Anesthesiology 2007; 107:427–36

Neonatal Exposure to a Combination of N-*Methyl-*D*-aspartate and γ-Aminobutyric Acid Type A Receptor Anesthetic Agents Potentiates Apoptotic Neurodegeneration and Persistent Behavioral Deficits*

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Background: **During the brain growth spurt, the brain develops and modifies rapidly. In rodents this period is neonatal, spanning the first weeks of life, whereas in humans it begins during the third trimester and continues 2 yr. This study examined whether different anesthetic agents, alone and in combination, administered to neonate mice, can trigger apoptosis and whether behavioral deficits occur later in adulthood.**

Methods: **Ten-day-old mice were injected subcutaneously with ketamine (25 mg/kg), thiopental (5 mg/kg or 25 mg/kg), propofol (10 mg/kg or 60 mg/kg), a combination of ketamine (25 mg/kg) and thiopental (5 mg/kg), a combination of ketamine (25 mg/kg) and propofol (10 mg/kg), or control (saline). Fluoro-Jade staining revealed neurodegeneration 24 h after treatment. The behavioral tests—spontaneous behavior, radial arm maze, and elevated plus maze (before and after anxiolytic)—were conducted on mice aged 55–70 days.**

Results: **Coadministration of ketamine plus propofol or ketamine plus thiopental or a high dose of propofol alone significantly triggered apoptosis. Mice exposed to a combination of anesthetic agents or ketamine alone displayed disrupted spontaneous activity and learning. The anxiolytic action of diazepam was less effective when given to adult mice that were neonatally exposed to propofol.**

Conclusion: **This study shows that both a γ-aminobutyric acid type A agonist (thiopental or propofol) and an *N*-methyl-D-aspartate antagonist (ketamine) during a critical stage of brain development potentiated neonatal brain cell death and resulted in functional deficits in adulthood. The use of thiopental, propofol, and ketamine individually elicited no or only minor changes.**

NUMEROUS pregnant women, newborn, and infants are exposed to anxiolytic, sedating, and anesthetic drugs to ease painful or unpleasant procedures. Often, several different drugs are used in combinations that give the most beneficial effects.[1,2] This exposure increases as advances are made in obstetric and pediatric surgery and intensive care.

This drug administration sometimes coincides with an important period of brain growth, the brain growth spurt (BGS), which proceeds from the third trimester until approximately 2 yr after birth.[3,4] During this time, the brain undergoes several fundamentally developmental phases, *e.g.*, maturation of axonal and dendritic outgrowth and the establishment of neural connections.[3,5,6] This stage of development is associated with numerous biochemical changes that transform the feto-neonatal brain into that of the mature adult. Simultaneously, the developing brain is not spared from physiologic apoptosis, programmed cell death, a naturally occurring process.[7–9]

Changes in the course of brain development threaten the structural and functional integrity with more or less permanent consequences.[10–12] In murine animals, the BGS is neonatal, spanning the first 2 to 3 weeks of life. The neonatal mouse brain has been shown to be vulnerable to low-dose exposure to neurotoxic agents that affect neuronal activity, such as nicotine, organophosphates, dichlorodiphenyltrichloroethane (DDT), and pyrethroids, as well as well-known toxic agents, *e.g.*, MPTP, polychlorinated biphenyl (PCB), and iron.[13–18] Such perturbations have been induced during a defined critical phase of the BGS, and can lead to persistent and/or permanent changes in adult brain function, and also to altered response to xenobiotics in adult life.[16,19,20]

The full mechanism of action of the substances used in anesthetic practice is not yet fully understood. However, most anesthetic agents have γ-aminobutyric acid type A ($GABA_A$) agonist and/or *N*-methyl-D-aspartate (NMDA) antagonist properties. The agents investigated in this article were thiopental and propofol, which act on the $GABA_A$ receptor, and ketamine, a potent NMDA receptor antagonist. Glutamic acid acts on the NMDA receptor and carries out trophic functions in the developing brain.[21] Transient blockade of glutamate NMDA receptors or the excessive activation of $GABA_A$ receptors during the BGS period triggers apoptotic neurodegeneration.[7,11,22,23]

N-Methyl-D-aspartate receptor antagonists given to neonatal rats produce spatial learning deficits in adulthood.[24] Several studies have demonstrated deficits in taste aversion learning and taste recognition memory tasks in animals neonatally exposed to ketamine and other NMDA antagonists.[25–28]

The most thoroughly investigated combined $GABA_A$ agonist and NMDA receptor antagonist is ethanol, which induces fetal alcohol syndrome (FAS) if the fetus is ex-

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Received from the Department of Neuroscience, Psychiatry Ulleråker, Uppsala University, Uppsala, Sweden. Submitted for publication January 18, 2007. Accepted for publication April 26, 2007. Supported by grants from Foundation for Strategic Environment Research, Stockholm, Sweden, and the Swedish Research Council for Environmental, Agricultural Sciences and Spatial Planning, Stockholm, Sweden.

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posed during the BGS.[29,30] In guinea pigs, fetal exposure to ethanol increases expression of $GABA_A$ receptor subunit protein in the adult brain.[31,32] Recent findings indicate that a combination of drugs incorporating an NMDA receptor antagonist and a $GABA_A$ agonist function is more detrimental and causes persistent learning deficits.[11]

The purpose of the current study was to establish whether the anesthetic agents thiopental, propofol, and ketamine, individually and in combination, when given to neonatal mice during the defined critical period of brain development, would modify apoptosis in the developing brain. Another aim was to study the consequences of neonatal exposure to these agents on adult spontaneous behavior, learning, and memory abilities and its influence on anxiety-like behavior.

Materials and Methods

Animals

Pregnant Naval Medical Research Institute (NMRI) mice were purchased from B&K (Sollentuna, Sweden). Each litter was adjusted within 48 h to 8–10 pups and to contain offspring of either sex in approximately equal numbers. Each litter was kept together with its mother in a plastic cage in a room at temperature of 22° ± 1°C and with a 12–12 h constant light–dark cycle (lights on between 06:00 and 18:00 h). Only the male offspring were used for the neurochemical and behavioral recordings. At the age of 4 weeks, the mice were weaned, and the males were raised in groups of 4–6 animals in a room for male mice only. They were kept as outlined above until behavioral testing (spontaneous motor activity [n = 10] followed by radial arm maze learning [n = 8] and elevated plus maze testing [n = 7] for anxiety) as young adults (55–70 days old). Mice were randomly picked from each treatment group for each different experiment (see further under Treatment and Drugs). Experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) after approval from the local ethics committee (Uppsala University and Agricultural Research Council, Uppsala, Sweden) and by the Swedish Committee for Ethical Experiments on Laboratory Animals (license S93/92 and S77/94, Stockholm, Sweden).

Treatment and Drugs

On postnatal day 10, pups were injected with 25 mg/kg body weight (BW) ketamine (Ketalar, 10 mg/ml; Pfizer Inc., New York, NY); 5 mg/kg BW or 25 mg/kg BW thiopental (Pentothal Natrium, 500 mg; Abbott, Solna, Sweden); 10 mg/kg BW or 60 mg/kg BW propofol (Diprivan, 10 mg/ml; Astra, Södertälje, Sweden); a combination of 25 mg/kg ketamine plus 5 mg/kg BW thiopental; a combination of 25 mg/kg BW ketamine plus 10 mg/kg BW propofol; or vehicle only (0.9% NaCl) in a volume of 10 ml/kg BW, subcutaneously in the neck. The study population consisted thus of eight different groups. Each treatment group was derived from at least four different litters. Diazepam (Stesolid novum, 5 mg/ml; Alpharma AB, Stockholm, Sweden), 1 mg/kg BW, was used in the elevated plus maze (EPM) test, to study the response in presence and absence of diazepam.

Six animals from each treatment group were assigned to Fluoro-Jade (Fluoro-Jade B; Histo-Chem Inc., Jefferson, AR) staining and were killed by cervical dislocation 24 h after injections. The brains were dissected on an ice-cold glass plate and stored at −70°C until staining analysis.

Fluoro-Jade Staining

We measured apoptosis with Fluoro-Jade staining. Other techniques used are different variants of silver staining methods. Fluoro-Jade produces results comparable to silver staining techniques for the detection of degenerating neurons.[33,34] Recently, Scallet *et al.* [35] used silver staining, caspase-3, and DAPI staining alongside Fluoro-Jade, confirming that the different techniques all reveal apoptosis in the same regions, albeit with differing sensitivity to the various stages of apoptosis. Hence, Fluoro-Jade is a reasonable choice of method for detecting apoptosis.

First, three brains from each of the combination groups (ketamine plus thiopental and ketamine plus propofol) and the vehicle group were examined. Slides from whole brains were collected, stained, and analyzed to select interesting regions of the brains. Sections from all the groups (including vehicle group) were processed in the same assay.

Two structures showing the most widespread neuronal degeneration were identified as stria terminalis and olfactory bulb (upper part). Only sections containing these two areas were collected and analyzed from the eight groups (n = 6). Whole brains stored at −70°C were removed from the freezer and mounted in the slicing apparatus (Cryocut, Jung CM 3000; Leica, Heidelberger, Germany). The Cryocut apparatus was set to a cutting temperature of −18°C, and the mouse brains were sliced in 10-μm-thick sections and mounted directly to the gelatin-coated slides. The brain regions were identified by the size and shape of the lateral ventricle and olfactory bulb.[36] Three consecutive images per region from each brain were selected for analysis. The tissue sections were left to dry, and the slides were then stored at −70°C. Fluoro-Jade staining was performed according to a previously described method,[33,34] well established in our group.[37] Sections from all the groups (including vehicle groups) were run in the same assay. Slides (10 μm thick) were examined using a Nikon Labophoto-2 (Nikon Corporation, Tokyo, Japan) microscope working with 20× magnification equipped with a filter system (Nikon B2A, with barrier filter at 520 nm)

suitable for visualizing fluorescence excitation and emission of the Fluoro-Jade spectra. The microscope was connected to a super high-pressure mercury lamp power supply (Nikon HB-1010AF). Photographs were taken using a digital camera (Nikon Coolpix 990) with 3.34 million–pixel capacity, attached to the microscope. Images were semiquantitatively analyzed for Fluoro-Jade positive (degenerative) cells, using Adobe PhotoShop 5.5 (Adobe Systems Incorporated, San Jose, CA) and Scion Image (Scion Corporation, Frederick, MD) software programs. The "magic wand tool" with a tolerance setting of 10 was used to mark all of the positive staining. Before the study, the Fluoro-Jade assay was run with a positive control, MPTP-treated adult male mice, and a negative control, ketamine-treated adult male mice.

Spontaneous Motor Activity and Habituation to Activity Test Chambers

Mice were observed for spontaneous behavior at an age of 55 days, measured in a specialized test cage, described and used previously.[16] The following parameters were measured: Locomotion was registered by a low grid of horizontal infrared beams. Rearing was registered throughout the time when at least one high-level infrared beam was interrupted, *i.e.*, the number of counts registered was proportional to the amount of time spent rearing. Total activity was measured by a sensor (a pickup similar to a gramophone needle, mounted on a lever with a counterweight) with which the cage was constantly in contact. The sensor registered all types of vibration received from the test cage, such as those produced by locomotion and rearing as well as shaking, tremors, scratching, and grooming. All three behavioral parameters were measured over three consecutive 20-min periods, on one occasion only.

Habituation is a relatively simple, nonassociative form of learning in situations where repeated measures of behavior are monitored. To assess the extent of habituation to the activity test chambers over the successive 20-min intervals, a habituation quotient for each mouse was derived by dividing the count during the third 20-min period by that obtained during the first 20-min period, multiplied by 100. In each case, the result of each division was multiplied by 100 to provide a quotient representing the reduction in activity counts from the first to the third period, for each mouse.[38]

Radial Arm Maze

The radial eight-arm maze (RAM), a device sensitive to deficits in spatial learning performance,[39,40] was modified and adapted to evaluate maze-learning performance in mice.[41] The radial maze consists of eight arms (35 cm long each, surrounded by a 1.5-cm border) radiating from a circular platform (diameter, 20 cm). The maze was placed 60 cm above the floor. Each arm was baited 3 cm from its outer end by placing a small food pellet (5 mg) behind a low barrier, preventing the animal from seeing whether a particular arm was still baited or not. Before testing, all mice were placed on food deprivation for 24 h, but with free access to water. After testing on day 1, they were given a controlled amount of food, and similarly after testing on day 2, but after testing on day 3, they were given free access to food. At the start of each test trial, the mouse was placed on the central hub and then monitored for its instrumental learning performance, *i.e.*, the latency until all eight pellets were eaten. The number of arms visited to find all eight pellets, subtracted by eight, gave the number of errors per animal. An error was defined as reentry to an arm already visited. Photocells registered all movements. The maze-learning test room was secluded, without any explicitly arranged extramaze cues. Each mouse was tested for one trial only on each of 3 consecutive days. Each animal was observed carefully to ensure that it ate each pellet.

Elevated Plus Maze Test

This test procedure gives a measure of anxiety level. It is based on the assumption that normally mice prefer a closed environment to an open space. Mice with an altered sense of anxiety show less inhibition to open areas. The procedure is based on the method of Luster.[42] The EPM apparatus was made of plywood and had two opposite open arms (white floor with no wall, 30 × 6 cm) and two opposite closed arms (black floor with walls, 30 × 6 × 30 cm) mounted 50 cm above the floor. The floor of the arms was smooth. Testing was conducted between 09:00 and 14:00 h. The animals were transferred to the testing laboratory in their home cages at least 60 min before they were submitted to the EPM. The mice were given diazepam (1 mg/kg) or vehicle (0.9% NaCl) subcutaneously 30 min before the test. A mouse was placed on the central platform of the apparatus, facing either of the closed arms. A video camera was used to monitor the animal's behavior. The number of entries into the open and enclosed arms and the time spent there were measured for 5 min. Arm entry was defined as all four paws present in the arm. The maze apparatus was cleaned after each trial.

Statistical Analysis

The locomotion, rearing, and total activity data over three consecutive 20-min periods (treatment, time, and treatment × time; between subjects, within subjects, and interaction factors, respectively) in the activity test chambers, the latency until all eight pellets were collected (total time), and the number of errors over 3 consecutive testing days (treatment, day, and treatment × day; between subjects, within subjects, and interaction factors, respectively) in the RAM were submitted to a split-plot analysis of variance (ANOVA) design.[43] The major advantages with a split-plot design compared with randomized block factorial design are that the estimates

of the within-block effects are usually more accurate than estimates of the between-block estimates. Because the average experimental error over all treatments is the same for both designs, the increased precision on within-block effects is obtained by sacrificing precision on between-block. The proportion of Fluoro-Jade staining of brain area from the two different brain regions, habituation quotients, and in the EPM the time spent in the open arms and the entries into the open arms, were submitted to one-way ANOVA design.[43] Pairwise testing between the different treatment groups was performed with the Tukey honestly significant difference (HSD) test at the level of 0.01 unless otherwise mentioned. In the case of Fluoro-Jade staining, testing was performed also between the different regions.[43]

Results

There were no obvious clinical signs of dysfunction in the treated mice throughout the experimental period, nor were there any significant deviations in the body weight in the anesthetic-treated mice, compared with the vehicle-treated mice.

Fluoro-Jade Visualization of Degenerating Neurons

Figure 1 presents Fluoro-Jade positive staining in the mouse brain at the level of the lateral ventricle and the olfactory bulb. Degenerating neurons are visualized 24 h after exposure to ketamine (25 mg/kg) plus propofol (10 mg/kg) in the stria terminalis (panels A and C) and the upper part of the olfactory bulb (panels B and D). Slides (10 μm thick) were semiquantitatively analyzed for Fluoro-Jade positive (degenerative) cells, using Adobe PhotoShop 5.5 and Scion Image software programs. Figure 1 illustrates the anatomical regions and the area of degeneration measured with Scion Image software.

Administration of ketamine, propofol, thiopental, a combination of ketamine and propofol, or a combination of ketamine and thiopental to 10-day-old mice resulted in the following alteration of the apoptotic process, revealed by Fluoro-Jade staining 24 h after treatment. Administration of a combination of ketamine and propofol triggered increased Fluoro-Jade staining plaques in olfactory bulb and stria terminalis, compared with the control group. Therefore, one-way ANOVA indicated significant between-groups effects for olfactory bulb ($F_{7,40} = 30.39$) and for stria terminalis ($F_{7,40} = 60.23$). Figure 2 illustrates the staining in each brain region *vis-à-vis* that in the controls (set to 100% in the figure).

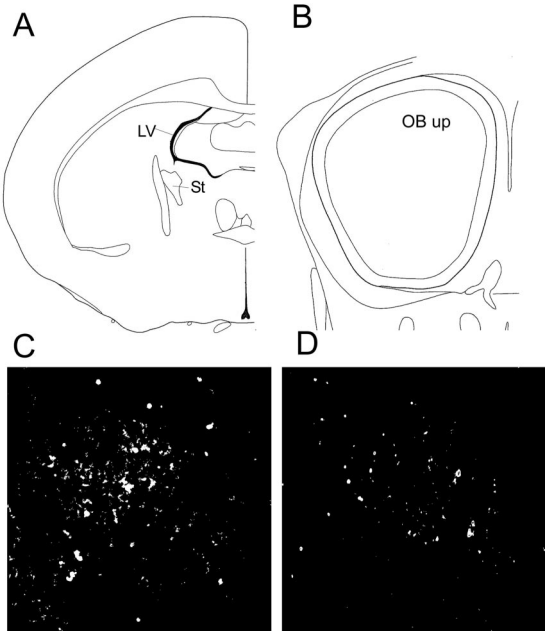

Fig. 1. Fluoro-Jade positive staining in the mouse brain at the level of the lateral ventricle (LV) and the olfactory bulb upper part (OB up). Degenerating neurons 24 h after exposure to ketamine (25 mg/kg) plus propofol (10 mg/kg) in the stria terminalis (St) (*A* and *C*) and OB up (*B* and *D*). Slides (10 μm thick) were semiquantitatively analyzed for Fluoro-Jade positive (degenerative) cells, using Adobe PhotoShop 5.5 (Adobe Systems Incorporated, San Jose, CA) and Scion Image (Frederick, MD) software programs. The figure illustrates the anatomical regions and the area of degeneration measured with Scion Image software.

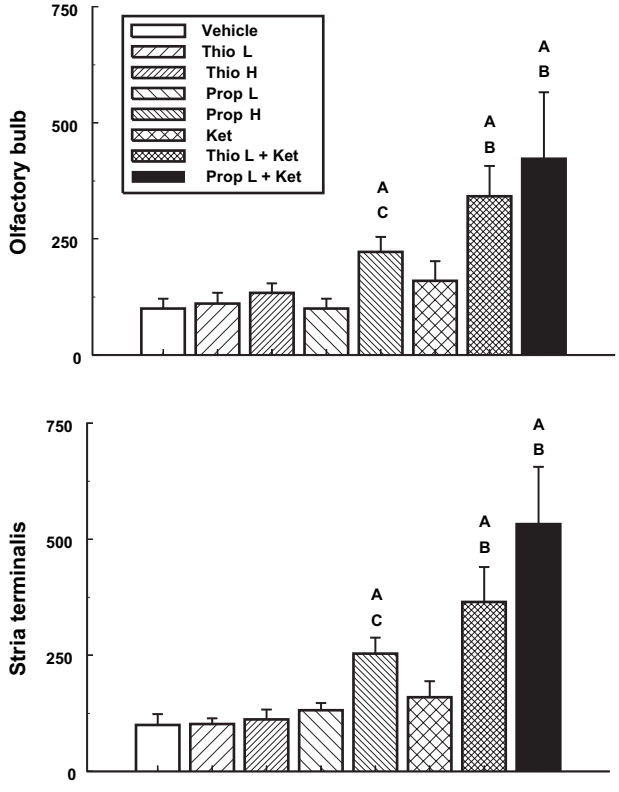

Fig. 2. Fluoro-Jade staining in olfactory bulb and stria terminalis of mouse pups (n = 6 in each group) treated with vehicle (0.9% NaCl), 5 mg/kg thiopental (Thio L), 25 mg/kg thiopental (Thio H), 10 mg/kg propofol (Prop L), 60 mg/kg propofol (Prop H), 25 mg/kg ketamine (Ket), 25 mg/kg ketamine plus 5 mg/kg thiopental (Thio L + Ket) or 25 mg/kg ketamine plus 10 mg/kg propofol (Prop L + Ket) on neonatal day 10 and killed 24 h later. Staining is expressed as a percentage of vehicle-treated mouse pups. *A* = significant difference *versus* vehicle; *B* = significant difference *versus* all monotherapy groups; *C* = significant difference *versus* Prop L, at 1% level of significance.

Pairwise testing using the Tukey HSD test revealed the following differences: ketamine plus thiopental and ketamine plus propofol induced increased Fluoro-Jade staining plaques in the olfactory bulb and stria terminalis more than did all monotherapy treatments and vehicle. High-dose propofol induced increased Fluoro-Jade staining plaques in the olfactory bulb and stria terminalis more than did low-dose propofol and vehicle. Low- and high-dose thiopental, low-dose propofol, and ketamine did not elicit any significant increase in apoptosis, compared with the control group.

Spontaneous Motor Activity and Habituation to Activity Test Chambers

Neonatal administration of ketamine plus propofol or ketamine plus thiopental on neonatal day 10 disrupted spontaneous motor activity in 55-day-old mice (fig. 3). Therefore, split-plot ANOVA indicated significant treatment × time period interaction effects: locomotion: $F_{14,144} = 40.15$; rearing: $F_{14,144} = 44.66$; and total activity: $F_{14,144} = 31.24$. Figure 3 presents mean and SD values for locomotion, rearing, and total activity.

Pairwise testing using the Tukey HSD test revealed differences between the different treatments and the control group. In control mice, there was a distinct decrease in activity in all spontaneous behavioral variables over the 60-min period. Such a decrease is a normal profile of spontaneous behavior, as has been reported in our previous studies.[37] Thiopental groups (high and low dose) and propofol groups (high and low dose) did not differ from the control group. Ketamine (25 mg/kg BW) differed from controls as follows. During the first period, there was a reduction of locomotion, rearing, and total activity. The ketamine group did not differ from the controls during the second and third test periods, with one exception: Rearing increased during the third period. The animals exposed to a combination of anesthetic agents displayed the following results in the spontaneous motor activity test:

First period: Reduced locomotion, rearing, and total activity in both groups, compared with vehicle and all monotherapy groups.

Second period: Both groups demonstrated more locomotion, rearing, and total activity than vehicle. Locomotion was also more pronounced in both combination groups than in all monotherapy groups except ketamine; the result was the same for the ketamine plus thiopental group regarding rearing. Ketamine plus propofol elicited more rearing than all monotherapy groups. Both groups showed increased total activity, compared with all monotherapy groups.

Third period: Both combination groups showed increased in locomotion, rearing, and total activity, compared with vehicle and all monotherapy groups.

The quotients that demonstrated a simple form of learning were subjected to a one-way ANOVA that indicated significant group effects, as follows: Locomotion: $F_{7,72} = 434.03$; rearing: $F_{7,72} = 102.86$; and total activity: $F_{7,72} = 505.39$. The mean quotient values per group for locomotion, rearing, and total activity are presented in table 1. The habituation quotient analysis reveals the following, from pairwise Tukey HSD testing: The habituation quotients were increased in the ketamine plus thiopental and ketamine plus propofol groups of mice. Compared with vehicle, an increase was evident by factors of quotient = 91, 289 and 7.63 (ketamine plus thiopental) and quotient = 153, 512 and 8.4 (ketamine plus propofol) for locomotion, rearing, and total activity, respectively. Tukey HSD pairwise testing indicated differences between the ketamine plus thiopental and ketamine plus propofol and the vehicle mice for locomotion, rearing, and total activity. The habituation

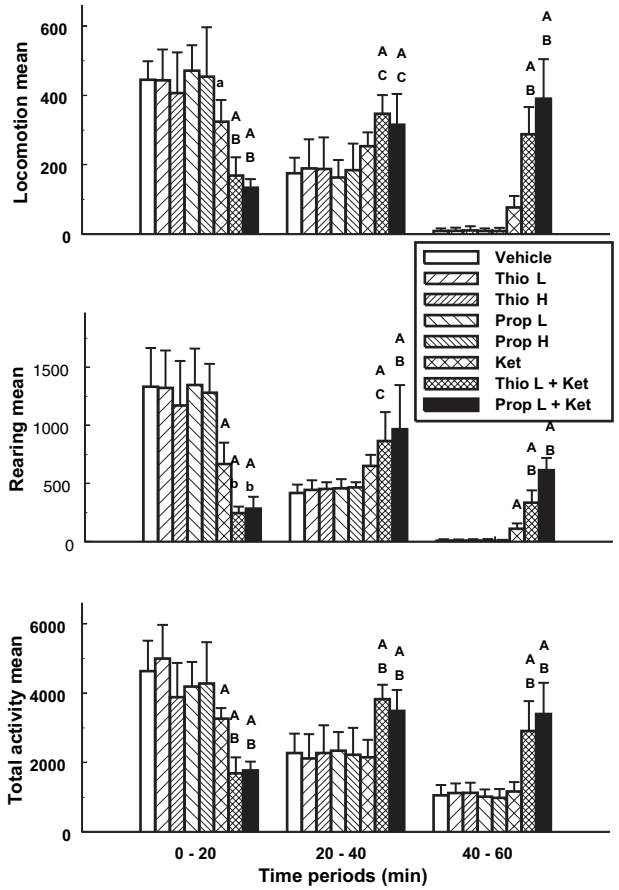

Fig. 3. Spontaneous motor activity of adult mice treated with vehicle (0.9% NaCl), 5 mg/kg thiopental (Thio L), 25 mg/kg thiopental (Thio H), 10 mg/kg propofol (Prop L), 60 mg/kg propofol (Prop H), 25 mg/kg ketamine (Ket), 25 mg/kg ketamine plus 5 mg/kg thiopental (Thio L + Ket), or 25 mg/kg ketamine plus 10 mg/kg propofol (Prop L + Ket) on neonatal day 10 and tested in the activity test chambers at an age of 55 days. Mean locomotion, rearing, and total activity counts of three consecutive 20-min periods. *A* = significant difference *versus* vehicle; *B* = significant difference *versus* all monotherapy groups; *C* = significant difference *versus* all monotherapy groups except ketamine. *Letters* in *uppercase* and *lowercase* represent 1% and 5% levels of significance, respectively.

Table 1. Spontaneous Motor Activity Habituation for Locomotion, Rearing, and Total Activity

	Quotient		
Group	Locomotion	Rearing	Total Activity
Vehicle	1.89 ± 1.37	0.46 ± 0.65	22.4 ± 2.57
Thio-L	1.84 ± 1.61	0.53 ± 0.61	22.2 ± 1.92
Thio-H	2.17 ± 2.05	0.58 ± 0.77	28.9 ± 2.06
Prop-L	1.77 ± 1.21	0.51 ± 0.73	24.0 ± 1.52
Prop-H	1.63 ± 1.43	0.47 ± 0.53	23.3 ± 3.25
Ket	22.7 ± 6.38	16.1 ± 2.70	35.2 ± 5.40
Thio-L + Ket	172 ± 8.55*	133 ± 16.9*	171 ± 13.3*
Prop-L + Ket	289 ± 45.5*	237 ± 76.6*	189 ± 24.0*

Mice were treated postnatally with thiopental (5 mg/kg [Thio-L] or 25 mg/kg [Thio-H]), propofol (10 mg/kg [Prop-L] or 60 mg/kg [Prop-H]), ketamine (25 mg/kg), a combination of ketamine (25 mg/kg) plus thiopental (5 mg/kg [Thio-L]), a combination of ketamine (25 mg/kg) plus propofol (10 mg/kg [Prop-L]), or vehicle (0.9% NaCl), through subcutaneous injection, on day 10 after birth, and were tested in the activity test chambers at 2 months of age. Habituation quotients pertaining to locomotion, rearing, and total activity counts from the first and third 20-min test periods in the motor activity test chambers, derived by dividing the respective number of counts per mouse during the third 20-min period by that obtained during the first 20-min period. In each case, the result was multiplied by 100. Motor activity was measured in 2-month-old Naval Medical Research Institute (NMRI) mice. Values are expressed as mean ± SD of 10 mice.

* $P < 0.01$ versus the vehicle group.

quotients of the other treatment groups were not significantly affected.

Radial Arm Maze

Mice that received vehicle, thiopental (low or high dose), or propofol (low or high dose) neonatally showed relatively linear improvements in RAM acquisition performance, whereas mice treated neonatally with ketamine, ketamine plus thiopental, or ketamine plus propofol did not improve over successive days of testing. Therefore, split-plot ANOVA indicated significant treatment × days interaction effects for number of errors ($F_{14,112} = 6.97$) and for total time in seconds ($F_{14,112} = 4.50$), but no differences in number of pellets taken ($F_{14,112} = 0.20$). Figure 4 illustrates the mean number of errors and the mean total time spent looking for pellets (in seconds) for the different groups.

Tukey HSD pairwise testing revealed the following significant differences:

Day 1: No differences between any of the groups.
Days 2 and 3: Ketamine, ketamine plus thiopental, and ketamine plus propofol groups made more errors and showed longer latency than all of the other groups.
Day 2: Ketamine plus propofol group made more errors than the ketamine group.
Day 3: Ketamine plus thiopental and ketamine plus propofol groups made more errors and showed longer latency than the ketamine group alone.

Elevated Plus Maze

Mice given saline 30 min before testing in an EPM all exhibited the same pattern of behavior, regardless of previous neonatal exposure to anesthetic agents or vehicle. All showed reluctance to enter the open arms and to remain there. Mice treated with diazepam 30 min before testing and neonatally exposed to vehicle showed marked behavioral differences. Therefore, one-way ANOVA indicated significant treatment effects for both percentage of entries into the open arms ($F_{15,96} = 14.79$) and percentage time spent on the open arms ($F_{15,96} = 16.20$) (fig. 5).

Pairwise testing showed that the anxiolytic effect of diazepam was reduced in animals neonatally exposed to propofol, whether in high or low dose or in combination with ketamine. The mice entered the open arms less often than the neonatally vehicle-exposed animals. Animals exposed neonatally to a high dose of propofol also spent less time on the open arms. Mice exposed to other anesthetic agents or combinations showed no difference *vis-à-vis* vehicle, after exposure to diazepam.

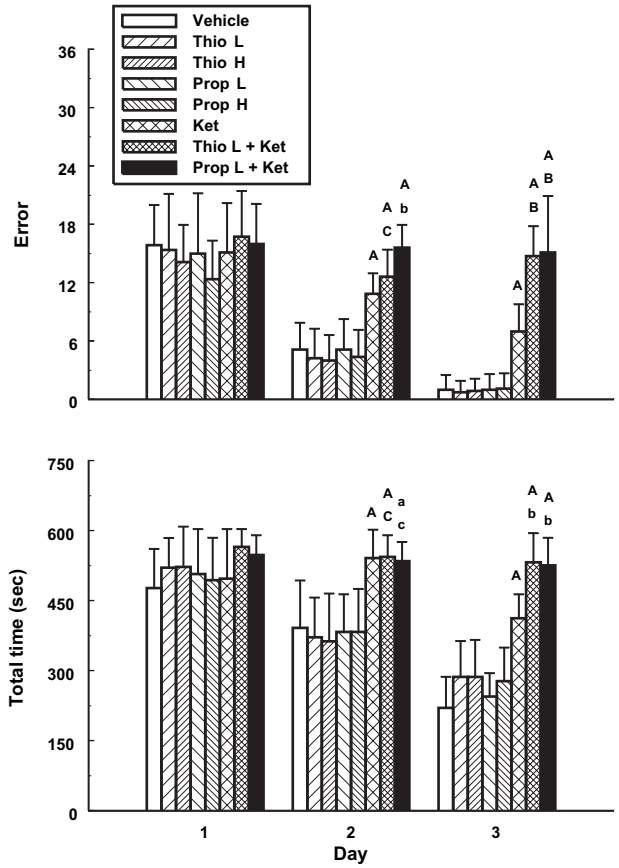

Fig. 4. Radial arm maze acquisition performance of adult mice treated with vehicle (0.9% NaCl), 5 mg/kg thiopental (Thio L), 25 mg/kg thiopental (Thio H), 10 mg/kg propofol (Prop L), 60 mg/kg propofol (Prop H), 25 mg/kg ketamine (Ket), 25 mg/kg ketamine plus 5 mg/kg thiopental (Thio L + Ket), or 25 mg/kg ketamine plus 10 mg/kg propofol (Prop L + Ket) on neonatal day 10 and tested in the radial arm maze at 63 days of age. Number of errors and total time (seconds) on 3 consecutive days of testing. *A* = significant difference *versus* vehicle; *B* = significant difference *versus* all monotherapy groups; *C* = significant difference *versus* all monotherapy groups except ketamine. *Letters* in *uppercase* and *lowercase* represent 1% and 5% levels of significance, respectively.

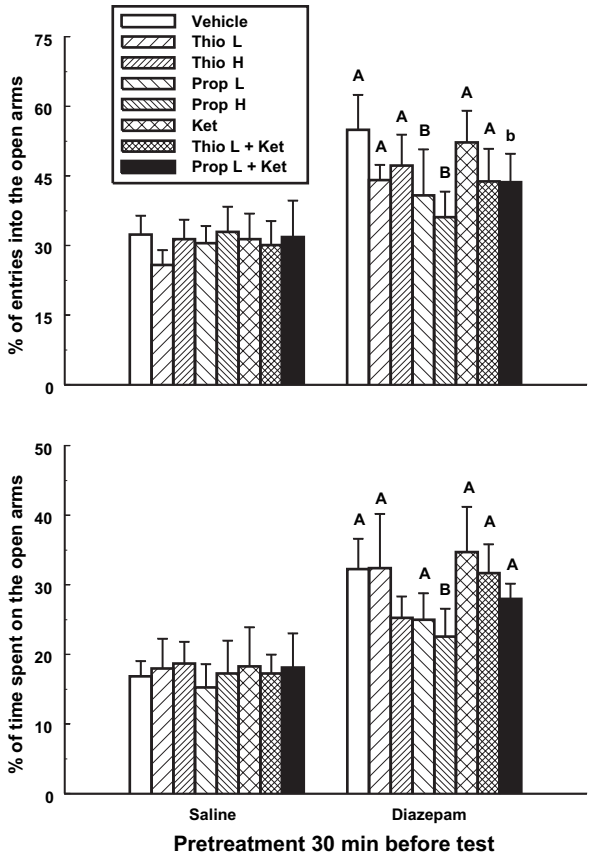

Fig. 5. Performance in an elevated plus maze of adult mice treated 30 min before testing with diazepam (1 mg/kg) or vehicle (0.9% NaCl). The animals were exposed neonatally, day 10 postpartum, to vehicle (0.9% NaCl), 5 mg/kg thiopental (Thio L), 25 mg/kg thiopental (Thio H), 10 mg/kg propofol (Prop L), 60 mg/kg propofol (Prop H), 25 mg/kg ketamine (Ket), 25 mg/kg ketamine plus 5 mg/kg thiopental (Thio L + Ket), or 25 mg/kg ketamine plus 10 mg/kg propofol (Prop L + Ket). Percentages of entries into open arms and time spent on open arms are shown. *A* = significant difference between diazepam- and saline-pretreated mice exposed neonatally to the same treatment; *B* = significant difference *versus* mice treated neonatally with vehicle and pretreated with diazepam before testing. *Letters* in *uppercase* and *lowercase* represent 1% and 5% levels of significance, respectively.

Discussion

This study showed that neonatal coadministration of a $GABA_A$ agonist and an NMDA antagonist, compared with monotherapy treatment, potentiates neuronal cell death in the neonatal brain and affects behavior in adulthood, manifested as deficit spontaneous behavior, lack of habituation, and impaired learning and memory functions.

The NMDA receptor antagonist ketamine was not found to cause apoptosis in the neonatal brain. However, we have seen in a previous study that a higher dose (50 mg/kg BW) can cause apoptosis in the parietal cortex of mice exposed on day 10.[37] Young *et al.* [44] demonstrated apoptosis in a dose-dependent manner in mice exposed on postnatal day 7 to ketamine at 20, 30, and 40 mg/kg BW.

Different brain regions are affected depending on the time of exposure during the brain development.[10] Despite differences in mouse strains and also exposure at different time points during the rapid development of the brain, the dose used in the current study might be close to one that triggers an apoptotic process. Regarding the $GABA_A$ agonists thiopental and propofol, only the high dose of propofol was shown to trigger apoptosis in the brain. Apoptosis triggered by thiopental and propofol is less thoroughly studied and less well described, but there are other $GABA_A$ agonists that can trigger apoptosis,[29] as did high-dose propofol in our experiment. However, coadministration of a $GABA_A$ agonist (thiopental or propofol) in a dosage not causing apoptosis, together with an NMDA antagonist (ketamine) also at a dose not causing apoptosis, results in a significant increase in neuronal cell death. This effect also seems to be synergistic because the effect was significantly more pronounced in the ketamine (25 mg) plus propofol (10 mg) group than in mice given the sixfold higher dose of propofol (60 mg/kg BW). Therefore, it is the combination of a $GABA_A$ agonist and an NMDA antagonist that more easily triggers neurodegeneration. Jevtovic *et al.*[11] demonstrated this with a cocktail of midazolam, nitrous oxide, and isoflurane, and Fredriksson *et al.*[37] demonstrated this with diazepam and ketamine. Ethanol, which has both NMDA antagonist and $GABA_A$ agonist properties, causes a widespread neurodegeneration.[9,29,30,45]

It is known that hypoxia and hypothermia can cause neuronal cell death.[46,47] In our study, the pups were separated from their mothers only for the brief time of injection (less than 10 s). The regulated environment rules out hypothermia. The control animals had the same treatment and environment. We have been unable to measure arterial blood saturation in these small animals, because there is no practical procedure to extract arterial blood or measure with pulse oximetry or transcutaneous measurements. However, the pups looked pink and well perfused, with no visible cyanosis. Other groups, investigating ketamine in rats, have not detected desaturation in blood gases.[48] In addition, Young *et al.*[49] have in a study differentiated between excitotoxic *versus* apoptotic mechanisms of neuronal cell death. During the period of neonatal brain development, it is not the time-specific areas of apoptosis due to NMDA antagonists or with $GABA_A$ agonists exposure, respectively, that are preferably affected by suppression of cardiorespiratory function due to excitotoxic neuronal cell death in perinatal hypoxia–ischemia. From our experience, dose-dependently produced hypoxia in neonatal mice on day 10 shows no effects on stria terminalis or the olfactory bulb visualized by Fluoro-Jade. It also seems unlikely that the animals with brain damage are animals exposed to ketamine and propofol— but not propofol or thiopental alone—if respiratory and ventilatory depres-

sion is suspected, because ketamine preserves spontaneous breathing.[1]

Neural loss, seen in the current study, may interfere with the development of an intact olfactory system in adulthood. It is well established that the olfactory system plays an important role for the outcome of behavior and learning performance both in humans and in animals. Early in life, the ability of olfactory recognition is necessary in the early stages of the mother–infant attachment process in humans[50] and neonatal rats.[51] Neonatal rats with early sensory deficits, treated with triethyl lead, show impaired olfactory discrimination together with changes in behavior and learning.[52] Hyperactivity and RAM acquisition deficits have also been reported related to olfactory mucosal metaplasia after treatment with methylsulphonyl-dichlorobenzene in mice and rats.[53,54] Both a subgroup of adults with residual attention deficit–hyperactivity disorder with hyperactivity[55] and one third of first-episode psychosis[56,57] have a measurable olfactory identification deficit on a smell identification test. Reduced olfactory sensitivity, discrimination, and identification in patients with alcohol dependence have also been reported.[58] Olfactory bulbectomy in rodents, a model of depression, causes marked hyperactivity, dissociation among different aggressive behaviors, and also cognitive impairment in a water maze.[59,60] Overall, the olfactory system seems intimately involved in the processes underlying the outcome of behavior and learning ability.

The behavioral tests also clearly revealed a significantly more pronounced effect in mice given both a $GABA_A$ agonist and an NMDA receptor antagonist, compared with any monodrug treatment. The combination of propofol or thiopental with ketamine elicited altered spontaneous behavior and complete lack of habituation. Mice exposed on neonatal day 10 to a combined low dose of propofol (10 mg/kg BW) or thiopental (5 mg/kg BW) together with ketamine (25 mg/kg BW) displayed significantly disrupted spontaneous behavior and lack of habituation. Habituation, defined here as a decrease in locomotion, rearing, and total activity variables in response to the diminishing novelty of the test chamber over 60 min, was evident in the control animals, whereas mice exposed to the combination propofol or thiopental and ketamine were clearly hypoactive early in the 60-min test period, becoming distinctly hyperactive toward the end. An altered spontaneous behavior was also observed after ketamine alone, but no significant effect on habituation was observed. In mice exposed only to propofol or thiopental, regardless of the fivefold to sixfold higher dose, no significant effects on spontaneous behavior or habituation were seen, compared with the controls.

The same groups that showed altered spontaneous behavior were also slower to learn and memorize as adults in the RAM test. Again, the effects were significantly more pronounced in mice given both $GABA_A$ agonist and NMDA antagonist. Learning difficulties have been demonstrated with ketamine in both RAM and the Morris water maze,[37] using a combination of midazolam, isoflurane, and nitrous oxide in the Morris water maze[11] and isoflurane–nitrous oxide in the RAM.[61] Again, we would like to emphasize that ethanol can be considered to be the equivalent of a combination of $GABA_A$ agonists and NMDA antagonists and that the FAS is associated with learning difficulties. In guinea pigs, where the BGS occurs prenatally,[3] prenatal ethanol exposure impairs spatial learning,[32] which concurs with our findings.

A known cause of behavioral disturbances in humans is FAS caused by exposure to ethanol *in utero* in humans. Ethanol has both $GABA_A$ agonist and NMDA antagonist properties given a similar drug action and adverse effects as our combination of anesthetics. Ethanol produces the same pattern of apoptosis as mentioned above, and FAS patients are also characterized by attention deficit and hyperactivity.[62,63] FAS patients also require more psychiatric care than healthy persons.[64] Wang *et al.*[65] produced apoptosis with phencyclidine, which blocks NMDA receptors. These animals showed long-lasting behavioral deficits. Early apoptosis seems to cause deficits later in adult life.

No changes in anxiety measured in the EPM were seen, regardless of neonatal exposure. However, the response to anxiolytic diminished in the groups exposed to propofol (low and high doses) and the combination ketamine plus propofol neonatally. The mechanism of this is not known, but GABA agonists administered neonatally alter GABA receptor expression,[66] and there is also evidence that GABA activation in newborn rats causes excessive calcium influx and cell death.[67] Nicosia *et al.* [68] have found adult male mice, exposed prenatally exposed to diazepam, to be more sensitive to convulsants, whereas females were less sensitive than control mice. Postnatal development and behavior in maturity are also affected in rats exposed prenatally to diazepam.[69,70]

Clinically, there is a wide variation in the drug requirements of patients. Some patients need much higher doses of anxiolytics, sedatives, or hypnotics than others do. Thiopental induction doses, for example, vary from 2.8 to 9.7 mg/kg,[71] midazolam varies from 0.05 to 0.15 mg/kg,[1] and other anesthetics show similar variation. Hypothetically, neonatal alteration of receptors may be one mechanism underlying this phenomenon, an effect seen in neonatal and adult animals exposed to agents affecting neuronal activity.[17,18]

Recently, the clinical relevance of experimental findings in animals treated with ketamine alone as an apoptotic agent has been debated.[35,48,72,73] The fact that ketamine in high or repeated doses in rodents causes neuroapoptosis is not challenged. Olney *et al.* reported apoptosis at 20, 30, and 40 mg/kg, but not at 10 mg/mg, whereas Scallet *et al.* reported no apoptosis after 20

mg/kg or 10 mg/kg ketamine, seven times during 9 h, whereas it did occur after 20 mg/kg, seven times in 9 h. The clinical relevance of the rodent results and whether they can be extrapolated to the human situation is still unanswered. However, mounting evidence of adverse effects in rodents gives rise to concern.

The current findings underline the potential hazards of perinatal interventions using combinations of anesthetic agents having the pharmacologic profiles of $GABA_A$ agonists (such as propofol or thiopental) and the NMDA antagonist ketamine. Both the accelerated rate of cell degeneration and the marked functional deficits in the adult animal imply a drastic potentiation by the combination treatment *vis-à-vis* to single treatment. In the anxiety test, the effect of diazepam in adults was influenced by neonatal $GABA_A$ agonist exposure. A reasonable standpoint in the clinical setting, based on available data, would be to avoid drugs and combinations thereof in doses that produce severe change, if any alternative exists. Whatever the case, the lowest effective dose should be used. With better knowledge in the future, we will be able to select single anesthetic agents—or combinations—that are the least harmful when we anesthetize newborns and infants with a developing brain. Until then, common sense and reasonable precautions should be applied in pediatric anesthesia.

References

1. Miller RD: Miller's Anesthesia, 6th edition. New York, Elsevier/Churchill Livingstone, 2004, pp 348–50
2. Prevention and management of pain and stress in the neonate. American Academy of Pediatrics. Committee on Fetus and Newborn. Committee on Drugs. Section on Anesthesiology. Section on Surgery. Canadian Paediatric Society. Fetus and Newborn Committee. Pediatrics 2000; 105:454–61
3. Davison AN, Dobbing J: Applied Neurochemistry. Oxford, Blackwell, 1968, pp 253–316
4. Bayer SA, Altman J, Russo RJ, Zhang X: Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. Neurotoxicology 1993; 14:83–144
5. Herschkowitz N, Kagan J, Zilles K: Neurobiological bases of behavioral development in the first year. Neuropediatrics 1997; 28:296–306
6. Byrnes ML, Reynolds JN, Brien JF: Effect of prenatal ethanol exposure during the brain growth spurt of the guinea pig. Neurotoxicol Teratol 2001; 23:355–64
7. Ikonomidou C, Bittigau P, Koch C, Genz K, Hoerster F, Felderhoff-Mueser U, Tenkova T, Dikranian K, Olney JW: Neurotransmitters and apoptosis in the developing brain. Biochem Pharmacol 2001; 62:401–5
8. Ishimaru MJ, Ikonomidou C, Tenkova TI, Der TC, Dikranian K, Sesma MA, Olney JW: Distinguishing excitotoxic from apoptotic neurodegeneration in the developing rat brain. J Comp Neurol 1999; 408:461–76
9. Dikranian K, Ishimaru MJ, Tenkova T, Labruyere J, Qin YQ, Ikonomidou C, Olney JW: Apoptosis in the *in vivo* mammalian forebrain. Neurobiol Dis 2001; 8:359–79
10. Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovska V, Turski L, Olney JW: Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 1999; 283:70–4
11. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF: Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 2003; 23:876–82
12. Bittigau P, Sifringer M, Genz K, Reith E, Pospischil D, Govindarajalu S, Dzietko M, Pesditschek S, Mai I, Dikranian K, Olney JW, Ikonomidou C: Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. Proc Natl Acad Sci U S A 2002; 99:15089–94
13. Eriksson P: Developmental neurotoxicity of environmental agents in the neonate. Neurotoxicology 1997; 18:719–26
14. Eriksson P, Talts U: Neonatal exposure to neurotoxic pesticides increases adult susceptibility: A review of current findings. Neurotoxicology 2000; 21: 37–47
15. Fredriksson A, Fredriksson M, Eriksson P: Neonatal exposure to paraquat or MPTP induces permanent changes in striatum dopamine and behavior in adult mice. Toxicol Appl Pharmacol 1993; 122:258–64
16. Fredriksson A, Schroder N, Eriksson P, Izquierdo I, Archer T: Maze learning and motor activity deficits in adult mice induced by iron exposure during a critical postnatal period. Brain Res Dev Brain Res 2000; 119:65–74
17. Talts U, Fredriksson A, Eriksson P: Changes in behavior and muscarinic receptor density after neonatal and adult exposure to bioallethrin. Neurobiol Aging 1998; 19:545–52
18. Talts U, Talts J, Eriksson P: Differential expression of muscarinic subtype mRNAs after exposure to neurotoxic pesticides. Neurobiol Aging 1998; 19:553–9
19. Eriksson P, Ahlbom J, Fredriksson A: Exposure to DDT during a defined period in neonatal life induces permanent changes in brain muscarinic receptors and behaviour in adult mice. Brain Res 1992; 582:277–81
20. Eriksson P, Ankarberg E, Fredriksson A: Exposure to nicotine during a defined period in neonatal life induces permanent changes in brain nicotinic receptors and in behaviour of adult mice. Brain Res 2000; 853:41–8
21. Raol YH, Lynch DR, Brooks-Kayal AR: Role of excitatory amino acids in developmental epilepsies. Ment Retard Dev Disabil Res Rev 2001; 7:254–60
22. Olney JW, Farber NB, Wozniak DF, Jevtovic-Todorovic V, Ikonomidou C: Environmental agents that have the potential to trigger massive apoptotic neurodegeneration in the developing brain. Environ Health Perspect 2000; 108 (suppl 3):383–8
23. Olney JW, Wozniak DF, Jevtovic-Todorovic V, Ikonomidou C: Glutamate signaling and the fetal alcohol syndrome. Ment Retard Dev Disabil Res Rev 2001; 7:267–75
24. Gorter JA, de Bruin JP: Chronic neonatal MK-801 treatment results in an impairment of spatial learning in the adult rat. Brain Res 1992; 580:12–7
25. Mickley GA, Remmers-Roeber DR, Crouse C, Peluso R: Ketamine blocks a taste recognition memory in fetal rats. Pharmacol Biochem Behav 2000; 67: 575–81
26. Mickley GA, Remmers-Roeber DR, Dengler CM, Kenmuir CL, Crouse C: Paradoxical effects of ketamine on the memory of fetuses of different ages. Brain Res Dev Brain Res 2001; 127:71–6
27. Mickley GA, Schaldach MA, Snyder KJ, Balogh SA, Len T, Neimanis K, Goulis P, Hug J, Sauchak K, Remmers-Roeber DR, Walker C, Yamamoto BK: Ketamine blocks a conditioned taste aversion (CTA) in neonatal rats. Physiol Behav 1998; 64:381–90
28. Roegge CS, Seo BW, Crofton KM, Schantz SL: Gestational-lactational exposure to Aroclor 1254 impairs radial-arm maze performance in male rats. Toxicol Sci 2000; 57:121–30
29. Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Horster F, Tenkova T, Dikranian K, Olney JW: Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 2000; 287:1056–60
30. Olney JW, Tenkova T, Dikranian K, Qin YQ, Labruyere J, Ikonomidou C: Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. Brain Res Dev Brain Res 2002; 133:115–26
31. Bailey CD, Brien JF, Reynolds JN: Chronic prenatal ethanol exposure increases GABA(A) receptor subunit protein expression in the adult guinea pig cerebral cortex. J Neurosci 2001; 21:4381–9
32. Iqbal U, Dringenberg HC, Brien JF, Reynolds JN: Chronic prenatal ethanol exposure alters hippocampal GABA(A) receptors and impairs spatial learning in the guinea pig. Behav Brain Res 2004; 150:117–25
33. Ye X, Carp RI, Schmued LC, Scallet AC: Fluoro-Jade and silver methods: application to the neuropathology of scrapie, a transmissible spongiform encephalopathy. Brain Res Brain Res Protoc 2001; 8:104–12
34. Schmued LC, Hopkins KJ: Fluoro-Jade B: A high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res 2000; 874:123–30
35. Scallet AC, Schmued LC, Slikker W Jr, Grunberg N, Faustino PJ, Davis H, Lester D, Pine PS, Sistare F, Hanig JP: Developmental neurotoxicity of ketamine: Morphometric confirmation, exposure parameters, and multiple fluorescent labeling of apoptotic neurons. Toxicol Sci 2004; 81:364–70
36. Paxinos G, Halliday G, Watson C, Koutcherov Y, Wang H: Atlas of the Developing Mouse Brain at E17.5, P0 and P6. London, United Kingdom, Academic Press, Elsevier Science & Technology, 2006, pp 104, 123
37. Fredriksson A, Archer T, Alm H, Gordh T, Eriksson P: Neurofunctional deficits and potentiated apoptosis by neonatal NMDA antagonist administration. Behav Brain Res 2004; 153:367–76
38. Fredriksson A, Dahlgren L, Danielsson B, Eriksson P, Dencker L, Archer T: Behavioural effects of neonatal metallic mercury exposure in rats. Toxicology 1992; 74:151–60
39. Olton DS, Werz MA: Hippocampal function and behavior: Spatial discrimination and response inhibition. Physiol Behav 1978; 20:597–605
40. Olton DS, Papas BC: Spatial memory and hippocampal function. Neuropsychologia 1979; 17:669–82
41. Fredriksson AA, Archer TT: Alpha-phenyl-tert-butyl-nitrone (PBN) reverses age-related maze learning performance and motor activity deficits in C57 BL/6 mice. Behav Pharmacol 1996; 7:245–53
42. Lister RG: The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 1987; 92:180–5

43. Kirk R: Experimental Design: Procedures for the Behavioural Sciences. Belmont, California, Brooks/Cole, 1995, pp 245–318

44. 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; 146:189–97

45. Wozniak DF, Hartman RE, Boyle MP, Vogt SK, Brooks AR, Tenkova T, Young C, Olney JW, Muglia LJ: Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. Neurobiol Dis 2004; 17:403–14

46. Lee HJ, Kim JW, Yim SV, Kim MJ, Kim SA, Kim YJ, Kim CJ, Chung JH: Fluoxetine enhances cell proliferation and prevents apoptosis in dentate gyrus of maternally separated rats. Mol Psychiatry 2001; 6 610:725–8

47. Johnston MV: Excitotoxicity in neonatal hypoxia. Ment Retard Dev Disabil Res Rev 2001; 7:229–34

48. Olney JW, Young C, Wozniak DF, Ikonomidou C, Jevtovic-Todorovic V: Anesthesia-induced developmental neuroapoptosis: Does it happen in humans? ANESTHESIOLOGY 2004; 101:273–5

49. Young C, Tenkova T, Dikranian K, Olney JW: Excitotoxic *versus* apoptotic mechanisms of neuronal cell death in perinatal hypoxia/ischemia. Curr Mol Med 2004; 4:77–85

50. Winberg J, Porter RH: Olfaction and human neonatal behaviour: Clinical implications. Acta Paediatr 1998; 87:6–10

51. Moriceau S, Sullivan RM: Unique neural circuitry for neonatal olfactory learning. J Neurosci 2004; 24:1182–9

52. Booze RM, Mactutus CF, Annau Z, Tilson HA: Neonatal triethyl lead neurotoxicity in rat pups: Initial behavioral observations and quantification. Neurobehav Toxicol Teratol 1983; 5:367–75

53. Carlsson C, Bahrami F, Fredriksson A, Brandt I: Behavioural changes related to olfactory mucosal metaplasia and bulbar glial fibrillary acidic protein (GFAP) induction in methylsulphonyl-dichlorobenzene-treated mice. Arch Toxicol 2002; 76:474–83

54. Carlsson C, Fredriksson A, Brandt I: 2,6-Dichlorophenyl methylsulphone induced behavioural impairments in rats and mice in relation to olfactory mucosal metaplasia. Pharmacol Toxicol 2003; 93:156–68

55. Gansler DA, Fucetola R, Krengel M, Stetson S, Zimering R, Makary C: Are there cognitive subtypes in adult attention deficit/hyperactivity disorder? J Nerv Ment Dis 1998; 186:776–81

56. Good KP, Whitehorn D, Rui Q, Milliken H, Kopala LC: Olfactory identification deficits in first-episode psychosis may predict patients at risk for persistent negative and disorganized or cognitive symptoms. Am J Psychiatry 2006; 163: 932–3

57. Rupp CI, Fleischhacker WW, Kemmler G, Oberbauer H, Scholtz AW, Wanko C, Hinterhuber H: Various bilateral olfactory deficits in male patients with schizophrenia. Schizophr Bull 2005; 31:155–65

58. Rupp CI, Kurz M, Kemmler G, Mair D, Hausmann A, Hinterhuber H, Fleischhacker WW: Reduced olfactory sensitivity, discrimination, and identification in patients with alcohol dependence. Alcohol Clin Exp Res 2003; 27:432–9

59. Leonard BE: The olfactory bulbectomized rat as a model of depression. Pol J Pharmacol Pharm 1984; 36:561–9

60. Mucignat-Caretta C, Bondi M, Caretta A: Time course of alterations after olfactory bulbectomy in mice. Physiol Behav 2006; 89:637–43

61. Culley DJ, Baxter MG, Yukhananov R, Crosby G: Long-term impairment of acquisition of a spatial memory task following isoflurane–nitrous oxide anesthesia in rats. ANESTHESIOLOGY 2004; 100:309–14

62. Mattson SN, Schoenfeld AM, Riley EP: Teratogenic effects of alcohol on brain and behavior. Alcohol Res Health 2001; 25:185–91

63. Hausknecht KA, Acheson A, Farrar AM, Kieres AK, Shen RY, Richards JB, Sabol KE: Prenatal alcohol exposure causes attention deficits in male rats. Behav Neurosci 2005; 119:302–10

64. Famy C, Streissguth AP, Unis AS: Mental illness in adults with fetal alcohol syndrome or fetal alcohol effects. Am J Psychiatry 1998; 155:552–4

65. Wang C, McInnis J, Ross-Sanchez M, Shinnick-Gallagher P, Wiley JL, Johnson KM: Long-term behavioral and neurodegenerative effects of perinatal phencyclidine administration: implications for schizophrenia. Neuroscience 2001; 107:535–50

66. Poulter MO, Ohannesian L, Larmet Y, Feltz P: Evidence that GABAA receptor subunit mRNA expression during development is regulated by GABAA receptor stimulation. J Neurochem 1997; 68:631–9

67. Nunez JL, Alt JJ, McCarthy MM: A new model for prenatal brain damage: I. GABAA receptor activation induces cell death in developing rat hippocampus. Exp Neurol 2003; 181:258–69

68. Nicosia A, Giardina L, Di Leo F, Medico M, Mazzola C, Genazzani AA, Drago F: Long-lasting behavioral changes induced by pre- or neonatal exposure to diazepam in rats. Eur J Pharmacol 2003; 469:103–9

69. Ryan CL, Pappas BA: Intrauterine diazepam exposure: Effects on physical and neurobehavioral development in the rat. Neurobehav Toxicol Teratol 1986; 8:279–86

70. Gai N, Grimm VE: The effect of prenatal exposure to diazepam on aspects of postnatal development and behavior in rats. Psychopharmacology (Berl) 1982; 78:225–9

71. Christensen JH, Andreasen F: Individual variation in response to thiopental. Acta Anaesthesiol Scand 1978; 22:303–13

72. Soriano SG, Anand KJ, Rovnaghi CR, Hickey PR: Of mice and men: Should we extrapolate rodent experimental data to the care of human neonates? ANESTHESIOLOGY 2005; 102:866–9

73. Olney JW, Young C, Wozniak DF, Ikonomidou C, Jevtovic-Todorovic V: Of mice and men: Should we extrapolate rodent experimental data to the care of human neonates? ANESTHESIOLOGY 2005; 102:868–9