neuronal densities ( $*P < 0.01$ , and  $**P < 0.05$ ), whereas the experimental animals show a significant decrease in neuronal densities in all vulnerable subcortical regions. (Number of fetuses per each data point: 4–5 for amygdaloid nuclei, 3–5 for subiculum, 4–5 for anterior thalamus and 4–6 for hippocampus). The representative microphotographs of the hippocampal CA1 region indicate a lower density of Nissl stained cells in the "true" control animals compared with the experimental animals.

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