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Response to neonatal anesthesia – effect of sex on anatomical and behavioral outcome

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Abstract

Numerous studies have documented the consequences of exposure to anesthesia in models of term and post-term infants, evaluating the incidence of cell loss, physiological alterations and cognitive dysfunction. However, surprisingly few studies have investigated the effect of anesthetic exposure on outcomes in newborn rodents, the developmental equivalent of premature human infants. This is critical given that one out of every eight babies born in the United States is premature, with an increased prevalence of surgical procedures required in these individuals. Also, no studies have investigated if the genetic sex of the individual influences the response to neonatal anesthesia. Using the newborn rat as the developmental equivalent of the premature human, we documented the effect of a single bout of exposure to either the inhalant isoflurane or the injectable barbiturate phenobarbital on hippocampal anatomy, hippocampal dependent behavioral performance and normal developmental endpoints in male and female rats. While both forms of anesthesia led to significant decrements in cognitive abilities, along with a significant reduction in volume and neuron number in the hippocampus in adulthood, the decrements were significantly greater in males than in females. Interestingly, the deleterious effects of anesthesia were manifest on developmental measures including surface righting and forelimb grasp, but were not evident on basic physiological parameters including body weight or suckling. These findings point to the hazardous effects of exposure to anesthesia on the developing central nervous system and the particular sensitivity of males to deficits.

Keywords

hippocampus; isoflurane; phenobarbital; surgery

Premature births are becoming increasingly common, with one in every eight babies born prematurely in the United States (Cockey, 2005; Green et al., 2005). While the normal gestational period for a human fetus is 39–40 weeks (Davidoff, 2006), babies as early as 24 weeks gestation have a 60% chance for survival (Weber et al., 2005). Unfortunately, their survival is fraught with difficulties complicated by the immature status of their central nervous system. Two of the most common concerns following premature birth are the prevention of respiratory distress and intraventricular hemorrhage, due to the juvenile developmental state of the lungs and heart (Andrews, et al., 2006; Bland et al., 2000; D'Angio and Maniscalco, 2004; Inder et al., 2005; McQuillen and Ferriero, 2005; Rees and Inder, 2005). If the dysfunction is sufficiently severe, the child will need to undergo surgery. While surgeries on

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premature human infants to repair these dysfunctions are essential, there has been relatively little exploration of the consequences of the anesthesia used during the surgical procedure themselves on the very immature central nervous system.

Evaluating the potential clinical significance of animal research requires placing it in the context of human development. A point of controversy is the relative developmental stage equivalency between the rodent and human. The newborn human, based on brain growth rate, acetylcholinesterase activity and reflex ontogeny is thought to be equivalent to the postnatal day 5–7 rat (Adlard et al., 1973). More recent work documenting synapse formation, glutamate decarboxylase activity, choline acetyltransferase activity and electrical activity in the rat cerebral cortex has suggested a later age equivalency, with the newborn human being equivalent to the postnatal day 12–13 rat (Romijn et al., 1991). This has been validated by a recent meta-analysis and web based program that allows one to translate developmental equivalency across species (Clancy et al., 2007a,b). From these data, it has been proposed that the developmental stage of the newborn rat is equivalent to the premature human infant. However, a critical caveat is that prematurity in human infants is often associated with altered or traumatic developmental events: transient decreases in thyroid hormone levels, high blood pressure, umbilical cord compression, seizures, hypoxia-ischemia, that do not occur following normal rodent birth (Inder et al., 2005; McQuillen and Ferriero, 2005; Nuñez et al., 2003a; Rees and Inder, 2005; Yager et al., 2005). Therefore, while newborn rats share developmental equivalence with premature humans, they do not reflect a complete model of human prematurity.

Anesthetic usage has been established based on their actions in the adult central nervous system (CNS), with less investigation of the action of these agents in the immature CNS. Many commonly used anesthetics act on the GABA_A receptor, the predominant inhibitory neurotransmitter system in the adult CNS (Rees and Inder, 2005). However, GABA_A receptor activation in the developing brain, including the hippocampus, cerebral cortex and hypothalamus, leads to chloride efflux and membrane depolarization sufficient to open voltage sensitive calcium channels (Leinekugel et al., 1995; LoTurco et al., 1995; Obrietan and van den Pol, 1995). GABA mediated excitation persists through the first postnatal week in the rodent hippocampus (Nuñez et al., 2005; Nuñez and McCarthy, 2007). Excessive calcium influx, which can occur following over-activation of the GABA_A receptor during this time period, results in cell death (Orrenius et al., 2003; Paschen, 2003; Siesjo, 1994; Tymianski, 1996). Work in fetal primates (Khazipov et al., 2001), along with data in humans documenting that benzodiazepines (GABA_A receptor agonists) can exacerbate seizures in premature and newborn human infants (Guerrini et al., 1998; Montenegro et al., 2001; Ng et al., 2002), has verified that the “excitatory” actions of developmental GABA_A receptor activation are not relegated to non-human species. Given this information, an investigation of the anatomical and behavioral consequences of early anesthetic exposure is warranted.

Previous work has documented that in a model of newborn human infants, the postnatal day 7 rat, surgery-specific durations of anesthesia lead to increased density of apoptotic and degenerating cells, along with behavioral deficits (Bittigau et al., 2002; Ikonomidou et al., 1999; Jevtovic-Todorovic et al., 2003; Thompson and Wasterlain, 2001; Yon et al., 2005; Young et al., 2005). However, there have been few investigations documenting the effect of anesthesia at earlier ages (Bittigau et al., 2002). Also, no studies have investigated the impact of genetic sex on the response to anesthetic exposure. In the current experiment, we examined the effects of two commonly used anesthetics that act on the GABA_A receptor, phenobarbital and isoflurane, on newborn male and female rat pups, the developmental equivalent of the premature human infant (Adlard et al., 1973; Clancy et al., 2001; Romijn et al., 1991). Focusing on hippocampal anatomy and hippocampal dependent behavior, these findings will give insight into the repercussions of anesthetic exposure on the immature CNS in males and females.

EXPERIMENTAL PROCEDURES

Subjects

Animals were first generation descendants of Sprague-Dawley albino rats purchased from Charles River Lab (Wilmington, MA) and housed under a 12:12 hour light/dark cycle, with free access to food and water. Females were bred with male breeders in the Michigan State University animal colony. Pregnant females were checked every morning for the presence of pups. Day of birth was designated as postnatal day 0 (PN0). All animal procedures were approved by the Michigan State University All-University Committee on Animal Use and Care, and followed National Institute of Health guidelines.

Treatment of animals

Rats were removed from their mothers two to four hours after birth (PN0). All animals were initially weighed by the experimenters then placed on a heated pad to avoid a drop in body temperature. Male and female rats were administered either: 1) phenobarbital (25mg/kg), 2) isoflurane (delivered at normobaric conditions using a calibrated isoflurane vaporizer) – 2% isoflurane for 3 min, followed by 1% isoflurane for 7 min, 3) saline (injection control), or 4) normobaric O₂/CO₂ for 10 min (inhalant control). All injections were administered subcutaneously in a volume of 0.05ml. The doses of phenobarbital and isoflurane were chosen based on previous reports of their efficacy as appropriate anesthetic agents in rats younger than postnatal day 3 (Yi and Barr, 1996; Bittigau et al., 2002). The duration of isoflurane exposure was chosen based on previous research by the experimenter on the average amount of time required to perform gonadectomies on rats on postnatal day 1 (Nuñez et al., 1998; Nuñez et al., 2000). This is significantly shorter than the 45 to 120 minute time period of anesthesia documented by other labs required to perform surgeries in neonatal rodents (Park et al., 1992; Yi and Barr, 1996; Karuri et al., 1998) and human infants (Messmer et al., 1976; Oates et al., 1995). The injection site was sealed with cyanoacrylate Vetbond Surgical Adhesive (3M Animal Care Products, St. Paul, MN). Pups were returned to the dam within 20 minutes.

Physiological Assessment

Body temperature (using a rectal probe) was monitored continuously in all animals over a 15 minute period, from 2 min prior anesthetic/control administration to 3 min following the cessation of inhalant/control administration. Upon return of the pups to their mothers, suckling was monitored every two hours over the first eight hours. Suckling was monitored four times daily over the next five days. Body weights were taken three times a week until PN25.

Behavioral Assessment

Neonatal Behaviors—Beginning on postnatal day 2, all pups underwent a battery of neonatal tests that were used to assess developmental milestones (Wu et al., 1997). Testing took place three times a week until PN15. The order of the neonatal test battery was randomized. Animals were removed from the dams, tested, and then immediately returned to the mothers.

Surface righting: Testing took place from PN2 through PN15. Pups were placed in the supine position, and the time to return to the prone position with all four paws on the ground was recorded.

Negative geotaxis: Testing took place from PN2 through PN15. Pups were placed head down on a 45° incline, and the amount of time required for the animals to turn 180° and to face up the incline was recorded.

Cliff aversion: Testing took place from PN5 through PN15. Pups were placed with their forepaws and nose on the edge of a 2” high block of plastic, and the amount of time required for the animals to turn around and crawl away from the edge was recorded.

Forelimb grasp: Testing took place from PN5 through PN15. Pups were made to grasp a thin wooden rod with their forelimbs. The amount of time pups remained suspended using only their forelimbs was recorded.

Adult Behaviors—Daily handling of all pups began on PN 15. Handling took place for three minutes each day over postnatal days 15 to 45 in order to make the rats more comfortable with behavioral testing and eliminate any potential confounding stress effects. Rat pups were weaned on PN20, with animals housed with one to two same sex littermates. All animals underwent adult behavioral testing. To assess effects on anxiety and motivation, a subset of animals underwent behavioral testing on hippocampal independent tasks including the elevated plus maze and open field task.

Balance beam testing began on PN25, while the remainder of behavioral testing began on PN45. The order of the adult behavior testing was fixed – first balance beam (PN25–30), then water maze (PN45–46), and finally radial arm maze (PN58–72). The subset of animals that underwent additional tests performed open field testing on PN47, and elevated plus maze testing on PN48. All testing took place in a single, isolated room. In order to minimize stress, low-light levels were maintained throughout testing. All behavioral testing occurred between 2 and 5pm (animals were on a 5am on/5pm off light: dark cycle). For females, testing (except for the radial arm maze) only took place during the diestrus phase of the estrous cycle. This avoided potential confounding effects of estrous cycle phase on behavioral performance. During testing, the experimenter sat at a computer station set up in one corner of the room, while an assistant was responsible for placing and retrieving animals. The room was filled with numerous cues (large shapes of different colors) and distinctive furniture pieces (cabinet, wall shelf) that could act as permanent extra maze cues. For data acquisition, an overhead low-light video camera connected to a computer with image analysis software (SMART video tracking system, San Diego Instruments, San Diego, CA) was used to track movement of rats in the various mazes.

Balance Beam: Pre-training: Animals underwent two days of pre-training. The setup is composed of two platforms (10 inch square and 36 inches high) separated by 48 inches. The distance between the platforms is spanned by two dowel rods, 1/4 inch diameter. The parallel beams are separated by 1 inch. The animal was placed on one platform for 3 minutes and allowed to investigate. The animal was then returned to its home cage, with this trial repeated 5 min later. On the second day of pre-training, the animal was placed on the platform for 10 sec, and then gently prodded until it crossed the beams between the two platforms. This trial was repeated 5 min later.

Testing: Testing took place one day following pre-training. Each animal was placed on the platform for 10 sec, then a static noise generator (80db) and bright light (100W), both located 6 inches from the platform, were turned on. If the animal did not begin to cross the beam within 10 sec of the initiation of the noise and light, it was gently prodded by the experimenter. If animals did not respond to a single bout of prodding, they were returned to their cage and tested 5 min later. By the second day of testing, no animal required prodding. Animals underwent two trials per day over three days. The following measures were taken: 1) latency to cross the balance beam, 2) front foot slips, 3) rear foot slips, and 4) falls.

Water Maze: Pre-training: Animals underwent one day of water maze pre-training. The maze, composed of blue industrial plastic, is circular with 30 inch high walls and a diameter of 6 feet.

Each animal was placed into the water maze filled with $26 \pm 1^\circ\text{C}$ water. A clear translucent plastic platform (4 inch diameter and 10 inches tall) was placed in the center of the pool, sticking one inch above the surface of the water. The animal was placed on the platform for 20 seconds, and then put into the pool at a random location. The animal was allowed 60 seconds to locate and escape onto the visible platform. If they did not find the platform in the allotted time, they were led there by the experimenter. The animal remained on the platform for twenty seconds. Each rat underwent three consecutive trials. After all trials were completed, animals were thoroughly dried and returned to their home cages.

Testing: The maze was filled to a depth of 21 inches with $26 \pm 1^\circ\text{C}$ water rendered opaque with 150 grams of powdered skim milk. A white plastic platform, 20 inches in height with a 4 inch diameter face, was used throughout the study and placed in the south-east corner of the pool (18 inches from the edge). The top of the platform was one inch below the surface of the water (therefore, the platform was invisible to the animal)

One day following pre-training, rats underwent one day of water maze testing. Each animal was placed on the platform for 20 seconds, and then put into the pool at one of four start locations: north, south, east or west. The animal was allowed 60 seconds to locate and escape onto the submerged platform. If they did not find the platform in the allotted time, they were led there by the experimenter. The animal remained on the platform for ten seconds. Each rat underwent twelve trials. There was a 2 to 5 minute inter-trial interval, with a 30 minute rest period between the 8th and 9th trials. After all trials were completed, animals were thoroughly dried and returned to their home cages. Data was obtained using the tracking software. The following measures were taken: 1) latency to find the platform, 2) pathlength, and 3) swim speed.

A subset of animals underwent open field maze and elevated plus maze testing immediately following water maze testing.

Open Field Maze: Testing: Rats underwent one trial of testing in the open field maze. The open field maze is a large square arena with 18 inch high walls. For the purpose of analysis, the open field enclosure is divided into two regions: an outer region (0 to 9 inches from the wall) and an inner region (9 to 27 inches from the wall). The trial begins with the rat being placed in the corner of the enclosure, facing the wall. Each trial lasts for 5 min, at which time the rat is returned to its home cage. The following measures are taken: 1) number of entries into the inner and outer regions of the maze, and 2) distance traveled.

Elevated Plus Maze: Testing: Rats underwent one trial of testing on the elevated plus maze. The maze consists of two sets of perpendicular arms. A central region separates the maze into two pairs of arms, one with twelve inch high walls (closed arms) and one without walls (open arms). The entire maze is elevated three feet off the ground. The trial began with the rat being placed in the central region of the maze facing an open arm. Each trial lasted 5 min, at which point the rat was removed and returned to its home cage. The following measures were taken: 1) number of entries into the open and closed arms, 2) distance traveled, and 3) amount of time in open arms.

Following water maze or elevated plus maze testing, the animals began food deprivation. Each animal was weighed, and fed 3 pellets (10 grams) per day. Over the next ten days, a feeding regiment was established in order to reduce each animal to 90–92% of the initial body weight. Along with the daily feeding, rats were given three pieces of whole grain cereal. The rats were encouraged to take the cereal from the experimenter. After a stable body weight was obtained, each animal began radial arm maze testing.

Radial Arm Maze: The maze, composed of black plastic, consists of eight arms 20 inches in length and 4 inches in width, with 8 inch high walls. A central circular region (6 inches in diameter) joins the arms of the maze. The entire maze is elevated 20 inches off the ground.

Pre-training: Prior to each trial, the maze was cleaned thoroughly with a soapy solution, followed by 70% alcohol. The pre-training trial began with the rat being placed in the central region of the maze. Each rat underwent one trial per day. On the first day of pre-training, a piece of the whole grain cereal was placed at the mouth of each of the eight arms. The animal was then allowed to eat the cereal, with the trial timed. After all cereal pieces were eaten, or 5 minutes had elapsed, the rat was removed and returned to its home cage. On the second day of pre-training, a piece of cereal was placed half-way down each of the eight arms, while for the third day of pre-training, a piece was placed at the end of each of the eight arms. In order to continue radial arm maze testing, animals had to eat all pieces of cereal within the allotted time.

Testing: One day after radial arm maze pre-training, rats began testing. Prior to each trial, the maze was cleaned thoroughly with a soap solution, followed by 70% alcohol. The testing trial began with the rat being placed in the central region of the maze. Each rat underwent one trial per day. A small piece of whole grain cereal was placed in a dish at the end of five of the eight arms. Each trial lasted for 5 minutes, at which point the rat was removed and returned to its home cage. Data was obtained using the tracking software and supplemented by experimenter observations. The following measures were taken: 1) number of entries into arms that never contained food (reference memory error), 2) number of entries into arms that contained food, but it was already consumed on the same trial (working memory errors), and 3) time to complete the task.

Histological processing and stereological investigation

Animals were euthanized on postnatal day 80 by barbiturate overdose, and their brains fixed overnight in 4% paraformaldehyde with 2.5% acrolein, then 24 hours in 4% paraformaldehyde. Brains were stored in 30% sucrose in paraformaldehyde for 72 hours, and then sectioned on a cryostat. Two sets of consecutive 60 μ m coronal sections were made through the entire hippocampus, with all tissue saved in the consecutive series. One set of tissue was mounted onto gelatin coated slides and used for cresyl violet staining. The remaining set of tissue sections were processed for free-floating neuronal nuclear antigen (NeuN) immunocytochemistry. Following immunocytochemistry, all consecutively labeled tissue was mounted onto gelatin coated slides. Using the StereoInvestigator program package (version 6.55, MicroBrightField, Colchester, VT) loaded onto a PC, with a Nikon Eclipse 80i microscope equipped with a motor-driven LEP stage and an Optronics Microfire color CCD camera, hippocampal volume and neuron number was quantified. The Cavalieri estimator allowed for quantification of hippocampal volume from the cresyl violet stained tissue, and the fractionator, a stereological tool, was used in conjunction with an unbiased disector frame to allow for correct estimation of NeuN immunoreactive neuron number counts in the hippocampal formation. We employed a systematic, random sampling strategy in order to eliminate potential bias that results from tissue processing, tissue/neuronal orientation or other sources of error. In all analyses, a distinction was made between the hippocampus and dentate gyrus (not investigated).

Volumetric analysis using Cavalieri estimator—The anterior to posterior extent of the hippocampal formation on the tissue-mounted slides was recorded, with the anterior-most tissue section containing the hippocampus and dentate gyrus noted. The first plane that underwent Cavalieri estimation was a randomly chosen tissue section within 120 μ m of the anterior-most tissue section containing the hippocampal formation. Each consecutive section analyzed was 180 μ m from the previous section. The last section analyzed for each animal was

the last plane in which the hippocampal formation was present. There were a total of 8 to 10 sections analyzed per animals. Cavalieri estimation was performed using a 4X objective and 75µm grid spacing.

Neuron number quantification with Fractionator—Eight tissue sections within the anterior-to-posterior extent of the hippocampal formation were selected for fractionator analysis. The first tissue section investigated was a randomly chosen tissue section within 180µm of the anterior-most tissue section containing the hippocampal formation. Each consecutive section analyzed was 180µm from the previous section. The disector counting frame, determined by preliminary analysis based on cell size and cell packing density, was 20 × 20µm. A systematic uniform spacing of sampling sites was determined based on obtaining fractionator error estimates (CE) for an individual animal of less than 0.01. For the hippocampus, this translated into 20 sampling sites per region per tissue section. The fractionator probe was then performed using the unbiased disector counting frame (of the 20 × 20µm predetermined size) and moving the disector across 20 systematic, uniform sampling sites and counting the number of neurons.

Statistical Analysis

Two-way analyses of variance (sex, treatment) for the effect of neonatal anesthesia on hippocampal volume and neuron number in male and females were performed. Effects of isoflurane and phenobarbital were investigated separately. Repeated measures analyses of variance were run on behavioral data. For all ANOVAs, post-hoc (Tukey) tests were performed, with a level of $p < 0.05$ required to obtain statistical significance. The non-parametric Kruskal-Wallis one-way ANOVA test was performed on radial arm maze data (males and females were analyzed separately).

RESULTS

Physiological Assessment

Body Temperature—There was no effect of exposure to neonatal anesthesia on body temperature as assessed using a rectal probe. Following both the injection and inhalant exposure, body temperatures did not consistently drop. In both control and anesthesia exposed animals, body temperature fluctuated over a 1–2 degree range during the time they were separated from their mothers.

Suckling—Food intake, manifest by milk bands visible through the ventral surface (belly area) of the pups, was unaffected by neonatal anesthetic exposure. All animals experienced at least one bout of feeding within 4 hours of being returned to their mothers. Consistent with this data, there was no long term effect of anesthesia on suckling and food intake. All animals ate normally over the entire duration.

Body Weight—There was no effect of neonatal anesthesia on body weights at any time point post exposure. As indicated by the normal suckling, anesthetic exposed animals showed no ill effects on the normal increase in body weight.

Behavioral Analysis

Surface Righting—There was a significant effect of anesthetic exposure on the time to perform surface righting in both males and females (Between group analyses - Isoflurane: $F_{3,11}=5.32$, $p=0.02$; Phenobarbital: $F_{3,11}=8.17$, $p=0.006$) (Figure 1). Animals treated neonatally with either anesthetic had longer latencies than control animals of the same sex ($p < 0.05$). All animals improved on the task across time (Within group analyses - Isoflurane:

$F_{3,22}=102.67$, $p<0.0001$; Phenobarbital: $F_{3,22}=98.63$, $p<0.0001$). All animals took less than one second to perform the task on the last day of investigation.

Negative Geotaxis—There was no significant effect of neonatal isoflurane and phenobarbital exposure on time to perform negative geotaxis. On the first day of testing, all animals took between 145 and 180 seconds to perform the task. This rapidly improved such that by the second postnatal week, all animals took between 21 to 34 seconds. By postnatal day 15, the last time point investigated, all animals took between 3 to 10 seconds to perform the task.

Cliff Aversion—As with negative geotaxis, there was no significant effect of neonatal isoflurane and phenobarbital exposure on the time to perform this task. On postnatal days 2 and 5, only two groups of the animals (control females and isoflurane treated males) were able to successfully perform the task. By postnatal day 7, all animals took between 5 and 15 seconds to move away from the edge of the cliff. On the last day of testing (postnatal day 15), animals took between 2 to 6 seconds to perform the task.

Forelimb Grasp—There was a significant effect of anesthetic exposure on the amount of time male and females could hang onto the wood bar with their forepaws (Between group analyses - Isoflurane: $F_{3,11}=12.42$, $p=0.003$; Phenobarbital: $F_{3,11}=16.32$, $p<0.001$) (Figure 2). Animals treated neonatally with either anesthetic had shorter hang times than control animals of the same sex ($p<0.05$). All animals improved on the task across time (Within group analyses - Isoflurane: $F_{3,22}=124.32$, $p<0.0001$; Phenobarbital: $F_{3,22}=151.87$, $p<0.0001$).

Balance Beam—There was a significant effect of isoflurane (but not phenobarbital) exposure on the number of front and rear foot slips in both males and females (Between group analyses – Front Foot Slips: $F_{3,10}=5.63$, $p=0.03$; Rear Foot Slips: $F_{3,10}=7.11$, $p=0.01$) (Figure 3). Animals treated neonatally with isoflurane had significantly more slips than control animals of the same sex ($p<0.05$). All animals improved on the task across time. While prodding (to initiate beam crossing) was common on the first day of testing (occurred in greater than 50% of the animals), all animals performed the beam crossing in the absence of prodding by the second day of testing. There was no sex or treatment difference in the incidence of prodding.

Morris Water Maze

Pretraining: There was a significant effect of neonatal isoflurane and phenobarbital exposure on the latency to find the platform across the three pretraining trials (Isoflurane: $F_{3,18}=17.559$, $p<0.0001$; Phenobarbital: $F_{3,19}=5.313$, $p=0.008$) (Figure 4). Post hoc analyses indicated that both anesthetics led to significant decrements in the latency to perform this procedural task in both male and females ($p<0.01$). Control animals performed at least 50% faster than the anesthesia treated animals across the three pretraining trials. There were no significant differences between control males and females.

Testing: There was a significant effect of both types of neonatal anesthetics on water maze performance in both males and females (Between group analyses - Isoflurane: $F_{3,18}=9.524$, $p=0.001$; Phenobarbital: $F_{3,19}=24.677$, $p<0.0001$) (Figure 4). Animals treated neonatally with either anesthetic had longer latencies than control animals of the same sex ($p<0.05$). Also, there was a sex difference in the effects of neonatal anesthesia, with male performance more affected than females ($p<0.05$). While all animals were able to learn the task (Within group analyses - Isoflurane: $F_{2,36}=123.165$, $p<0.0001$; Phenobarbital: $F_{2,38}=65.924$, $p<0.0001$), there was a significant difference in the rate of learning between the control and anesthesia-treated groups (Between group by treatment analyses - Isoflurane: $F_{6,36}=6.189$, $p=0.0009$; Phenobarbital: $F_{6,38}=2.694$, $p=0.028$). The decrement due to anesthesia was the most pronounced on the first

trial block, however all animals were able to attain asymptotic performance by the final trial block. Control animals obtained maximal performance by the second trial block. Pathlength data (not shown) was consistent with latency data, with a lack of effect of sex and treatment on swim speed (data not shown).

Due to the small sample size ($n=3$ per group), statistical analysis was not performed on open field or elevated plus maze data. However, the variance amongst animals within an individual group was small (less than 12% of the group mean); therefore the data are estimated to be indicative of effects on a larger population of animals.

Open Field Maze—From the small subset of animals, it appears that vehicle treated animals make a greater percentage of inner region entries, defined as the number of inner region entries/total number of region entries*100, as compared to both isoflurane (control males: $7.09\pm0.82\%$, isoflurane treated males: $0.59\pm0.06\%$, control females: $11.46\pm0.97\%$, isoflurane treated females: $1.80\pm0.21\%$) and phenobarbital treated animals (control males: $7.86\pm0.77\%$, phenobarbital treated males: $5.86\pm0.49\%$, control females: $6.13\pm0.57\%$, phenobarbital treated females: $3.48\pm0.31\%$).

Elevated Plus Maze—From the small subset of animals, it appears that there is no effect of anesthetic treatment on the percent of open arm entries, defined as number of open arm entries over total number of arm entries*100 (control males: $40.57\pm3.12\%$, isoflurane treated males: $38.78\pm4.13\%$, control females: $42.22\pm3.97\%$, isoflurane treated females: $44.44\pm4.53\%$) and phenobarbital treated animals (control males: $48.10\pm5.02\%$, phenobarbital treated males: $29.57\pm3.64\%$, control females: $48.65\pm4.54\%$, phenobarbital treated females: $41.94\pm4.56\%$). In contrast, there appears to be a difference in the amount of time spent in the open arms control males: $43.3\pm5.11\%$, isoflurane treated males: $23.5\pm2.76\%$, control females: $62.0\pm7.11\%$, isoflurane treated females: $24.7\pm2.12\%$) and phenobarbital treated animals (control males: $36.7\pm3.98\%$, phenobarbital treated males: $19.83\pm2.14\%$, control females: $62.6\pm6.84\%$, phenobarbital treated females: $12.86\pm2.06\%$).

Radial Arm Maze—There was a significant effect of treatment on the number of reference memory errors on the last two trials in males (Isoflurane – Trial 5: $p=0.046$; Trial 6: $p=0.048$; Phenobarbital – Trial 5: $p=0.029$; Trial 6: $p=0.026$) and the last trial in isoflurane treated females ($p=0.046$) (Figure 5). Although not significant, there trend was similar on the fourth trial in phenobarbital treated males ($p=0.061$), and the fifth trial in isoflurane treated females ($p=0.053$). In the above cases, anesthesia treated animals made more reference memory errors than their control counterparts of the same sex.

There was no effect of treatment on the time required to finish the radial arm maze (data not shown). The time to perform the task improved across the testing days (Within group analyses – Isoflurane: $F_{5,40}=10.621$, $p=0.0003$; Phenobarbital: $F_{5,30}=2.644$, $p=0.043$), but this was unaffected by treatment with neonatal anesthesia.

Anatomical Analysis

Volume—Hippocampal volume was significantly smaller in animals that experienced a single bout of exposure to either form of neonatal anesthesia in both males and females (Isoflurane: $F_{1,23}=24.272$, $p<0.0001$; Phenobarbital: $F_{1,21}=15.993$, $p=0.001$) (Figure 6). The percent difference in hippocampal volume was equivalent across both forms of anesthesia in males (25.8–25.9%). However, females were less affected, with a 20.12% reduction in hippocampal volume following isoflurane and only a 12.93% reduction in hippocampal volume following a single injection of phenobarbital. Of additional interest is the effect of anesthesia on the sex difference in hippocampal volume. In the absence of anesthetic exposure, the hippocampus is

10–15% larger in males than in females. However, following neonatal exposure to either isoflurane or phenobarbital, the sex difference is eliminated – from 0.5% larger in males to 1% larger in females.

Neuron Number—Hippocampal neuron number was significantly less in animals that experienced a single bout of exposure to either form of neonatal anesthesia in both males and females (Isoflurane: $F_{1,19}=16.153$, $p<0.001$; Phenobarbital: $F_{1,19}=14.174$, $p<0.001$) (Figure 7). Hippocampal neuron number was reduced between 15–20% following exposure to both types of anesthesia. Consistent with data obtained from hippocampal volume analyses, females were less affected than males. While hippocampal neuron number was reduced 13–15% following neonatal isoflurane and phenobarbital in females, there was a larger 17–22% reduction in males. Likewise consistent with volumetric analyses, neonatal anesthesia eliminated the sex difference in adult hippocampal neuron number.

DISCUSSION

This is the first documentation that isoflurane and phenobarbital have deleterious effects on the newborn rodent, and that males are more sensitive to the damaging effects of these agents as compared to females. We have found that a single injection of phenobarbital or ten minute exposure to isoflurane on the day of birth, what is routinely administered as anesthesia for surgical procedures in both animal research and human operations, results in a significant decrement in hippocampal neuron number and impairs performance on hippocampal dependent cognitive tasks in adulthood. Deficits were likewise observed on fundamental motor abilities including forelimb grasp and traversing a balance beam. This is in contrast to the lack of effects of these anesthetics on basic developmental parameters such as suckling, increases in body weight and neonatal reflexes including negative geotaxis and cliff aversion. The present findings highlight the deleterious nature of anesthesia when administered to the immature central nervous system.

Males more sensitive than females to neonatal anesthesia

The anatomical and behavioral deficits following neonatal isoflurane and phenobarbital exposure are more pronounced in males as compared to females. The increased sensitivity of males to the deleterious effects of anesthesia is consistent with numerous reports in both humans and animals (Hall et al., 1991; Hurn et al., 2005; Rogers and Wagner, 2006; Yager et al., 2005). Across both sexes, isoflurane had the most pronounced negative repercussions, resulting in a 20–26% reduction in hippocampal volume and neuron number. In contrast, phenobarbital injected animals displayed a 13–25% reduction in hippocampal volume and neuron number. With regard to sex, the decrement in neuron number in females was 23.5% less following isoflurane treatment than in males. This sex difference was more pronounced in the phenobarbital treated animals, with the female decrement in neuron number as 50% less than in males. Given that the majority of neurogenesis in the hippocampal formation (excluding the dentate gyrus) occurs prior to the day of birth (Bayer, 1980a; Bayer, 1980b; Reznikov, 1991), we hypothesize that exposure to these anesthetics results in a significant increase in cell death. We hypothesize that the more severe anatomical deficits in males contribute to the more pronounced behavioral deficits in these animals.

Interestingly, the sex difference in response to neonatal anesthesia may represent a “floor effect:” while the decrement in male hippocampal neuron number is greater, ultimately, the number of neurons following insult arrives at a “floor” value that is equivalent for both male and females. While an intriguing possibility, this stands as a difficult hypothesis to test. Also, the rationale for such a “floor” stands in opposition to the fact that the neonatal hippocampus can endure a significantly greater loss of cells than in the present experiment (Nuñez et al.,

2007). The “floor” may represent the specific population of cells that is sensitive to isoflurane and phenobarbital-mediated injury. Regardless, hippocampal cell loss following neonatal anesthetic exposure is greater in males than in females.

Neonatal anesthetic action results in GABA-mediated excitation

We hypothesize that the negative effects of these anesthetic agents results in part from their ability to activate GABA_A receptor (Hall et al., 2004; Hasenader et al., 2002). In the neonatal mammalian hippocampus, activation of the GABA_A receptor leads to chloride efflux and membrane depolarization sufficient to open voltage sensitive calcium channels (Leinekugel et al., 1995; LoTurco et al., 1995; Obrietan and van den Pol, 1995). This cellular excitation is in stark contrast to chloride influx and membrane hyperpolarization (cellular inhibition) that occurs following GABA_A receptor activation in the hippocampus in adulthood (Duggan, 1978). Phenobarbital, a potent anticonvulsant/barbiturate, binds directly to the GABA_A receptor, resulting in enhanced receptor activation (Czapinski et al., 2005). The inhalant isoflurane has been documented to enhance GABA_A receptor activity and increase the number of GABA_A-mediated postsynaptic potentials (Hall et al., 2004; Hasenader et al., 2002). Findings from the present study are consistent with work documenting the ability of excessive activation of the GABA_A receptor in the developing brain to induce hippocampal cell death, decrements in hippocampal-dependent behavioral performance, and enhanced calcium influx in primary cultures of hippocampal neurons (Nuñez et al., 2003a; Nuñez et al., 2003b; Nuñez and McCarthy, 2003; Nuñez et al., 2005). Recent work has also demonstrated that males are more sensitive to GABA_A receptor-mediated hippocampal damage and the persistent repercussions of GABA-mediated excitation (Nuñez et al., 2003a, Nuñez and McCarthy, 2007), consistent with the current results of the increased sensitivity of males to phenobarbital and isoflurane mediated-injury.

Actions of isoflurane and phenobarbital outside of the GABAergic system

Isoflurane is a potent analgesic and muscle relaxant. The actions of this agent are diffuse, acting at glutamate receptors leading to decreased phosphorylation of NMDA and AMPA receptors (Snyder et al., 2007), and attenuated glutamate receptor-mediated population spikes recorded from CA1 hippocampal pyramidal neurons (Asahi et al., 2006). Isoflurane also inhibits sodium channel and voltage dependent glutamate release, resulting in attenuated spread of excitation (Wu et al., 2004). The barbiturate phenobarbital likewise acts on the glutamatergic system. This anesthetic inhibits NMDA receptor-mediated calcium influx (Daniell, 1994) and binding (Silva-Brum and Elisabetsky, 2000). Concomitant with phenobarbital binding to the GABA_A receptor is an inhibition of AMPA receptor function (Joo et al., 1999). Reduction in normal developmental glutamate receptor activation has been shown by Olney and colleagues to be a potent instigator of cell loss (Ikonomidou et al., 1999; Bittigau et al., 2002; Jevtovic-Todorovic et al., 2003).

Behavioral deficits following exposure to neonatal anesthesia

Neonatal anesthetic exposed animals displayed attenuated hippocampal dependent behavioral performance on tasks including the Morris water maze and radial arm maze. This is consistent with previous work from our lab documenting the deleterious effects of cryoanesthesia (Nuñez et al., 2000). While both males and females who encountered phenobarbital and isoflurane on the day of birth displayed increased latency to find the escape platform (especially evident across the first two trial blocks), the water maze deficits were significantly more pronounced in males than in females. These deficits were not due to motor impairments, given equivalent swim speed between the groups (data not shown). Likewise, radial arm maze performance was more affected in males than in females.

CONCLUSIONS

Brief exposure of newborn rats, the developmental equivalent of premature human infants, to an injectable and an inhalant form of anesthesia has permanent, detrimental effects on the hippocampus and hippocampal dependent behavioral expression. The response to the deleterious effects is sex specific; greater damage, both anatomically and behaviorally, is experienced in males as compared to females. While it can be anticipated that chronic exposure to deleterious agents affects the developing brain, recent research consistent with the present experiment emphasizes the damaging effects of a single bout of anesthesia to the postnatal day 7 brain (Bittigau et al., 2002; Ikonomidou et al., 1999; Jevtovic-Todorovic et al., 2003; Thompson and Wasterlain, 2001; Yon et al., 2005; Young et al., 2005). Studies such as these are essential given the necessity for surgical procedures in early life. This is especially critical for premature human infants who are often compromised by severe conditions such as immature heart and lung status (Andrews et al., 2006; Bland et al., 2000; D'Angio and Maniscalco, 2004; Inder et al., 2005; Rees and Inder, 2005). While surgical procedures cannot be avoided, care needs to be taken with the choice of anesthesia. As stated previously, activation of the GABA_A receptor in the immature mammalian hippocampus results in membrane depolarization and cellular excitation (Leinekugel et al., 1995; Nuñez et al., 2005). This is the opposite of what occurs in the adult. Therefore, the actions of GABAergic drugs in the adult CNS cannot be inferred to have the same effect during development. We hypothesize that the actions of isoflurane and phenobarbital on the immature GABAergic system play a prominent role in the ontogeny of the injury documented in the present experiment. This study highlights the deleterious effects of commonly used anesthetics on the developing brain, and the need for alternative forms of anesthesia in the developing CNS that do not act via the GABAergic system.

REFERENCES

- Adlard BP, Dobbing J, Smart JL. An alternative animal model for the full-term small-for-dates human baby. *Biol Neonate* 1973;23:95–108. [PubMed: 4773522]
- Andrews RE, Simpson JM, Sharland GK, Sullivan ID, Yates RW. Outcome after preterm delivery of infants antenatally diagnosed with congenital heart disease. *J Pediatr* 2006;148:213–216. [PubMed: 16492431]
- Asahi T, Hirota K, Sasaki R, Mitsuaki Y, Roth SH. Intravenous anesthetics are more effective than volatile anesthetics on inhibitory pathways in rat hippocampal CA1. *Anesth Analg* 2006;102:772–778.
- Bayer SA. Development of the hippocampal region in the rat. I. Neurogenesis examined with 3H-thymidine autoradiography. *J Comp Neurol* 1980a;190:87–114. [PubMed: 7381056]
- Bayer SA. Development of the hippocampal region in the rat. II. Morphogenesis during embryonic and early postnatal life. *J Comp Neurol* 1980b;190:115–134. [PubMed: 7381049]
- Bittigau P, Siffringer M, Genz K, Reith E, Pospischil D, Govindarajulu S, Dzietko M, Pesditschek S, Mai I, Dikranian K, Olney JW, Ikonomidou C. Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proc Natl Acad Sci U S A* 2002;99:15089–15094. [PubMed: 12417760]
- Bland RD, Albertine KH, Carlton DP, Kullama L, Davis P, Cho SC, Kim BI, Dahl M, Tabatabaei N. Chronic lung injury in preterm lambs: abnormalities of the pulmonary circulation and lung fluid balance. *Pediatr Res* 2000;48:64–74. [PubMed: 10879802]
- Clancy B, Darlington RB, Finlay BL. Translating developmental time across mammalian species. *Neuroscience* 2001;105:7–17. [PubMed: 11483296]
- Clancy B, Finlay BL, Darlington RB, Anand KJ. Extrapolating brain development from experimental species to humans. *Neurotoxicology* 2007a;28:931–937. [PubMed: 17368774]
- Clancy B, Kersh B, Hyde J, Darlington RB, Anand KJ, Finlay BL. Web-based method for translating neurodevelopment from laboratory species to humans. *Neuroinformatics* 2007b;5:79–94. [PubMed: 17426354]

- Cockey CD. Premature births hit record high. AWHONN Lifelines 2005;9:365–370. [PubMed: 16359074]
- Czapinski P, Blaszczyk B, Czuczwar SJ. Mechanisms of action of antiepileptic drugs. *Curr Top Med Chem* 2005;5:3–14. [PubMed: 15638774]
- D'Angio CT, Maniscalco WM. Bronchopulmonary dysplasia in preterm infants: pathophysiology and management strategies. *Paediatr Drugs* 2004;6:303–330. [PubMed: 15449969]
- Daniell LC. Effect of anesthetic and convulsant barbiturates on N-methyl-D-aspartate receptor-mediated calcium flux in brain membrane vesicles. *Pharmacology* 1994;49:296–307. [PubMed: 7862741]
- Duggan AW. Pharmacology of mammalian central inhibition. *Int Rev Physiol* 1978;17:119–148. [PubMed: 29845]
- Green NS, Damus K, Simpson JL, Iams J, Reece EA, Hobel CJ, Merkatz IR, Greene MF, Schwarz RH. Research agenda for preterm birth: recommendations from the March of Dimes. *Am J Obstet Gynecol* 2005;193:626–635. [PubMed: 16150253]
- Guerrini R, Belmonte A, Genton P. Antiepileptic drug-induced worsening of seizures in children. *Epilepsia* 1998;39:S2–S10. [PubMed: 9593229]
- Hall AC, Rowan KC, Stevens RJ, Kelley JC, Harrison NL. The effects of isoflurane on desensitized wild-type and alpha 1(S270H) gamma-aminobutyric acid type A receptors. *Anesth Analg* 2004;98:1297–1304. [PubMed: 15105205]
- Hall ED, Pazara KE, Linseman KL. Sex differences in postischemic neuronal necrosis in gerbils. *J Cereb Blood Flow Metab* 1991;11:292–298. [PubMed: 1997500]
- Haseneder R, Rammes G, Zieglgansberger W, Kochs E, Hapfelmeier G. GABA(A) receptor activation and open-channel block by volatile anaesthetics: a new principle of receptor modulation? *Eur J Pharmacol* 2002;451:43–50. [PubMed: 12223227]
- Hurn PD, Vannucci SJ, Hagberg H. Adult or perinatal brain injury: does sex matter? *Stroke* 2005;36:193–195. [PubMed: 15625289]
- Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovskia V, Turski L, Olney JW. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* 1999;283:70–74. [PubMed: 9872743]
- Inder T, Neil J, Kroenke C, Dieni S, Yoder B, Rees S. Investigation of cerebral development and injury in the prematurely born primate by magnetic resonance imaging and histopathology. *Dev Neurosci* 2005;27:100–111. [PubMed: 16046843]
- Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci* 2003;23:876–882. [PubMed: 12574416]
- Joo DT, Xiong Z, MacDonald JF, Jia Z, Roder J, Sonner J, Orser BA. Blockade of glutamate receptors and barbiturate anesthesia: increased sensitivity of pentobarbital-induced anesthesia despite reduced inhibition of AMPA receptors in GluR2 null mutant mice. *Anesthesiology* 1999;91:1329–1341. [PubMed: 10551584]
- Karuri AR, Engelking LR, Kumar MSA. Effects of halothane and methoxyflurane on the hypothalamic-pituitary-adrenal axis in rat. *Brain Res Bull* 1998;47:205–209. [PubMed: 9865851]
- Khazipov R, Esclapez M, Caillard O, Bernard C, Khalilov I, Tyzio R, Hirsch J, Dzhalal V, Berger B, Ben-Ari Y. Early development of neuronal activity in the primate hippocampus in utero. *J Neurosci* 2001;21:9770–9781. [PubMed: 11739585]
- Leinekugel X, Tseeb V, Ben-Ari Y, Bregestovski P. Synaptic GABAA activation induces Ca²⁺ rise in pyramidal cells and interneurons from rat neonatal hippocampal slices. *J Physiol* 1995;487(Pt 2): 319–329. [PubMed: 8558466]
- LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR. GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron* 1995;15:1287–1298. [PubMed: 8845153]
- McQuillen PS, Ferriero DM. Perinatal subplate neuron injury: implications for cortical development and plasticity. *Brain Pathol* 2005;15:250–260. [PubMed: 16196392]
- Messmer BJ, Schallberger U, Gattiker R, Senning A. Psychomotor and intellectual development after deep hypothermia and circulatory arrest in early infancy. *J Thoracic Cardiovasc Surg* 1976;72:495–502.

- Montenegro MA, Guerreiro MM, Caldas JP, Moura-Ribeiro MV, Guerreiro CA. Epileptic manifestations induced by midazolam in the neonatal period. *Arq Neuropsiquiatr* 2001;59:242–243. [PubMed: 11400034]
- Ng E, Klinger G, Shah V, Taddio A. Safety of benzodiazepines in newborns. *Ann Pharmacother* 2002;36:1150–1155. [PubMed: 12086545]
- Núñez JL, Kim BY, Juraska JM. Neonatal cryoanesthesia affects the morphology of the visual cortex in the adult rat. *Develop Brain Res* 1998;111:89–98.
- Núñez JL, Koss WA, Juraska JM. Hippocampal anatomy and water maze performance are affected by neonatal cryoanesthesia in rats of both sexes. *Horm Behav* 2000;37:169–178. [PubMed: 10868480]
- Núñez JL, Alt JJ, McCarthy MM. A new model for prenatal brain damage: I. GABA_A receptor activation induces cell death in developing rat hippocampus. *Exper Neurol* 2003a;181:258–269.
- Núñez JL, Alt JJ, McCarthy MM. A novel model for prenatal brain damage: II. Long-term deficits in hippocampal cell number and hippocampal-dependent behavior following neonatal GABA_A receptor activation. *Exper Neurol* 2003b;181:270–280.
- Núñez JL, McCarthy MM. Estradiol exacerbates hippocampal damage in a model of preterm infant brain injury. *Endocrinology* 2003;144:2350–2359. [PubMed: 12746295]
- Núñez JL, Bambrick LL, Krueger BK, McCarthy MM. Prolongation and enhancement of GABA_A receptor mediated excitation by chronic treatment with estradiol in developing rat hippocampal neurons. *Eur J Neurosci* 2005;21:3251–3261. [PubMed: 16026463]
- Núñez JL, McCarthy MM. Evidence for an extended duration of GABA-mediated excitation in the developing male versus female hippocampus. *Dev Neurobiol* 2007;67:1879–1890. [PubMed: 17823921]
- Núñez JL, Yang Z, Jiang Y, Grandy T, Mark I, Levison SW. 17beta-Estradiol protects the neonatal brain from hypoxia-ischemia. *Exp Neurol* 2007;208:269–276. [PubMed: 17950281]
- Oates RK, Simpson JM, Cartmill TB. The relationship between intelligence and duration of circulatory arrest with deep hypothermia. *J Thoracic Cardiovasc Surg* 1995;110:786–792.
- Obrietan K, van den Pol AN. GABA neurotransmission in the hypothalamus: developmental reversal from Ca²⁺ elevating to depressing. *J Neurosci* 1995;15:5065–5077. [PubMed: 7623135]
- Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* 2003;4:552–565. [PubMed: 12838338]
- Park CM, Clegg KE, Harvey-Clark CJ, Hollenberg MJ. Improved techniques for successful neonatal rat surgery. *Lab Anim Sci* 1992;42:508–513. [PubMed: 1460853]
- Paschen W. Mechanisms of neuronal cell death: diverse roles of calcium in the various subcellular compartments. *Cell Calcium* 2003;34:305–310. [PubMed: 12909077]
- Rees S, Inder T. Fetal and neonatal origins of altered brain development. *Early Hum Dev* 2005;81:753–761. [PubMed: 16107304]
- Reznikov KY. Cell proliferation and cytogenesis in the mouse hippocampus. *Adv Anat Embryol Cell Biol* 1991;122:1–74. [PubMed: 1927657]
- Rogers E, Wagner AK. Gender, sex steroids, and neuroprotection following traumatic brain injury. *J Head Trauma Rehabil* 2006;21:279–281. [PubMed: 16717505]
- Romijn HJ, Hofman MA, Gramsbergen A. At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? *Early Hum Dev* 1991;26:61–67. [PubMed: 1914989]
- Siesjo BK. Calcium-mediated processes in neuronal degeneration. *Ann N Y Acad Sci* 1994;747:140–161. [PubMed: 7847668]
- Silva-Brum LF, Elisabetsky E. Antiepileptogenic properties of phenobarbital: behavior and neurochemical analysis. *Pharmacol Biochem Behav* 2000;67:411–416. [PubMed: 11164067]
- Snyder GL, Galdi S, Hendrick JP, Hemmings HC. General anesthetics selectively modulate glutamatergic and dopaminergic signaling via site-specific phosphorylation in vivo. *Neuropharmacology* 2007;53:619–630. [PubMed: 17826804]
- Thompson KW, Wasterlain CG. Urethane anesthesia produces selective damage in the piriform cortex of the developing brain. *Brain Res Dev Brain Res* 2001;130:167–171.

- Tymianski M. Cytosolic calcium concentrations and cell death in vitro. *Adv Neurol* 1996;71:85–105. [PubMed: 8790793]
- Weber C, Weninger M, Klebermass K, Reiter G, Wiesinger-Eidenberger G, Brandauer M, Kraschl R, Lingitz K, Grassl-Jurek R, Sterniste W, Balluch B, Kolmer M, Bruckner R, Schweintzger G, Salzer H, Rath I, Kubitsch P, Zissler W, Muller W, Urlesberger B. Mortality and morbidity in extremely preterm infants (22 to 26 weeks of gestation): Austria 1999–2001. *Wien Klin Wochenschr* 2005;117:740–746. [PubMed: 16416355]
- Wu JY, Henins KA, Gressens P, Gozes I, Fridkin M, Brenneman DE, Hill JM. Neurobehavioral development of neonatal mice following blockade of VIP during the early embryonic period. *Peptides* 1997;18:1131–1137. [PubMed: 9396053]
- Wu XS, Sun JY, Evers AS, Crowder M, Wu LG. Isoflurane inhibits transmitter release and presynaptic action potential. *Anesthesiology* 2004;100:663–670. [PubMed: 15108983]
- Yager JY, Wright S, Armstrong EA, Jahraus CM, Saucier DM. A new model for determining the influence of age and sex on functional recovery following hypoxic-ischemic brain damage. *Dev Neurosci* 2005;27:112–120. [PubMed: 16046844]
- Yi DK, Barr GA. The suppression of formalin-induced Fos expression by different anesthetic agents in the infant rat. *Develop Psychobiol* 1996;29:497–506.
- Yon JH, Daniel-Johnson J, Carter LB, Jevtovic-Todorovic V. Anesthesia induces neuronal cell death in the developing rat brain via the intrinsic and extrinsic apoptotic pathways. *Neuroscience* 2005;135:815–827. [PubMed: 16154281]
- Young C, Jevtovic-Todorovic V, Qin YQ, Tenkova T, Wang H, Labruyere J, Olney JW. Potential of ketamine and midazolam, individually or in combination, to induce apoptotic neurodegeneration in the infant mouse brain. *Br J Pharmacol* 2005;146:189–197. [PubMed: 15997239]

Figure 1. Deleterious effect of single bout of exposure to both (A) isoflurane and (B) phenobarbital on surface righting reflex in neonatal male and female rats. By the postnatal day 12, performance was equivalent across all animals. * indicates significant difference from anesthetic treated animals of the same sex at the same age (Tukey; $p < 0.05$).

Figure 2.

Forelimb grasp in neonatal male and female rats is compromised following exposure to the anesthetics (A) isoflurane and (B) phenobarbital. In males who encountered either anesthetic and phenobarbital exposed females, the deficits persist to the last time point examined. * indicates significant difference from anesthetic treated animals of the same sex at the same age (Tukey; $p < 0.05$).

Figure 3.

The number of front and rear foot slips while traversing a balance beam is significantly increased in adolescent male and female rats that were neonatally exposed to a single bout of (A) isoflurane, but not (B) phenobarbital. * indicates significant difference from isoflurane treated animals of the same sex on the same trial (Tukey; $p < 0.05$).

Figure 4.

A single bout of isoflurane anesthesia (A) and phenobarbital injection (B) administered to the newborn male and female rat leads to profound deficits on the hippocampal and non-hippocampal components of the Morris water maze. Anesthesia treatment led to latencies at least 50% greater than in control animals of the same sex on the initial cued version of the Morris water maze. Likewise, performance on the place version of the Morris water maze was significantly compromised in the neonatal anesthesia treated animals. * indicates significant difference from vehicle treated animals of the same sex on the same trial (Tukey; $p < 0.05$).

Figure 5.

Neonatal (A) isoflurane inhalation and (B) phenobarbital injection led to significant deficits on the reference memory component of the radial arm maze task. Especially evident across the last three testing days, control males and females made significantly fewer errors than anesthetic treated animals. * indicates significant difference from anesthetic treated animals of the same sex on the same trial (Kruskal-Wallis; $p < 0.05$).

Figure 6.

A single bout of exposure to either (A) isoflurane or (B) phenobarbital on the first day of life results in smaller hippocampal volume in male and female rats in adulthood. * indicates significant difference from anesthetic treated animals of the same sex (Tukey; $p < 0.05$).

Figure 7.

Hippocampal neuron number is significantly reduced following a single bout of exposure to both A) neonatal isoflurane and B) phenobarbital. There was a profound 15–20% reduction in neuron number that was documented by unbiased stereological analysis (fractionator) 80 days following administration of anesthesia. Of additional interest is that females were less affected than males. * indicates significant difference from vehicle treated animals of the same sex (Tukey; $p < 0.05$).