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Developmental Neurotoxicity of Methanol Exposure by Inhalation in Rats

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 150 projects at institutions in North America and Europe.

Typically, HEI receives half its funds from the U.S. Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the United States. Occasionally, funds from other public or private organizations either support special projects or provide resources for a portion of an HEI study. This study was part of a larger methanol program that received initial support from the American Petroleum Institute. However, in all cases HEI exercises complete autonomy in setting its research priorities and in reaching its conclusions. An independent Board of Directors governs HEI. The Institute's Research Committee and the Review Committee serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigators' Report and the Review Committee's evaluation of the work's scientific quality and regulatory relevance.

HEI Statement

Synopsis of Report Number 73

Developmental Neurotoxicity of Inhaled Methanol in Rats

BACKGROUND

Methanol was designated a "clean alternative fuel" by the Clean Air Act Amendments of 1990. The use of methanol as an alternative motor vehicle fuel could have important remedial effects on air pollution by decreasing hydrocarbon emissions and thereby potentially reducing atmospheric ozone concentrations. As a result, its use could help address the continued inability to meet air quality standards in many urban areas of the United States.

If methanol is used as an alternative motor vehicle fuel, humans will be exposed to relatively low levels of methanol vapors through inhalation. Although methanol is clearly poisonous when ingested at high levels, it is not known whether it would pose health risks when inhalated at low levels. Observations of methanol's effects on pregnant rodent suggest the potential for methanol vapors to have adverse effects on the fetus. To determine whether exposure to methanol vapors may influence development of the nervous system, HEI funded the study described by Dr. Weiss and his colleagues, which examined the effects of prenatal and early postnatal inhalation of methanol on selected measures of neurobehavior in rats.

APPROACH

Dr. Weiss and his colleagues conducted a controlled series of experiments in which they exposed pregnant rats and their newborn offspring to 4,500 parts per million (ppm) methanol by inhalation, and then submitted them to tests of behavioral function.

RESULTS AND IMPLICATIONS

Exposure to 4,500 ppm methanol vapors did not affect the amount of time it took newborn rats to attach to their mothers' nipples (suckling test) or their ability to be trained to avoid specific odors (conditioned olfactory aversion test); however, methanol-exposed newborns were less active on postnatal day 18 and more active on postnatal day 25 than control newborns (motor activity test). The lack of effect of methanol on suckling contrasted with what the investigators had observed in an earlier study in which high doses of methanol were administered via drinking water. In adult rats that had been exposed to methanol in utero and as newborns, small effects were observed in tests of coordination on a running wheel and persistence in obtaining food rewards for this activity (fixed-ratio wheel-running test), but only when the genders were analyzed separately. In addition, when adult rats were trained to press levers in a specific sequence to obtain food rewards (stochastic spatial discrimination test), there were some relatively subtle performance differences in their rates of adjustment to a new pattern of lever-pressing; specifically, after extended training the control animals performed better than the methanol-treated animals on the second of two lever-press sequence patterns.

The HEI Review Committee noted that the investigators conducted many tests and found only isolated positive results that were small and variable. Because no compensation was made for multiple testing, care must be taken not to ascribe too much significance to these results. This is particularly true in light of the fact that the methanol exposure level investigated in this study (4,500 ppm) was appreciably higher than the ambient exposures predicted for the use of methanol fuels (1 to 10 ppm in typical traffic situations and as high as 200 ppm in a worst-case scenario such as a malfunctioning vehicle in an enclosed garage). Overall, the investigators' results point to the neurobehavioral measures that should be studied when evaluating the potential neurologic effects of inhaled methanol exposure.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI from 1990 to 1994. This study was conducted by Dr. Bernard Weiss at the University of Rochester School of Medicine and Dentistry. This Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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ABSTRACT

The possibility of widespread methanol exposure via inhalation stemming from its adoption as an automotive fuel or fuel component arouses concerns about the potential vulnerability of the fetal brain. This project was designed to help address such concerns by studying the behavior of neonate and adult rats following perinatal exposure to methanol vapor. Four cohorts of pregnant Long-Evans hooded rats, each cohort consisting of an exposure and a control group, were exposed to 0 parts per million (ppm)* (control) or 4,500 ppm methanol vapor for six hours daily beginning on gestation day (GD) 6 with both dams and pups then being exposed through postnatal day (PND) 21. Exposures took place in 2-m^3 Rochester-type inhalation chambers while the animals remained in their plastic breeder cages. Prenatal and postnatal blood methanol concentrations were determined by gas chromatography. Blood methanol concentrations of the dams, measured immediately following a six-hour exposure, were approximately 500 to 800 μg/mL throughout gestation and lactation. Average blood methanol concentrations of the pups were about twice those of the dams. Because such results appeared consistently across the other cohorts, we decided to obtain additional data with Cohort 4. Once it had undergone the standard exposure protocol, we selected sets of extra pups from those that had not been assigned previously to the adult phase of behavioral testing. Each set was exposed once, at ages that extended out to PND 52, for one additional six-hour session of exposure to 4,500 ppm methanol. The blood methanol concentrations of these pups declined until about PND 48, at which time they approximated those of the dams. These findings might be accounted for by a process of metabolic maturation in the pups that remains to be identified.

Neurotoxicity was assessed primarily by behavioral tests used previously to reveal adverse effects following developmental exposures to ethanol, cocaine, heavy metals, and many other agents. Male-female pairs from identical litters were entered into the statistical analysis, whenever appropriate, to examine gender differences. Suckling latency and attachment, odor discrimination, and spontaneous activity were measured before weaning. At 90 to 267 days of age, depending on the cohort, the offspring received training in either of two types of schedule-controlled operant behavior. One schedule required them to rotate a running wheel a specified number of revolutions to secure food pellets, which reinforced the behavior. It was included both as an index of motor function and as an index of responsiveness to the contingencies built into the reinforcement schedule. The primary measure of performance consisted of the number of responses (revolutions) per one-hour session. The other schedule consisted of a complex stochastic spatial discrimination task and was included as an index of cognitive function. In an operant test chamber containing three levers, contingencies were arranged so that the probability of food pellet reinforcement for a response on any particular lever depended on which of the three levers had been the site of the previous response. The primary measure was based on the extent to which the subjects maximized payoffs for lever-pressing.

Exposure to methanol did not affect suckling latency and attachment on PND 5, or performance on the conditioned olfactory aversion test on PND 10. Exposure to methanol did alter performances in the motor activity test. Methanol-exposed neonates were less active on PND 18, but more active on PND 25 than the equivalent control-group pups. Schedule-controlled running displayed a complex interaction with treatment. Changes in performance over the course of training differed between males and females depending on exposure to methanol. The results of the complex stochastic reinforcement schedule also revealed behavioral differences due to methanol exposure; these were relatively

* A list of abbreviations appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report Number 73, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Bernard Weiss, University of Rochester School of Medicine and Dentistry, Department of Environmental Medicine, 575 Elmwood Avenue, Box EHSC, Rochester, NY 14642.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Agreement 816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The contents of this document also have not been reviewed by private party institutions, includiug those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

subtle in nature and were determined by asymptotic performance after the introduction of a new pattern of contingencies.

Light-microscopic analysis showed no significant abnormalities in the brains of the methanol-treated animals. Analyses of neural cell adhesion molecules (NCAMs) in the brains of pups killed on PND 4 showed staining for both NCAM 140 and NCAM 180 to be less intense in the cerebellum of exposed animals. Such differences were not apparent in animals killed 15 months after their final exposure.

This research program was directed toward detecting effects of developmental exposures to methanol. Although it was more focused than a battery of exploratory tests for a novel agent, it did not test restrictive hypotheses about mechanisms. No assumptions were made, therefore, concerning the likelihood of joint outcomes across procedures. In addition, relations of exposure concentration to response were not evaluated. Developmental exposure to 4,500 ppm methanol did alter some of the endpoints studied, and it appeared that gender played a role. These results should serve as guides for future evaluations of methanol exposure.

INTRODUCTION

Concerns about the toxicity of methanol have grown in parallel with the prospect that it may be widely adopted as an additive to or replacement for gasoline (Kavet and Nauss 1990). Its potential neurotoxicity, especially during early brain development, has aroused special uncertainties about the effects of low levels of exposure, or prolonged exposures, or both. Developmental neurotoxicity may emerge insidiously under such circumstances (Needleman and Bellinger 1994), unlike the well-known effects of severe acute methanol poisoning.

Acute poisoning by methanol has been documented by a long and often dismal history. In the workplace, toxic episodes have resulted both from inhalation of vapor and from dermal exposure to methanol-soaked clothing or footwear (Hunter 1975). Most instances of poisoning, however, can be traced to ingestion, typically in the form of adulterated alcoholic beverages. Hunter (1975) estimated that some brandies and whiskeys sold in New York City early in this century contained from 24% to 43% methanol.

Usually, only the most seriously affected victims of methanol exposure come under medical observation. Those not fatally afflicted may suffer severe visual deficits including blindness arising from optic nerve edema (Hayreh et al. 1980). Some victims are left with a residue of motor dysfunction arising from basal ganglia damage (LeWitt and Martin 1988). Others seem to enjoy a full recovery, at least by clinical criteria. Milder effects, attributed to low-level inhalation exposure in the workplace, include complaints of headache and dizziness, blurred vision, and other subjective indices of health effects (Frederick et al. 1984).

The biochemical mechanisms of severe acute poisoning are now recognized to follow from excessive accumulation of formic acid and the lapse of victims into metabolic acidosis, as described at length by Kavet and Nauss (1990). Optic nerve damage seems to be due to formate itself rather than to reduced pH (Hayreh et al. 1980).

Except for accidents, however, and deliberate adulteration of alcoholic beverages, the primary health questions kindled by methanol fuels arise from much lower environmental exposures, especially by inhalation. Only a scant literature is devoted to questions posed in such a context, although most observers agree that a predominant issue would be neurobehavioral impairment. Cook and associates (1991) examined a broad range of neuropsychological endpoints in subjects exposed to 200 ppm methanol (the threshold limit value) and observed some hints of adverse effects, especially on subjective measures.

Developmental neurotoxicity has emerged as a major element in evaluating the health risks posed by environmental exposure to chemicals. One concern is the recognized vulnerability of the fetal brain to heavy metals such as lead (Bellinger and Stiles 1993) and methylmercury (Cox et al. 1989). Together with analogous data for other agents, such results have led the EPA to formulate developmental neurotoxicity guidelines. Even more compelling for methanol, surely, is the huge volume of information devoted to the fetal alcohol syndrome, coupled with findings that indicate much more subtle consequences at lower levels of maternal consumption than those evoking malformations and mental retardation (Streissguth et al. 1986).

Evidence that methanol is a potential developmental toxicant for humans comes from a sparse array of animal studies. Nelson and coworkers (1985) exposed pregnant rats by inhalation for six hours daily to concentrations of methanol and ethanol ranging from 5,000 to 20,000 ppm. At equivalent concentrations in air, methanol produced more malformations and induced more weight depression than ethanol. In a study of malformations in mice, Rogers and colleagues (1993) exposed the pregnant dams for seven hours daily to concentrations ranging from 1,000 to 15,000 ppm and observed an elevated incidence of exencephaly in those exposed at 5,000 ppm and above. Some skeletal abnormalities occurred in those exposed at 2,000 ppm and above. Bolon and colleagues (1993), in a mouse study aimed at the identification of critical gestational periods, found increased resorptions, reduced fetal weights, fetal malformations, or all of these in mice exposed at 10,000 and

15,000 ppm. Exposures at 5,000 ppm yielded no observable adverse effects. At the higher methanol exposure levels, neural tube defects and ocular lesions occurred after exposure during GD 7 through 9. Limb anomalies were seen when exposures occurred during GD 9 through 11. Exposure during either three-day period produced cleft palate and hydronephrosis. These data indicate that specific developmental abnormalities induced by methanol, as is typical of teratogenic assays, depend on both the stage of development and the timing and magnitude of exposure.

Data on functional effects are equally sparse. Infurna and Weiss (1986) exposed pregnant rats to methanol in drinking water (2% v/v) during GD 15 through 17 or GD 17 through 19. Both groups of exposed offspring showed disrupted suckling behavior on PND 1 and difficulties in locating nesting material from the home cage on PND 10. Stanton and associates (1991) exposed rats via inhalation seven hours daily during GD 7 through 19 at a concentration of 15,000 ppm. Maternal blood concentrations measured about 3 mg/mL. An extensive battery of behavioral tests administered to the offspring failed to reveal any persistent adverse effects.

Two inhalation studies were conducted by the New Energy Development Organization (NEDO) in Japan. Rats whose dams were exposed to 1,000 ppm methanol for about 20 hours daily from before gestation until weaning showed lower brain weights than those of controls. The report claimed that histopathological examination revealed no effects of treatment, but no other details were offered. Neither the relatively simple behavioral tests conducted after 5 weeks of age, nor evaluations of simple reflexes before weaning, yielded evidence of adverse treatment effects. Other treatment-related effects reported to occur at 1,000 ppm included earlier testes descent and lower pituitary and thymus weights.

The current project consisted of a set of procedures selected to detect neurobehavioral effects arising from developmental exposure to methanol in the rat. Methanol administration was accomplished by exposing pregnant rats, and then both dams and their litters until weaning, to 4,500 ppm methanol vapor. Inhalation served as the exposure medium because it is projected to be the major source of exposure for the general population arising from the adoption of methanol fuels. The pharmacokinetic properties of inhalation differ from those of oral administration, the other alternative for experimental work.

Four such cohorts, each consisting of an exposure group and a control group, were assayed. Pairs of male-female littermates were studied under three different procedures as neonates and two different procedures as adults. The three neonatal tests were selected on the basis of their utility for assessing different behaviors at different ages in developing rats. These tests have been used previously, with other agents, to detect neurobehavioral toxicity. The two adult-phase tests were selected because they assess different behaviors under schedule-controlled conditions. They enabled us to investigate transitional and steady-state performances in great detail, a property useful not only for detecting an effect, but also for stimulating hypotheses about mechanisms.

In addition to behavioral observations, we examined morphological indices of brain development. One such index was neuropathology. Another consisted of assays of NCAMs. The NCAM is a developmentally regulated surface glycoprotein that serves a central role in the formation and maintenance of the nervous system (Edelman 1985; Tomasiewicz et al. 1993; Lagunowich et al. 1994) and is sensitive to developmental neurotoxicants such as lead (Cookman et al. 1987).

The scope of the project, therefore, was more focused than that of a broad screening program directed at a novel agent, but less narrow than a protocol designed for testing a single hypothesis. Such protocols are typically aimed at identifying mechanisms of action, but it would have been premature to aim for such a goal given the limited data available. Because we treated each procedure as an independent opportunity to assess the potential neurotoxicity of the methanol exposures, we made no predictions about the likelihood of joint outcomes across procedures; a modification of the behavior under one procedure would not have led to the prediction of an effect under any of the other procedures. There was no rationale for offering such predictions with methanol exposure as the independent variable. The statistical approaches adopted for the analyses recognized the exploratory nature of the project.

Although meager, the existing data suggest that developmental neurotoxicity may arise from maternal methanol exposure. The current project was framed to further explore the functional (behavioral) consequences. Originally, on the basis of the data published by Nelson and coworkers (1985) and the unpublished report by NEDO, as well as the projected environmental levels stemming from methanol use in automobiles, it was designed to include ambient concentrations of 500, 1,500, and 4,500 ppm. After the results of Stanton and colleagues (1991) became available, the project focus shifted. The absence of detectable behavioral effects in that study, even after a maternal exposure as high as 15,000 ppm, guided the decision to eliminate the lower concentrations and to commit the available experimental resources to the highest of the originally planned exposure levels.

The choice of rats as the test species evolved from the prevailing literature. Until the recent reports of Rogers and associates (1993) and Bolon and associates (1993), all of the developmental studies had relied on rats, and even the ethanol literature was dominated by rat experiments. At least superficially, rats may seem to be far from an optimal choice. Unlike humans and other primates, they rapidly degrade formate, the metabolite presumed to underlie the most severe effects of methanol poisoning, unless their normal mechanisms, dependent on folate, are impaired (Eells 1991). At exposure levels short of those promoting the accumulation of formic acid, however, rats and monkeys metabolize methanol at comparable rates (Horton et al. 1992). Because the current effort was directed at the consequences of low and moderate exposure concentrations, the rat was deemed a suitable species for such an exploration.

The experimental design was predicated on the basis that adverse neurobehavioral effects arising from early developmental exposure often do not unfold until maturity. As a result, behavioral assays spanned the period from shortly after birth to adulthood. The design also took account of the asynchrony in development between the human and the rodent brain. At birth, the rat brain is roughly equivalent in maturity to the second trimester of the human and primate brain; its growth spurt occurs postnatally (Bayer et al. 1993). To accommodate this feature of rat development, methanol exposures continued until weaning. Given the plasticity of the developing nervous system, and the possibility of silent damage not expressed in behavioral measures (Weiss and Reuhl 1994), morphological markers were also examined. Finally, a biological index of exposure, the methanol concentration in blood, was evaluated throughout exposure.

SPECIFIC AIMS

This study sought to determine the effects of methanol exposure in utero and through weaning on neurobehavioral function in Long-Evans hooded rats. Originally, it had been planned to expose pregnant rats, and then both suckling mothers and their litters, to 0, 500, 1,500, and 4,500 ppm methanol vapor for six hours daily. The neurobehavioral consequences of this exposure schedule were to be examined in both neonates and adult offspring. However, while the current study was under way, Stanton and colleagues (1991) reported finding no behavioral effects in offspring following maternal methanol exposure to 15,000 ppm. Therefore, the investigators and the HEI Research Committee agreed to a single methanol exposure level of 4,500 ppm.

METHODS

SUBJECTS

Virgin female Long-Evans hooded rats, 170 to 200 g, were obtained from Charles River Breeding Laboratories (Wilmington, MA). They were housed individually, in the University of Rochester Medical Center Vivarium, a facility certified by the American Association for the Accreditation of Laboratory Animal Care, in polycarbonate breeder cages (45 × 22 × 20 cm) with a wire cover and filter top. All experimental procedures had been approved by the University Committee on Animal Research. The bedding consisted of aspen chips (Northeastern Products Corp., Warrensburg, NY) selected for their methanol absorbance properties (see below). Fresh bedding was provided weekly. The cages were located on racks with solid metal shelves. Cages were distributed across shelves by random assignment to help ensure that exposure to any potentially significant variables associated with location in the room was balanced across the exposure and control groups, Males weighing 250 to 300 g, also from Charles River, were housed in wire-mesh cages (36 × 17.5 × 17 cm) in the same room. No other animals were housed in the room, which was maintained at a temperature of 24° ± 2°C and a humidity setting of approximately 50%. Both males and females were allowed free access to Purina RMH 2000 Lab Chow and tap water except when pregnant animals were in the exposure chambers. The rats were adapted to these conditions for approximately two weeks. A separate group of test dams was bred at the same time as the other groups. These females were used in the suckling test. As exposure and control group subjects were adapted to the chambers before breeding, the suckling test dam group was necessarily assigned before other group assignments or treatments. Such assignment was based on a list of random numbers generated for that purpose.

Females not assigned to the test dam group were adapted to the exposure chambers over the next two weeks. The handling procedure duplicated the procedure initiated on GD 6 except that no exposures to methanol occurred during this adaptation period. For this purpose, the females were removed from the vivarium daily and taken to the inhalation chamber room. They were then placed into the chambers, where they remained for six hours. Afterward, they were returned to the vivarium.

BREEDING

For breeding, the females were placed individually with males in the hanging wire-mesh cages at approximately 3 to 4 p.m. Vaginal smears obtained at 8:30 a.m. were exam-

ined microscopically for the presence of sperm. A sperm-positive smear determined GD 0. Following such a determination, the female was assigned to one of the treatment conditions. Assignments were randomized within blocks of two females, with the first of the two dams randomly assigned to one of the treatments and the second specifically assigned to the other. This ensured that the date of conception was counterbalanced across treatment conditions. Litter number within a treatment group was also designated at this time as the ordinal position to which the subject was assigned in that group. The female was then returned to a polycarbonate breeder cage and maintained under standard conditions.

In total four cohorts were bred. When a litter was discovered in the morning prior to transport from the vivarium to the exposure chambers, that date was designated PND 1. All litters of more than eight were culled to eight offspring on PND 4 using an assignment procedure based on a series of random numbers generated in our Biostatistics Shared Facility. Whenever possible, four male and four female pups were selected. An India ink injection of the paw identified pups, who were randomly assigned identification numbers by an equivalent list. These numbers determined subsequent treatments of the pups.

From GD 6 through PND 21, pregnant and lactating dams and litters were transported from the vivarium to the inhalation chambers in their home cages. The cages were then placed in the inhalation chambers according to a preassigned location, and the experimental group was exposed to the nominal methanol vapor concentration of 4,500 ppm. Control dams and litters were placed in an adjoining exposure chamber and exposed to air. The number of rats in the chamber varied, depending on gestational and postnatal dates, with a maximum of 12 cages per exposure chamber. Cage location within chambers was rotated systematically across days of exposure. Exposures were conducted six hours daily, and the animals remained in the chambers for degassing for 30 minutes after vapor generation was terminated. They were then returned to the vivarium. Maternal weights were measured on GD 7, 14, and 19.

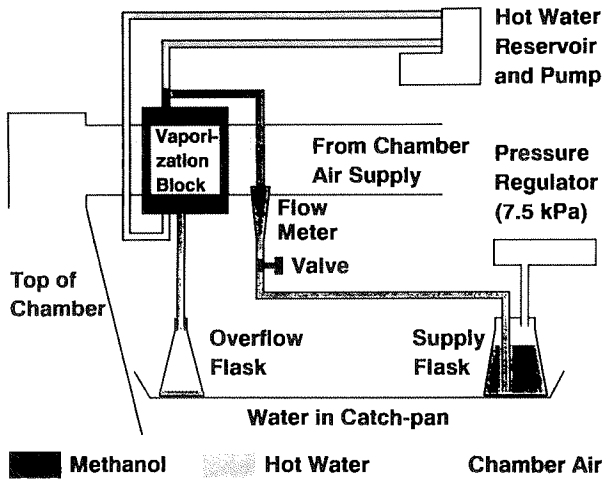

Figure 1. Diagram of the system for delivering methanol vapor to the exposure chamber.

INHALATION FACILITY AND PROCEDURES

Exposures were conducted in hexagonal Rochester-type chambers (Leach et al. 1959; Cheng and Moss 1989) with a volume of 2 m^3. Each chamber was supplied with filtered, warmed outside air pumped into the chamber through an intake duct at the top and exhausted at the bottom. Humidity was not regulated. High-performance liquid chromatography (HPLC)-grade methanol (J.T. Baker, Phillipsburg, NJ; Catalog No. 9093-03) was introduced into the airstream by passing liquid methanol through a heated aluminum vaporization block mounted on the intake duct adjacent to each chamber (Figure 1). The vaporization block held aluminum tubes of 1/8-inch o.d. seated in the 4-inch-square aluminum block (Figure 2). The block exposed 2.5 inches

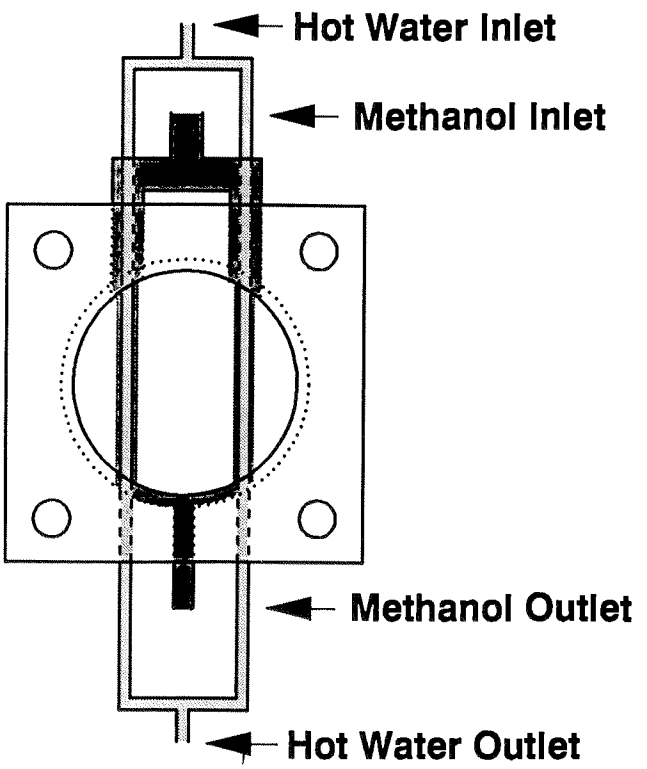

Figure 2. Schematic of the vaporization block. The methanol (solid bars) is vaporized by contact with aluminum tubes heated by hot water (stippled bars).

of each tube to the airstream. A hot solution of propylene glycol in water circulated through the tubing, heating its surfaces to an average temperature of 95°C. Methanol entered the block through four male tees, where it came into contact with the hot tubing. As the liquid passed down the tubing, some of it was converted to vapor, picked up by the airstream, and carried into the chamber. The remaining methanol exited the block through an overflow line and was collected in a waste receptacle. Static or chamber pressure, as read from a gauge connected to the chamber, was negative and indicated a slight leak into the chamber from the room; it was usually no more than 0.02 inch of water. Airflow into each chamber was determined by a calibrated Venturi meter, recorded daily, and monitored regularly. A turnover of 12 chamber volumes per hour was the nominal airflow rate.

Methanol concentration within the chamber was monitored continuously by a gas infrared analyzer (Miran 1A, Foxboro Corp., Foxboro, MA) calibrated from gas chromatographic results and connected to a chart recorder. Chamber temperature was measured by thermometers inserted into the chamber.

Chamber airflow was approximately 160 L/min. The gas infrared analyzer sampled the methanol concentration by pulling air through the chamber, with the result being an increase in total airflow equal to the sample flow. As part of our initial calibration of the system, a bubble meter (Gilibrator, Primary Flow Calibrator P/N D-800268 and Bubble Generator P/N D800285, Gilian Instrument Corp., West Caldwell, NJ) was used to measure the airflow through the gas infrared analyzer. The mean of 11 determinations was 6.77 L/min. This value was less than 5% of the total airflow.

The rat cages were placed on two large mesh shelves located in the center region of the chamber. The chambers were illuminated by fluorescent lighting in the room housing them.

Exposure Chamber Calibration

The infrared spectrophotometer used to monitor chamber concentration was calibrated against duplicate silica gel samples (silica gel tubes; SKC No. 226-10, SKC, Eighty Four, PA) obtained at five-minute intervals, simultaneously with the spectrophotometer readings at three different chamber concentrations of methanol (500, 1,500, and 4,500 ppm). The silica gel samples were analyzed for methanol content with a gas chromatograph using the National Institute for Occupational Safety and Health (1984) Analytic Method 2000. Passive dosimeters, consisting of a distilled-water-filled SKC developing vial (No. 226-02A) and capped with a 5.0-μm pore, LS-type filter (No. LSWP 01300; Millipore Corp., Bedford, MA), were positioned at various chamber locations and subsequently analyzed for methanol content. These samples were obtained simultaneously with the silica gel samples used to calibrate the spectrophotometer. The dosimeters provided a means for determining methanol concentrations in quality control experiments in which it was not feasible or appropriate to use the silica gel sampling method.

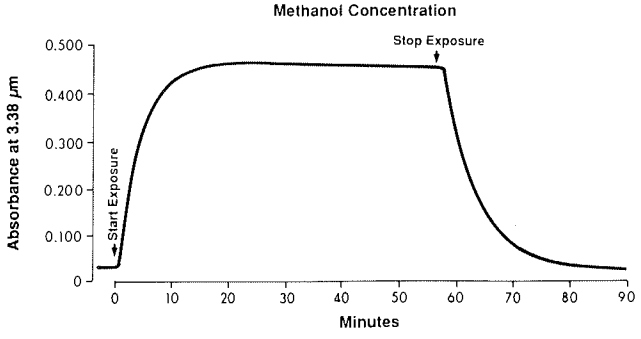

Figure 3. Infrared spectrophotometer (Miran 1A) tracing showing onset, steady state, and termination of methanol exposure conditions during a one-hour test of the exposure system.

Table 1. Methanol Exposure Concentration Summary

	Cohort			
	1	2	3	4
Exposure sessions (*n*)	40[a]	42	44	41
Mean concentration (ppm)	4,352	4,382	4,582	4,471
SEM (ppm)	63	26	46	17
Maximum concentration (ppm)	5,363	4,880	5,209	4,776
Minimum concentration (ppm)	3,558	4,033	3,552	4,197

[a] System failures resulted in two sessions that were less than six hours; therefore 38 sessions were included in the summary statistics.

Once the system for generating methanol vapor was turned on, about 15 minutes was required to reach steady state corresponding to 4,500 ppm. Figure 3 shows a tracing of a chart record from a brief one-hour system test. An equivalent amount of time was required for the chamber concentration to fall to baseline values after the generating system was turned off. During an exposure, the strip chart and other instruments were monitored frequently to ensure that no systematic deviations were taking place.

Table 1 summarizes the methanol exposure concentrations attained for each cohort. The excursion of the chart record at the end of each hour was measured by an investigator, and then the concentration (in ppm) was calculated based on the original calibration. Generally, the exposure concentrations closely approximated the nominal value of 4,500 ppm. The differences in the number of exposure sessions across cohorts reflect the outcome of the breeding program, as not all females became pregnant on the same day. From the perspective of experimental design, this is an advantage because several gestation days of a cohort are distributed within each exposure day shown in Table 1 (with the exception being the first exposure day in each cohort). A main effect of exposure, therefore, is unlikely to be due to any single exposure session, particularly within the generally small range of values established in the present experiment. (Appendix B, Table B.1 shows the daily mean concentrations for each exposure session for each cohort.)

Characterization of Methanol Exposure

For inhalation studies, whole-body exposures are conventionally performed in mesh cages to allow relatively unimpeded flow of the exposure materials. Such enclosures would pose problems for lactating dams and their litters. In addition, we were concerned about the consequences of the handling of the dams and the pups that would be required for transferring them on a daily basis between the home cages and wire-mesh enclosures. An extensive literature in psychobiology indicates that variation in the early environment, including the process of handling pregnant dams and neonates, leads to significant modifications of subsequent behavioral and neuroendocrine responses (Smythe et al. 1994). Our concern about introducing such variables into the experiment led us to ascertain the possibility of exposing the animals in their home cages.

To assess whether conventional breeder cages were suitable, we first compared methanol concentrations from passive dosimeters placed in the breeder cages containing aspen-chip bedding with dosimeter placed on the chamber racks. Two cages were placed on the upper shelf and two on the lower. Two dosimeters were placed in each cage; four additional dosimeters were placed on each shelf. The ratio of methanol concentration inside the cage to that outside was 0.77. Our next step was to determine whether any differences could be discerned in rats, whose movements and positions in the cages might modify their exposures. Blood methanol concentrations of rats housed in breeder cages were compared with those of rats housed in standard wire-mesh cages. Eight female rats, not used in any of our other experiments, were exposed to methanol at 4,500 ppm for six hours. Four of the rats were housed individually in breeder cages with aspen-chip bedding; four rats were housed individually in wire-mesh cages with a solid stainless-steel top. Blood was obtained by tail nick immediately following exposure. Blood methanol concentrations between the two groups did not differ, yielding levels of 1.26 mg/mL (SD 0.15) for the breeder cages and 1.28 mg/mL (SD 0.08) for the wire-mesh cages. These levels were higher, probably because of the novel environment, than those obtained later from animals acclimated to the chambers. Cooper and associates (1992) found that following a single six-hour exposure to 5,000 ppm methanol, blood methanol concentrations were higher in unacclimated rats than in acclimated rats. We did not pursue this observation further because we were concerned with differences in blood methanol concentrations between cage types rather than absolute values. Although the methanol concentration in the cage with no rat in it was lower than outside the cage, blood methanol concentrations of rats housed in the breeder cages matched those of rats housed in the wire-mesh cages. This experiment, therefore, confirmed the validity of using breeder cages in the inhalation chambers.

Other independent experiments evaluating blood methanol concentration were conducted as part of our characterization of exposure to methanol. Twelve female rats were exposed to 4,500 ppm methanol for six hours daily over three consecutive days. Blood samples were obtained by tail nick from groups of three rats at the end of the first hour of exposure, at the termination of the 6-hour exposure, at 1-hour postexposure, and at 17 hours postexposure. (Note that the 17-hour postexposure samples were obtained the following morning; for Days 1 and 2, therefore, they were obtained just prior to the exposures on Days 2 and 3, respectively.) Gas chromatographic analyses were used to determine the concentration of methanol in blood. The results appear in Figure 4. They show higher elevations on the first exposure day, perhaps because of greater exploratory activity in a novel setting. Within our limits of detection at 10 ng/μL, we did not detect any more than trace amounts of methanol in the blood at the 17-hour postexposure time; those data were not plotted. We also did not detect methanol in the blood immediately prior to the first exposure session (data not shown).

Degassing of Methanol

Any bedding material absorbs some quantity of methanol; therefore degassing of methanol from the bedding must be assumed to occur following termination of methanol vapor generation in the exposure system. An initial experiment using passive dosimeters found that aspen chips absorbed less methanol than the standard hardwood chips used in our vivarium, and less than bedding made from paper and corn cobs. The magnitude of the degassing was further evaluated in two experiments using passive dosimeters. In the first of these experiments, three breeder cages containing aspen chips, the material selected for bedding, were exposed to 4,500 ppm methanol for six hours. Thirty minutes after methanol vapor generation ceased, passive dosimeters were placed on top of the bedding in each cage for one hour. Then, one cage remained in the inhalation chamber and the other two were returned to the vivarium. One of the two cages in the vivarium had a filter hood on its top; the other did not. Passive dosimeters again were placed into the three cages 17 hours after termination of the exposure. In the first hour following exposure, methanol concentrations averaged 17% of the 4,500 ppm exposure; 17 hours later, the concentration was about 1% for the cages without the filter hood, and about 5% for the cage with a filter hood.

A second experiment was conducted to determine whether the presence of animals would modify the results reported above. Two rats were placed in the inhalation chamber and exposed to 4,500 ppm methanol for six hours. After one hour of exposure, the rats were removed and the dosimeters were placed on the bedding in each cage while the cages remained in the inhalation chamber. The dosimeters were removed one hour later, and the rats were returned to their respective cages, which remained in the inhalation chamber. One hour after termination of exposure, the rats were returned to the vivarium and then removed from those cages for one hour, during which dosimeters again were placed in the cages. Upon removal of the dosimeters, the rats were returned to their cages. The next morning, 16 hours after termination of exposure, the procedure was repeated to obtain another one-hour sample with the dosimeters. These 1-hour and 16-hour postexposure assessments were conducted also following the second and third consecutive days of exposure to 4,500 ppm methanol for six hours. Fresh bedding was used at the start of the first day of exposure only. Cage concentrations during the first hour of exposure averaged 4,400 ppm. Across all three days, during the one-hour postexposure period, they fell to 224 ppm, and 16 hours later, the values had fallen to a range of 0.3 to 2.6 ppm. The results showed that, although the bedding did absorb some methanol, degassing occurred rapidly. The lower degassing values obtained in the second experiment probably reflect the presence of the rat; both heat and convection currents produced by movement would facilitate the dispersion of the methanol. This means that the nominal specification of a six-hour exposure to methanol corresponds closely to our measurements. It means also that by the time the rats were removed from the inhalation chambers for return to the vivarium, potential exposure of the control group to methanol degassing from the other group would be insignificant.

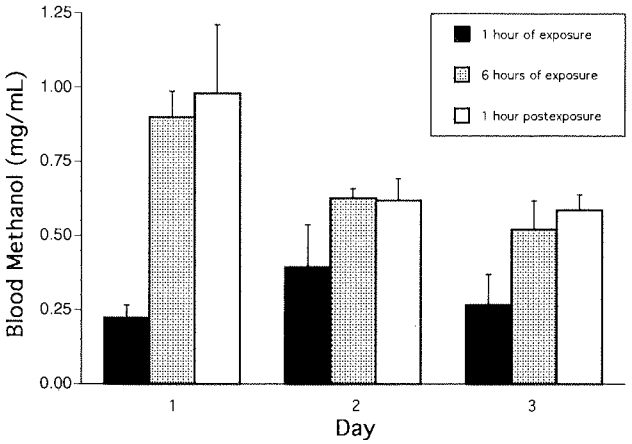

Figure 4. Mean (± SD) blood methanol concentrations attained by a group of 12 female rats exposed for three successive days to 4,500 ppm methanol for six hours daily. Blood samples were drawn at 1 and 6 hours during exposure, and at 1 hour following the cessation of exposure. Methanol was not detected in the blood samples obtained 30 minutes prior to the first day of exposure, or at 17 hours in the morning following each exposure (data from shown).

If degassing produced experimentally significant levels of methanol that could compromise the control group, they would be reflected in blood methanol concentrations. To examine this possibility, the following experiment was conducted. First, at the end of a standard six-hour exposure, blood was obtained from subjects of both the 4,500 ppm exposure and control groups. Then, following the standard procedure, all rats were returned to the vivarium. Two cages, each containing a spare lactating female and her pups that had never been removed from the vivarium (vivarium controls), were placed between the cages of the subjects from the exposure group. One hour later, blood was obtained from dams of the standard control group and from both dams and pups of the vivarium control group. Although the blood methanol concentrations obtained from the methanol-exposed dams and pups were 0.48 and 1.33 mg/mL, respectively, within the limits of the gas chromatographic analysis (10 ng/μL), no methanol was detected in the blood of either the standard control group or the vivarium control group. Environmental degassing of methanol, therefore, did not compromise the validity of the control group used in these experiments.

DETERMINATION OF BLOOD METHANOL CONCENTRATIONS

Sample Preparation and Blood Collection

Blood samples during the experimental phase were collected by tail nick from selected methanol-exposed and control dams on GD 7, 13, and 19; from both dams and pups on PND 7, 14, and 21; and at various times up to PND 52 from dams and pups of Cohort 4. For Cohorts 1 and 2, 100 μL of blood was collected from either the nicked tail vein of adult rats or the cut jugular vein of pups into a 1-mL microfuge tube previously prepared by adding 200 μL of 10% trichloroacetic acid and 100 μL of the 1-propanol (20 mg/mL) internal standard. It was vortexed and then spun at 3,000 rpm for five minutes in a table-top microfuge centrifuge. Next, 0.2 mL of the supernatant was pipetted out into two microfuge tubes to produce duplicate samples. Tubes were then frozen (–20°C) for later methanol determinations. Although gas chromatographic analysis was carried out at variable periods following sample collection, the consistency of the results confirmed the adequacy of the storage procedure. Apparatus problems precluded the analyses of blood levels from Cohort 3.

For Cohort 4, because of the acquisition of a new gas chromatograph, the procedures were modified. After the blood was collected into calibrated 100-μL heparinized capillary tubes, it was expelled directly into cold 1.5-mL capped conical centrifuge tubes containing 200 μL of 10% trichloroacetic acid and 100 μL of internal standard acetonitrile. The tubes were spun at 5,000 × *g*, and the trichloroacetic acid extract was removed and stored frozen in tightly capped 0.5-mL microfuge tubes at –20°C until analysis by gas chromatography. Sets of standards made from blood spiked with varying, known amounts of methanol and blood standards without methanol were prepared each time an experiment was conducted. These samples were stored under the same conditions and for the same duration as extracts obtained from the methanol-treated animals. Analyses of the standards demonstrated that storage at –20°C did not degrade the experimental samples.

When the results from Cohorts 1 and 2 showed that the blood methanol concentration of the pups exceeded that of the dams, we decided to examine more closely the relation between blood methanol concentration and age using subjects from Cohort 4. At the time of weaning, we had already designated subjects for the adult phase of the behavioral testing. We constructed the following design for the remaining subjects.

On six different days, pups selected from those remaining were exposed once to 4,500 ppm methanol for six hours. Across all six days, the same two dams, one from the exposure group and one from the control group, were exposed. On any one day, male-female littermates from both the originally exposed and control groups were exposed; i.e., in this experiment only, "control" designates the history of such subjects. All subjects were exposed to 4,500 ppm methanol in this procedure. A total of 9 pairs from six different exposure-group litters, and 13 pairs from eight different control-group litters formed our sample. No litter contributed more than one pair to an individual exposure day. No litter contributed more than two pairs to the protocol. Because pups were not all born on the same day, on any given exposure day during this procedure, ages spanned a range of three to five days. Dates of exposure and litters for that date were selected to provide postweaning ages that extended to 52 days. This procedure, therefore, allowed us to examine how blood methanol concentrations changed as age increased. It also provided an opportunity to evaluate the potential roles of gender and previous exposure history. In addition, because the weight of the subjects was known, possible relations between blood methanol concentration, weight, and an estimate of surface area based on weight could be examined.

Standards Preparation

For Cohorts 1 and 2, stock solutions of HPLC-grade methanol (20 mg/mL) and propanol (20 mg/mL) were prepared with double-distilled water. From these stock solutions, a set of working standards of methanol at 50, 100, 200, 500, and 800 μg/mL, combined with the same concentration of propanol, was prepared weekly. The standards for methanol and propanol at 5, 10, and 20 μg/mL were prepared by diluting to 1:9 the standards for 50, 100, and 200 μg/mL, respectively. The standards for 1 and 2 μg/mL were prepared by diluting to 1:9 the standards for 10 and 20 μg/mL, respectively.

A stock solution of propanol internal standard at 789 μg/mL was prepared by using HPLC-grade propanol and diluting it with double-distilled water. We added 100 μL of this solution to each blood sample as an internal standard. During the gas chromatographic analysis, the peak area of the internal standard was monitored and recorded for each injection. Deviations of more than 10% from the expected value for the internal standard resulted in rejection of the methanol concentration data obtained for that injection.

For Cohort 4, an initial methanol stock standard (10 mg/mL) was prepared by weighing 1 g of HPLC-grade methanol in a 100-mL volumetric flask and adding double-distilled water to a final volume of 100 mL. Serial dilutions of the methanol stock standard were made to produce aqueous standards ranging from 2,000 to 100 μg/mL of

methanol. The internal standard of acetonitrile was prepared by dissolving 30 μL of acetonitrile in 100 mL of glass-distilled water.

Gas Chromatography Conditions

For samples from Cohorts 1 and 2, blood methanol was analyzed on a Packard 2000 gas chromatograph equipped with a flame ionization detector, chart recorder, and reporting integrator. A column (15 m × 0.53 mm i.d.) of wide-bore fused silica BP-20 with a film thickness of 1.2 μm (SGE, Austin, TX) and a precolumn SGE Retention Gap Kit with tubing measuring 2 m × 0.53 mm i.d. (Fisher Scientific, Pittsburgh, PA, Catalog No. SG-052296) was used in the chromatographic separation of methanol and ethanol from whole blood. The carrier gas (hydrogen) was delivered to the column at the rate of 8.5 cc/min. Nitrogen was delivered as the detector makeup gas at the rate of 30 cc/min, and hydrogen and air were delivered for flame ionization detector combustion at the rate of 30 and 240 cc/min, respectively. The injector and detector temperatures were maintained at 150°C and 250°C, respectively. Chromatography was conducted by ramping the column temperature. The initial column temperature was 45°C, where it was held for two minutes; the temperature was then ramped to the final temperature of 150°C at the rate of 15°C/min; the final hold time at this temperature was one minute. Assay standard curves for the exposed group were constructed from the standards of 50, 100, 200, 500, and 800 μg/mL by injecting 1.0 μL of each standard in the column. For the control group, standards utilized were 2, 5, 10, 20, and 50 μg/mL. Peaks were traced by the recorder, and the counts for peak area were cumulated by the integrator. Duplicate determinations were made on each sample and standard. The means ± SD of the values were used for the calculations.

A linear regression analysis provided a best-fit line to the peak area counts: concentration data of methanol standard solutions. The concentration of methanol in each sample was then calculated from its peak area using the regression equation.

The trichloroacetic acid blood extracts from Cohort 4 samples were analyzed with a Hewlett-Packard Model 5890 Series-II gas chromatograph by direct aqueous injection of 1 μL onto a 6 ft × 2 mm i.d. (dimensions as given in the manual) glass column (Supelco 60/80 Carbopack B/5% CARBOWAX 20M). The column temperature was 80°C; the helium carrier gas flow rate was 21 mL/min; the injection port temperature was 150°C; and the flame ionization detector was kept at 260°C.

Blood methanol concentrations were calculated by substituting the integrated peak areas from duplicate blood samples into the regression equation developed from aqueous methanol standards ranging from 100 to 2,000 μg/mL. The peak area of the internal standard acetonitrile was used as an indicator of injection precision. Samples were rerun if the acetonitrile peak area varied more than 10% from the expected value.

Interlaboratory Quality Control Assessments

As part of the quality control assurances of the research program, this laboratory participated in three HEI-sponsored interlaboratory studies designed to confirm the overall adequacy of the blood methanol determinations and to estimate the variation in the measurements among the four participating laboratories. Both gas chromatographs in our laboratory were used, the Packard 2000 in the first two assessments, the Hewlett-Packard 5890 in the third. Methanol concentrations ranging from 1 to 100 μg/mL in both blood and water samples were produced by direct mixing by an investigator from one of the laboratories, or blood methanol concentrations were produced by exposing rats for six hours to either 1,500 ppm or 4,500 ppm. Coded samples were analyzed by investigators. At values greater than 10 μg/mL, the coefficient of variation fell below 25%. No systematic attempt was made to identify interlaboratory sources of variation.

BEHAVIORAL PROCEDURES

The behavioral testing was divided into two phases. During the neonatal phase, which also coincided with continuing exposure, we examined relatively simple endpoints that could be measured in pups. During the adult phase, we studied complex motor and cognitive performance to assess the long-term legacy of perinatal methanol exposure. Throughout this phase, the maintenance diet consisted of Purina RMH 5001.

Subject Assignments

Litters consisted of four male-female pairs that had been assigned subject numbers 1 through 4 in the process of culling. This subject number determined the test assignment. Only one pair from a litter was assigned to an individual test. For neonatal testing, all litters were tested from Cohorts 3 and 4. Pups from Cohort 1 were used to develop our procedures for the suckling test and the conditioned olfactory aversion test; their results were not included in subsequent analyses. Subjects from Cohort 2 were used to develop the motor activity test system. For the adult phase, only litters not used for the blood sampling conducted

during exposures were studied. From those litters, half were assigned to the fixed-ratio wheel-running test, and the other half to the stochastic spatial discrimination test.

Neonatal Phase

Suckling Test The suckling test was selected because it simultaneously tests several functional capacities of the very young neonate. It was used by Infurna and Weiss (1986) for a prenatal methanol exposure study in which methanol was administered via the dam's drinking water, and by Chen and associates (1982) for studies of prenatal ethanol effects. On PND 5, immediately preceding the daily exposure session, a pup was placed with its snout in contact with the ventral surface of a dam anesthetized by an intraperitoneal injection of pentobarbital at 65 mg/kg of body weight, which also inhibits milk release. These dams came from the group bred specifically to serve in suckling tests, so that their own pups were approximately the same age as the tested pup. The latency to nipple attachment was determined with a maximum of three 2-minute trials allowed. Upon attachment, the observer signaled the person recording the data, who registered the latency. Attachment was confirmed by gently tugging on the pup to ensure that the nipple had been grasped in the mouth. If not confirmed, the trial was repeated a maximum of two more times. Latency to confirmed attachment, defined as the time between the start of a trial and attachment, was the dependent variable. Latency could take between 1 and 120 seconds. Two values were recorded for each trial: "latency" and "confirm."

Conditioned Olfactory Aversion Test Neonatal rats undergo rapid changes in sensory and other behavioral capacities directly after birth. Because Infurna and Weiss (1986) found pup orientation to the odor of the home cage to be diminished as a result of oral methanol treatment of the dam, we elected to examine odor discrimination in the current experiment. A procedure that offers both more control over stimulus qualities and additional measures, olfactory aversive conditioning has proved to be a sensitive way to assess odor discrimination capacities, learning, and memory in the neonate (Spear et al. 1989). The general procedure, carried out on PND 10, consisted of a training condition followed by a testing condition. During training, a pup was placed first into one chamber containing an olfactory stimulus or odorant, then into a second, identical chamber with a different odorant. While in the second chamber, the pup received brief aversive electrical stimulation through the grid floor. Placement into each chamber occurred four times in an alternating sequence with each placement lasting 20 seconds. After training, the pup was placed in the center region of a special three-compartment chamber containing each of the original odorants on opposite sides. Over three 60-second trials, the time spent in the center region, in the region containing the odorant paired with the shock, and in the region containing the odorant unpaired with the shock was recorded.

On PND 10, one male and one female from each litter were removed before the methanol exposure session. They were housed together in one breeder cage kept at approximately 31° to 33°C until testing, then placed in a different cage after testing. They were returned to their litters after all subjects had been tested that day, and after the methanol exposure had been terminated for that day. The PND 10 pups were tested in a randomized order.

The apparatus consisted of two conditioning chambers (12.7 × 9.5 × 12.8 cm). Each consisted of two aluminum walls and two plastic walls and top. The floor consisted of 2-mm metal rods aligned 0.6 cm apart. The odorant was held in a tray 3.7 cm below the floor surface. In one chamber, the floor was connected to an electrical-stimulus source (Coulbourn Programmable Shocker Model E13-35, Coulbourn Instruments, Lehigh Valley, PA) with alternate rods wired in common to the two poles of the source, which provided the conditioned stimulus (CS^+). Each chamber was housed in a larger wooden chamber with a ventilation blower used to exhaust the chamber air into an exhaust hood.

The test chamber (38 × 20 × 14 cm) was divided into an upper and a lower section. The lower section was constructed of plastic with a divider evenly separating its length. Window screening, which was mounted on a wooden frame resting on the bottom section, served as a floor for the pup. The floor was marked into three regions, a central 2-cm region and two side regions, identified as the CS^+ (paired) and CS^- (unpaired) regions. The upper 10-cm-high section was made of plastic and was placed on the screen floor to enclose the test chamber.

Two odorants, orange oil and methyl salicylate (Humco, Walmead Industries, Texarkana, TX), were used as stimuli. A 0.5-cc volume of odorant was injected onto a 5 × 5 cm pad of nesting material (Neslets, Ancare Corp., Manhasset, NY). The methyl salicylate was used as the paired odorant in the CS^+ chamber; the orange oil was used as the unpaired odorant in the CS^- chamber. A pad of each odorant was placed also into the test chamber at the end of the designated region. The locations of the odorant pads were not counterbalanced across either trials or subjects. Such switching most likely would have produced increased mixing of the airborne odorants, an outcome that would have prevented assessing the odor preference of the pup.

Training A pup was placed into the CS^- chamber, where it remained for 20 seconds. It was removed, held for 5 seconds, and then placed into the CS^+ chamber for 20 seconds. The electrical shocker was programmed to provide 0.5 mA of cutaneous electrical stimulation through the grid floor during seconds 8 to 10 and 18 to 20. The sequence of exposures to CS^- followed by CS^+ constituted one training trial. Four such sequences of trials were conducted, with the time between trials being about 2 to 5 seconds for Cohort 2 and 20 seconds for Cohorts 3 and 4.

Testing Testing began immediately following the final CS^+ trial. The pup was placed in the middle of the center region of the test chamber, oriented toward a side wall, and the lid was placed on top. Regardless of its location, at the end of 60 seconds the pup was removed momentarily from the chamber and placed again in the center region of the chamber, and the next trial was started. Three 60-second trials were conducted in this manner. Pup location was defined as that region where both the head and torso were located (the presence of only one leg or the tail in a section was not sufficient for designating that section as the pup's location). Time spent in each section was recorded by the investigator. Cumulative seconds in each region summed across trials constituted the dependent variable.

Preliminary Assessment Olfactory aversion conditioning (or learning) is demonstrated during the posttraining test by a preference for the region sited over the stimulus unpaired with electrical shock. In a preliminary investigation, we examined the roles of several training variables on test performance: presence versus absence of the odor during training; shock intensity; number of training trials; and duration of the intertrial interval. The degree of conditioning was presumed to depend on the combined influence of these variables. For these studies, PND 10 pups obtained from the dams used as test dams for the suckling test served as the subjects.

A subject was tested once under one of the eight conditions evaluated for effectiveness of training. The number of subjects tested, the number that left the center region, and the mean difference in time between the side on which odor was not paired with shock and the side on which odor was paired with shock (Unpaired – Paired) are shown in Table 2. Throughout the preliminary investigations the test conditions remained essentially equivalent to those used in the standard procedure. The test for learning always consisted of three 60-second trials. The stimuli also remained fixed, as in the standard procedure, with methyl salicylate assigned as the odor paired with shock and orange oil as the neutral (unpaired) odor, including the test for subjects trained under the no odor condition.

Table 2 shows that learning occurred under the conditions used in our standard procedure. When the odor was not present, so that a learned association could not have occurred, or when the shock intensities were lower, the subjects either did not move from the center region of the test chamber or showed a slight bias toward the stimulus paired with shock (methyl salicylate) in the experimental protocol. The fact that learning was demonstrated with the parameters selected for the experiment proper showed that the preparation was adequate for determining whether our conditions of methanol exposure affected learning in the neonatal rat.

Motor Activity Procedure We introduced a test to measure general motor activity into the general protocol following the recommendations of the HEI Research Committee

Table 2. Conditioned Olfactory Aversion Test: Training Variables

Shock Intensity (mA)	Number of Trials	Intertrial Interval (sec)	Odor Present	Number of Rats	Rats Spending Less Than 180 Seconds in the Center	Mean Difference (Unpaired 7 – Paired)
0	2	2	No	3	1	–28
0.50	2	2	No	3	1	–77
0.35	4	2	Yes	6	4	–3.25
0.40	4	2	Yes	2	1	–11
0.50	4	2	Yes	14	10	29.2
0.75	4	2	Yes	4	4	48.5
0.40	4	20	Yes	5	5	41.4
0.50	4	20	Yes	5	4	52.5
0.60	4	20	Yes	5	5	47.6

after a site visit. By that time, subjects from Cohorts 1 and 2 were older than stipulated by the test protocol. For Cohorts 3 and 4, one male and one female from each litter were assigned for testing. Subjects were removed from their litter on PND 18 before that day's methanol exposure session. The subjects to be tested were held in a single breeder cage and transported to the room in which the test was conducted. After the completion of testing, they were returned to the vivarium. They rejoined their litters after the exposure session for that day had ended, and the home cages were transported back to the vivarium. The same subjects were retested on PND 25. On this occasion, because the subjects were housed individually after weaning on PND 21, they were transported in their home cages to the testing room.

The test apparatus consisted of an annulus-shaped Plexiglas chamber (17.8 cm high, 13.3 cm i.d., and 25.3 cm o.d.). The subject was contained in the annulus, which was 62 cm long (calculated as the circumference at the midpoint between the inner and outer walls). A Plexiglas lid covered the chamber. The floor of the chamber was constructed of 3-mm metal rods evenly spaced between the chamber walls and perpendicular to the outer wall. Photodiode emitters and detectors evenly spaced 2.2 cm above the chamber floor divided the chamber into 47 equal segments. Any interruption of a beam following 100 msec since the last beam break was recorded as a movement. The activity chamber rested in a sound-attenuating enclosure.

Data acquisition and control were accomplished by the SKED operating system (Snapper et al. 1982) implemented on a PDP-11 computer system (Digital Equipment Corp., Maynard, MA). The rat was placed into the chamber, the door was closed, and the 15-minute session was started. Data were recorded as counts (i.e., photodetector events) for each minute of the 15-minute session. A 15-minute period is typically long enough to encompass an early phase of heightened activity succeeded by gradual diminution to a relatively sedentary phase (Stanton 1994).

Adult Phase

Subjects were randomly assigned to the two adult-phase tests with the restriction that, whenever possible, equal numbers of male-female littermate pairs were tested under the two procedures. Four test chambers, or apparatuses, were used in each test. Within each test, two were assigned exclusively to subjects of each gender. Assignments were structured to ensure as much as possible that equal numbers of methanol-exposed and control subjects were tested in each chamber. Each subject was tested in its designated chamber at its designated time of day, which remained fixed throughout the experiment. Male-female littermate assignments to time-of-day of testing were balanced across treatment groups.

Fixed-Ratio Wheel-Running Procedure A long history of research in psychobiology shows that the daily activity cycles of rodents can be monitored by giving them access to running wheels (Munn 1950). Because running is such a prepotent activity, it is also easy to use as a trained response (Tepper and Weiss 1986). In our study, schedule-controlled operant running served two roles. First, it provided a measure of motor function intactness, an important endpoint because many developmental neurotoxicants, such as ethanol (Streissguth et al. 1986) and methylmercury (Cox et al. 1989), produce deficits in coordination and strength. Second, it provided a means for assessing schedule-controlled operant behavior, considered by the U.S. EPA (1991) to be a particularly useful index for evaluating neurotoxicity.

The wheels were designed specifically to be sensitive to motor deficits produced by neurotoxicants rather than as instruments for monitoring locomotor activity. Kulig and colleagues (1985) devised a similar modification of the conventional wheel to assess impaired coordination. The rat was required to grip bars spaced every 15 degrees around the internal circumference. To propel the wheel, the rat had to grip one rod with a front paw and thrust against another rod with a hind paw in a coordinated series of movements. The wheel diameter of 60 cm was chosen to approximate more closely a level surface than the smaller (45-cm) conventional wheels. The wheel rotated around a bearing with adjustable friction to prevent coasting. Pellet feeders and cups were mounted on each wheel support frame together with levers, an audio device, and lights. Each set of two wheels was enclosed in a separate chamber for light and sound attenuation and provided with broad-spectrum noise to mask irrelevant sounds. The wheels were adjusted to allow rotation in only one direction. Each complete 360° rotation was recorded by the closing of a reed switch activated by a small magnet attached to the wheel. Each activation counted as a single response. The time between switch closures (designated as an interresponse time or IRT) was recorded with 10-msec resolution.

The SKED system controlled experimental events and data acquisition. Each event and response was recorded in real time. The rats were trained in steps. At the beginning pellets were placed in the pellet cups to encourage exploration. Response requirements were gradually increased until the final criterion of 20 full rotations on the wheel for each reinforcing event (a fixed ratio [FR] 20 schedule of reinforcement) was imposed for females and an FR 28 schedule was imposed for males. These ratios were selected

on the basis of our experience with the procedure, which showed that stable performance could be maintained at these values within gender.

During testing, each rat assigned to this task was placed into one of the four running-wheel chambers for one hour daily, five days per week. Each time a rat attained the criterion number of rotations, it received two 45-mg food pellets delivered to a tray reached through a small port in the side of the running-wheel chamber. Body weight for the males was maintained at 300 g, and for the females at 220 g. At these weights, Long-Evans rats exhibit substantial rates of behavior to obtain food reinforcements. The gender difference in FR criterion is at least partly a function of these differences in body weight.

Performance engendered by this procedure was evaluated by calculating the overall rate of responding reported as responses per minute. Differences due to methanol treatment were expected to emerge as differences in the rate of responding. Compensatory adjustments in attaining the steady-state baseline performance (i.e., responding under FR 20 for females and FR 28 for males), however, may mask potential underlying functional deficits. To provide further opportunities to detect such covert deficits, behavioral challenges were introduced by raising the criterion number of rotations for food-pellet delivery at specified times. The increase in criterion value consisted of an elevation in the baseline FR value once per week, expressed as increments across successive weeks of 25%, 50%, 75%, and 100%. The standard baseline condition was reintroduced the next day. Finally, during each daily session of the fifth week, no food pellets were delivered (extinction).

Stochastic Spatial Discrimination Test To provide an assessment of cognitive functioning, a complex spatial discrimination test was based on the rat's ability to learn and to modify its behavior in accordance with transition probabilities determined by sequences of responses. Learning of response sequences, such as delayed spatial alternation, is frequently used as a measure of memory (Eckerman and Bushnell 1992). The current scheme provided another level of complexity by introducing stochastic relations. The data were collected in standard operant chambers (Coulbourn) containing three levers on the front wall and a 45-mg pellet dispenser on the back wall.

After preliminary lever-press training, the rats were allowed to respond on any of the three levers in the experimental chamber. Pellet delivery for pressing any one of the three levers was determined by a relation in which the probability of pellet delivery after pressing a particular lever depended on the location of the previous lever-press (Weiss and Heller 1969). As shown in Figure 5, certain sequences maximized the probability of pellet delivery. After responding had stabilized under the first payoff matrix (Matrix 1), the matrix entries were changed as shown in the second payoff matrix (Matrix 2). The primary meas-

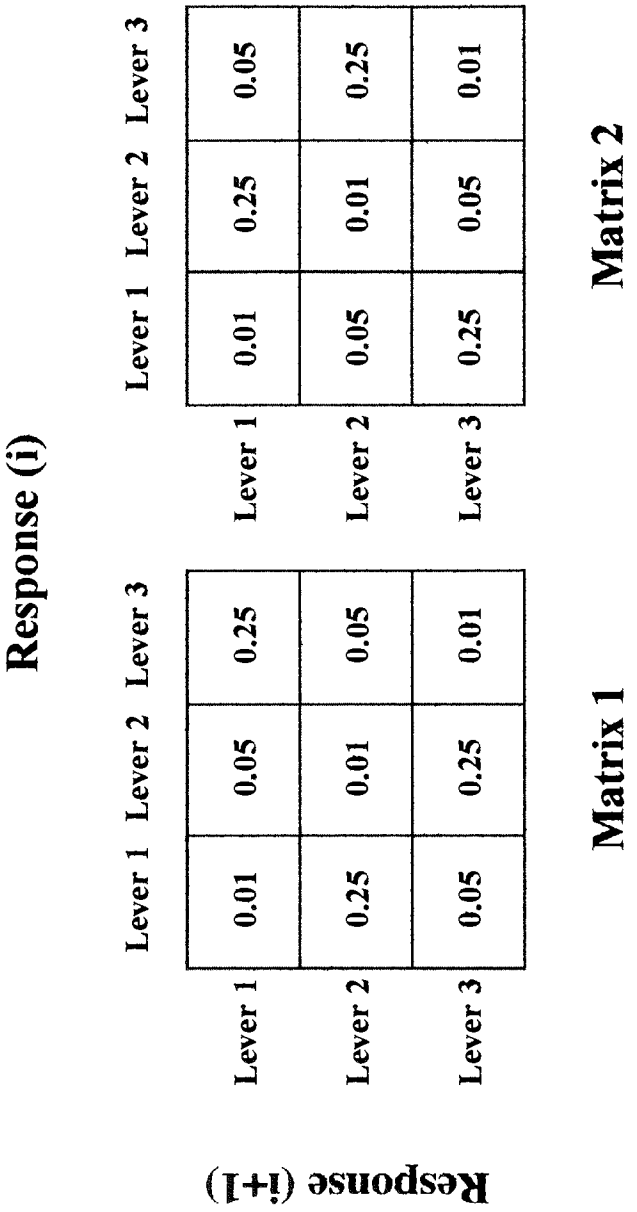

Figure 5. Scheme governing the stochastic spatial discrimination test. In the operant test chamber (Coulbourn) three levers were arrayed on the front wall. A pellet delivery device and cup receptacle were built into the back wall. Each time the rat pressed a lever, a random number generator was used to determine, on the basis of the location of the previous lever-press, and its accompanying probability, whether a pellet would be delivered for the response. Under Matrix 1, the optimal sequence would be Lever 1→Lever 2→Lever 3→Lever 1, etc. Under Matrix 2, it would be Lever 1→Lever 3→Lever 2→Lever 1, etc.

ure of performance consisted of a function describing the rat's adjustment to the reinforcement contingencies over the course of successive sessions.

MORPHOLOGICAL ASSESSMENT

Neuropathology

Selected pups from Cohorts 2 and 3 were killed on either PND 1 or PND 21. Both full and half brains were taken for analysis. Samples were fixed by immersion in Bouin's solution for a minimum of one week prior to processing. Bouin's solution is a rapid fixative well suited to brain and to tissues of juvenile animals. All samples were received at Rutgers University with the codes assigned at the University of Rochester. Upon receipt, the samples were assigned accession numbers by the pathologist (KRR), who revealed the code only after the slides had been read. Full brains were cut down the sagittal midline. The left hemisphere was then cut into three sagittal sections with the aid of a Plexiglas template to yield reproducible sections. The right half was cut into four sections along coronal landmarks. The samples were then embedded to provide one Paraplast (Oxford Labware, St. Louis, MO) block each of the right and left hemispheres. For half brains, the samples were cut in coronal sections. The blocks were cut serially with every tenth section at 6 μm, until 40 sections had been collected. They were not serially sectioned in their entirety because such sectioning is unnecessarily time-consuming and does not leave tissue available should other techniques requiring thicker sections be necessary at a later time. Every fourth section was stained by hematoxylin/eosin/phloxine, yielding 10 hematoxylin and eosin–stained slides per animal. Two slides were stained with Luxol fast blue/periodic acid–Schiff (LFB/PAS), and other techniques were used as suggested by preliminary study. All slides for each animal were read without knowledge of treatment assignment. The absence of significant anatomic differences obviated the need for detailed damage assessment scales.

Three features were specifically examined: (1) delayed neuronal migration, which would be evident as heterotopic rests, thickened germinal zones, neurons arrested within the migratory zones, or all of these; (2) increased numbers of apoptotic cells in the cortex or germinal zones; and (3) defective myelination. The first two features are evident in hematoxylin and eosin–stained tissue, provided the effect is sufficiently robust, and LFB/PAS is a reasonably good myelin stain.

Analysis of Neural Cell Adhesion Molecules

Development and maintenance of the nervous system depends on the proper temporal and spatial expression of several families of membrane glycoproteins collectively termed cell adhesion molecules. Cell adhesion molecules mediate the complex cell-cell and cell-substrate adhesion systems responsible for migration, axonal outgrowth, and establishment of the complex connective patterning that underlies mature neuronal function. The NCAM is the best characterized of the adhesion molecules. A growing body of evidence indicates that specific isoforms of NCAM, such as NCAM 140 and NCAM 180, appear at specific developmental stages and that perturbation of their temporal expression may be involved in brain dysgenesis (Edelman and Chung 1982).

Neural cell adhesion molecules are members of the immunoglobulin superfamily (Yoshihara et al. 1991) and function as homophilic receptors in forming cell-cell bonds. They are encoded by a single gene, but several isoforms of differing molecular weight are formed by differential splicing (Cunningham et al. 1987). Three major isoforms have been identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE); these three isoforms have identical extracellular domains and differ only in the length of the intracellular component. The longest intracellular domain, which may communicate directly with elements of the cytoskeleton, belongs to NCAM 180. The intracellular domain of NCAM 140 is short, while NCAM 120 lacks an intracellular domain entirely and is attached to the plasma membrane via a phosphatidylinositol linkage (He et al. 1987).

During development, the extracellular domain of NCAM is heavily glycosylated; these polysialylic acid residues may contribute up to 30% of the molecule's weight. The sialylic acids impart to the molecule a negative charge that inhibits close membrane-membrane apposition of cells. This embryonic NCAM state is essential for cell migration and for permitting movement of cells relative to one another without static adhesion (Rutishauser et al. 1988). When neurons reach their final postmigratory destinations, the embryonic NCAM isoform is replaced by a poorly sialylated adult NCAM. This isoform lacks the repulsive negative charges and consequently favors the establishment of persistent cell-cell contacts.

We chose to examine NCAMs for the following reasons: First, temporal disturbance of brain NCAM has been documented following developmental exposure to ethanol and to neurotoxic heavy metals (Regan 1989, 1993; Dey et al. 1994; Reuhl et al. 1994). Second, persistent expression of a juvenile NCAM isoform in the brain of treated animals after its normal down-regulation might serve as a marker of injury even in the absence of pathological evidence. Finally, NCAM assays are sensitive to diminished or defective synaptogenesis; migration disturbances are generally assayed by light microscopy.

Frozen samples of rat brain from neonates killed on PND 4 and at 15 months of age were divided into cerebellum and cerebrum and homogenized in 1× sample buffer of 0.0625 M tris(hydroxymethyl)aminomethane (Tris)–HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% mercaptoethanol, as described by Laemmli (1970). The total protein concentration of each sample was measured on a COBAS FARA II (Roche Instruments, Nutley, NJ), using the Bradford (1976) dye-binding assay. Split samples were run with or without boiling for five minutes. Boiling cleaves the sialic acids from NCAM, giving more compact bands on electrophoresis and permitting better assessment of sialic acid status. Samples were run on a 7.5% polyacrylamide gel with a Bio-Rad Mini Protein II system. After protein separation, the gels were transferred onto nitrocellulose membranes (Towbin et al. 1979) prior to overnight incubation in primary antibody. Antibodies 5B8 (University of Iowa Developmental Studies Hybridoma Registry) and 0B11 (Sigma, St. Louis, MO), recognizing the different portions of the intracellular domain, were employed. Blots were then incubated with alkaline phosphate–linked goat antimouse IgG (Fisher Scientific), with cross-reaction to rat NCAM. Visualization of the bands was obtained using the 5-bromo-4-chloro-3-indole phosphate/nitroblue tetrazolium substrate system (Bio-Rad, Melville, NY).

Immunoblots were digitized using a Presage CV-6 Image Analysis System (Advanced Imaging Concepts, Princeton, NJ) into black-and-white images encoded as a sequence of integer values between 0 and 255. Images were acquired with a NEC TI-23 EX CCD camera, and numerical values were placed on the gray scale calculation by in-house algorithms.

STATISTICAL METHODS

Data for this series of experiments were analyzed primarily by repeated measures analysis of variance (ANOVA). Each such analysis included both between animal (grouping) factors and within animal factors. Between animal factors in these analyses were treatment (methanol or control) and, for some analyses, cohort (corresponding to replicate experiments). Within animal factors represented mainly repeated measurements on each animal (either of the same variable over time or of different variables corresponding to different conditions). In addition, in those analyses in which one male and one female were included from each of a number of litters, gender was included as a within animal factor (in which case the unit of analysis was really the litter). Any other within animal factors were crossed with the gender factor. For within animal factors having more than two levels (e.g., time, which had five levels in some analyses of motor activity), the ANOVA also included single-degree-of-freedom trend tests for linear, quadratic, and higher-order polynomial trends. (The highest degree is one less than the number of levels of the factor; for a factor with five levels, one can compute first- through fourth-degree trend tests.) Tests for interactions of these trends with between animal factors were also included.

Each analysis of variance is summarized by an appropriate ANOVA table (Appendix A), which lists sums of squares and mean squares for each of the main effects and interactions in the model, as well as appropriate F tests for the statistical significance of the corresponding effects. The Greenhouse-Geisser correction for degrees of freedom was used when appropriate. The Huynh-Feldt p value is also reported. In addition, each analysis included an examination of residuals as a check on the required assumptions of normally distributed errors with constant variance. The BMDP statistics software (BMDP2V) was used for all analyses except for one aspect of the stochastic spatial discrimination test. For this analysis, a special program had to be written to extract parameter estimates based on the shape of the acquisition function.

QUALITY CONTROL CONSIDERATIONS

Several features that helped ensure the quality of the research program have been discussed and are highlighted here. First, randomization procedures were used extensively to eliminate bias in selecting subjects for procedures. The randomization included the selection of the females for breeding and then exposure or control treatments, as well as assignments to home-cage locations in the vivarium. Subject selection for culling from litters was randomized as was the assignment of numbers that determined the subsequent treatments. Each male-female pair was assigned to only one test, so each litter contributed only once to the outcome of the behavioral test.

Interlaboratory studies evaluated methanol concentration analyses. Whenever possible, investigator-blind procedures were used in the behavioral testing. Data were not analyzed until after a behavioral procedure had been completed, thus keeping the investigator blind about outcomes. The neonatal motor activity test and the long-term behavioral tests were controlled by online computers, thus ensuring maintenance of standard conditions across days within cohorts and across cohorts, as well as eliminating any potential effect of interaction with the investigator during testing. In addition, standard computer-controlled procedures were implemented for data collection, analysis, and storage.

Statistical evaluations of the data were directed by a statistician and were conducted using standardized com-

mercially available computer programs. Standard operating procedures including those for data collection were examined by an independent auditor at the request of the sponsor. The HEI Research Committee consisted of an independent group of scientists with the expertise required for evaluating the program. An on-site visit by the group (July 31, 1991) produced useful feedback.

RESULTS

This section is organized, in general, in a sequence that proceeds from early to later developmental indices. It first presents the breeding data, then the blood methanol measures during the gestational and postnatal periods, succeeded by the results of the neonatal behavioral tests. It next presents the results of the behavioral assays conducted during the juvenile and adult stages. Finally, it discusses the morphological assessments. The influence of neonatal age on methanol uptake is particularly noteworthy, and significant interactions of treatment and gender are a recurrent theme.

BREEDING

Table 3 summarizes the breeding data for all four cohorts. Although differences between cohorts, especially between the first and subsequent cohorts, are readily apparent, control and methanol-exposed litters showed virtually identical litter sizes, gender distributions, and dam and pup weight gains. Cohort 4 exhibited the highest incidence of litters containing more than five pups.

BLOOD METHANOL CONCENTRATIONS

Samples were drawn from dams before parturition and from both dams and pups after parturition on the occasions shown in Figure 6. (Operational difficulties with the gas chromatograph left us with no data for the Cohort 2 dams during gestation; a replacement instrument became available only after the Cohort 3 exposures had ceased.) Maternal blood concentrations remained fairly constant during gestation (mean 0.55 mg/mL; SD 0.07) and lactation (mean 0.56 mg/mL; SD 0.09), although, for both Cohorts 1 and 4, values for GD 19 appeared lower than those secured earlier during gestation. Nelson and associates (1985) reported a similar decline, but in our case the difference was not statistically significant. The levels themselves are not substantially different from those reported by Nelson and coworkers for exposures of 5,000 ppm methanol.

For all three cohorts analyzed, the pups, before weaning, exhibited blood methanol concentrations (mean 1.26 mg/mL; SD 0.23) approximately twice those attained by their dams. One explanation for such differences might be the position of the pups in the breeder cages and their more intimate contact with the bedding; however, such an explanation seems unlikely because differences in pup size and activity between PND 7 and 21 are substantial. In addition to marked growth in size, the processes of sensory and motor development equip the neonates with rapidly expanding visual, auditory, and locomotor capacities.

Figure 7 supports another explanation for the difference in blood methanol concentrations. Subjects from Cohort 4 not used in the adult-phase behavioral testing were used to evaluate how blood methanol concentration, following a six-hour exposure to 4,500 ppm, varies with age up to PND 52. Even after weaning and assignment of pups to individual cages, the dam-pup differences persisted until about PND 48.

These data induced us to pursue further analyses of the relation between blood methanol concentration and age. The data from both the preweaning and postweaning blood methanol determinations in Cohort 4 are shown in Figure 8. (Data from PND 21 were not included because values for the dams fell substantially below those recorded for all the other days, and those for the pups also showed a major departure from the trend across time that was observed graphically.) For the postweaning assays, subjects from both the methanol-exposed and control groups that were not designated for later behavioral testing were exposed for one 6-hour session to 4,500 ppm methanol at a designated age. Data were available for age, weight, an estimate of surface area (9.85 $\times$ body weight squared), gender, and exposure history (treatment). We initially examined the question of whether any of these variables would predict the level of methanol using multiple linear regression. The results of the series of regressions indicated that age, weight, and surface area were highly correlated ($r > 0.90$), so that the independent effects of these three variables could not be determined. Second, no indication of any effect of gender or treatment appeared.

Two regression models were then considered to evaluate a possible relation between methanol blood concentration and age. (Age was selected because, in the absence of a known mechanism for the decline in blood methanol concentration, developmental changes based on age seem likely candidates.) The first model was a straight line; i.e., the data were analyzed by ordinary linear regression. The second, a four-parameter nonlinear regression model, hypothesized an initial plateau in the concentration of methanol, followed by a linear decline, which in turn is followed by a plateau at older ages. The four parameters were the

Table 3. Rat Breeding and Litter Summary[a]

	Cohort 1		Cohort 2		Cohort 3		Cohort 4		Mean of Cohorts (n = 4)	
Methanol (ppm)	4,500	0	4,500	0	4,500	0	4,500	0	4,500	0
Females designated pregnant (n)	10	10	12	12	12	12	12	12	11	11
Mean weight gain (g)[a]	78.17	99.83	86.75	93.67	103.28	105.25	93.81	99.54	90.50	99.57
Litters with more than 5 pups (n)	6	6	8	7	7	4	11	11	8	7
Mean pups per litter (n)										
M	6.00	7.33	6.75	7.86	7.43	7.25	6.36	6.36	6.64	7.20
F	6.33	5.83	5.25	6.28	7.00	6.75	5.91	7.73	6.12	6.65
Mean weight and 1 SEM (g)										
PND 1 M & F	6.20[b]	6.00[b]								
	0.32	0.21								
M			6.60	6.31	6.33	6.19	6.30	6.36		
			0.25	0.27	0.25	0.27	0.15	0.14		
F			6.38	5.80	6.01	5.60	6.01	5.95		
			0.20	0.22	0.22	0.21	0.17	0.11		
PND 4										
M	7.68	7.79	9.53	8.93	9.12	9.22	9.44	9.34	8.94	8.82
	0.38	0.54	0.36	0.27	0.37	0.68	0.39	0.26	0.43	0.36
F	7.31	7.38	9.19	8.36	8.81	8.40	8.98	8.78	8.57	8.23
	0.42	0.62	0.33	0.26	0.32	0.42	0.41	0.16	0.43	0.30
PND 11										
M	17.94	20.36	24.22	24.35	23.31	22.54	24.69	25.24	22.54	23.12
	2.39	1.29	0.58	0.47	0.43	1.26	1.07	0.41	1.56	1.08
F	17.31	18.93	23.49	23.23	22.88	21.38	23.67	24.86	21.84	22.10
	2.36	1.54	0.48	0.48	0.52	0.96	0.99	0.31	1.52	1.27
PND 18										
M	32.16	39.22	36.63	38.52	38.24	37.64	41.49	43.21	32.13	39.65
	4.22	3.09	0.80	1.12	0.96	2.14	1.43	0.66	1.94	1.23
F	34.37	36.76	36.48	37.28	37.56	36.07	40.21	42.10	37.16	38.05
	3.41	3.48	0.991	0.74	1.37	2.08	1.25	0.63	1.22	1.37

[a] Weight gain between GD 7 and GD 19 for females with litters of more than 5 pups.

[b] For Cohort 1 only, gender distribution was not determined until PND 4.

initial level, the threshold for the start of the decline, the slope of the linear decline, and the threshold for the second plateau.

The initial plateau was estimated to be 1.4 mg/mL (SD 0.05); the onset of the linear decline was estimated to occur at 11.22 days (SD 3.49); the estimated slope was −19.26 (SD 2.08); and the second inflection in the function was estimated to occur at 47.67 days. The final plateau was estimated to be 0.71 mg/mL, as determined by the threshold for the second plateau. From a statistical point of view, the four-parameter nonlinear model provides only a small improvement in fit to the data over the two-parameter linear regression in which the slope was −17.18 (SD 1.27) and the intercept was 1.56 (SD 0.04). More data with a greater range of ages would be required to provide a definitive description of the relation between age and blood methanol concentration.

Mean blood methanol concentrations for the two dams exposed during all six of the postweaning exposures were 0.58 mg/mL (SD 0.06) and 0.69 mg/mL (SD 0.06), respectively. These values showed no trends across the six exposures, which occurred from 2 to 31 days after weaning. The final concentration plateau of the offspring was above these values. Again, more data would be required to determine whether blood methanol concentrations would decline further with increasing age and the extent to which individual differences could account for this difference.

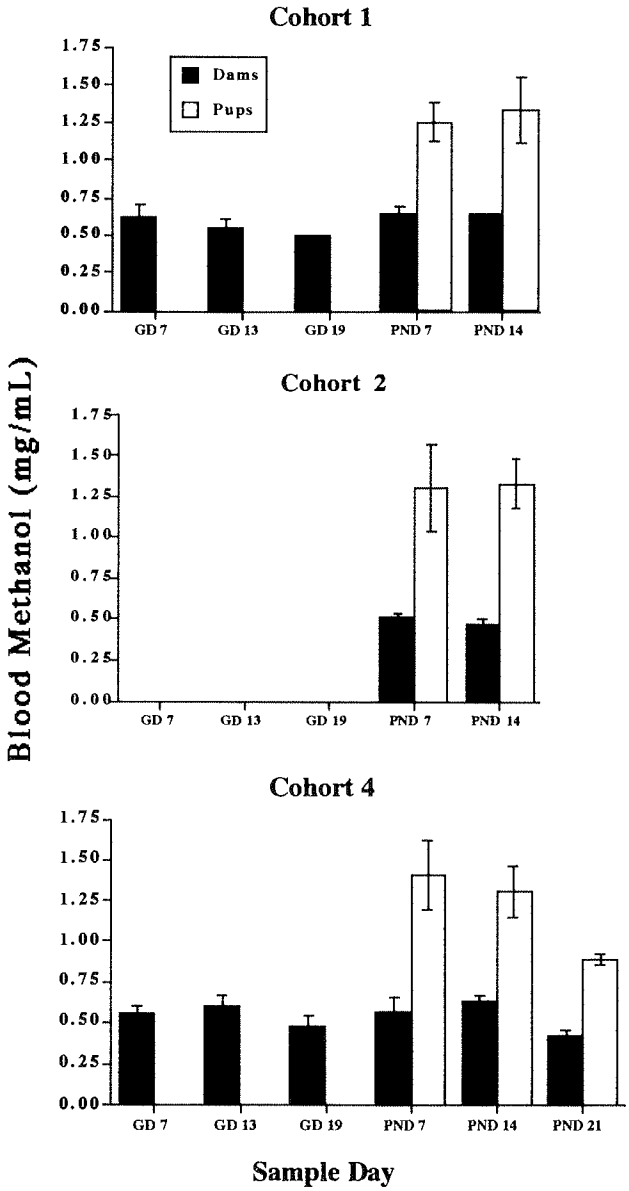

Figure 6. Blood methanol concentrations (mean ± SD) achieved at the end of the six-hour exposure period in dams and pups during gestation and lactation, on the days indicated, for Cohorts 1, 2, and 4.

BEHAVIORAL INDICES

Table 4 summarizes the results of the various test procedures, and for each, Table 5 shows the number of subjects studied from each cohort. An outcome was considered compelling when it occurred reliably across cohorts. Because the variety of outcome measures necessitated many statistical analyses, it might be argued that, on the basis of chance alone, more conservative estimates of significance than those relied on here would have been more appropriate. Such an argument normally arises with multiple tests of a single hypothesis. The current project, in contrast, offered a different rationale for selecting each of the different test situations. Such an approach is the dominant strategy in neurobehavioral assessment. Most typically, investigators rely on a battery, or collection, of tests whose aim is to provide a comprehensive survey of many different functions (for example, see Paule 1984). For the current

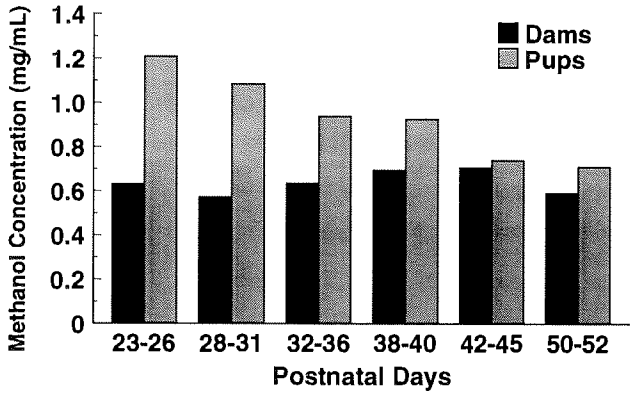

Figure 7. Postweaning blood methanol concentrations achieved at the end of the six-hour exposure period in dams and pups from Cohort 4 on the days indicated. Offspring had been maintained in individual cages since PND 21 (weaning).

investigation, each test situation was deliberately selected to represent an evaluation, to the degree possible, of an independent behavioral process. We did not hypothesize that if methanol affected behavior under one test, it necessarily should have done so under the others. Such independence was considered to be an asset in the design of the research program because we sought to determine which endpoints were sensitive to methanol exposure during development. Implementing a mechanistic study would have been premature in the absence of widely recognized and documented effects at the concentrations proposed for assessment.

Statistically significant differences are defined as those yielding a value of $p < 0.05$. When the term "differences" is used in this section of the report, it refers to those that are statistically significant. Complete ANOVA tables are given in Appendix A. In each ANOVA table this p value is shown in the column heading "p" and under the column headings for the two corrections to degrees of freedom (Greenhouse-Geisser and Huynh-Feldt) when these have been applied. Within each ANOVA table, "Cohort" refers to differences between the four cohorts, and "TRT" (treatment) refers to methanol exposure or control. To facilitate examining the ANOVA tables, when p values are mentioned below, the corresponding row label of the ANOVA table is included with that p value in parentheses. A complete key of abbreviations is given with each table.

Neonatal Phase

Suckling Test Latencies to nipple attachment are plotted in Figure 9. Considerable variability within exposure conditions is apparent. There was no effect of methanol expo-

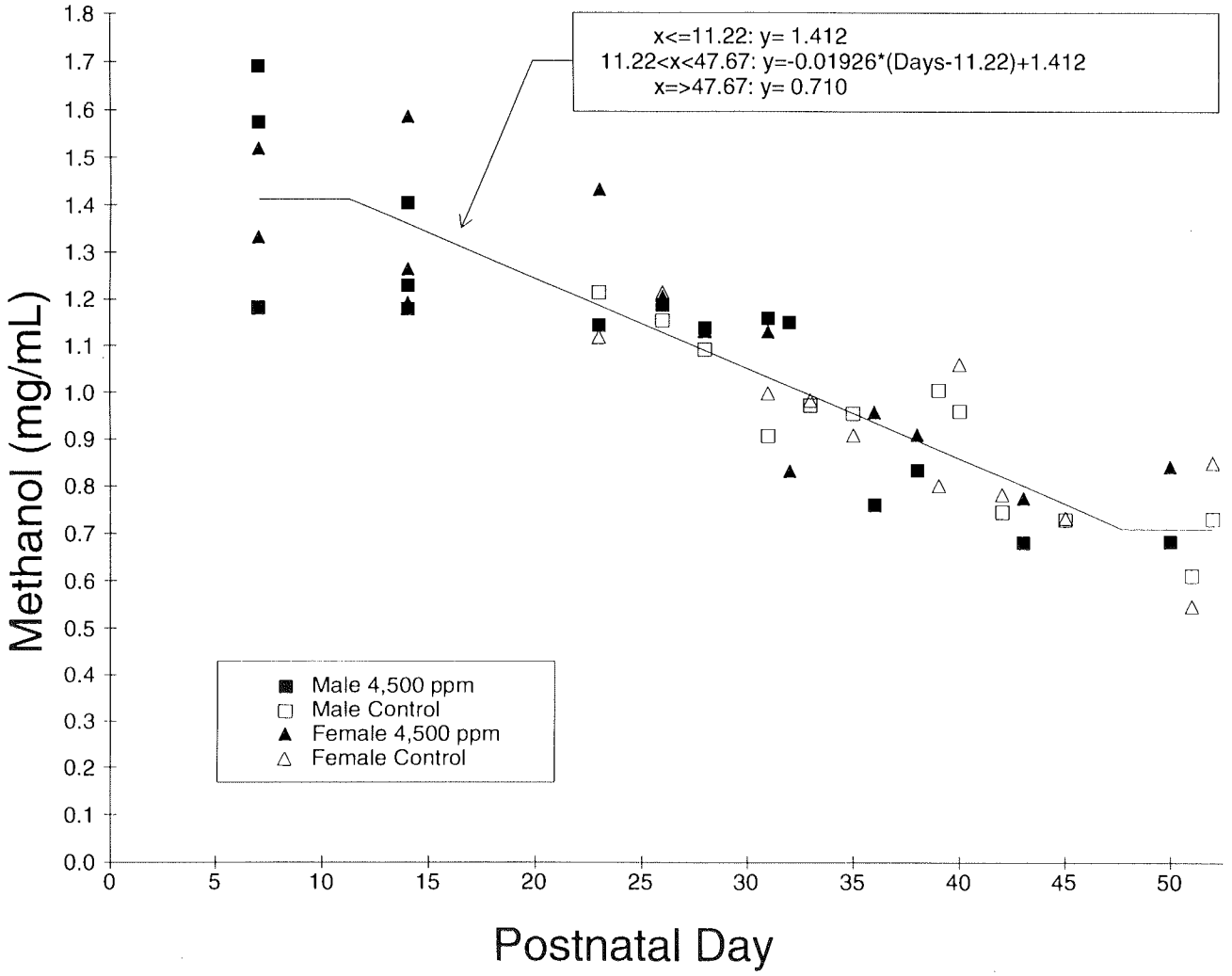

Figure 8. Nonlinear regression function fitted to offspring blood methanol values between PND 7 and PND 52. Exposures to 4,500 ppm methanol lasted six hours.

sure on the pup's latency to attach to the nipple of the test dam (TRT, $p = 0.59$; Table A.1). Comparisons between cohorts were not conducted because the number of pups attaching was not sufficient for such an evaluation. The within-litter comparison showed no difference between males and females, and there was no interaction between exposure history and gender.

Table 6 shows the proportion of subjects that attached to the nipple. Attachment rates for males and females were analyzed separately by logistic regression. Each analysis included a test for consistency of methanol effect across the three cohorts (test for interactions). There was no effect of methanol in males ($p > 0.05$), and the effect was not consistent across cohorts in females ($p = 0.03$); i.e. there was no consistent effect of methanol ($p = 0.24$).

Conditioned Olfactory Aversion Test Figure 10 plots the amount of time spent in the three test areas. Mean data are shown in Table 7. "Paired" designates the test chamber area above the pad containing the odor present during the aversive electrical stimulation. "Unpaired" designates the test chamber area, on the opposite side, above the pad containing the neutral odor. For all cohorts, the 10-day-old pups spent most of their time in the center area. They displayed a bias, however, in favor of the unpaired, or neutral odor, indicating that the association of the paired odor with shock had been learned. No exposure-related differences emerged, however.

Two questions were addressed in the statistical analysis. The first was whether exposure to methanol affected the comparative distribution of times spent in the shock-paired and the unpaired, neutral odor regions of the test apparatus. The analysis indicated that exposure to methanol did not affect the relative preferences (TRT, $p = 0.82$; Table A.2). No differences between cohorts emerged, nor was the cohort × treatment interaction significant. Similarly, a within-litter (i.e., gender) effect was not apparent, nor did other significant interactions appear. We conclude that methanol exposure did not affect olfactory aversion learning in the 10-day-old pup.

The second question was whether exposure to methanol affected the amount of time spent in the center region. Methanol exposure might have affected the overall level of

Table 4. Effects of Developmental Exposure to 4,500 ppm Methanol

Test	Outcome
Blood Methanol Concentration	
Dams vs pups	Concentration in the pups was twice that in the dams through PND 21.
PND 7 to PND 52	Concentration in the pups declined until about PND 48.
Neonatal Behavioral Tests	
Suckling (PND 5)	No effect on likelihood of attachment; no effect on latency to attachment.
Conditioned olfactory aversion (PND 10)	No effect.
Motor activity (PND 18, PND 25)	Relative activity levels of methanol-exposed males vs females reversed between PND 18 and PND 25.
Adult Behavioral Tests	
Fixed-ratio wheel running	Relative rates of running between methanol-exposed males and females reversed (1) during changes in the FR criterion, and (2) when initial baseline rates were subtracted from the performance measure.
Stochastic spatial discrimination	Methanol effects appeared in the form of a gender × treatment interaction.
NCAM 140 and NCAM 180 Assays	Brains from PND 4 offspring showed NCAM decreases.

motor activity, or the level of activity following the olfactory training procedure itself, which included exposure to electric shock, a variable known to alter motor activity (these two cases cannot be distinguished in the present analysis). Methanol did not affect the time spent in the center region (TRT, $p = 0.32$; Table A.3). Similarly, the analysis found no differences between cohorts and no treatment × cohort interaction. Gender difference was not significant, nor was there a significant treatment × gender interaction. Although the gender × cohort interaction was significant (GC, $p = 0.04$), the interaction between treatment and gender × cohort was not.

The first two analyses uncovered no differences between methanol-exposed and control pups. The test was selected as part of the protocol because it could be used to assess learning in the young neonatal rat. Learning was demonstrated by more time being spent on the unpaired side than on the paired side; that is, the pups learned to move away from the odor that was paired with the shock. Our preliminary study (described in the Methods section) demonstrated that in the absence of electrical shock or odor stimuli, the pups showed either no odor preference on testing or showed a bias to move toward the methyl salicylate odor. We now asked whether the methanol-exposed and control subjects showed different patterns of behavior.

Figure 11 shows how the difference between time spent on the unpaired side and on the paired side was distributed among individual subjects that moved out of the center region. The differences were rank ordered in magnitude ignoring exposure history. Figure 11 and the statistical analysis show that most subjects spent more time on the unpaired side ($p < 0.01$; Table A.4), and that there were no differences between the methanol-exposed and control rats ($p = 0.41$). The PND 10 pups, therefore, learned to move away from the stimulus paired with the shock, although exposure to 4,500 ppm methanol did not alter their performances.

Motor Activity Test Methanol exposure affected performance on the motor activity test. The same effects were seen in Cohorts 3 and 4. Offspring from Cohorts 3 and 4 were tested on PND 18 and 25. Figure 12 shows the activity counts per minute for each minute of the test. The declines seen over the 15-minute period are common in tests of this nature and are generally interpreted as habituation, which is often viewed as a form of learning (Stanton 1994). The older pups exhibited more activity during the 15-minute test period than the younger ones (Session, $p < 0.01$; Table A.5). A complex pattern of exposure-related effects appeared. The formidable size of the data set led us to summarize these performances for all 15 minutes and then for each of the successive 5-minute segments of the test session. The overall results are summarized in Table 8.

Table 5. Number of Pairs in Male-Female Littermate Behavioral Tests

	Cohort 1		Cohort 2		Cohort 3		Cohort 4		Total	
Methanol (ppm)	4,500	0	4,500	0	4,500	0	4,500	0	4,500	0
Neonatal Tests										
Suckling (PND 5)	*[a]	*	8	7	7	4	11	10	26	21
Conditioned olfactory aversion (PND 10)	*	*	7	7	7	4	8	8	22	19
Motor activity (PND 18; PND 25)	*	*	*	*	6	7	7	8	13	15
Adult Tests										
Fixed-ratio wheel running	2	2	3	3	4	3	4	4	13	12
Stochastic spatial discrimination[b]	2	2	4	3	3	3	—[c]	—[c]	8	9

[a] An asterisk indicates the test was not conducted.

[b] Additional subjects from Cohorts 1, 2, and 3 were included in the analysis of the fitted functions shown in Figure 17 (Appendix A, Tables A.12, A.13, A.14, A.15). The total number of subjects was 21 for the 4,500 ppm group and 19 for the 0 ppm control group.

[c] Cohort 4 data were excluded because the number of sessions was insufficient for determining asymptotic performances.

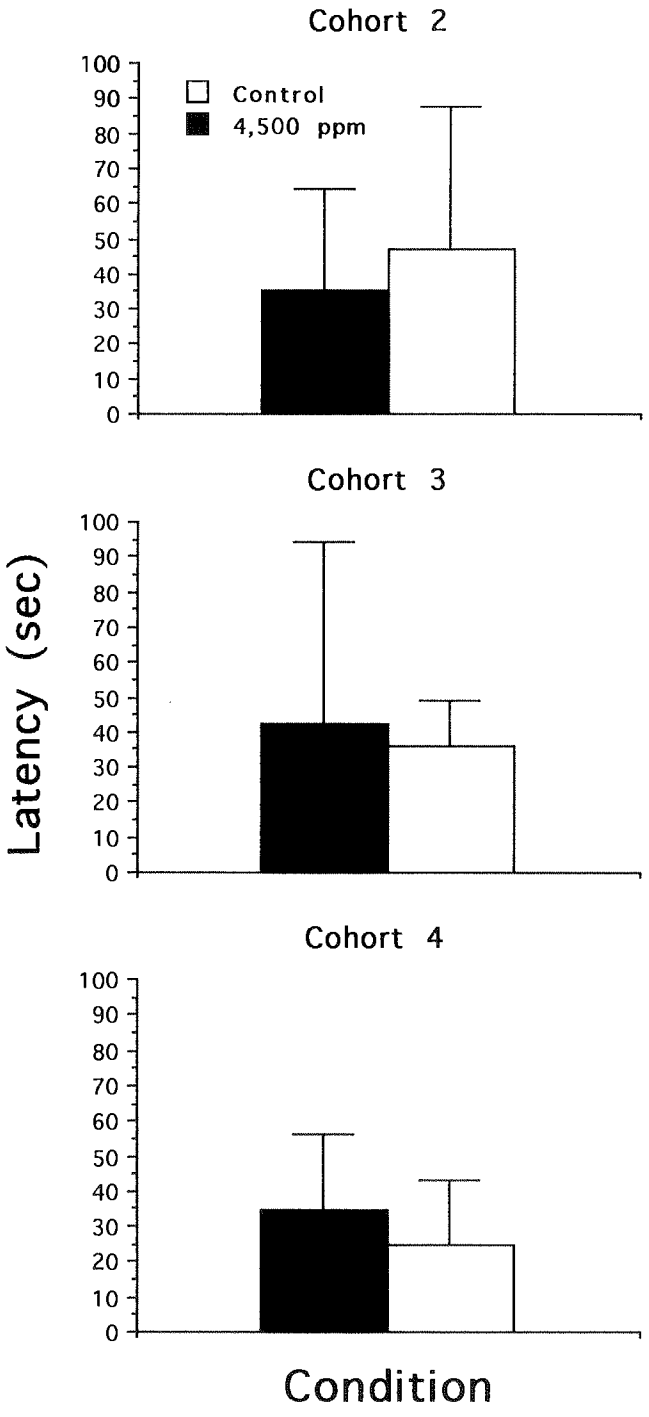

Figure 9. Latencies to nipple attachment (mean ± SD) on the suckling test. The test was conducted on PND 5; rats had been exposed to 4,500 ppm methanol or 0 ppm (control) since GD 6.

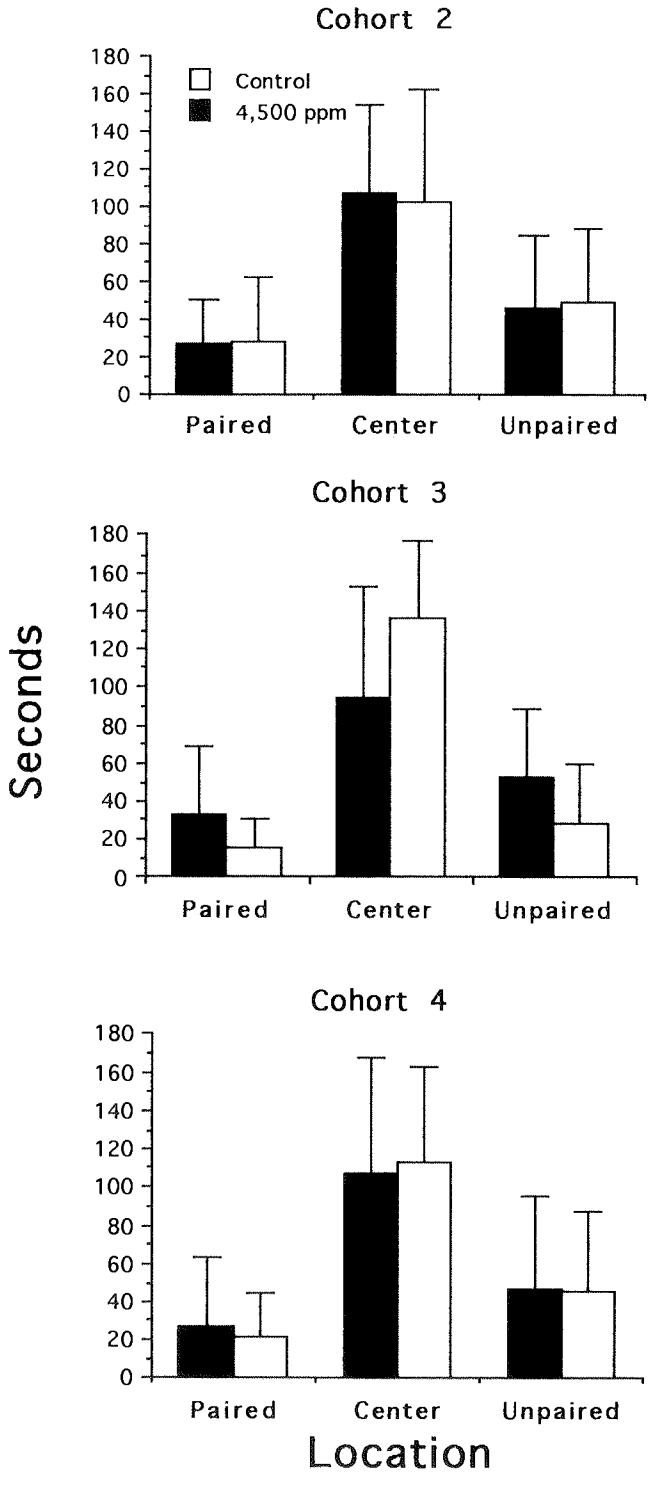

Figure 10. Conditioned olfactory aversion test: time spent in the three sections of the test box (mean ± SD) after odor conditioning on PND 10. "Paired" refers to the side of the box above the pad with the odor present during the aversive electrical stimulation.

No main effect of methanol was observed across the entire 15-minute period (TRT, $p = 0.508$), nor did the cohorts differ (Cohort, $p = 0.97$). A clearly significant session × treatment interaction emerged for PND and methanol exposure (ST, $p < 0.01$). The activity levels of methanol-exposed pups were below those of control pups on PND 18 and above them on PND 25. The data appear in both Table 8 and Figure 13.

Activity levels changed across time (Minutes, $p < 0.01$), as expected, but significant interactions of time (minutes) appeared with PND (session) and cohort (SMC, $p < 0.01$). The fact that other interactions with time were not significant indicates that the interaction between methanol exposure history and PND altered overall levels of activity. The form of the declines seen across minutes of the session was not different between genders (GM, $p = 0.55$) or between sessions (SM, $p = 0.25$). Figure 12 reveals two aspects of these findings. First, activity levels of the methanol-exposed and control groups, on both PND 18 and 25, generally

Table 6. Suckling Test: Proportion of Subjects Successfully Attaching to the Dam's Nipple

	Methanol (4,500 ppm)		Control (0 ppm)	
Cohort	Male	Female	Male	Female
Cohort 2	1.00	1.00	0.71	0.57
Cohort 3	0.43	0.14	0.25	0.50
Cohort 4	0.18	0.27	0.30	0.10

Table 7. Conditioned Olfactory Aversion Test Summary for Cohorts 2, 3, and 4: Mean Time (Seconds) Spent in Each Region of the Test Chamber

	Paired Side	Center	Unpaired Side
Methanol (4,500 ppm)			
Mean	28.99	102.75	48.26
SEM	2.18	4.28	2.10
Control (0 ppm)			
Mean	21.44	117.33	41.23
SEM	3.67	9.84	6.47

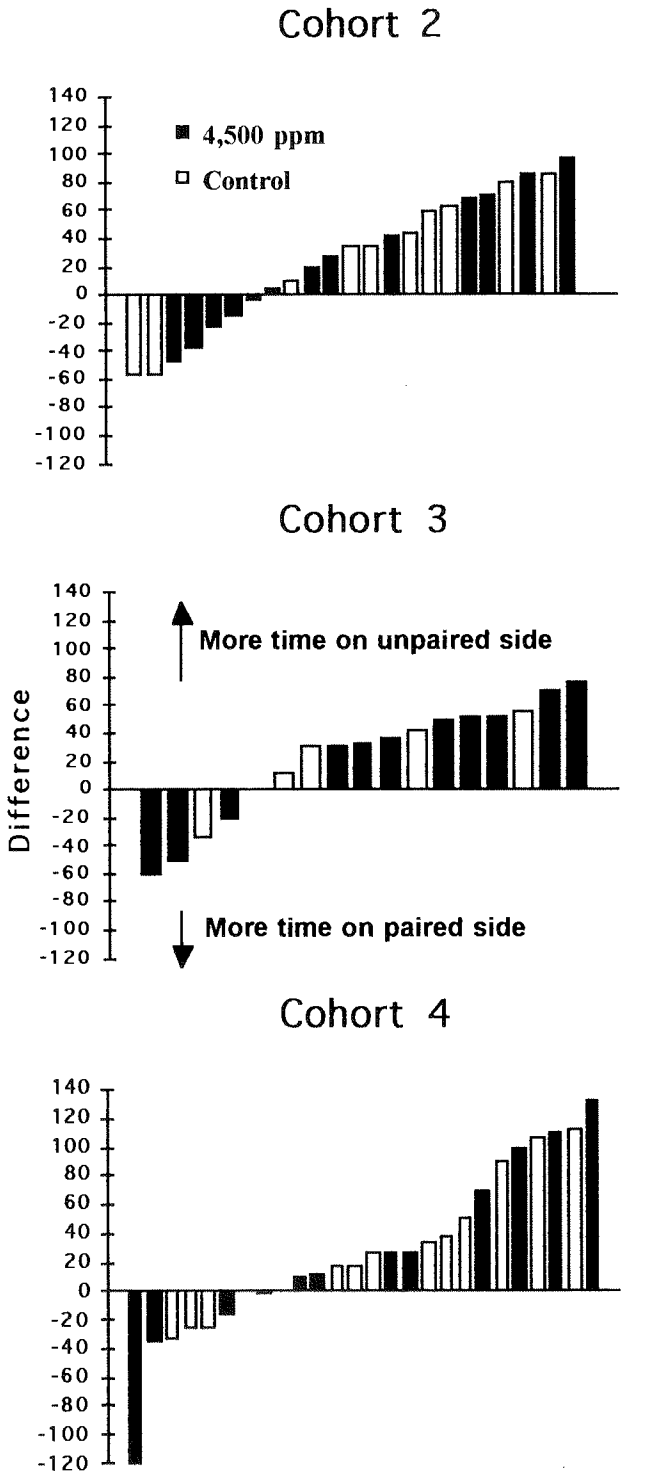

Figure 11. Difference in seconds between time spent on the unpaired and on the paired sides of the test box for subjects that moved out of the center section.

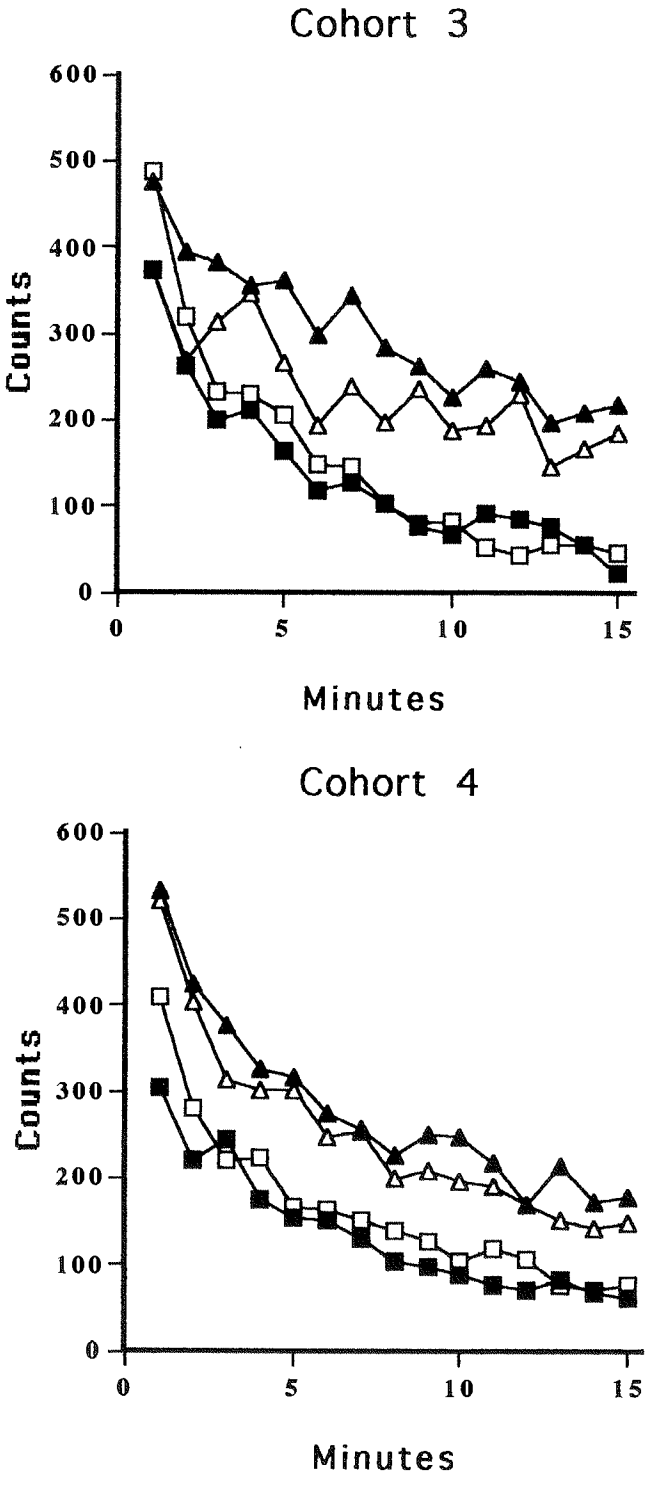

Figure 12. Activity counts per minute (mean) during the 15-minute test period for methanol-exposed and control males and females in Cohorts 3 and 4: (■) 4,500 ppm methanol PND 18; (▲) 4,500 ppm methanol PND 25; (□) control PND 18; (Δ) control PND 25.

remained parallel. Second, it shows how treatment interacted with test day (session) so that the direction of the difference reversed.

As well as analyzing the entire 15-minute session, we decided to analyze the results for successive 5-minute segments. This seemed a useful approach given the distribution of activity across session minutes. The results of these analyses basically confirm the earlier ones without adding any further findings. These findings are summarized in Appendix A, Tables A.6, A.7, and A.8.

Altogether, the results show that on PND 18, during the period when litters and dams were still undergoing exposure, methanol-exposed pups displayed significantly less activity than control pups. The groups reversed roles on PND 25, four days after methanol exposure had ceased. Hence, the methanol exposure effect emerged in the form of an interaction.

Adult Phase

Testing of Cohort 1 began on PND 96 and, to accommodate a fuller testing schedule, extended over 127 sessions. As a result of testing and breeding logistics, Cohorts 2, 3, and 4 began testing at ages of 205, 243, and 267 days, with testing duration extended over 95, 122, and 93 days, respectively. Although we had originally decided to limit testing to a period of three months, we extended the duration, consistent with the schedule for the next cohort and other boundary dates, for as long as possible to obtain further data.

Fixed-Ratio Wheel-Running Test Methanol affected how wheel-running performances responded to changes in the FR criterion when those performances were scaled to their initial values. These differences depended on gender. Although fixed-ratio wheel-running baseline performances were maintained under baseline values of FR 20 for females and FR 28 for males, we included both genders in the same analyses. This inclusive approach allowed us to examine possible interactions of methanol exposure history (treatment) with gender. Although varying interpretations of such interactions may be entertained, the same situation would have prevailed had the FR values for males and females been equal, because, at the higher FR values, they still would not have been functionally equivalent.

Results for all four cohorts are plotted in Figure 14 as responses per minute. A response was defined as a complete rotation of the wheel. The overall rate of responding (total responses per session duration in minutes) was obtained for the following segments of this assay: the mean of the first 5 sessions of the baseline FR values; the mean of the last 15 sessions of the baseline FR values; the rate for

Table 8. Motor Activity Test: Cohorts 3 and 4[a]

	Cohort 3		Cohort 4	
	Mean	SD	Mean	SD
All 15 Minutes				
PND 18: 4,500 ppm methanol	2,039	467	2,029	678
PND 18: Control	2,292	899	2,441	747
PND 25: 4,500 ppm methanol	4,511	1,060	4,183	814
PND 25: Control	3,542	524	3,753	1,156
PND 18: 4,500 – Control	–253		–412	
PND 25: 4,500 – Control	969		430	
First 5 Minutes				
PND 18: 4,500 ppm methanol	1,213	199	1,098	326
PND 18: Control	1,477	320	1,299	286
PND 25: 4,500 ppm methanol	1,970	535	1,977	355
PND 25: Control	1,571	247	1,844	528
PND 18: 4,500 – Control	–264		–201	
PND 25: 4,500 – Control	399		133	
Second 5 Minutes				
PND 18: 4,500 ppm methanol	493	168	569	221
PND 18: Control	562	343	691	306
PND 25: 4,500 ppm methanol	1,415	351	1,257	311
PND 25: Control	1,053	163	1,104	356
PND 18: 4,500 – Control	-69		–122	
PND 25: 4,500 – Control	362		153	
Third 5 Minutes				
PND 18: 4,500 ppm methanol	333	239	362	278
PND 18: Control	253	286	451	269
PND 25: 4,500 ppm methanol	1,127	308	949	250
PND 25: Control	919	302	805	318
PND 18: 4,500 – Control	80		–89	
PND 25: 4,500 – Control	208		144	

[a] Mean and standard deviation of total counts for all 15 minutes, and for the first 5-minute, and third 5-minute periods for subjects exposed to 4,500 ppm methanol and control subjects on PND 18 and 25. The difference in total counts (4,500 – Control) for each postnatal test day also is shown.

individual sessions in which the FR criterion became the baseline value raised by 25%, 50%, 75%, and 100%; and the rate for the fifth session of extinction, during which pellets were never presented. The data for Cohort 1 are based on only two animals in each group, which probably accounts for the large variability shown.

Three separate repeated measures ANOVAs were conducted. In the first, all of the data shown in Figure 14 were examined (Table A.9). There was no main effect of methanol on the rate of running (TRT, $p = 0.62$). Males differed from females (Gender, $p < 0.01$), and gender differences interacted with cohort (GC, $p = 0.04$). Performances differed across the fixed-ratio conditions (FR, $p < 0.01$), indicating that the subjects were sensitive to the change in conditions. Also, the FR outcome differed across cohorts (FC, $p < 0.01$). The interaction of FR condition × cohort × treatment was significant (FCT, $p = 0.04$), indicating that the methanol exposure history altered performances across the FR conditions, but that the effect was not consistent across cohorts. There was a significant gender × FR condition × cohort interaction (GFC = 0.04). Sensitivity to methanol exposure history also was shown in the gender × FR condition × treatment interaction (GFT, $p < 0.01$). This outcome also interacted with cohort (GFCT, $p < 0.01$).

In the second analysis, we focused more closely on performance changes occurring in response to changes in the FR value from baseline. That analysis (Table A.10) examined the data from the single sessions in which the FR criterion was raised 25%, 50%, 75%, and 100% (Conditions 3, 4, 5, and 6 in Table A.10) above the baseline value. Although no main effect of methanol appeared (TRT, $p = 0.40$), there was a significant interaction between cohort performances and treatment (CT, $p = 0.05$). The gender difference was seen again (Gender, $p < 0.01$) but not the gender × cohort interaction (GC, $p = 0.10$). A significant interaction of gender with treatment (GT, $p = 0.04$) appeared again, as shown for those four conditions in Figure 15 (baseline and extinction data are shown also for context). (Table B.2 provides supplemental information about variability in the data that is not shown in Figure 15 to maintain clarity.) Duplicating the first analysis, performances differed across FR conditions (FR, $p < 0.01$) as did the interaction with cohort (FC, $p < 0.01$). As in the first analysis, the gender × FR condition × treatment interaction was significant (GFT, $p = 0.02$), but in this case that outcome did not interact with cohort (GFCT, $p = 0.06$).

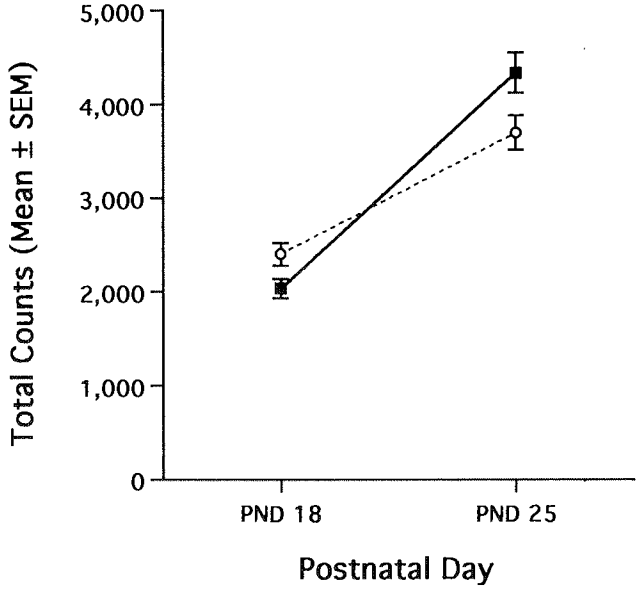

Figure 13. Total counts (mean ± SEM) for the entire 15-minute motor activity test averaged across both Cohorts 3 and 4 and both genders for the exposed (■) and control (○) groups on PND 18 and PND 25. Exposure was to 4,500 ppm methanol.

In a different analysis (Table A.11), the initial values (the first five sessions) were subtracted from the later measures as shown in Figure 16. (Table B.3 provides supplemental information about variability in the data that is not shown in Figure 16 to maintain clarity.) Again, there was no main effect of methanol exposure (TRT, $p = 0.57$). Performances differed among cohorts (Cohort, $p < 0.01$) and among FR conditions (FR, $p < 0.01$), and the FR condition × cohort interaction was significant (FC, $p < 0.01$). The main effect of gender here was not significant (Gender, $p = 0.37$), but there was a significant interaction between gender and treatment (GT, $p < 0.01$). The interaction of gender × FR condition × treatment was also significant (GFT, $p = 0.03$).

Overall, the results show that the rate of responding of males differed from that of females, that as the FR conditions changed, the behavior changed, and that the changes across conditions depended jointly on gender and treatment. One interpretation of that outcome is that behavior undergoing a transition differs between males and females depending on exposure to methanol. In the first five days of responding under the final baseline FR condition (FR 20 for females and FR 28 for males), males from both groups responded similarly but at a rate greater than that of the females. As training progressed, the rates of responding for male and females exposed to methanol converged to values intermediate between those seen in the rats not exposed to methanol. Viewed another way, methanol exposure moved males and females in opposite directions. In several, but not all, instances in which the treatment × gender interaction was statistically significant, the three-way interaction of gender × cohort × treatment was also significant. Only future studies can resolve the question of robustness of the gender × treatment outcome.

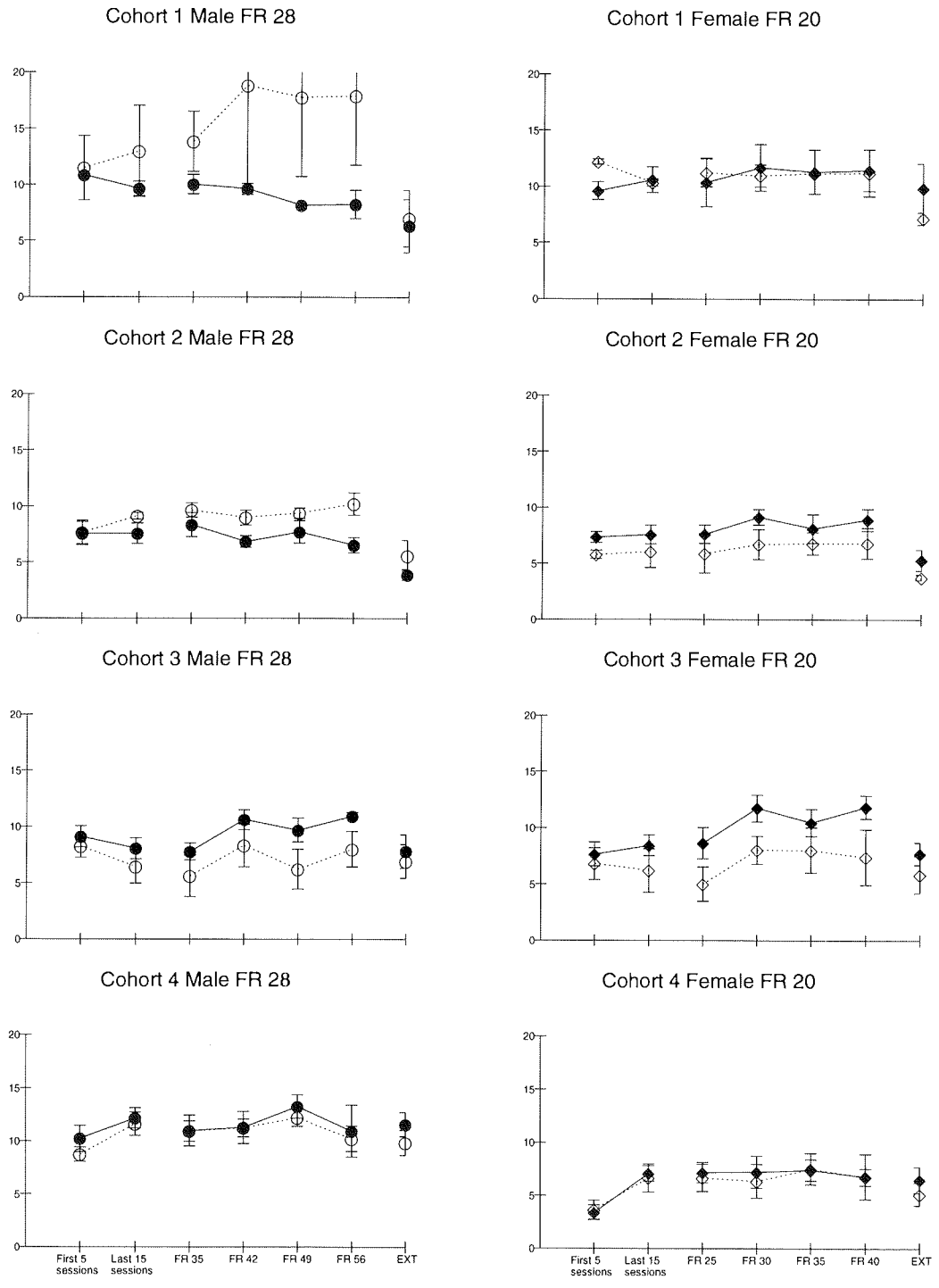

Figure 14. Rates of running in the wheels for food reinforcement (mean ± SD). Control (○) and methanol-exposed (●) males were required to rotate the wheel 28 times (FR 28) to achieve food pellet delivery; control (◊) and methanol-exposed (♦) females were required to rotate the wheel 20 times (FR 20) for the same reinforcement. Shown here are data from the first 5 sessions at the final training ratio; the last 15 sessions at the final training ratio; performance when the FR requirements were raised by 25%, 50%, 75%, and 100%, respectively; and during the fifth session of extinction (EXT).

Stochastic Spatial Discrimination Test Recall that this test was designed to ascertain the degree to which rats chose optimal sequences of responding. Because the probability of reinforcement for a response on any particular lever depended on the location of the previous response, the optimal lever would be that which offered the greatest probability of food delivery.

In accord with these contingencies, as the primary measure of performance on this task, we chose an index of efficiency. It was calculated as the number of reinforcements (pellet deliveries) actually earned during a session divided by the maximum number of reinforcements possible during the session given the number of responses emitted by the rat. For the first condition, shown by the entries in the Matrix 1 of Figure 5, payoffs would be maximized by repeating the sequence Lever 1→Lever 2→Lever 3→Lever 1, etc., corresponding, in the test chambers, to left, center, right, left, etc. Following the change to Matrix 2 of Figure 5, which occurred after 45 sessions on Matrix 1, the optimal sequence became Lever 1→Lever 3→Lever 2→Lever 1, etc.

Figure 17 shows how both control and methanol-exposed groups approached the maximizing criterion during training with both matrices. The response measure, percentage of maximum reinforcements earned, was calculated without including repeated (i.e., successive) responses on the same lever. Such double responses are often caused by simple mechanical bounce of the lever mechanism and, on other reinforcement schedules, have been shown to reflect simple position artifacts rather than deliberate responses. The general pattern of acquisition was similar for both groups and both matrices. Response sequences rapidly be-

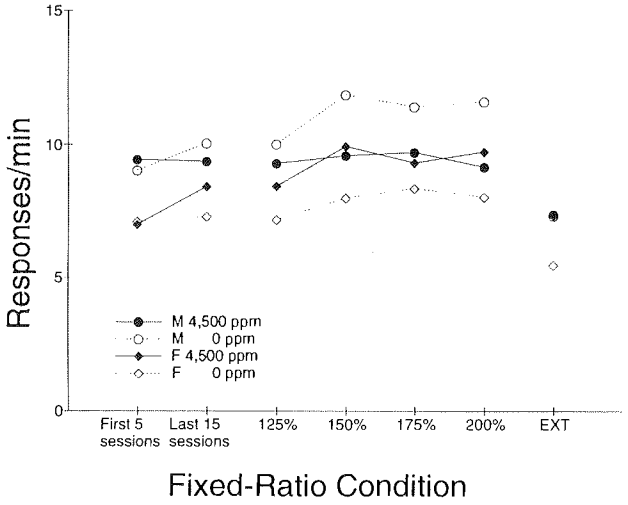

Figure 15. Means of the mean rates of wheel running (responses/minute) for the four cohorts under different fixed-ratio conditions. The data were averaged across those shown in Figure 14.

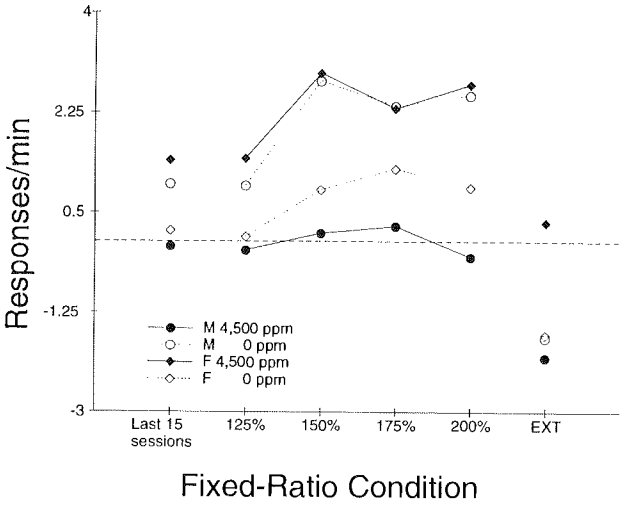

Figure 16. Means of the mean rates of wheel running for the four cohorts corrected by subtracting the mean rate of running during the first five sessions of FR 28 for males and FR 20 for females from the other conditions shown. The data were obtained from the same sessions shown in Figures 14 and 15.

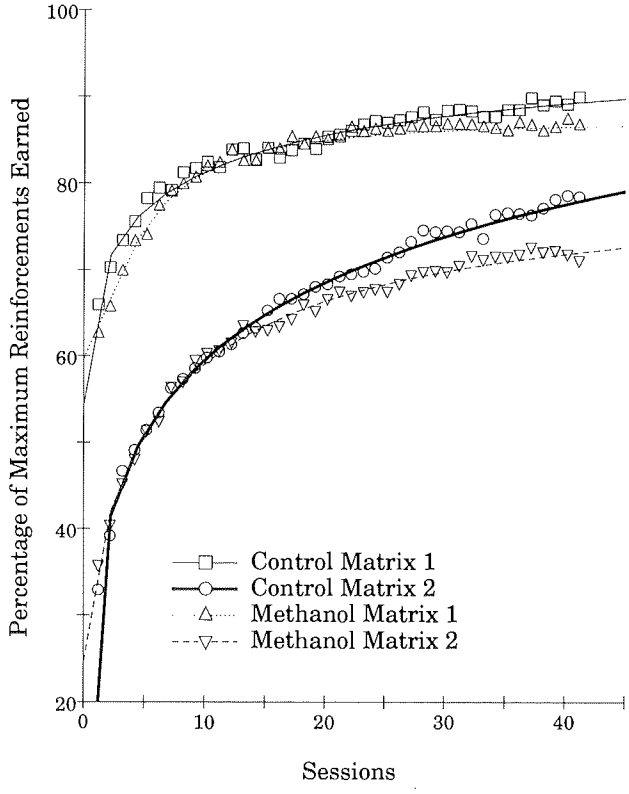

Figure 17. Performance on the stochastic spatial discrimination test: group means and medians, and a fitted hyperbolic function are plotted. Matrix 1 refers to the original set of transition probabilities on which the subjects were trained (see Figure 5). Matrix 2 refers to the second set. The ordinates define the degree to which optimal performance was approached.

came more efficient over the first 10 sessions or so and then began approaching asymptotic performance more slowly. Asymptotic levels were lower in Matrix 2 than in Matrix 1 for both groups, presumably owing to interference by the previously learned response sequence. The fitted curves are empirical functions of the form

$$f(x) = \frac{dx^n + ai^n}{x^n + i^n}$$

fitted by a nonlinear iterative fitting technique known as Marquardt's compromise method (see Statistical Tools from RS/1 documentation; BBN Software Products Corp. 1994). The variable x represents the number of training sessions. The parameter a represents the performance level at the start of each matrix (the intercept when the number of training sessions is zero). The parameter d represents asymptotic performance. The parameter i represents the number of training sessions before efficiency scores improved half the distance between a and d (analogous to a 50% median effective dose). The parameter n is a rate change parameter comparable to a straight-line slope constant. The curves in Figure 17 were fitted to the group mean daily efficiency scores shown.

The effects of methanol exposure were analyzed by fitting the same function to the daily efficiency scores from each rat in each matrix and then comparing the parameter estimates from the two groups. (Preliminary evaluation of the data suggested that methanol effects might be apparent only after extended training. Because Cohort 4 received too few experimental sessions in Matrix 2, their data were excluded from the analysis. The data shown are from the first 41 sessions in each matrix for each of Cohorts 1, 2, and 3.) Parameter estimates for the two groups and two matrices are given in Table 9.

Four separate repeated measures ANOVAs (treatment × matrix for each of four parameters) were conducted to determine the significance of group differences in the parameter estimates (Tables A.12 through A.15). Consistent with the apparent effects in Figure 17, control and methanol-treated animals differed only in the level of asymptotic performance reached (d; $F(1,39) = 5.59$, $p = 0.02$). There was no significant effect of treatment on the intercept (a; $F(1,39) = 0.29$, $p = 0.59$), learning rate (n; $F(1,39) = 0.745$, $p = 0.39$), or sessions to half asymptote (i; $F(1,39) = 0.741$, $p = 0.40$). Further analysis of the simple main effects suggested that control and methanol-exposed rats differed primarily in the asymptote obtained in Matrix 2.

In short, the performance of control animals was superior to that of methanol-exposed animals after extended training in the reversal condition of Matrix 2. It is unlikely that this effect reflects simple response perseveration in which the methanol-exposed rats were less able to inhibit the sequence previously learned in Matrix 1. There was no apparent difference in performance in the two groups over the first 10 to 15 sessions of Matrix 2. Neither the rate parameter nor the half-asymptote parameter could be shown to differ for the two groups.

These effects were confirmed with additional ANOVAs examining the efficiency scores from the final five sessions under each matrix for each litter, using treatment and cohort as additional factors (matrix, session, and gender

Table 9. Stochastic Spatial Discrimination Test: Estimates for Percentage of Correct Parameters for Matrix 1 and Matrix 2[a]

	a		d		i		n	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Matrix 1								
Control	63.26	2.45	98.40	4.95	21.22	8.30	3.20	0.74
Methanol	60.05	2.53	90.54	3.48	9.40	2.81	3.37	0.81
Matrix 2								
Control	27.42	3.92	99.99	7.96	19.95	4.45	2.12	0.49
Methanol	26.89	5.15	81.55	6.75	19.94	10.10	3.16	0.72

[a] Parameter a = performance level at the start of each matrix (the intercept when the number of training sessions is 0); d = asymptotic performance; i = number of training sessions before efficiency scores improved half the distance between a and d; n = rate change comparable to a straight-line slope constant.

were repeated-measure factors), again excluding the data from Cohort 4. (A total of 17 litters were used in the analyses, one male and one female from each.) Figure 18 shows the mean efficiency scores of both treatment groups in the final five sessions of both Matrix 1 (top panel) and Matrix 2 (bottom panel). Table A.16 shows that there were significant session × treatment (ST, $p = 0.014$) and matrix × session × treatment (MST, $p = 0.05$) interactions. Subsequent analyses for each matrix separately detected significant session × treatment effects ($p = 0.01$) in Matrix 2 (Table A.17) but not in Matrix 1 (Table A.18). Again, performance of control animals was superior to that of methanol-exposed animals in that their efficiency scores continued to improve even at the end of extended training in the procedure.

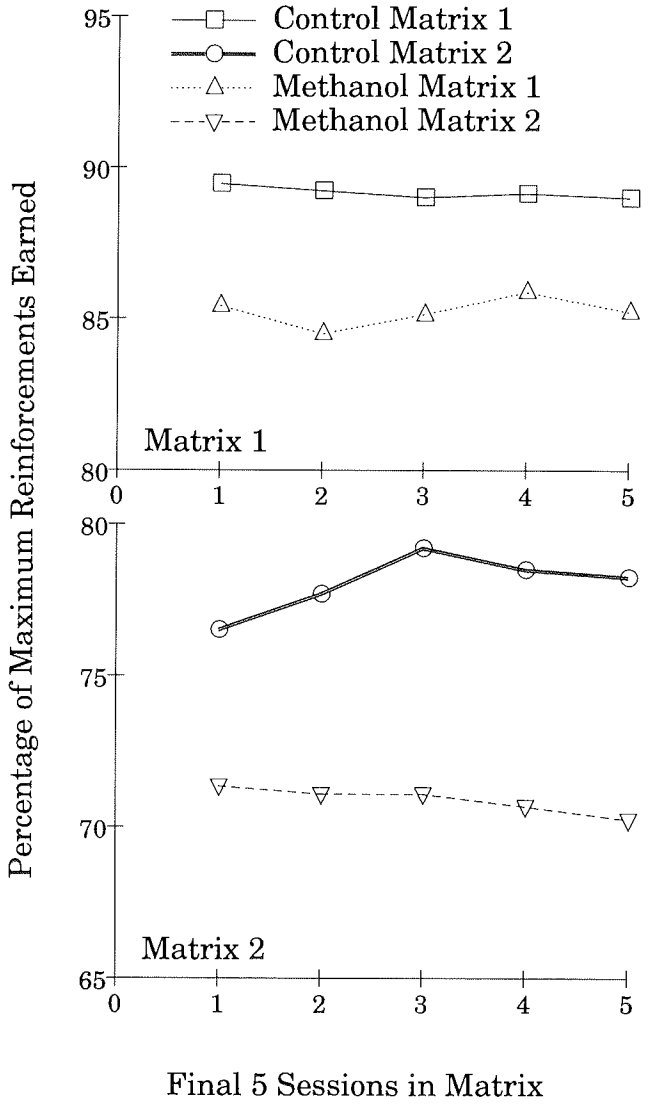

Figure 18. Performance on the stochastic spatial discrimination test: the mean efficiency scores from the final five sessions in each matrix are shown for both control and methanol-exposed animals.

MORPHOLOGY

The neuropathological examination was based on the hypothesis that damage arising from methanol exposure during development would be characterized by three features: one is delayed neuronal migration expressed as heterotopic rests, thickened germinal zones, neurons arrested within the migratory zones, or all of these; the second is increased numbers of apoptotic cells in the cortex or germinal zones; and the third is defective myelination. The first two features are typically evident on hematoxylin and eosin–stained tissue provided the effect is sufficiently robust; LFB/PAS is a reasonably good myelin stain.

Light-microscopic surveys of the brains of methanol-exposed pups revealed no evidence of neuropathology according to the criteria listed above.

In contrast, NCAM indices revealed a significant treatment difference in the brains of neonates killed on PND 4. The brains of offspring killed at 15 months of age showed no NCAM differences. Figure 19 plots the results of densitometer readings based on Western blots. The NCAM 140 and 180 assays were performed on the same brains. Each plot is based on determinations from brains of six control and eight methanol-exposed rats. Four of the methanol-exposed brains represent the average of two separate determinations performed to check assay reliability. Student's t tests performed on each set of assays produced p values of 0.01 (NCAM 140) and 0.001 (NCAM 180).

DISCUSSION

Rats were exposed to 4,500 ppm methanol vapor during the developmental period between GD 6 and PND 21. The dams, and then both dams and their offspring, were exposed via inhalation for six hours daily. Four cohorts of methanol-exposed and control rats constituted the subject populations. Performances of male-female littermate pairs were evaluated across behavioral tests, with each litter contributing once to each test. Neuropathological examinations assessed morphological endpoints, and NCAMs also were assayed.

Statistically significant differences at early ages were found for motor activity tested at PND 18 and 25. The two adult assays, the fixed-ratio wheel-running test and the stochastic spatial discrimination test, yielded evidence of a significant methanol effect. For wheel-running performance, the influence of methanol exposure appeared in the

form of a significant gender × treatment interaction. A significant effect of methanol exposure appeared in the analysis of stochastic spatial discrimination in the form of reduced efficiency after the transition to a new set of contingencies. Methanol effects were not observed in neonates on two other tests: the suckling test and the conditioned olfactory aversion test. No evidence of brain damage emerged on the basis of neuropathology. Differences in NCAMs arising from methanol exposure, however, were observed in neonatal cerebellum. Blood methanol determinations up to PND 21, using subjects not included in the behavioral tests, showed that the concentrations in pups reached values approximately twice those assayed in the dams. To study that outcome further, blood was sampled from those Cohort 4 subjects not designated for the adult-phase behavioral tests. Samples were drawn immediately following exposure at ages ranging from PND 23 to 52. Those results, together with the preweaning data, showed that neonatal blood methanol concentrations declined from about PND 11 to 48.

Most of the preweaning behavioral functions failed to disclose differences between control and methanol-exposed pups. Suckling behavior and olfactory learning had been selected as criteria because of previous findings by ourselves and others. Spear and her collaborators (1989) noted impairment of olfactory discrimination in rats exposed prenatally to high levels of cocaine. Several studies (e.g., Chen et al. 1982) found changes in suckling in pups exposed to ethanol, but only with blood concentrations in the dams resulting from substituting ethanol for 35% of total calories. Our own past work found differences due to prenatal methanol in latency to suckling and in locating odors from the home nest (Infurna and Weiss 1986). These findings, however, were based on giving pregnant rats a 2% (v/v) solution of methanol as their drinking fluid. When, in a recent experiment, we examined the morning blood methanol concentrations achieved by such a regimen, we found the results depicted in Figure 20. In three of the six rats studied in this experiment, the concentrations exceeded, by a considerable margin, those attained by exposure to 4,500 ppm methanol vapor at the end of six hours.

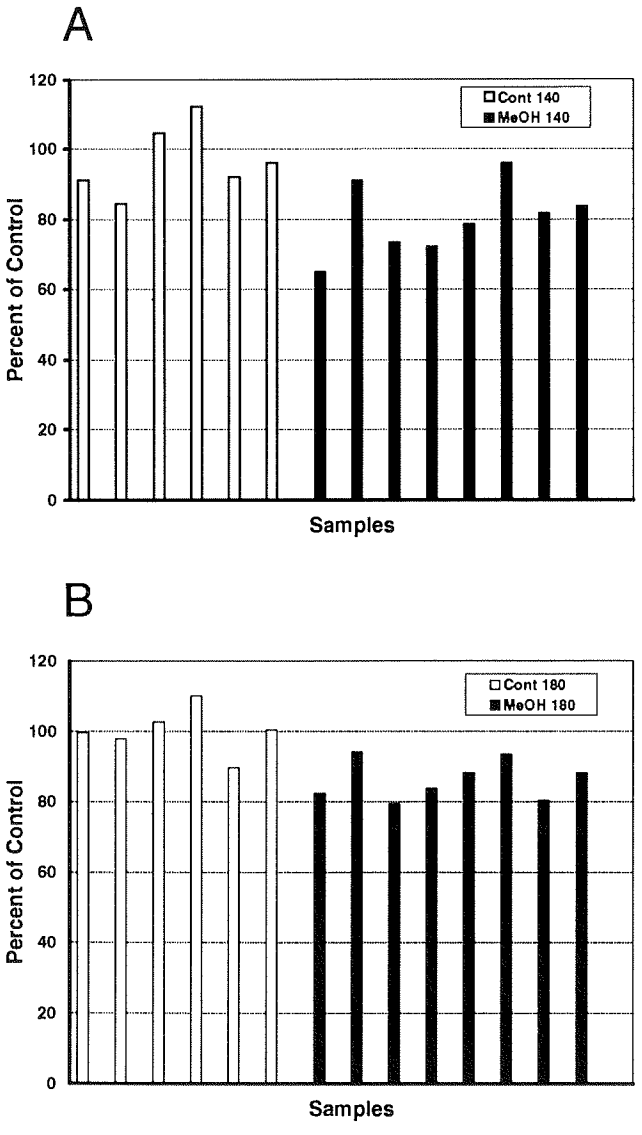

Figure 19. Neural cell adhesion molecule assays: results of densitometer readings based on Western blots. Assays of NCAM 140 (A) and NCAM 180 (B) were performed on the same brains. Each plot is based on determinations from six control brains and eight methanol-exposed brains. Additional control brain assays were identical to those shown here.

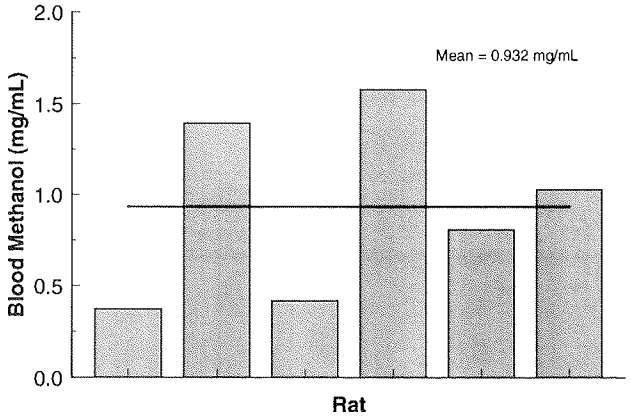

Figure 20. Blood methanol values in six female rats given access for three successive days to drinking water containing 2% (v/v) methanol. Blood samples were obtained on the morning of Day 4. This schedule duplicated that used by Infurna and Weiss (1986).

Motor activity measures, however, did indicate some methanol-induced alterations in neonatal behavior. In the current experiment, they demonstrated decreased locomotor activity in methanol-exposed pups on PND 18 and increased activity on PND 25. Interpretation of this observation is complicated by the fact that, on PND 18, the pups were being tested within 24 hours of exposure. By PND 25, several days had elapsed since the last exposure. The time course of the blood methanol decline in pups was not studied in this project. Although unlikely, residual methanol might have contributed to the gender × treatment × session (PND) interaction. Because spontaneous motor activity varies systematically through the neonatal period, reaching a peak at approximately PND 14 to 17 (depending on the test situation and apparatus) and then declining again, the interpretation of the current findings most consistent with the literature is that methanol exposure modified the normal developmental patterns of activity (Kellogg et al. 1980).

Operant running is sensitive to acute methanol administration (Youssef et al. 1993) and to ozone exposure (Tepper and Weiss 1986). The current experiment also revealed it to be a useful endpoint for assessing developmental changes induced by methanol. Its sensitivity depended on two factors. One was gender. The other was the imposition of an added challenge in the form of increments in the response (FR) requirement. With both factors combined, the influence of methanol emerged in the form of a gender × treatment interaction. One possible source of the interaction is the smaller size and, accordingly, lower FR requirement selected as the baseline value for the female offspring.

The significant treatment effect in the stochastic spatial discrimination test implies rather subtle cognitive deficits as a consequence of methanol exposure during early development. These deficits were apparent only toward the latter stages of performance, and were reflected, in essence, by the level of asymtomatic efficiency attained. The original investigation of this task viewed it as a means of ascertaining the ability of subjects to discriminate serial patterning in reinforcement schedules (Weiss and Heller 1969). It was applied to a study of how anticholinergic hallucinogens modified complex learned behavior in monkeys. Since then, many investigators specializing in the experimental analysis of behavior have turned to quantitative models to describe how reinforcement schedules control behavior. A subject typically is given a choice between two concurrently available reinforcement schedules, each in effect on a different response lever (e.g., leftmost or rightmost lever). Much of this research revolves around what is called the matching law (Davison and McCarthy 1988), a quantitative model of the average correspondence between the distribution of reinforcements from the two sources and the resultant allocation of behavior to the two spatially distinct levers. Generally, the source (e.g., lever) with the greater frequency of payoff attracts a greater percentage of the total responses.

The schedule studied in the current experiments introduced another level of complexity. After each response, it offered three alternative choices — respond again on the same lever or respond on one of the other two levers. A second response on the same lever only rarely led to food delivery. Each of the other two levers was associated with a higher designated probability of reinforcement. The situation was designed to combine the stochastic essence of the natural environment with a discrimination complicated by its dependence on behavioral transitions rather than on fixed positions. Figure 17 shows that adjustment to this procedure was slow and apparently difficult, especially in Matrix 2. It seems that the very difficulty of the task may have played a critical role in revealing the neurotoxic effects of methanol. That methanol effects were statistically significant only toward the end of Matrix 2 indicates the ease with which neurotoxic effects might be overlooked with less complex behavioral tasks.

One potentially significant pharmacokinetic finding from the present experiment is the difference between methanol blood concentrations attained by dams and pups. The differences persisted until about PND 48, which suggests that they might be accounted for by maturation of metabolic processes during development. According to Card and associates (1989), due to low alcohol dehydrogenase activity in fetal and neonatal liver of the guinea pig, the fetus has no capacity to oxidize ethanol during gestation. In the rat, liver alcohol dehydrogenase was first detected on GD 18 (Tietjen et al. 1994).

In rodents, however, catalase is the predominant substrate for the conversion of methanol to formaldehyde (Tephly and McMartin 1984). At least in rat lung, catalase activity increases progressively during late prenatal development and postnatally as well (Chen and Frank 1993). A more extended longitudinal assessment of catalase levels in various tissues during development might help explain the differences between dams and pups observed in the current project.

One source of evidence that methanol vapor exposure under the conditions of the present experiment could result in significant structural modifications during development is the assay of NCAMs. Although many potential biochemical markers of toxicity might have been studied, NCAM assays based on Western immunoblot analysis were selected because of their sensitivity to developmental neurotoxicity in the form of temporal disturbances of brain

NCAM expression, persistent expression of juvenile NCAM isoforms after their normal down-regulation, and impaired synaptogenesis.

These determinations indicate that methanol treatment caused a decrease of expression in both NCAM 140 and NCAM 180. Because NCAM 180 concentrations peak at about PND 14, assays based on later developmental ages might be even more revealing. Specific reductions of NCAM 180 have been noted in hippocampus following excitotoxic damage (Le Gal La Salle et al. 1992).

The primary isoform expressed during the stages of neuronal migration is NCAM 140 and NCAM 180 is expressed during synaptogenesis. Decreases in NCAM 140 might be expected to result in abnormal cell movement following methanol treatment. The absence of evidence of migratory disturbance by light microscopy is not readily reconciled with the NCAM data. However, NCAM is not the only cell adhesion molecule involved in cell movement, and it may be that other adhesion molecules, combined with the residual NCAM 140, may have been sufficient to mediate normal migration. In addition, interrupted movement of single cells, or even small groups of cells, would be difficult to detect by conventional light-microscopic techniques, even if such changes were anticipated and specifically sought. No heterotopias or gross migration arrest were noted in the older animals. Such studies would require use of isotopic tagging of neuron populations. The reduction in NCAM 180 is particularly intriguing given the indications of subtle behavioral changes. Primarily localized at synapses, NCAM 180 is critical to neuronal plasticity, learning, and memory (Regan 1993). Even subtle alterations of this molecule at critical times of life could potentially alter the learning profile of an individual.

IMPLICATIONS FOR RISK ASSESSMENT

This project originated in the possibility that inhalation of methanol, arising from its adoption as a replacement for or additive to gasoline, might pose serious health risks during early development. Although our findings pointed to significant functional consequences in rats resulting from exposure to 4,500 ppm methanol, these consequences are subtle in character and, in one instance, also entwined with gender differences in responsiveness. The morphological findings were also subtle, and appeared in the form of a molecular marker governing cell-cell connections rather than as overt pathology. Both sets of findings emphasize the necessity of using advanced procedures for assessing neurotoxicity.

One viewpoint would argue that, because the estimated concentrations to which the general public would be exposed (see Kavet and Nauss 1990) are far below the concentration selected for the research reported here, our results suggest that the use of methanol fuels does not pose a grave health risk in terms of developmental neurotoxicity.

Excessive complacency is not justified either. First, although methanol concentrations in the communal environment should be quite modest even with widespread adoption of methanol fuels, the delivery chain from producer to consumer may offer opportunities for exposure to much higher concentrations. Furthermore, the marked differences in blood concentrations noted here between dams and pups suggest correspondingly marked pharmacokinetic differences that deserve investigation. The finding that developmental exposure to methanol altered performance on the two complex behavioral endpoints is also a candidate for systematic investigation in future studies. Because developing mice may be more sensitive to methanol than rats (Rogers et al. 1993), similar behavioral assays in mice would be revealing.

The interactions of methanol exposure with gender on behavioral endpoints also warrant further exploration. Sexual dimorphism in response to toxic chemicals is often observed. For example, adult female rats exposed perinatally to cocaine were less active than controls while males showed no effect of exposure (Dow-Edwards 1989). Greater female responsiveness to cocaine, in fact, is a common finding. In the case of methylmercury, male children seem to be more sensitive than females (McKeown-Eyssen et al. 1983).

The discourse surrounding methanol-associated health risks, although clarified somewhat by the current and other recent findings, is now positioned to move to another plane. One lesson from the neurobehavioral assessment of developmental lead exposure is that performance deficits in appropriate animal models appear at about the same blood concentrations as those that yield lowered IQ scores in children (Cory-Slechta 1990). If further research on the developmental toxicity of methanol is undertaken, complex functional endpoints deserve a major role in it.

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APPENDIX A. Analysis of Variance Tables

Table A.1. Analysis of Variance for Suckling Test Latency[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse–Geisser[b] *p*	Huynh-Feldt[b] *p*
Between Animal Factors[c]							
TRT	253.12500	1	253.12500	0.30	0.5932		
1 Error	8315.33333	10	831.53333				
Within Animal Factors[d]							
Gender	238.34722	1	238.34722	0.23	0.6392		
GT	550.01389	1	550.01389	0.54	0.4795		
2 Error	10197.11111	10	1019.71111	2.04	0.1619		

[a] The dependent variable was latency to attachment.

[b] For a description of the Greenhouse–Geisser and Huynh-Feldt corrections, see Crowder and Hand (1990).

[c] TRT = treatment (methanol vs. control).

[d] Gender = male vs. female; GT = gender × treatment.

Table A.2. Analysis of Variance for the Conditioned Olfactory Aversion Test, Paired – Unpaired Time[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
Cohort	696.22810	2	348.11405	0.21	0.8148		
TRT	84.61607	1	84.61607	0.05	0.8242		
CT	587.56197	2	293.78099	0.17	0.8412		
1 Error	59147.73214	35	1689.93520				
Within Animal Factors[c]							
Gender	322.26992	1	322.26992	0.14	0.7142		
GC	12330.91492	2	6165.45746	2.61	0.0879		
GT	4826.78640	1	4826.78640	2.04	0.1619		
GCT	1638.50541	2	819.25271	0.35	0.7095		
2 Error	82724.16071	35	2363.54745				

[a] The dependent variable was the difference in seconds spent in the region of the test chamber that contained the olfactory stimulus paired with shock minus the seconds spent in the region of the test chamber that contained the stimulus not paired with the shock.

[b] Cohort = Cohorts 2, 3, and 4; TRT = treatment (methanol vs control); CT = cohort × treatment.

[c] Gender = male vs female; GC = gender × cohort; GT = gender × treatment; GCT = gender × cohort × treatment.

Table A.3. Analysis of Variance for the Conditioned Olfactory Aversion Test, Time in Center[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
Cohort	1070.18544	2	535.09272	0.14	0.8737		
TRT	3952.74794	1	3952.74794	1.00	0.3239		
CT	6947.07620	2	3473.53810	0.88	0.4239		
1 Error	138189.37500	35	3948.26786				
Within Animal Factors[c]							
Gender	322.26992	1	322.26992	0.16	0.6905		
GC	14254.81149	2	7127.40574	3.57	0.0390		
GT	1127.03365	1	1127.03365	0.56	0.4578		
GCT	1538.78951	2	769.39476	0.38	0.6834		
2 Error	69965.37500	35	1999.01071				

[a] The dependent variable was total seconds spent in the center region of the test chamber.x

[b] Cohort = Cohorts 2, 3, and 4; TRT = treatment (methanol vs control); CT = cohort × treatment.

[c] Gender = male vs female; GC = gender × cohort; GT = gender × treatment; GCT = gender × cohort × treatment.

Table A.4. Analysis of Variance for the Conditioned Olfactory Aversion Test, Unpaired Versus Paired Time When Center Time Was Less Than 180 Seconds[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
TRT	957.32783	1	957.32783	0.72	0.4061		
Cohort	89.18164	2	44.59082	0.03	0.9671		
CT	1688.37544	2	844.18772	0.63	0.5404		
1 Error	27972.16429	21	1332.00782				
Within Animal Factors[c]							
Gender	12.68858	1	12.68858	0.02	0.9005		
GT	25.80686	1	25.80686	0.03	0.8584		
GC	1120.77971	2	560.38985	0.71	0.5040		
GCT	1937.49288	2	968.74644	1.22	0.3142		
2 Error	16623.21429	21	791.58163				
Paired	11492.29311	1	11492.29311	10.41	0.0041		
PT	916.44365	1	916.44365	0.83	0.3727		
PC	142.83015	2	71.41507	0.06	0.9376		
PTC	273.76038	2	136.88019	0.12	0.8841		
3 Error	23191.27857	21	1104.34660				
GP	450.32350	1	450.32350	0.27	0.6117		
GPT	1144.45970	1	1144.45970	0.68	0.4205		
GPC	4274.00739	2	2137.00370	1.26	0.3041		
GPTC	583.356.23	2	291.67811	0.17	0.8431		
4 Error	35599.15714	21	1695.19796				

[a] The dependent variable was the amount of time spent on the unpaired side versus the time spent on the paired side when the time in the center was less than 180 seconds.

[b] TRT = treatment (methanol vs control); Cohort = Cohorts 2, 3, and 4; CT = cohort × treatment.

[c] Gender = male vs female; GT = gender × treatment; GC = gender × cohort; GCT = gender × cohort × treatment. Paired = paired vs unpaired sec; PT = paired – unpaired sec × treatment; PC = paired – unpaired sec × cohort; PTC = paired – unpaired sec × treatment × cohort. GP = gender × paired – unpaired sec × treatment; GPT = gender × paired – unpaired sec × treatment; GPC = gender × paired – unpaired sec × cohort; GPTC = gender × paired – unpaired sec × treatment × cohort.

Table A.5. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, All 15 Minutes[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
Cohort	120.99067	1	120.99067	0.00	0.9715		
TRT	43956.36845	1	43956.36845	0.48	0.4978		
CT	43122.81693	1	43122.81693	0.47	0.5019		
1 Error	1748228.59206	19	92012.03116				
Within Animal Factors[c]							
Session	4407539.13810	1	4407539.13810	254.65	0.0000		
SC	4791.74416	1	4791.74416	0.28	0.6049		
ST	354400.70077	1	354400.70077	20.48	0.0002		
SCT	13480.57148	1	13480.57148	0.78	0.3885		
2 Error	328852.88571	19	17308.04662				
Gender	14725.58966	1	14725.58966	0.25	0.6253		
GC	94570.67249	1	94570.67249	1.58	0.2237		
GT	2563.95387	1	2563.95387	0.04	0.8381		
GCT	59892.85084	1	59892.85084	1.00	0.3294		
3 Error	1135517.30159	19	59764.06850				
SG	24619.78721	1	24619.78721	0.77	0.3904		
SGC	12209.16902	1	12209.16902	0.38	0.5433		
SGT	2688.33939	1	2688.33939	0.08	0.7746		
SGCT	33890.52121	1	33890.52121	1.06	0.3154		
4 Error	605488.10000	19	31867.79474				
Minutes	9365984.29000	14	668998.87786	158.00	0.0000	0.0000	0.0000
MC	68777.27989	14	4912.66285	1.16	0.3062	0.3335	0.3219
MT	77056.14165	14	5504.01012	1.30	0.2070	0.2658	0.2365
MCT	62482.23862	14	4463.01704	1.05	0.4002	0.3933	0.3998
5 Error	1126286.78889	266	4234.16086				
SM	78809.00620	14	5629.21473	1.29	0.2115	0.2531	0.2115
SMC	346398.82439	14	24742.77317	5.68	0.0000	0.0000	0.0000
SMT	117959.31655	14	8425.66547	1.93	0.0232	0.0609	0.0232
SMCT	71869.14281	14	5133.51020	1.18	0.2914	0.3164	0.2914
6 Error	1158480.94762	266	4355.19153				
GM	51755.50774	14	3696.82198	0.83	0.6378	0.5489	0.6043
GMC	56932.24310	14	4066.58879	0.91	0.5470	0.4886	0.5256
GMT	97665.46388	14	6976.10456	1.56	0.0894	0.1652	0.1179
GMCT	57851.47600	14	4132.24829	0.93	0.5311	0.4783	0.5119
7 Error	1186888.69841	266	4461.98759				

(Table continues on next page)

Table A.5. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, All 15 Minutes[a] *(Continued)*

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
SGM	49934.77701	14	3566.76979	0.82	0.6507	0.5558	0.6125
SGMC	52727.75883	14	3766.26849	0.86	0.6006	0.5221	0.5692
SGMT	43580.55094	14	3112.89650	0.71	0.7610	0.6355	0.7114
SGMCT	95434.91457	14	6816.77961	1.56	0.0902	0.1675	0.1213
8 Error	1161678.06667	266	4367.21078				

[a] The dependent variable was activity counts per minute across the entire 15-minute test.

[b] Cohort = Cohorts 3 and 4; TRT = treatment (methanol vs control); CT = cohort × treatment.
Session = PND 18 vs PND 25; SC = session × cohort; ST = session × treatment; SCT = session × cohort × treatment.

[c] Gender = male vs female; GC = gender × cohort; GT = gender × treatment; GCT = gender × cohort × treatment.
SG = session × gender; SGC = session × gender × cohort; SGT = session × gender × treatment; SGCT = session × gender × cohort × treatment.
Minutes = minutes; MC = min × cohort; MT = min × treatment; MCT = min × cohort × treatment.
SM = session × min; SMC = session × min × cohort; SMT = session × min × treatment; SMCT = session × min × cohort × treatment.
GM = gender × min; GMC = gender × min × cohort; GMT = gender × min × treatment; GMCT = gender × min × cohort × treatment.
SGM = session × gender × min; SGMC = session × gender × min × cohort; SGMT = session × gender × min × treatment; SGMCT = session × gender × min × cohort × treatment.

Table A.6. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, First Five Minutes[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
Cohort	0.65747	1	0.65747	0.00	0.9971		
TRT	696.19224	1	696.19224	0.01	0.9060		
CT	12082.48921	1	12082.48921	0.25	0.6238		
1 Error	923321.80357	19	48595.88440				
Within Animal Factors[c]							
Session	1302706.37955	1	1302706.37955	105.23	0.0000		
SC	80039.41591	1	80039.41591	6.47	0.0199		
ST	259178.46757	1	259178.46757	20.94	0.0002		
SCT	25381.51605	1	25381.51605	2.05	0.1684		
2 Error	235208.66071	19	12379.40320				
Gender	11257.29527	1	11257.29527	0.32	0.5766		
GC	10579.56800	1	10579.56800	0.30	0.5882		
GT	47057.63669	1	47057.63669	1.35	0.2598		
GCT	34558.60032	1	34558.60032	0.99	0.3320		
3 Error	662656.66071	19	34876.66635				
SG	18332.85891	1	18332.85891	0.81	0.3807		
SGC	2405.02255	1	2405.02255	0.11	0.7487		
SGT	14264.54636	1	14264.54636	0.63	0.4383		
SGCT	92690.28575	1	92690.28575	4.07	0.0579		
4 Error	432374.73690	19	22756.56510				
M(1)	1610631.17605	1	1610631.17605	126.67	0.0000		
M(1)C	28480.33362	1	28480.33362	2.24	0.1509		
M(1)T	13286.06782	1	13286.06782	1.04	0.3195		
M(1)CT	6315.63752	1	6315.63752	0.50	0.4895		
Error	241589.07381	19	12715.21441				
M(2)	221790.47640	1	221790.47640	39.61	0.0000		
M(2)C	59.66687	1	59.66687	0.01	0.9189		
M(2)T	13395.11874	1	13395.11874	2.39	0.1384		
M(2)CT	6968.17069	1	6968.17069	1.24	0.2785		
Error	106391.04592	19	5599.52873				
M(3)	70967.27318	1	70967.27318	20.60	0.0002		
M(3)C	9308.47924	1	9308.47924	2.70	0.1167		
M(3)T	12414.77679	1	12414.77679	3.60	0.0730		
M(3)CT	1868.90406	1	1868.90406	0.54	0.4704		
Error	65457.81607	19	3445.14821				
M(4)	283.60410	1	283.60410	0.07	0.7979		
M(4)C	1785.72878	1	1785.72878	0.42	0.5225		
M(4)T	9804.14474	1	9804.14474	2.33	0.1433		
M(4)CT	9186.67461	1	9186.67461	2.18	0.1558		
Error	79916.08801	19	4206.10990				

(Table continues on next page.)

Table A.6. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, First Five Minutes[a] *(Continued)*

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Minutes	1903672.52973	4	475918.13243	73.31	0.0000	0.0000	0.0000
MC	39634.20851	4	9908.55213	1.53	0.2030	0.2211	0.2066
MT	48900.10808	4	12225.02702	1.88	0.1220	0.1486	0.1268
MCT	24339.38687	4	6084.84672	0.94	0.4470	0.4226	0.4430
5 Error	493354.02381	76	6491.50031				
Session × Minutes Interactions[d]							
SM(1,1)	40317.62778	1	40317.62778	4.75	0.0422		
SM(1,1)C	95412.20960	1	95412.20960	11.23	0.0034		
SM(1,1)T	36477.28896	1	36477.28896	4.29	0.0521		
SM(1,1)CT	1000.05260	1	1000.05260	0.12	0.7353		
Error	161411.74286	19	8495.35489				
SM(1,2)	1208.75330	1	1208.75330	0.29	0.5990		
SM(1,2)C	36314.00871	1	36314.00871	8.59	0.0086		
SM(1,2)T	11510.66919	1	11510.66919	2.72	0.1153		
SM(1,2)CT	5719.76010	1	5719.76010	1.35	0.2590		
Error	80283.91156	19	4225.46903				
SM(1,3)	272.80521	1	272.80521	0.05	0.8207		
SM(1,3)C	6799.10218	1	6799.10218	1.32	0.2656		
SM(1,3)T	4358.71257	1	4358.71257	0.84	0.3699		
SM(1,3)CT	10613.31257	1	10613.31257	2.05	0.1681		
Error	98194.96845	19	5168.15623				
SM(1,4)	410.97619	1	410.97619	0.06	0.8136		
SM(1,4)C	9942.59351	1	9942.59351	1.38	0.2541		
SM(1,4)T	51.23346	1	51.23346	0.01	0.9336		
SM(1,4)CT	1455.30619	1	1455.30619	0.20	0.6579		
Error	136601.04379	19	7189.52862				
SM	42210.16248	4	10552.54062	1.68	0.1626	0.1721	0.1626
SMC	148467.91400	4	37116.97850	5.92	0.0003	0.0007	0.0003
SMT	52397.90418	4	13099.47605	2.09	0.0904	0.1011	0.0904
SMCT	18788.43146	4	4697.10786	0.75	0.5616	0.5446	0.5616
6 Error	476491.66667	76	6269.62719				
Gender × Minutes Interactions[e]							
GM(1,1)	5926.26039	1	5926.26039	0.60	0.4492		
GM(1,1)C	14694.18766	1	14694.18766	1.48	0.2386		
GM(1,1)T	3069.95159	1	3069.95159	0.31	0.5846		
GM(1,1)CT	9139.61219	1	9139.61219	0.92	0.3493		
Error	188547.61429	19	9923.55865				
GM(1,2)	2176.71619	1	2176.71619	0.52	0.4801		
GM(1,2)C	135.41749	1	135.41749	0.03	0.8593		
GM(1,2)T	5.78360	1	5.78360	0.00	0.9708		
GM(1,2)CT	3467.80092	1	3467.80092	0.83	0.3747		
Error	79716.56803	19	4195.60884				

(Table continues on next page.)

Table A.6. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, First Five Minutes[a] *(Continued)*

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
GM(1,3)	3778.27794	1	3778.27794	0.90	0.3560		
GM(1,3)C	9615.83552	1	9615.83552	2.28	0.1477		
GM(1,3)T	2249.31257	1	2249.31257	0.53	0.4743		
GM(1,3)CT	609.92469	1	609.92469	0.14	0.7081		
Error	80203.66845	19	4221.24571				
GM(1,4)	410.71590	1	410.71590	0.08	0.7827		
GM(1,4)C	3828.37823	1	3828.37823	0.73	0.4038		
GM(1,4)T	18230.76883	1	18230.76883	3.47	0.0779		
GM(1,4)CT	5917.39394	1	5917.39394	1.13	0.3017		
Error	99741.60162	19	5249.55798				
GM	12291.97042	4	3072.99260	0.52	0.7205	0.6767	0.7205
GMC	28273.81890	4	7068.45473	1.20	0.3184	0.3188	0.3184
GMT	23555.81659	4	5888.95415	1.00	0.4137	0.4022	0.4137
GMCT	19134.73175	4	4783.68294	0.81	0.5219	0.4970	0.5219
7 Error	448209.45238	76	5897.49279				
Session × Gender × Minutes Interactions[f]							
SGM(1,1,1)	2585.29553	1	2585.29553	0.30	0.5926		
SGM(1,1,1)C	1826.91371	1	1826.91371	0.21	0.6525		
SGM(1,1,1)T	2637.38211	1	2637.38211	0.30	0.5890		
SGM(1,1,1)CT	11732.57605	1	11732.57605	1.34	0.2607		
Error	165857.89762	19	8729.36303				
SGM(1,1,2)	398.86230	1	398.86230	0.05	0.8296		
SGM(1,1,2)C	2102.99650	1	2102.99650	0.25	0.6222		
SGM(1,1,2)T	3645.02680	1	3645.02680	0.43	0.5175		
SGM(1,1,2)CT	14249.14368	1	14249.14368	1.70	0.2079		
Error	159265.25340	19	8382.38176				
SGM(1,1,3)	4422.87664	1	4422.87664	1.69	0.2093		
SGM(1,1,3)C	2071.68270	1	2071.68270	0.79	0.3849		
SGM(1,1,3)T	940.52744	1	940.52744	0.36	0.5561		
SGM(1,1,3)CT	286.72744	1	286.72744	0.11	0.7444		
Error	49767.65893	19	2619.35047				
SGM(1,1,4)	1181.94158	1	1181.94158	0.19	0.6700		
SGM(1,1,4)C	1008.67405	1	1008.67405	0.16	0.6937		
SGM(1,1,4)T	5113.01027	1	5113.01027	0.81	0.3792		
SGM(1,1,4)CT	4819.82066	1	4819.82066	0.76	0.3929		
Error	119834.44719	19	6307.07617				

(Table continues on next page.)

Table A.6. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, First Five Minutes[a] *(Continued)*

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
SGM	8588.97605	4	2147.24401	0.33	0.8571	0.7630	0.8208
SGMC	7010.26696	4	1752.56674	0.27	0.8969	0.8076	0.8635
SGMT	12335.94661	4	3083.98665	0.47	0.7548	0.6638	0.7183
SGMCT	31088.26782	4	7772.06696	1.19	0.3204	0.3180	0.3207
8 Error	494725.25714	76	6509.54286				

[a] The dependent variable was activity counts per minute over the first five minutes of the test.

[b] Cohort = Cohorts 3 and 4; TRT = treatment (methanol and control); CT = cohort × treatment.
Session = PND 18 vs PND 25; SC = session × cohort; ST = session × treatment; SCT = session × cohort × treatment.

[c] Gender = male vs female; GC = gender × cohort interaction; GT = gender × treatment interaction; GCT = gender × cohort × treatment interaction; SG = session × gender; SGC = session × gender × cohort; SGT = session × gender × treatment; SGCT = session × gender × cohort × treatment.
M(1) = min (linear); M(1)C = min (linear) × cohort; M(1)T = min (linear) × treatment; M(1)CT = min (linear) × cohort × treatment; M(2) = min (quadratic); M(2)C = min (quadratic) × cohort; M(2)T = min (quadratic) × treatment; M(2)CT = min (quadratic) × cohort × treatment; M(3) = min (3rd order); M(3)C = min (3rd order) × cohort; M(3)T = min (3rd order) × treatment; M(3)CT = min (3rd order) × cohort × treatment; M(4) = min (4th order); M(4)C = min (4th order) × cohort; M(4)T = min (4th order) × treatment; M(4)CT = min (4th order) × cohort × treatment; Minutes = minutes; MC = min × cohort; MT = min × treatment; MCT = min × cohort × treatment.

[d] SM(1,1) = session × min (linear); SM(1,1)C = session × min (linear) × cohort; SM(1,1)T = session × min (linear) × treatment; SM(1,1)CT = session × min (linear) × cohort × treatment; SM(1,2) = session × min (quadratic); SM(1,2)C = session × min (quadratic) × cohort; SM(1,2)T = session × min (quadratic) × treatment; SM(1,2)CT = session × min (quadratic) × cohort × treatment; SM(1,3) = session × min (3rd order); SM(1,3)C = session × min (3rd order) × cohort; SM(1,3)T = session × min (3rd order) × treatment; SM(1,3)CT = session × min (3rd order) × cohort × treatment; SM(1,4) = session × min (4th order); SM(1,4)C = session × min (4th order) × cohort; SM(1,4)T = session × min (4th order) × treatment; SM(1,4)CT = session × min (4th order) × cohort × treatment; SM = session × min; SMC = session × min × cohort; SMT = session × min × treatment; SMCT = session × min × cohort × treatment.

[e] GM(1,1) = gender × min (linear); GM(1,1)C = gender × min (linear) × cohort; GM(1,1)T = gender × min (linear) × treatment; GM(1,1)CT = gender × min (linear) × cohort × treatment; GM(1,2) = gender × min (quadratic); GM(1,2)C = gender × min (quadratic) × cohort; GM(1,2)T = gender × min (quadratic) × treatment; GM(1,2)CT = gender × min (quadratic) × cohort × treatment; GM(1,3) = gender × min (3rd order); GM(1,3)C = gender × min (3rd order) × cohort; GM(1,3)T = gender × min (3rd order) × treatment; GM(1,3)CT = gender × min (3rd order) × cohort × treatment; GM(1,4) = gender × min (4th order); GM(1,4)C = gender × min (4th order) × cohort; GM(1,4)T = gender × min (4th order) × treatment, GM(1,4)CT = gender × min (4th order) × cohort × treatment; GM = gender × min; GMC = gender × min × cohort; GMT = gender × min × treatment; GMCT = gender × min × cohort × treatment.

[f] SGM(1,1,1) = session × gender × min (linear); SGM(1,1,1)C = session × gender × min (linear) × cohort; SGM(1,1,1)T = session × gender × min (linear) × treatment; SGM(1,1,1)CT = session × gender × min (linear) × cohort × treatment; SGM(1,1,2) = session × gender × min (quadratic); SGM(1,1,2)C = session × gender × min (quadratic) × cohort; SGM(1,1,2)T = session × gender × min (quadratic) × treatment; SGM(1,1,2)CT = session × gender × min (quadratic) × cohort × treatment; SGM(1,1,3) = session × gender × min (3rd order); SGM(1,1,3)C = session × gender × min (3rd order) × cohort; SGM(1,1,3)T = session × gender × min (3rd order) × treatment; SGM(1,1,3)CT = session × gender × min (3rd order) × cohort × treatment; SGM(1,1,4) = session × gender × min (4th order); SGM(1,1,4)C = session × gender × min (4th order) × cohort; SGM(1,1,4)T = session × gender × min (4th order) × treatment; SGM(1,1,4)CT = session × gender × min (4th order) × cohort × treatment; SGM = session × gender × min; SGMC = session × gender × min × cohort; SGMT = session × gender × min × treatment; SGMCT = session × gender × min × cohort × treatment.

Table A.7. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, Second Five Minutes[a]

Source[b]	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors							
Cohort	2524.28662	1	2524.28662	0.09	0.7734		
TRT	26805.66670	1	26805.66670	0.91	0.3531		
CT	17560.46064	1	17560.46064	0.59	0.4505		
1 Error	562006.47976	19	29579.28841				
Within Animal Factors							
Session	1635105.79398	1	1635105.79398	166.27	0.0000		
SC	21458.27276	1	21458.27276	2.18	0.1560		
ST	119078.18214	1	119078.18214	12.11	0.0025		
SCT	8023.00032	1	8023.00032	0.82	0.3777		
2 Error	186843.88929	19	9833.88891				
Gender	15.76194	1	15.76194	0.00	0.9776		
GC	69812.70133	1	69812.70133	3.58	0.0740		
GT	5547.79224	1	5547.79224	0.28	0.6002		
GCT	21882.67103	1	21882.67103	1.12	0.3030		
3 Error	370941.39405	19	19523.23127				
SG	21139.20812	1	21139.20812	1.75	0.2018		
SGC	2047.11721	1	2047.11721	0.17	0.6853		
SGT	1241.76367	1	1241.76367	0.10	0.7521		
SGCT	407.51519	1	407.51519	0.03	0.8563		
4 Error	229737.51786	19	12091.44831				
M(1)	160650.28636	1	160650.28636	31.20	0.0000		
M(1)C	435.55909	1	435.55909	0.08	0.7743		
M(1)T	547.62085	1	547.62085	0.11	0.7479		
M(1)CT	9959.52388	1	9959.52388	1.93	0.1804		
Error	97841.88571	19	5149.57293				
M(2)	1881.97531	1	1881.97531	0.74	0.3988		
M(2)C	9277.25670	1	9277.25670	3.67	0.0705		
M(2)T	675.71846	1	675.71846	0.27	0.6110		
M(2)CT	6061.32885	1	6061.32885	2.40	0.1379		
Error	47998.24490	19	2526.22342				
M(3)	6853.84288	1	6853.84288	3.65	0.0713		
M(3)C	3582.47924	1	3582.47924	1.91	0.1832		
M(3)T	17.50045	1	17.50045	0.01	0.9241		
M(3)CT	2546.14288	1	2546.14288	1.36	0.2587		
Error	35680.03988	19	1877.89684				
M(4)	20151.50353	1	20151.50353	13.66	0.0015		
M(4)C	284.36154	1	284.36154	0.19	0.6656		
M(4)T	1070.14956	1	1070.14956	0.73	0.4051		
M(4)CT	50.86644	1	50.86644	0.03	0.8547		
Error	28039.15332	19	1475.74491				

(Table continues on next page.)

Table A.7. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, Second Five Minutes[a] (*Continued*)

Source[b]	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Minutes	189537.60808	4	47384.40202	17.18	0.0000	0.0000	0.0000
MC	13579.65657	4	3394.91414	1.23	0.3048	0.3068	0.3048
MT	2310.98932	4	577.74733	0.21	0.9324	0.8848	0.9324
MCT	18617.86205	4	4654.46551	1.69	0.1615	0.1813	0.1615
5 Error	209559.32381	76	2757.35952				
Session × Minutes Interactions							
SM(1,1)	4100.75909	1	4100.75909	0.72	0.4068		
SM(1,1)C	299.37727	1	299.37727	0.05	0.8212		
SM(1,1)T	1420.41623	1	1420.41623	0.25	0.6233		
SM(1,1)CT	19994.41623	1	19994.41623	3.51	0.0765		
Error	108270.14286	19	5698.42857				
SM(1,2)	1535.80092	1	1535.80092	0.81	0.3796		
SM(1,2)C	8329.22949	1	8329.22949	4.39	0.0498		
SM(1,2)T	202.24495	1	202.24495	0.11	0.7477		
SM(1,2)CT	398.00253	1	398.00253	0.21	0.6522		
Error	36060.48299	19	1897.92016				
SM(1,3)	1.20997	1	1.20997	0.00	0.9803		
SM(1,3)C	714.31907	1	714.31907	0.37	0.5504		
SM(1,3)T	105.89699	1	105.89699	0.05	0.8174		
SM(1,3)CT	4402.56365	1	4402.56365	2.28	0.1477		
Error	36716.63988	19	1932.45473				
SM(1,4)	10854.46147	1	10854.46147	6.01	0.0241		
SM(1,4)C	572.91775	1	572.91775	0.32	0.5799		
SM(1,4)T	788.51023	1	788.51023	0.44	0.5167		
SM(1,4)CT	1.23750	1	1.23750	0.00	0.9794		
Error	34317.98189	19	1806.20957				
SM	16492.23146	4	4123.05786	1.45	0.2243	0.2415	0.2314
SMC	9915.84358	4	2478.96089	0.87	0.4831	0.4457	0.4705
SMT	2517.06840	4	629.26710	0.22	0.9253	0.8502	0.9026
SMCT	24796.21991	4	6199.05498	2.19	0.0783	0.1110	0.0898
6 Error	215365.24762	76	2833.75326				
Gender × Minutes Interactions							
GM(1,1)	8358.02085	1	8358.02085	1.68	0.2106		
GM(1,1)C	3782.97843	1	3782.97843	0.76	0.3943		
GM(1,1)T	593.87886	1	593.87886	0.12	0.7336		
GM(1,1)CT	11228.40007	1	11228.40007	2.25	0.1496		
Error	94611.46190	19	4979.55063				
GM(1,2)	3582.02273	1	3582.02273	1.81	0.1949		
GM(1,2)C	5842.02273	1	5842.02273	2.94	0.1024		
GM(1,2)T	1068.32658	1	1068.32658	0.54	0.4720		
GM(1,2)CT	178.10147	1	178.10147	0.09	0.7677		
Error	37690.56122	19	1983.71375				

(Table continues on next page.)

Table A.7. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, Second Five Minutes[a] *(Continued)*

Source[b]	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
GM(1,3)	1203.10045	1	1203.10045	0.29	0.5994		
GM(1,3)C	606.99136	1	606.99136	0.14	0.7086		
GM(1,3)T	1533.00795	1	1533.00795	0.36	0.5537		
GM(1,3)CT	892.69886	1	892.69886	0.21	0.6506		
Error	80112.63036	19	4216.45423				
GM(1,4)	196.33952	1	196.33952	0.09	0.7725		
GM(1,4)C	2.58194	1	2.58194	0.00	0.9735		
GM(1,4)T	420.27015	1	420.27015	0.18	0.6727		
GM(1,4)CT	2169.91950	1	2169.91950	0.95	0.3418		
Error	43374.63699	19	2282.87563				
GM	13339.48355	4	3334.87089	0.99	0.4178	0.4036	0.4178
GMC	10234.57446	4	2558.64361	0.76	0.5544	0.5208	0.5544
GMT	3615.48355	4	903.87089	0.27	0.8973	0.8474	0.8973
GMCT	14469.11991	4	3617.27998	1.07	0.3750	0.3670	0.3750
7 Error	255789.29048	76	3365.64856				
Session × Gender × Minutes Interactions							
SGM(1,1,1)	462.19055	1	462.19055	0.14	0.7099		
SGM(1,1,1)C	8897.62085	1	8897.62085	2.75	0.1139		
SGM(1,1,1)T	766.31176	1	766.31176	0.24	0.6323		
SGM(1,1,1)CT	454.19055	1	454.19055	0.14	0.7123		
Error	61573.13810	19	3240.69148				
SGM(1,1,2)	331.70321	1	331.70321	0.16	0.6972		
SGM(1,1,2)C	6911.95429	1	6911.95429	3.25	0.0872		
SGM(1,1,2)T	1729.48098	1	1729.48098	0.81	0.3784		
SGM(1,1,2)CT	610.20826	1	610.20826	0.29	0.5983		
Error	40391.62245	19	2125.87487				
SGM(1,1,3)	5643.95240	1	5643.95240	1.74	0.2029		
SGM(1,1,3)C	12595.24937	1	12595.24937	3.88	0.0636		
SGM(1,1,3)T	371.61834	1	371.61834	0.11	0.7387		
SGM(1,1,3)CT	1390.80016	1	1390.80016	0.43	0.5205		
Error	61645.85893	19	3244.51889				
SGM(1,1,4)	455.60248	1	455.60248	0.31	0.5838		
SGM(1,1,4)C	2721.23018	1	2721.23018	1.86	0.1890		
SGM(1,1,4)T	0.41849	1	0.41849	0.00	0.9867		
SGM(1,1,4)CT	11.03061	1	11.03061	0.01	0.9318		
Error	27860.07100	19	1466.31953				
SGM	6893.44863	4	1723.36216	0.68	0.6052	0.5671	0.6052
SGMC	31126.05469	4	7781.51367	3.09	0.0206	0.0336	0.0206
SGMT	2867.82958	4	716.95740	0.28	0.8871	0.8386	0.8871
SGMCT	2466.22958	4	616.55740	0.24	0.9120	0.8669	0.9120
8 Error	191470.69048	76	2519.35119				

[a] The dependent variable was activity counts per minute over the second five minutes of the test.

[b] See Table A.6, footnotes b through g, for definitions of the abbreviations in this column.

Table A.8. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, Third Five Minutes[a]

Source[b]	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors							
Cohort	1024.08312	1	1024.08312	0.04	0.8478		
TRT	29938.70361	1	29938.70361	1.11	0.3059		
CT	13745.68543	1	13745.68543	0.51	0.4845		
1 Error	513724.51429	19	27038.13233				
Within Animal Factors							
Session	1479185.06724	1	1479185.06724	223.71	0.0000		
SC	65701.33391	1	65701.33391	9.94	0.0052		
ST	31309.22078	1	31309.22078	4.74	0.0424		
SCT	2283.47532	1	2283.47532	0.35	0.5637		
2 Error	125628.28571	19	6612.01504				
Gender	11675.32828	1	11675.32828	0.57	0.4604		
GC	27412.85556	1	27412.85556	1.33	0.2626		
GT	2996.57273	1	2996.57273	0.15	0.7069		
GCT	8110.39091	1	8110.39091	0.39	0.5375		
3 Error	390801.50000	19	20568.50000				
SG	81.38543	1	81.38543	0.01	0.9361		
SGC	9427.83391	1	9427.83391	0.76	0.3931		
SGT	31.47027	1	31.47027	0.00	0.9603		
SGCT	33.38543	1	33.38543	0.00	0.9591		
4 Error	234576.71905	19	12346.14311				
M(1)	69472.72224	1	69472.72224	24.19	0.0001		
M(1)C	5.08588	1	5.08588	0.00	0.9669		
M(1)T	368.67679	1	368.67679	0.13	0.7241		
M(1)CT	16740.91315	1	16740.91315	5.83	0.0260		
Error	54567.60893	19	2871.97942				
M(2)	1973.05485	1	1973.05485	0.99	0.3312		
M(2)C	72.18472	1	72.18472	0.04	0.8507		
M(2)T	694.31923	1	694.31923	0.35	0.5611		
M(2)CT	137.51403	1	137.51403	0.07	0.7952		
Error	37692.90434	19	1983.83707				
M(3)	1015.15159	1	1015.15159	0.52	0.4782		
M(3)C	1377.21219	1	1377.21219	0.71	0.4099		
M(3)T	3554.57150	1	3554.57150	1.83	0.1917		
M(3)CT	188.18362	1	188.18362	0.10	0.7588		
Error	36856.94762	19	1939.83935				
M(4)	494.28597	1	494.28597	0.22	0.6472		
M(4)C	10680.89550	1	10680.89550	4.67	0.0436		
M(4)T	7743.28263	1	7743.28263	3.39	0.0814		
M(4)CT	2192.56055	1	2192.56055	0.96	0.3397		
Error	43431.77483	19	2285.88289				

(Table continues on next page.)

Table A.8. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, Third Five Minutes[a] (*Continued*)

Source[b]	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Minutes	72955.21465	4	18238.80366	8.03	0.0000	0.0000	0.0000
MC	12135.37828	4	3033.84457	1.34	0.2643	0.2674	0.2643
MT	12360.85014	4	3090.21254	1.36	0.2554	0.2589	0.2554
MCT	19259.17136	4	4814.79284	2.12	0.0864	0.0941	0.0864
5 Error	172549.23571	76	2270.38468				
Session × Minutes Interactions							
SM(1,1)	680.67188	1	680.67188	0.19	0.6645		
SM(1,1)C	0.67188	1	0.67188	0.00	0.9891		
SM(1,1)T	682.00218	1	682.00218	0.19	0.6642		
SM(1,1)CT	3881.30521	1	3881.30521	1.11	0.3060		
Error	66635.64702	19	3507.13932				
SM(1,2)	7149.71461	1	7149.71461	1.26	0.2761		
SM(1,2)C	4923.77088	1	4923.77088	0.87	0.3637		
SM(1,2)T	2773.87303	1	2773.87303	0.49	0.4933		
SM(1,2)CT	22.75615	1	22.75615	0.00	0.9502		
Error	108019.04719	19	5685.21301				
SM(1,3)	978.67886	1	978.67886	0.52	0.4815		
SM(1,3)C	9905.34553	1	9905.34553	5.22	0.0340		
SM(1,3)T	1063.34206	1	1063.34206	0.56	0.4634		
SM(1,3)CT	2126.55418	1	2126.55418	1.12	0.3032		
Error	36077.19524	19	1898.79975				
SM(1,4)	1839.44425	1	1839.44425	0.94	0.3437		
SM(1,4)C	10778.00009	1	10778.00009	5.53	0.0297		
SM(1,4)T	3359.95697	1	3359.95697	1.72	0.2050		
SM(1,4)CT	46.45567	1	46.45567	0.02	0.8790		
Error	37064.19388	19	1950.74705				
SM	10648.50960	4	2662.12740	0.82	0.5186	0.4898	0.5186
SMC	25607.78838	4	6401.94710	1.96	0.1086	0.1299	0.1086
SMT	7879.17424	4	1969.79356	0.60	0.6608	0.6146	0.6608
SMCT	6077.07121	4	1519.26780	0.47	0.7605	0.7067	0.7605
6 Error	247796.08333	76	3260.47478				
Gender × Minutes Interactions							
GM(1,1)	7770.02210	1	7770.02210	1.84	0.1914		
GM(1,1)C	1799.78573	1	1799.78573	0.43	0.5222		
GM(1,1)T	15634.42426	1	15634.42426	3.69	0.0698		
GM(1,1)CT	6084.89699	1	6084.89699	1.44	0.2453		
Error	80430.95655	19	4233.20824				
GM(1,2)	8650.01960	1	8650.01960	4.01	0.0597		
GM(1,2)C	3387.52609	1	3387.52609	1.57	0.2254		
GM(1,2)T	104.13638	1	104.13638	0.05	0.8284		
GM(1,2)CT	11200.85066	1	11200.85066	5.19	0.0344		
Error	40990.85332	19	2157.41333				

(Table continues on next page.)

Table A.8. Analysis of Variance for the Motor Activity Test, Cohorts 3 and 4, Third Five Minutes[a] (*Continued*)

Source[b]	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
GM(1,3)	50.90909	1	50.90909	0.03	0.8684		
GM(1,3)C	1.14545	1	1.14545	0.00	0.9802		
GM(1,3)T	1530.68081	1	1530.68081	0.85	0.3688		
GM(1,3)CT	11.08687	1	11.08687	0.01	0.9384		
Error	34318.85000	19	1806.25526				
GM(1,4)	1430.30715	1	1430.30715	0.71	0.4099		
GM(1,4)C	0.94005	1	0.94005	0.00	0.9830		
GM(1,4)T	186.87450	1	186.87450	0.09	0.7640		
GM(1,4)CT	2291.97840	1	2291.97840	1.14	0.2994		
Error	38267.04252	19	2014.05487				
GM	17901.25794	4	4475.31448	1.75	0.1471	0.1671	0.1471
GMC	5189.39733	4	1297.34933	0.51	0.7298	0.6762	0.7298
GMT	17456.11595	4	4364.02899	1.71	0.1566	0.1759	0.1566
GMCT	19588.81291	4	4897.20323	1.92	0.1160	0.1376	0.1160
7 Error	194007.70238	76	2552.73293				
Session × Gender × Minutes Interactions							
SGM(1,1,1)	14623.22744	1	14623.22744	6.98	0.0161		
SGM(1,1,1)C	5587.93653	1	5587.93653	2.67	0.1189		
SGM(1,1,1)T	3492.55456	1	3492.55456	1.67	0.2121		
SGM(1,1,1)CT	1973.13638	1	1973.13638	0.94	0.3439		
Error	39795.19464	19	2094.48393				
SGM(1,1,2)	4677.08731	1	4677.08731	1.30	0.2680		
SGM(1,1,2)C	2260.23017	1	2260.23017	0.63	0.4374		
SGM(1,1,2)T	5403.38900	1	5403.38900	1.50	0.2350		
SGM(1,1,2)CT	45.48857	1	45.48857	0.01	0.9116		
Error	68254.28189	19	3592.33063				
SGM(1,1,3)	0.84156	1	0.84156	0.00	0.9852		
SGM(1,1,3)C	1970.49610	1	1970.49610	0.83	0.3735		
SGM(1,1,3)T	1898.76623	1	1898.76623	0.80	0.3822		
SGM(1,1,3)CT	387.00260	1	387.00260	0.16	0.6908		
Error	45078.27857	19	2372.54098				
SGM(1,1,4)	217.53078	1	217.53078	0.13	0.7197		
SGM(1,1,4)C	3101.96974	1	3101.96974	1.89	0.1850		
SGM(1,1,4)T	4732.62404	1	4732.62404	2.89	0.1056		
SGM(1,1,4)CT	234.12447	1	234.12447	0.14	0.7097		
Error	31153.49014	19	1639.65738				
SGM	19518.68709	4	4879.67177	2.01	0.1012	0.1164	0.1012
SGMC	12920.63254	4	3230.15813	1.33	0.2657	0.2713	0.2657
SGMT	15527.33384	4	3881.83346	1.60	0.1828	0.1949	0.1828
SGMCT	2639.75202	4	659.93801	0.27	0.8951	0.8605	0.8951
8 Error	184281.24524	76	2424.75323				

[a] The dependent variable was activity counts per minute over the third five minutes of the test.

[b] See Table A.6, footnotes b through g, for definitions of the abbreviations in this column.

Table A.9. Analysis of Variance for the Fixed-Ratio Wheel-Running Test for Males and Females[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
Cohort	374.85926	3	124.95309	2.88	0.0646		
TRT	10.71263	1	10.71263	0.25	0.6253		
CT	286.16311	3	95.38770	2.20	0.1235		
1 Error	781.04324	18	43.39129				
Within Animal Factors[c]							
Gender	378.88320	1	378.88320	13.52	0.0017		
GC	291.70116	3	97.23372	3.47	0.0379		
GT	83.65077	1	83.65077	2.99	0.1011		
GCT	56.23184	3	18.74395	0.67	0.5820		
2 Error	504.25087	18	28.01394				
FR	339.76832	6	56.62805	26.93	0.0000	0.0000	0.0000
FC	206.09395	18	11.44966	5.45	0.0000	0.0000	0.0000
FT	21.03922	6	3.50654	1.67	0.1359	0.1618	0.1359
FCT	71.54448	18	3.97469	1.89	0.0239	0.0446	0.0239
3 Error	227.09462	108	2.10273				
GF	11.71456	6	1.95243	1.35	0.2400	0.2659	0.2464
GFC	55.78997	18	3.09944	2.15	0.0084	0.0381	0.0119
GFT	48.31675	6	8.05279	5.58	0.0000	0.0018	0.0001
GFCT	43.23531	18	2.40196	1.67	0.0570	0.0672	0.0672
4 Error	155.78775	108	1.44248				

[a] The dependent variable was the number of complete wheel revolutions per minute. Fixed ratio (FR) conditions were the following: (1) first 5 sessions at the final training ratio; (2) final 15 sessions at the final training ratio; (3–6) single sessions in which tbe ratio was increased by 25%, 50%, 75%, and 100%; and (7) the fifth session of extinction. Three separate analyses were conducted and are shown in Tables A.9., A.10., and A.11. Data for the entire experiment are analyzed in this table.

[b] Cohort = Cohorts 1, 2, 3, and 4; TRT = treatment (methanol vs control); CT = cohort × treatment.

[c] Gender = male vs female; GC = gender × cohort; GT = gender × treatment; GCT = gender × cohort × treatment.
FR = fixed-ratio (FR) condition; FC = FR condition × cohort; FT = FR condition × treatment; FCT = FR condition × cohort × treatment.
GF = gender × FR condition; GFC = gender × FR condition × cohort; GFCT = gender × FR condition × cohort × treatment.

Table A.10. Analysis of Variance for the Fixed-Ratio Wheel-Running Test for Males and Females, Conditions 3, 4, 5, and 6 Only[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
Cohort	243.21381	3	81.07127	2.48	0.0945		
TRT	24.60705	1	24.60705	0.75	0.3975		
CT	309.25257	3	103.08419	3.15	0.0506		
Error	589.50736	18	32.75041				
Within Animal Factors[c]							
Gender	238.90646	1	238.90646	10.59	0.0044		
GC	160.65029	3	53.55010	2.37	0.1042		
GT	103.81937	1	103.81937	4.60	0.0458		
GCT	53.26845	3	17.75615	0.79	0.5167		
2 Error	406.07637	18	22.55980				
FR	40.91977	3	13.63992	6.73	0.0006	0.0011	0.0006
FC	67.02155	9	7.44684	3.67	0.0012	0.0021	0.0012
FT	2.40846	3	0.80282	0.40	0.7563	0.7316	0.7563
FCT	8.76869	9	0.97430	0.48	0.8812	0.8625	0.8812
3 Error	109.42787	54	2.02644				
GF	2.16552	3	0.72184	0.58	0.6335	0.5785	0.6335
GFC	18.19202	9	2.02134	1.61	0.1353	0.1663	0.1353
GFT	15.12690	3	5.04230	4.02	0.0118	0.0237	0.0118
GFCT	24.60530	9	2.73392	2.18	0.0379	0.0623	0.0379
4 Error	67.72188	54	1.25411				

[a] The dependent variable was the number of complete wheel revolutions per minute. Fixed ratio (FR) conditions were the following: (1) first 5 sessions at the final training ratio; (2) final 15 sessions at the final training ratio; (3–6) single sessions in which the ratio was increased by 25%, 50%, 75%, and 100%; and (7) the fifth session of extinction. Three separate analyses were conducted and are shown in Tables A.9., A.10., and A.11. Data from fixed-ratio conditions 3, 4, 5, and 6 only are analyzed in this table. These conditions correspond to the 25%, 50%, 75%, and 100% increases in the fixed-ratio criterion above the baseline values (FR 28 for males; FR 20 for females).

[b] Cohort = Cohorts 1, 2, 3, and 4; TRT = treatment (methanol vs control); CT = cohort × treatment.

[c] Gender = male vs female; GC = gender × cohort; GT = gender × treatment; GCT = gender × cohort × treatment.
FR = fixed-ratio (FR) condition; FC = FR condition × cohort; FT = FR condition × treatment; FCT = FR condition × cohort × treatment.
GF = gender × FR condition; GFC = gender × FR condition × cohort; GFCT = gender × FR condition × cohort × treatment.

Table A.11. Analysis of Variance for the Fixed-Ratio Wheel-Running Test for Males and Females, Difference from Mean of First Five Sessions' Baseline Values[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	p	Greenhouse-Geisser p	Huynh-Feldt p
Between Animal Factors[b]							
Cohort	391.92975	3	130.64325	6.74	0.0031		
TRT	6.35844	1	6.35844	0.33	0.5740		
CT	107.94525	3	35.98175	1.86	0.1733		
1 Error	349.01790	18	19.38988				
Within Animal Factors[c]							
Gender	9.37911	1	9.37911	0.84	0.3727		
GC	91.53093	3	30.51031	2.72	0.0751		
GT	161.13057	1	161.13057	14.36	0.0013		
GCT	75.75279	3	25.25093	2.25	0.1174		
2 Error	202.02745	18	11.22375				
FR	318.98551	5	63.79710	32.40	0.0000	0.0000	0.0000
FC	150.10400	15	10.00693	5.08	0.0000	0.0000	0.0000
FT	20.13087	5	4.02617	2.04	0.0799	0.1045	0.0799
FCT	56.12373	15	3.74158	1.90	0.0333	0.0558	0.0333
3 Error	177.23493	90	1.96928				
GF	10.37469	5	2.07494	1.47	0.2071	0.2389	0.2201
GFC	42.71412	15	2.84761	2.02	0.0221	0.0707	0.0347
GFT	25.29810	5	5.05962	3.59	0.0053	0.0278	0.0101
GFCT	32.41348	15	2.16090	1.53	0.1105	0.1784	0.1330
4 Error	126.92666	90	1.41030				

[a] The dependent variable was the number of complete wheel revolutions per minute. Fixed ratio (FR) conditions were the following: (1) first 5 sessions at the final training ratio; (2) final 15 sessions at the final training ratio; (3–6) single sessions in which the ratio was increased by 25%, 50%, 75%, and 100%; and (7) the fifth session of extinction. Three separate analyses were conducted and are shown in Tables A.9., A.10., and A.11. Data from condition 1, i.e., the first five sessions at the final training value, are subtracted from the other data in this table.

[b] Cohort = Cohorts 1, 2, 3, and 4; TRT = treatment (methanol vs control); CT = cohort × treatment.

[c] Gender = male vs female; GC = gender × cohort; GT = gender × treatment; GCT = gender × cohort × treatment.
FR = fixed-ratio (FR) condition; FC = FR condition × cohort; FT = FR condition × treatment; FCT = FR condition × cohort × treatment.
GF = gender × FR condition; GFC = gender × FR condition × cohort; GFCT = gender × FR condition × cohort × treatment.

Table A.12. Asymptotic Efficiency (d)

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	p	Greenhouse-Geisser p	Huynh-Feldt p
TRT[a]	3542.174	1	3542.174	5.590	0.0231		
Subject (Group)	24711.674	39	633.633				
Matrix	281.412	1	281.412	0.334	0.5667	0.5667	0.5667
Matrix × TRT	573.475	1	573.475	0.680	0.4145	0.4145	0.4145
Matrix × Subject (Group)	32873.191	39	842.902				

[a] TRT = treatment.

Table A.13. Sessions Required to Improve $(d - a)/2$, or i

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	p	Greenhouse-Geisser p	Huynh-Feldt p
TRT[a]	716.559	1	716.559	0.741	0.3945		
Subject (Group)	37699.157	39	966.645				
Matrix	439.451	1	439.451	0.406	0.5277	0.5277	0.5277
Matrix × TRT	714.087	1	714.087	0.660	0.4215	0.4215	0.4215
Matrix × Subject (Group)	42197.831	39	1081.996				

[a] TRT = treatment.

Table A.14. Learning Rate (n)

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	p	Greenhouse-Geisser p	Huynh-Feldt p
TRT[a]	7.490	1	7.490	0.745	0.3933		
Subject (Group)	391.959	39	10.050				
Matrix	8.558	1	8.558	0.836	0.3663	0.3663	0.3663
Matrix × TRT	4.021	1	4.021	0.393	0.5346	0.5346	0.5346
Matrix × Subject (Group)	399.428	39	10.242				

[a] TRT = treatment.

Table A.15. Intercept (a)

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	p	Greenhouse-Geisser p	Huynh-Feldt p
TRT[a]	71.907	1	71.907	0.289	0.5941		
Subject (Group)	9714.583	39	249.092				
Matrix	24391.859	1	24391.859	77.726	0.0001	0.0001	0.0001
Matrix × TRT	36.801	1	36.801	0.117	0.7339	0.7339	0.7339
Matrix × Subject (Group)	12238.940	39	313.819				

[a] TRT = treatment.

Table A.16. Analysis of Variance for the Stochastic Spatial Discrimination Test: Matrix 1 and Matrix 2[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
Cohort	400.20810	2	200.10405	0.19	0.8294		
TRT	2015.19112	1	2015.19112	1.92	0.1938		
CT	458.29737	2	229.14868	0.22	0.8077		
1 Error	11571.80608	11	1051.98237				
Within Animal Factors[c]							
Matrix	11592.97965	1	11592.97965	35.59	0.0001		
MC	2408.88481	2	1204.44241	3.70	0.0591		
MT	214.27678	1	214.27678	0.66	0.4345		
MCT	1394.96489	2	697.48245	2.14	0.1639		
2 Error	3582.76220	11	325.70565				
Session	13.16991	4	3.29248	1.62	0.1857	0.2004	0.1857
SC	12.84678	8	1.60585	0.79	0.6133	0.5886	0.6133
ST	32.48242	4	8.12060	4.00	0.0075	0.0139	0.0075
SCT	57.44718	8	7.18090	3.54	0.0031	0.0070	0.0031
3 Error	89.33031	44	2.03023				
MS	24.78968	4	6.19742	1.54	0.2062	0.2332	0.2062
MSC	11.28316	8	1.41040	0.35	0.9401	0.8570	0.9401
MST	53.02236	4	13.25559	3.30	0.0189	0.0497	0.0189
MSCT	70.43450	8	8.80431	2.19	0.0465	0.0946	0.0465
4 Error	176.64062	44	4.01456				
Gender	34.86120	1	34.86120	0.06	0.8082		
GC	919.87659	2	459.93830	0.82	0.4674		
GT	1068.28160	1	1068.28160	1.89	0.1961		
GCT	748.23186	2	374.11593	0.66	0.5345		
5 Error	6202.31229	11	563.84657				
MG	300.00772	1	300.00772	0.34	0.5730		
MGC	3745.10184	2	1872.55092	2.11	0.1680		
MGT	484.41672	1	484.41672	0.55	0.4758		
MGCT	2135.03986	2	1067.51993	1.20	0.3374		
6 Error	9776.69348	11	888.79032				
SG	11.05678	4	2.76419	1.01	0.4113	0.3903	0.4113
SGC	23.72382	8	2.96548	1.09	0.3905	0.3898	0.3905
SGT	12.55976	4	3.13994	1.15	0.3457	0.3400	0.3457
SGCT	31.71542	8	3.96443	1.45	0.2025	0.2385	0.2025
7 Error	120.11564	44	2.72990				

(Table continues on next page.)

Table A.16. Analysis of Variance for the Stochastic Spatial Discrimination Test: Matrix 1 and Matrix 2[a] *(Continued)*

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
MSG	4.80862	4	1.20216	0.42	0.7929	0.6662	0.7767
MSGC	16.30642	8	2.03830	0.71	0.6785	0.5945	0.6670
MSGT	11.10764	4	2.77691	0.97	0.4327	0.3955	0.4283
MSGCT	30.31607	8	3.78951	1.33	0.2561	0.2907	0.2618
8 Error	125.74489	44	2.85784				

[a] The dependent variable was the efficiency score, or percentage of maximum reinforcements secured. That is, the degree to which the rat's selection of successive lever positions approached optimal selection. This analysis is based on performances during the last 5 sessions.

[b] Cohort = all 4 cohorts; TRT = treatment (methanol vs control); CT = cohort × treatment.

[c] Matrix = Matrix 1 vs Matrix 2; MC = matrix × cohort; MT = matrix × treatment; MCT = matrix × cohort × treatment.
Session = sessions; SC = sessions × cohort; ST = sessions × treatment; SCT = sessions × cohort × treatment.
MS = matrix × session; MSC = matrix × session × cohort; MST = matrix × session × treatment; MSCT = matrix × session × cohort × treatment.
Gender = male vs female; GC = gender × cohort; GT = gender × treatment; GCT = gender × cohort × treatment.
MG = matrix × gender; MGC = matrix × gender × cohort; MGT = matrix × gender × treatment; MGCT = matrix × gender × cohort × treatment.
SG = session × gender; SGC = session × gender × cohort; SGT = session × gender × treatment; SGCT = session × gender × cohort × treatment.
MSG = matrix × session × gender; MSGC = matrix × session × gender × cohort; MSGT = matrix × session × gender × treatment; MSGCT = matrix × session × gender × cohort × treatment.

Table A.17. Analysis of Variance for the Stochastic Spatial Discrimination Test: Matrix 1[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
Cohort	1650.94990	2	825.47495	1.26	0.3217		
TRT	457.61245	1	457.61245	0.70	0.4212		
CT	1415.62385	2	707.81192	1.08	0.3731		
1 Error	7209.92348	11	655.44759				
Within Animal Factors[c]							
Session	12.41586	4	3.10396	1.24	0.3071	0.3103	0.3071
SC	10.68505	8	1.33563	0.53	0.8241	0.7526	0.8241
ST	14.17028	4	3.54257	1.42	0.2440	0.2594	0.2440
SCT	24.91670	8	3.11459	1.25	0.2959	0.3141	0.2959
2 Error	109.95710	44	2.49903				
Gender	65.16702	1	65.16702	0.09	0.7721		
GC	3070.07062	2	1535.03531	2.08	0.1717		
GT	56.97897	1	56.97897	0.08	0.7864		
GCT	2588.89043	2	1294.44521	1.75	0.2187		
3 Error	8131.31716	11	739.21065				
SG	10.82535	4	2.70634	0.92	0.4611	0.4373	0.4611
SGC	14.86443	8	1.85805	0.63	0.7470	0.6937	0.7470
SGT	9.52834	4	2.38209	0.81	0.5259	0.4909	0.5259
SGCT	30.84706	8	3.85588	1.31	0.2637	0.2837	0.2637
4 Error	129.49617	44	2.94309				

[a] The dependent variable was the efficiency score, or percentage of maximum reinforcements secured. That is, the degree to which the rat's selection of successive lever positions approached optimal selection. This analysis is based on performances during the last 5 sessions.

[b] Cohort = all 4 cohorts; TRT = treatment (methanol vs control); CT = cohort × treatment.

[c] Session = sessions; SC = sessions × cohort; ST = sessions × treatment; SCT = sessions × cohort × treatment.
Gender = male vs female; GC = gender × cohort; GT = gender × treatment; GCT = gender × cohort × treatment.
SG = session × gender; SGC = session × gender × cohort; SGT = session × gender × treatment; SGCT = session × gender × cohort × treatment.

Table A.18. Analysis of Variance for the Stochastic Spatial Discrimination Test: Matrix 2[a]

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*p*	Greenhouse-Geisser *p*	Huynh-Feldt *p*
Between Animal Factors[b]							
Cohort	1158.14301	2	579.07150	0.80	0.4731		
TRT	1771.85545	1	1771.85545	2.45	0.1456		
CT	437.63841	2	218.81920	0.30	0.7446		
1 Error	7944.64480	11	722.24044				
Within Animal Factors[c]							
Session	25.54373	4	6.38593	1.80	0.1457	0.1786	0.1457
SC	13.44489	8	1.68061	0.47	0.8679	0.7884	0.8679
ST	71.33450	4	17.83363	5.03	0.0020	0.0100	0.0020
SCT	102.96498	8	12.87062	3.63	0.0025	0.0127	0.0025
2 Error	156.01382	44	3.54577				
Gender	269.70190	1	269.70190	0.38	0.5512		
GC	1594.90781	2	797.45391	1.12	0.3615		
GT	1495.71935	1	1495.71935	2.10	0.1755		
GCT	294.38130	2	147.19065	0.21	0.8167		
3 Error	7847.68861	11	713.42624				
SG	5.04005	4	1.26001	0.48	0.7528	0.6544	0.7528
SGC	25.16581	8	3.14573	1.19	0.3272	0.3412	0.3272
SGT	14.13906	4	3.53476	1.34	0.2715	0.2827	0.2715
SGCT	31.18442	8	3.89805	1.47	0.1943	0.2351	0.1943
4 Error	116.36436	44	2.64464				

[a] The dependent variable was the efficiency score, or percentage of maximum reinforcements secured. That is, the degree to which the rat's selection of successive lever positions approached optimal selection. This analysis is based on performances during the last 5 sessions.

[b] Cohort = all 4 cohorts; TRT = treatment (methanol vs control); CT = cohort × treatment.

[c] Matrix = Matrix 1 vs Matrix 2; MC = matrix × cohort; MT = matrix × treatment; MCT = matrix × cohort × treatment.
Session = sessions; SC = sessions × cohort; ST = sessions × treatment; SCT = sessions × cohort × treatment.
MS = matrix × session; MSC = matrix × session × cohort; MST = matrix × session × treatment; MSCT = matrix × session × cohort × treatment.
Gender = male vs female; GC = gender × cohort; GT = gender × treatment; GCT = gender × cohort × treatment.
MG = matrix × gender; MGC = matrix × gender × cohort; MGT = matrix × gender × treatment; MGCT = matrix × gender × cohort × treatment.
SG = session × gender; SGC = session × gender × cohort; SGT = session × gender × treatment; SGCT = session × gender × cohort × treatment.
MSG = matrix × session × gender; MSGC = matrix × session × gender × cohort; MSGT = matrix × session × gender × treatment; MSGCT = matrix × session × gender × cohort × treatment.

APPENDIX B. Supplementary Data Tables

Table B.1. Daily Mean Methanol Concentration (ppm)

	Cohort 1		Cohort 2		Cohort 3		Cohort 4	
Day	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	5363	146	4136	43	4480	192	4449	102
2	5273	202	4248	92	4067	212	4510	152
3	4859	452	4691	324	3552	566	4410	91
4	4921	421	4880	154	4704	247	4497	175
5	4627	81	4663	119	4369	331	4371	434
6	4378	44	4294	294	4520	446	4515	487
7	4571	105	4413	124	4956	151	4471	129
8	4175	117	4328	121	4451	49	4539	402
9	4245	77	4457	119	4964	161	4597	92
10	4169	55	4497	239	4735	444	4581	49
11	4467	176	4263	240	4747	29	4550	200
12	4400	50	4411	85	4517	47	4456	354
13	4418	132	4367	128	4223	597	4622	314
14	3697	37	4560	89	4595	235	4464	179
15	*[a]		4492	172	4672	190	4523	103
16	4623	621	4460	96	5209	169	4556	120
17	4170	141	4341	131	5021	137	4477	190
18	4440	170	4336	63	5043	214	4776	121
19	*[b]		4282	148	4209	151	4544	144
20	4778	149	4358	91	4427	155	4376	111
21	4633	233	4395	246	4308	389	4424	168
22	4523	152	4253	123	4709	548	4599	159
23	4110	108	4328	130	4771	176	4687	107
24	3945	102	4398	263	4661	92	4621	190
25	4065	391	4518	109	4832	148	4399	157
26	4250	321	4689	321	4638	700	4288	149
27	4388	175	4033	108	4441	395	4462	195
28	4369	168	4172	337	4905	189	4197	442
29	4364	62	4082	237	4639	203	4531	128
30	4422	183	4302	196	4984	215	4348	328
31	4494	86	4379	52	4725	712	4533	146
32	4308	181	4354	151	4550	187	4412	245
33	4416	147	4411	118	4906	241	4341	170
34	4238	75	4145	164	4429	164	4436	121
35	4082	147	4214	108	4432	226	4414	383
36	3772	20	4469	172	4615	147	4428	38
37	3902	309	4514	98	4549	181	4373	243
38	3558	253	4474	61	4435	87	4317	185
39	3649	66	4321	411	4435	228	4389	770
40	4308	283	4593	298	4490	298	4399	232
41			4310	114	4470	372	4430	191
42			4197	167	4229	271		
43					4131	251		
44					4846	266		

[a] System failure: two-hour exposure.

[b] System failure: four-hour exposure.

Table B.2. Fixed-Ratio Wheel-Running Responses per Minute: Summary Measures for Methanol-Exposed and Control Males (M) and Females (F), All Four Cohorts

	Condition[a]						
	First 5	Last 15	125%	150%	175%	200%	EXT
M 4,500 ppm Mean	9.46	9.38	9.31	9.60	9.73	9.19	7.41
M 4,500 ppm SEM	0.72	1.04	0.74	0.98	1.27	1.09	1.61
M Control Mean	9.05	10.06	10.03	11.86	11.42	11.61	7.36
M Control SEM	0.85	1.44	1.71	2.41	2.45	2.17	0.90
F 4,500 ppm Mean	7.02	8.44	8.46	9.96	9.34	9.76	7.36
F 4,500 ppm SEM	1.31	0.78	0.72	1.10	0.94	1.20	0.99
F Control Mean	7.13	7.32	7.21	8.03	8.39	8.07	5.49
F Control SEM	1.82	1.02	1.39	1.06	0.98	1.08	0.72

[a] Data are for the first 5 baseline sessions, the last 15 baseline sessions, single sessions when the FR requirements were raised by 25%, 50%, 75%, and 100%, and during the fifth session of extinction (EXT).

Table B.3. Fixed-Ratio Wheel-Running Mean Responses per Minute Minus the Mean Responses per Minute During the First Five Baseline Sessions: Summary Measures for Methanol-Exposed and Control Males (M) and Females (F), All Four Cohorts

	Condition[a]					
	Last 15	125%	150%	175%	200%	EXT
M 4,500 ppm Mean	−0.08	−0.15	0.14	0.27	−0.27	−2.04
M 4,500 ppm SEM	0.72	0.54	0.67	1.18	0.98	1.32
M Control Mean	1.01	0.97	2.81	2.37	2.55	−1.69
M Control SEM	0.99	1.21	1.59	1.74	1.41	1.15
F 4,500 ppm Mean	1.42	1.44	2.94	2.32	2.74	0.34
F 4,500 ppm SEM	0.77	0.79	0.58	0.70	0.62	1.05
F Control Mean	0.20	0.08	0.90	1.26	0.94	−1.63
F Control SEM	1.03	1.05	0.81	1.00	0.84	1.34

[a] Data are for the last 15 baseline sessions, the single sessions when FR requirements were raised by 25%, 50%, 75%, and 100%, and during the fifth session of extinction (EXT).

ABOUT THE AUTHORS

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Ray Preston is a Research Scientist at the Department of Environmental Medicine at the University of Rochester School of Medicine and Dentistry. Before coming to the University of Rochester in 1991, he was a Visiting Scientist at the Walter Reed Army Institute of Research in Washington, DC, and a Visiting Instructor at the University of Maryland in College Park. He received his Ph.D. at the University of California at San Diego in 1989. He has undertaken research on basic animal behavior and on behavioral pharmacology and toxicology.

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Robert Gelein received his B.S. in chemistry from the University of Wisconsin at Eau Claire. He was a research associate at Duke University from 1968 to 1978. While there, he also pursued graduate studies at the University of North Carolina–Chapel Hill in biochemistry and physiology. He has been at the University of Rochester since 1978 and is now an Associate in the Department of Environmental Medicine, School of Medicine and Dentistry. His research interests are in the areas of analytical chemistry and inhalation toxicology.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Sharma A, Stern S, Soderholm S, Weiss B. 1992. Blood methanol concentrations in the pregnant rat, dams and pups exposed by inhalation. Presented at a meeting of the Society of Toxicology, Seattle, WA, February 23–27, 1992.

Weiss B. 1993. Neurobehavioral toxicity of methanol. Presented at a meeting of the Behavioral Toxicology Society, Annapolis, MD, May 1993.

ABBREVIATIONS

ANOVA	analysis of variance
EPA	U.S. Environmental Protection Agency
FR	fixed ratio
GD	gestational day
HPLC	high-performance liquid chromatography
IRT	interresponse time
LFB/PAS	Luxol fast blue/periodic acid–Schiff stain
NCAM	neural cell adhesion molecule
NEDO	New Energy Development Organization (Japan)
NIOSH	National Institute for Occupational Safety and Health
PND	postnatal day
ppm	parts per million
r	estimate of the Pearson correlation coefficient from a sample
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

The Clean Air Act Amendments of 1990 designated methanol a "clean alternative fuel" for motor vehicles. Using methanol as an alternative fuel may offer important advantages to human health and quality of life by decreasing emissions of hydrocarbons that help form atmospheric ozone. As a result, its use could help address the continued inability of many urban areas of the United States to meet air quality standards, which is due, in large part, to the atmospheric ozone produced when hydrocarbons are released through evaporative emissions, exhaust emissions, and refueling operations associated with the use of motor vehicles. Although methanol substitution for, or its use in, gasoline could have significant remedial effects on pollution, its use raises substantial concerns about potential health risks posed by increased exposure of the general population to methanol vapors.

When methanol was first proposed as an alternative fuel, the Health Effects Institute evaluated the existing information on the health effects of methanol from the peer-reviewed literature to determine what health problems might emerge if it were to become more widely used (Health Effects Institute 1987). At that time, the HEI concluded that the low-level, brief, and intermittent exposure to methanol vapors that would occur from evaporative emissions or exhaust emissions from methanol-fueled vehicles probably would not result in adverse health effects. However, the HEI recognized that sufficient research had not been done to eliminate the possibility of health effects in individuals chronically exposed to low levels of methanol. Because methanol continues to be considered seriously as a gasoline additive or replacement in the United States, the HEI has continued to support research on the potential health effects of exposure to methanol (Cook et al. 1991). The study described in this report was funded to examine the effects of prenatal exposure to methanol on the neurobehavior of rats.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA)* set standards for air pollutants under Section 202 of the Clean Air Act Amendments of 1990. Section 202 (a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." The Act imposes specific requirements for reducing motor vehicle emissions of certain oxidants (and other pollutants) and, in some cases, gives the EPA limited discretion to modify those requirements.

Several legislative changes instituted by the Clean Air Act Amendments of 1990 relate to methanol. Although described in Section 112 as a "hazardous air pollutant," methanol is also defined in Section 219 as a "low-polluting fuel" and in Section 241 as a "clean alternative fuel." The use of low-polluting fuels is required for all "urban buses" used in "all metropolitan statistical areas or consolidated metropolitan statistical areas with a 1980 population of 750,000 or more" if certain targets are not met for the level of particulate matter in the air. In addition, the Administrator may extend the use of low-polluting fuels for urban buses in areas where the 1980 population was less than 750,000 if "a significant benefit to public health could be expected to result from such extension." Sections 241 through 250 of the Act, as amended by Section 229 of the Amendments, also requires the Administrator to implement a program for "clean-fuel vehicles" to be used by some "centrally fueled fleets" in various areas that do not attain the standards for air pollutants.

The Energy Policy Act of 1992 helped to determine how government and private industry would proceed to fulfill the low-polluting and clean-fuel mandates of the Clean Air Act. The Energy Policy Act was designed to stimulate the private sector to manufacture and provide alternative fuels for a variety of vehicles. High-octane alcohol fuels were developed as a result of the incentives in this Act. Given the activities supported by the Energy Policy Act, alcohol fuels are expected to make a significant contribution to the combination of fuels that will be used by the year 2000.

SCIENTIFIC BACKGROUND

Concern about the potential toxicity of methanol originates from the well-documented and severe effects of acute methanol poisoning from both accidental and intentional ingestion (Hunter 1975; Hayreh et al. 1980; LeWitt and Martin 1988). In those individuals who do not die from such exposures, the most severe effects are blindness due to optic

* A list of abbreviations appears at the end of the Investigators' Report for your reference.

nerve damage (Hayreh et al. 1980) and residual motor dysfunction resulting from basal ganglia damage (LeWitt and Martin 1988). The biochemical mechanisms responsible for methanol toxicity during acute exposures are well understood. Toxicity results from the excessive accumulation of formic acid (a methanol metabolite), which leads to metabolic acidosis (reviewed by Kavet and Nauss 1990).

Most analysts predict that the levels of human exposure to methanol via vapors from motor vehicles would be much lower than those experienced by individuals suffering acute methanol poisoning by ingestion. On the basis of limited emissions data, Gold and Moulis (1988) projected exposure concentrations for methanol vapors from evaporative and motor vehicle exhaust emissions. As reviewed by Kavet and Nauss (1990), maximal exposures to evaporative emissions are expected to be 1 to 10 ppm in typical traffic situations, and they may be as high as 200 ppm in a worst-case scenario such as a malfunctioning vehicle in an enclosed garage. However, these latter high exposures would usually last only a matter of minutes. In light of this, at even the highest estimated levels of exposure to methanol vapors from auto emissions, formate, the most toxic metabolite of methanol, should be completely metabolized (Kavet and Nauss 1990). Therefore, based on current knowledge of methanol metabolism, even levels experienced in a worst-case scenario would not be high enough to cause toxicity.

The low levels of exposure to methanol anticipated during refueling suggest that there will be little risk of human mortality from vapor inhalation; however, some animal studies have shown that exposure to high concentrations of methanol can have effects on mammalian development. Pregnant rats exposed to methanol by inhalation at concentrations ranging from 5,000 to 20,000 ppm for 7 hours daily on days 1 through 19 of gestation reportedly gave birth to offspring showing developmental malformations and reduced weights (Nelson et al. 1985). In a study of malformations in offspring of pregnant mice that inhaled methanol concentrations ranging from 1,000 to 15,000 ppm for 7 hours daily on days 6 through 15 of gestation, Rogers and colleagues (1993) observed an elevated incidence of exencephaly in mice exposed to 5,000 ppm methanol and above, as well as skeletal abnormalities in mice exposed to 2,000 ppm methanol and above. In addition, Bolon and coworkers (1993) showed that the induction of developmental abnormalities (such as neural tube defects, ocular lesions, limb abnormalities, hydronephrosis, and cleft palate) in neonatal mice exposed in utero to 10,000 to 15,000 ppm methanol was dependent on both the stage of fetal development at exposure and the timing and magnitude of exposure. However, species differences in methanol metabolism (McMartin et al. 1977) make direct comparison of exposure levels difficult.

The possibility of developmental neurotoxicity, which has been shown to result from lower and more prolonged exposures to some environmental neurotoxins (Needleman and Bellinger 1994), is of greatest concern when considering scenarios of low-level methanol exposure. For example, subtle neurotoxic effects occur in the offspring of women who consume alcohol at levels far lower than those necessary to cause the characteristic malformations of the fetal alcohol syndrome (Streissguth et al. 1986). These observations suggest that the developing human fetus is a subpopulation at risk when pregnant women are exposed to methanol vapors or other pollutants. In an attempt to study this possibility, Infurna and Weiss (1986) exposed pregnant rats to methanol in drinking water (2% v/v) during gestation. The offspring of the exposed rats showed disrupted suckling behavior on postnatal day 1 and difficulties in locating nesting material from the home cage on postnatal day 10. The Health Effects Institute supported Dr. Weiss and his colleagues in order to extend this work to the effects of inhaled methanol at environmentally relevant concentrations.

JUSTIFICATION FOR THE STUDY

As a result of the concerns relating to the potential developmental neurotoxicity of inhaled methanol, in 1989 the HEI Research Committee issued RFA 89-1, titled "Health Effects of Methanol Exposure: Metabolism and Pharmacokinetics; Fetal and Perinatal Neurotoxicity; Reproductive Toxicity," requesting applications on these topics in order to improve the understanding of the exposure-dose–response relation for methanol. The HEI Research Committee was interested in methanol because of its potential use as a fuel additive or alternative. The RFA was broadly based because, as described above, little information was available on the metabolism and health effects of low-level exposures to methanol although the literature on the acute toxicity of high-level exposures was substantial.

In response to this RFA, Dr. Bernard Weiss and colleagues at the University of Rochester submitted an application titled "Developmental Neurotoxicity of Methanol Exposure." The rationale for the study was to confirm and extend the results of two previous studies showing that prenatal exposure to methanol caused teratogenic and behavioral effects in the offspring of rats (Nelson et al. 1985; Infurna and Weiss 1986). This four-year project, which began in June 1990, had total expenditures of $200,947. The

investigators submitted their report in June 1994, and the Review Committee accepted their revised report in April 1995.

During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the HEI Review Committee's Commentary. This Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the studies and by placing the Investigators' Report into scientific and regulatory perspective.

OBJECTIVES AND STUDY DESIGN

The objective of the study was to determine the effects of methanol exposure in utero and through weaning on the behavioral development of Long-Evans hooded rats. The original specific aims were to (1) expose pregnant rats, and then suckling mothers and their litters until weaning, to 0, 500, 1,500, or 4,500 ppm methanol vapor, and (2) evaluate the neurotoxicity of this exposure regimen on pairs of male-female littermates by using behavioral test procedures on neonates and on the adult rats that had been exposed to methanol as newborns. The investigators also analyzed blood methanol concentrations in the mothers and neonates.

The investigators selected three neonatal tests on the basis of their utility for assessing different behaviors at different ages in developing rats. First, the suckling test, which measured the latency time to nipple attachment on postnatal day 5, simultaneously evaluated several functional capacities of the very young neonate. Second, the conditioned olfactory aversion test paired a brief electrical shock with an odorant under an aversive conditioning regimen and then measured the relative time that conditioned pups on postnatal day 10 spent in different areas of specially designed cages in which odorants could be localized; this test evaluates the sensory capacities of neonates, which are known to undergo rapid changes in the period immediately following birth. Third, the motor activity test measured the level of activity of neonates on postnatal day 18 by recording their movements in a specially designed sound-attenuated cage.

When offspring became adults two behavioral tests were performed to measure the neurotoxicity of methanol exposure during their development and weaning. First, in the fixed-ratio wheel-running test rats were conditioned to obtain a food pellet reward on completion of a fixed number of rotations on a running wheel. The wheel was designed to be sensitive to motor deficits produced by neurotoxicants. This test provides a measure of motor function intactness, which is important because neurotoxicants produce deficits in coordination and strength. Also, it provides a means for assessing schedule-controlled operant behavior, which is considered to be a particularly useful index for evaluating neurotoxicity (U.S. Environmental Protection Agency 1991). Second, in the stochastic spatial discrimination test, rats that had undergone preliminary lever-press training were conditioned to operate three levers in a specific sequence in order to maximize the food pellet reward, and then the sequence that maximized the reward was changed. This test assesses cognitive functioning by complex spatial discrimination based on the ability of the rat to modify its behavioral sequence in response to changing probabilities of obtaining a food reward.

Brain tissue sections from experimental animals were subsequently examined, using standard light-microscopic techniques. The levels of neural cell adhesion molecule (NCAM), a protein that facilitates the association of certain brain cells, were measured in brain tissue by Western blotting.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

Dr. Weiss and his colleagues collected a considerable amount of information on the neonatal effects of exposure to 4,500 ppm methanol vapor. However, they did not obtain any data at lower methanol levels, which are more environmentally relevant. Originally, the investigators also planned to test methanol exposure levels of 500 and 1,500 ppm. However, while this study was under way, Stanton and colleagues (1991) reported no behavioral effects in rats exposed to methanol levels below 4,500 ppm. Therefore, the investigators and the HEI Research Committee agreed to test only the higher 4,500 ppm level. In retrospect, and in light of the results found in this study, it may have been informative to test lower exposure levels, particularly as part of a pattern of multiple, repetitive exposures.

ASSESSMENT OF METHODS AND STUDY DESIGN

The investigators selected behavioral tests that were appropriate to address their objectives. They analyzed a complex set of behavioral test data with great care and precision and were careful to match test animals by litter. In general, the data were meticulously obtained, the methods and data analyses are thoroughly described, and the results are presented clearly. In light of the investigators' acknowledgment that this study was directed primarily toward generating hypotheses, the results were cautiously interpreted.

STATISTICAL METHODS

The choice of statistical techniques was well justified, and the investigators provide good descriptions of the intricate randomization procedures and the repeated measures analysis. The narrative of experimental results is well integrated with the statistical analyses and tests of hypotheses. The graphical presentations are effective. The documentation and tabulation of the analysis of variance (ANOVA) in Appendix A are very thorough.

One limitation of the study is that no multiple testing compensation was made for the large number of statistical tests that were performed. Given the investigators' statement that the emphasis of this study was to conduct a battery of exploratory tests after exposure to high levels of inhaled methanol, rather than an investigation of effects over a range of exposure levels, the concern about the possibility of Type I statistical error is lessened as long as care is taken not to overinterpret the results.

RESULTS AND INTERPRETATION

In the tests of neonatal behavior, neither the suckling test (latency to nipple attachment) nor the conditioned olfactory aversion test showed any consistent and interpretable effect of methanol exposure. It is noteworthy that, using the inhalation route of exposure, the investigators were not able to confirm the effect of methanol exposure on suckling behavior that had been observed in an earlier study in which methanol was administered via drinking water (Infurna and Weiss 1986). In the motor activity test, methanol-exposed neonates were less active on postnatal day 18, but more active on postnatal day 25, than the equivalent control group pups. Why this pattern emerged is unclear.

Two behavioral tests were performed on adult rats that had been exposed to methanol as newborns. In the fixed-ratio wheel-running test, small effects of methanol were observed on motor coordination and on the rats' persistence in obtaining food rewards, but only when the genders were analyzed separately. In the stochastic spatial discrimination test, there were some relatively subtle performance differences in the rates at which the rats adjusted to a new pattern of lever-pressing. Specifically, the control animals performed better than the methanol-treated ones after extended training on the second of two sequence patterns of lever-pressing.

In total, the behavioral effects observed were small enough to be close to the limit of statistical significance. In addition, the small effects emerged from a large number of statistical tests in which no compensation procedure for multiple testing was performed. Therefore, some concern about ascribing a coherent pattern of effects to the results obtained is justified. Although the results from treatment with 4,500 ppm methanol by inhalation do not allow definitive conclusions about physiological doses of methanol, the small effects observed do give important information about directions for future research on prenatal and neonatal methanol exposure.

In addition, although no significant abnormalities were seen by light-microscopic analysis of tissue sections from the brains of methanol-treated animals, NCAM levels in brain tissue showed some difference due to methanol exposure on postnatal day 4. The investigators also observed that the average blood methanol concentrations of pups were approximately twice those of their suckling mothers when measured on postnatal days 7 and 14. A measurable difference in blood methanol concentrations persisted until approximately postnatal day 48. This is an interesting result that may warrant further research because it indicates a potentially important difference in methanol pharmacokinetics between neonates and their mothers.

In summary, the results of this study contribute to our understanding of methanol neurotoxicity in rats at high levels of exposure. However, care must be taken not to over emphasize the small and variable effects seen. Moreover, the methanol exposure level of 4,500 ppm used in this study is much higher than the exposure levels of less than 1 to approximately 500 ppm anticipated if methanol is used as an alternative fuel. The value of this study can be more readily appreciated if it is viewed as one in which the future direction of research in this area is at least partially delineated.

IMPLICATIONS FOR FUTURE RESEARCH

Given the results of this study, two areas of research on methanol exposure may prove to be particularly useful to pursue. First, it may be important that the blood methanol concentrations of pups is twice that of their suckling mothers, apparently indicating pharmacokinetic differences between neonates and mothers and reflecting maturation of the metabolic pathways that handle methanol elimination. The possibility that blood levels of methanol could be significantly higher in developing humans than in their mothers may be a critical factor in evaluating the effects of methanol exposure. Experiments in which the persistence of this pharmacokinetic difference is explored and the effect that different exposure patterns have on blood methanol concentrations and associated behavioral testing could be useful for further understanding the potential toxicity of methanol.

Second, both adult tests, the fixed-ratio wheel-running test and stochastic spatial discrimination test, indicated

possible gender-treatment interactions in response to methanol exposure. These results suggest that the influence of gender on the response to methanol exposure should be explored further in future studies.

CONCLUSIONS

Dr. Weiss and colleagues exposed pregnant rats to 4,500 ppm methanol vapor beginning on gestational day 6; after birth, both pups and suckling mothers were exposed to the same regimen through postnatal day 21. Blood methanol concentrations in the pups were approximately twice those in their suckling mothers when measured on postnatal days 7 and 14. Of the three behavioral tests performed on neonates exposed to methanol, two (the suckling and conditioned olfactory aversion tests) showed no neurotoxic effect of methanol exposure; one (the motor activity test) showed decreased activity at postnatal day 18, and increased activity on postnatal day 25. Of the two behavioral tests performed on adults, one (the fixed-ratio wheel-running test) showed methanol-induced effects, but only when the genders were analyzed separately; the other test (stochastic spatial discrimination) showed some relatively subtle performance differences in the rate of adjustment to a new pattern of lever-pressing in methanol-exposed animals.

The HEI Review Committee noted that the investigators performed many tests, finding some isolated positive results that were small and variable and emerged from a large number of statistical tests in which no compensation was made for multiple testing. In addition, the effects were seen at a level of methanol exposure higher than levels likely to be experienced by the general public. In light of these facts, care must be taken in ascribing a coherent pattern of effects to the positive results. Overall, the investigators' results point to the neurobehavioral mechanisms that should be studied when evaluating the potential neurologic effects of methanol exposure by inhalation. Such investigations would help to clarify and focus questions surrounding the potential neurotoxic effects of methanol exposure and, thus, suggest a fruitful avenue of approach to future experimentation.

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