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Perinatal Methanol Exposure in the Rat

II. Behavioral Effects in Neonates and Adults

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The use of methanol as a component of automobile fuel will increase perinatal exposures in the general population. Few studies have addressed questions concerning neurotoxicity stemming from such exposures. In the current study, four cohorts of pregnant Long–Evans rats, each cohort consisting of an exposure and a control group, were exposed to 4500 ppm methanol vapor in Rochester-type inhalation chambers for 6 hr daily beginning on Gestation Day 6. Exposure continued for both dams and pups through Postnatal Day 21 (PND 21) to model gestational and neonatal toxicity in humans. Several behavioral procedures were used to assess exposure effects in the offspring. Male–female littermates were studied whenever possible to examine sex differences, with one pair from a litter for each procedure. Exposure to methanol did not affect suckling latency and nipple attachment on PND 5 or performance on an aversive olfactory conditioning procedure on PND 10. Exposure to methanol did alter performances in a motor activity procedure. Methanol-exposed neonates were less active on PND 18, but more active on PND 25 than the equivalent control group pups. Two operant conditioning procedures, not used previously in this context, assayed other littermates as adults. A fixed ratio schedule required the rat to rotate a running wheel a specified number of revolutions to obtain food-pellet reinforcers. When the fixed ratio requirement changed, number of responses (revolutions) per 1-hr session displayed a complex interaction with treatment. Changes in performance over the course of training differed between males and females depending on exposure to methanol. Compared to initial baseline performances, methanol-exposed males showed decreases, and methanol-exposed females increases, in the rate of running. A stochastic spatial discrimination procedure permitted subjects to respond on any three levers, with the probabilities of food-pellet delivery determined by the location of the preceding response. A reinforcement matrix defined the response sequence required to maximize reinforcements. When the matrix was changed, the methanol-exposed subjects responded less efficiently at asymptotic levels of performance than controls. Across procedures, developmental exposure to 4500 ppm methanol vapor was associated with subtle behavioral changes in both neonates and adults. © 1997 Society of Toxicology.

Use of methanol as an automotive fuel blended with gasoline, or replacing it, may result in long-term, low-concentration exposures via inhalation in the general population. Estimated concentrations to which the general public would be exposed under various scenarios range from <1 to ~100 ppm (cf., Kavet and Nauss, 1990, pp. 23 and 24). Although neurotoxic consequences of high-dose, acute exposures to methanol are well-documented and understood, its potential neurotoxicity at much lower concentrations for extended durations, especially during early brain development, has received little evaluation.

Our recognition of the Fetal Alcohol Syndrome (FAS), especially findings that indicate subtle consequences following maternal consumption at doses below those producing malformations and mental retardation in humans (Streissguth, 1986), provokes concerns about potentially similar outcomes following prenatal exposure to methanol. Animal models have provided useful tools for increasing our understanding of this phenomenon (e.g., Meyer and Riley, 1986; West and Goodlett, 1990).

Evidence that methanol might be a potential developmental toxicant for humans comes from a limited number of animal studies. Nelson *et al.* (1985) exposed pregnant rats by inhalation, for 6 hr daily, to concentrations of methanol and ethanol ranging from 5000 to 20,000 ppm. For equivalent air concentrations, methanol produced more malformations and induced more weight depression than ethanol. A study of malformations in mice by Rogers *et al.* (1993) exposed the pregnant dams, for 7 hr daily, to concentrations ranging from 1000 to 15,000 ppm and observed an elevated

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incidence of exencephaly at 5000 ppm and above; some skeletal abnormalities occurred at 2000 ppm and above. These results indicate that the mouse is more sensitive to some toxic effects of methanol than the rat. Bolon *et al.* (1993), in a mouse study aimed at the identification of critical periods, found increased resorptions, reduced fetal weights, and/or fetal malformations at 10,000 and 15,000 ppm. Exposures at 5000 ppm yielded no observable adverse effects. At the higher exposure levels, neural tube defects and ocular lesions occurred after methanol exposure during Gestation Days (GD) 7–9. Limb anomalies were seen when exposures occurred during GD 9–11. Exposure during either 3-day period produced cleft palate and hydronephrosis. These data indicate that specific developmental abnormalities induced by methanol, as is typical of teratogenic assays, depend upon both the stage of development and the timing and magnitude of exposure.

Few studies have examined functional consequences of such exposures. Infurna and Weiss (1986) exposed pregnant rats to methanol in drinking water (2%, v/v) during GD 15–17 or 17–19. Both groups of exposed offspring showed disrupted suckling behavior on Postnatal Day (PND) 1 and difficulties in locating nesting material from the home cage on PND 10. Stanton *et al.* (1991, 1995) exposed rats via inhalation exposure, 7 hr daily, during GD 7–19, to a concentration of 15,000 ppm. Maternal blood levels measured about 3 mg/ml. An extensive battery of behavioral tests administered to the offspring failed to reveal any persistent adverse effects.

In the current project (Weiss *et al.,* 1996), pregnant rats, and then both the dams and their litters, were exposed to 4500 ppm methanol vapor from GD 6 through PND 21. Four cohorts, each consisting of an exposure group and a control group, were studied. Although we had originally planned on including lower methanol-vapor concentrations in this study, when Stanton *et al.* (1991) reported that significant functional effects were not observed in the offspring of pregnant rats exposed to 15,000 ppm methanol vapor, we adopted the current design. We rejected going to a concentration higher than 4500 ppm, because (1) we wished to use concentrations below those producing indications of teratogenicity (Nelson *et al.* (1985) reported malformations at 10,000 ppm) and (2) we deemed it important to expose the neonates during the preweaning period when significant rodent brain development occurs.

We previously reported (Stern *et al.,* 1996) that blood methanol concentrations of the pups at the end of a 6-hr exposure were about twice those of the 500–800 μg/ml found in the dams. Supplementary observations with additional offspring demonstrated declines in post-6-hr exposure levels as the pups matured, but the levels exceeded the dam's until at least PND 48. Neural-cell adhesion molecules (NCAMs) for both the 140 and 180 kDa isoforms showed less intense staining on PND 4 in the methanol-exposed pups than the controls. Those outcomes raise questions about the risks posed by perinatal methanol exposures.

In this report, we describe the results of the tests used to evaluate possible behavioral effects at several ages following those exposures. Different tests were used to study pairs of male–female littermates both as neonates and adults. The selected tests may be considered as "apical" (Geyer *et al.,* 1985) in that each simultaneously assesses several functional capacities. The tests, therefore, were not designed to examine a single potential effect of methanol exposure, or the mechanism underlying such an effect, although the results may provoke useful hypotheses about controlling variables (Evans, 1994). This exploratory approach was adopted in view of the limited data available.

Neonatal rats undergo rapid changes in sensory and other behavioral capacities during the period directly following birth. The three neonatal tests, a test of suckling behavior, olfactory conditioning, and general motor activity, were selected on the basis of their utility for assessing behaviors at different ages in developing rats. Furthermore, the tests have been used previously, with other agents, to detect neurobehavioral toxicity.

The suckling test, conducted on PND 5, was selected because it simultaneously tests the development of several functional capacities of the very young neonate that may be altered by prenatal pretreatments (e.g., Barron *et al.,* 1991). It was used by Infurna and Weiss (1986) for a prenatal methanol study in which methanol was administered via the dam's drinking water and by Chen *et al.* (1982) for studies of prenatal ethanol effects.

Olfactory aversive conditioning, conducted on PND 10, has been used for assessing the development of odor discrimination capacities, learning, and memory in the neonate (e.g., Miller *et al.,* 1989), and it has proven to be sensitive to prenatal treatments such as ethanol (e.g., Barron *et al.,* 1988) and other agents (e.g., Stanton, 1991). The procedure offers greater control over experimental variables than that used by Infurna and Weiss (1986), where pup orientation to the odor of the home cage was diminished as a result of oral methanol treatment of the dam.

A test of general motor activity was used on PND 18 and PND 25. The USEPA developmental neurotoxicity guidelines recommend such evaluation. Motor activity consists of a number of components, which can be defined on the basis of temporal and spatial characteristics. There is no standard protocol for such measurement; different laboratories have used different measuring devices and response definitions. We arbitrarily selected a device available for the assessment, since there was no compelling reason for choosing any other.

Schedule-controlled operant wheel running was studied

in the adult offspring. This provided a measure of motor function in the adult, an important endpoint because many developmental neurotoxicants such as ethanol (Streissguth, 1986) produce deficits in coordination and strength. In addition, schedule-controlled operant behavior is considered to be particularly useful for evaluating neurotoxicity (e.g., USEPA 1991).

Stochastic spatial discrimination learning, a different operant behavior, was used to provide an assessment of cognitive functioning (Weiss and Heller, 1969). The complex spatial discrimination test was based on the rat's ability to learn and 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). This procedure adds another level of complexity by introducing stochastic relations. As task complexity increases, we expect its sensitivity to increase to other variables (Thompson *et al.*, 1975), including chemical ones such as methanol.

MATERIALS AND METHODS

Weiss *et al.* (1996) and Stern *et al.* (1996) provide detailed descriptions of the subjects, the breeding protocol, the maintenance conditions, the subject-sampling procedures, and the exposure environment and procedures.

Animals

Virgin female Long–Evans hooded rats (Charles River Breeding Laboratories, Wilmington, MA) were bred with Long–Evans males (also from Charles River). A separate group of test dams was bred at the same time as the other groups. These females were used in the suckling test. A sperm-positive vaginal smear determined GD 0. Following such a determination the female was assigned randomly to one of the treatment conditions.

A total of four cohorts was bred. When a litter was discovered in the morning that date was designated PND 1. All litters greater than eight were culled to eight offspring on PND 4; whenever possible, four male and four female pups were selected randomly and, within gender, assigned numbers 1 through 4. This subject number determined the subsequent test assignment. Only one pair from a litter was assigned to an individual test. A small, subcutaneous India ink injection uniquely marked each pup in a litter.

Inhalation Facility and Procedures

Exposures were conducted in 2-m^3 hexagonal "Rochester" chambers (Cheng and Moss, 1989; Leach *et al.*, 1959). HPLC grade methanol (J.T. Baker, Phillipsburg, NJ; Catalog No. 9093-03) was introduced into the air stream by passing liquid methanol through a heated aluminum block mounted on the intake duct adjacent to each chamber from which the vaporized portion then flowed into the chamber. The rat cages were placed on two large mesh shelves located in the mid-region of the chamber. Methanol concentration within the chamber was monitored continuously by a Miran 1A (Foxboro Corporation, Foxboro, MA) gas infrared analyzer calibrated from gas chromatographic results and connected to a chart recorder.

Prior to breeding, females that later provided the methanol and control litters were adapted to the exposure session protocol by being placed into the chambers for 6 hr daily over several days; all were exposed to air during this period. Then, 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 experimental group was exposed to the nominal concentration of 4500 ppm methanol vapor. Control dams and litters were placed in an adjoining exposure chamber and exposed to air. The animals remained in the chambers for degassing of methanol for 30 min after vapor generation was terminated. They were then returned to the vivarium.

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 schedule-controlled behavior to assess long-term consequences of perinatal methanol exposure.

Table 1 lists the behavioral procedures and shows the number of male–female littermate pairs that were studied in each cohort. For neonatal testing, pups from all litters were tested. Pups from Cohort 1 were used to develop our procedures for the suckling and olfactory aversion tests; those results were not included in subsequent analyses. The motor activity test was introduced later into the general protocol following the recommendations of a project review committee, and so only pups from Cohorts 3 and 4 were studied. For the adult phase, litters were limited to those not used for the blood sampling (Stern *et al.*, 1996) procedures conducted during exposures. From those litters, half were assigned randomly to the fixed-ratio wheel running test, and the other half to a the stochastic spatial discrimination procedure.

Neonatal Phase

Suckling Test

This test was conducted on PND 5 immediately preceding the daily exposure session. A test dam was anesthetized by an ip injection of 65 mg/kg pentobarbital, which also inhibits milk release, and placed on her side in a different plastic breeder cage with the floor covered by an absorbent paper. The surface of the dam was maintained at about 35°C by using a heating pad and an infrared lamp. These dams came from the group bred specifically to serve in suckling tests, so that their own pups were about the same age as that of the tested pup; the dams were maintained in the same vivarium room assigned to the methanol exposure and control groups. Immediately prior to the first test that day, several of her pups were placed with her for a few minutes to suckle and thereby "prime" the nipples: they were then removed. A pup from one of the two treatment groups was then placed on the paper-covered, test-cage floor with its snout in contact with the ventral surface of the test dam. The latency to nipple attachment, defined as the time between the start of a trial and confirmed attachment, was determined with a maximum of three 2-min trials allowed. 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 additional times. Following the test, the pup was returned to its litter prior to that day's scheduled exposure.

Conditioned Olfactory Aversion Test

The general design was similar to that employed by Stanton (1991).

Subjects. On PND 10, one male and one female were removed from their litter prior to the methanol exposure session. All pups were held in one polycarbonate cage kept at approximately 31 to 33°C prior to testing and placed in a different one following 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 pups were tested in a randomized order.

Apparatus. Two training chambers (12.7 × 9.5 × 12.8 cm), designated as CS^+ or CS^-, were used. 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.

TABLE 1
Number of Pairs of Male–Female Littermates Included in Each Behavioral Test

	Cohort									
	1		2		3		4		Total	
Methanol (ppm):	0	4500	0	4500	0	4500	0	4500	0	4500
Neonates										
Suckling (PND 5)	*a*	*a*	7	8	4	7	10	11	21	26
Olfactory aversion (PND10)	*a*	*a*	7	7	4	7	8	8	19	22
Motor activity (PND 18; PND 25)	*a*	*a*	*a*	*a*	7	6	8	7	15	13
Adults										
Fixed ratio running wheel	2	2	3	3	3	4	4	4	12	13
Stochastic spatial discrimination	2	2	2	3	4	5	(4)[b]	(3)[b]	8	9

[a] Test not conducted.
[b] Excluded from parameter estimation analysis due to insufficient data from the second matrix.

In the CS^+ chamber, the floor was connected to a Coulbourn Programmable Shocker Model E13-35 (Coulbourn Instruments, Inc., Lehigh Valley, PA) with alternate rods wired in common to the two poles of the source. 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 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^+ and CS^- 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 odorant in the CS^+ chamber; the orange oil was used as the odorant in the CS^- chamber. A pad of each odorant was placed also into the test chamber at the end of the its designated region. Position was not counterbalanced to avoid confounding by the presence of lingering odors.

Training. A pup was placed into the CS^- chamber where it remained for 20 sec. It was then removed, held for 5 sec, and then placed into the CS^+ chamber for 20 sec. A Coulbourn Programmable Shocker provided 0.5 mA cutaneous electrical stimulation through the grid floor during sec 8–10 and 18–20. The sequence of exposures to CS^- followed by CS^+ constituted one training trial. Four such sequences of trials were conducted.

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. At the end of 60 sec, the pup was removed momentarily from the chamber regardless of its location, placed again in the center region of the chamber, and the next trial started. Three 60-sec trials were conducted in this manner. Pup location was defined as that region where both the head and torso were located. Time spent in each section was recorded by the investigator. Cumulative seconds in each region summed across trials constituted the dependent variable. Learning is demonstrated if, following repeated exposures to the paired vs unpaired conditions, the subject tends to move away from the CS^+ odorant when given the opportunity to do so.

ACTIVITY TEST

Subjects were removed from their litter on PND 18 prior to 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. They rejoined their litters in the vivarium after the exposure session for that day had ended. 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 a 62-cm long annulus-shaped Plexiglas chamber 17.8 cm high with an inside wall measuring 13.3 cm in diameter and the outside wall measuring 25.3 cm. 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 Corporation).

The rat was placed into the chamber, the door closed, and the 15-min session started. Data were recorded as counts (i.e., photodetector events) for each minute of the 15-min session. A 15-min period is typically long enough in duration to encompass an early phase of heightened activity succeeded by gradual diminution to a relatively sedentary phase (e.g., Stanton, 1994).

Adult Phase

Subjects were randomly assigned to the adult-phase tests with the restriction that, whenever possible, equal numbers of male–female littermate pairs were tested under the procedures. After weaning, the rats were maintained under a 12-hr light–dark cycle commencing at 6:00 AM, with body weights held constant through restricted postsession feeding of Purina RMH 5001 Lab Chow. The rats were housed individually in acrylic cages lined with pine bedding. All rats had continuous access to water.

Four test chambers were used in the operant running wheel procedure, and four were used in the stochastic spatial discrimination procedure. Within each procedure, 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 for 1 hr daily, 5 days per week, 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. As a result of testing and breeding logistics, testing of the successive cohorts began at 96, 205, 243, and 267 Days of age, with testing duration extended over 127, 95, 122, and 93 days, respectively.

Fixed-Ratio Running Wheel

Body weights were maintained at approximately 220 g for the females and 300 g for the males.

Apparatus. The 60-cm-diameter wheels were designed specifically to be sensitive to motor deficits produced by neurotoxicants rather than as instruments for monitoring locomotor activity. Youseff *et al.* (1993) provide additional details, including photographs. A pellet feeder and receptacle cup were mounted on each wheel support frame. The wheels were adjusted to allow rotation in only one direction. Kulig *et al.* (1985) devised a similar modification of the conventional wheel to assess impaired coordination. Each complete 360° rotation was recorded as a single response. The SKED system controlled experimental events and data acquisition. Each event and response was recorded in real time with 10 msec resolution.

Procedure. The rats were trained in steps, beginning with pellets placed in the pellet cups to encourage exploration, followed by gradually increasing response requirements for pellet delivery until the final criterion of 20 full rotations for each reinforcing event (a Fixed Ratio or FR 20 schedule of reinforcement) was imposed for females, and a criterion of FR 28 was imposed for males. These ratios were selected on the basis of our experience with the procedure, which showed that stable performances could be maintained at these values within gender. Each time a criterion number of rotations was attained, the rat received three 45 mg of food pellets delivered to the food cup accessible through a small port in the side of the running wheel chamber.

Behavioral challenges were introduced during the final 5 weeks of testing. During the first 4, the criterion number of rotations for food pellet delivery was increased for one session. Increments of 25, 50, 75, and 100% over the baseline ratio value were introduced in ascending order across weeks. The standard baseline condition was reintroduced the next day. Finally, during each daily session of the fifth week, no food pellets were delivered (extinction). This was accomplished by setting the ratio to an unattainable value.

Overall rate of responding, calculated as responses per minute, was the dependent variable.

Stochastic Spatial Discrimination Learning

Body weights were maintained between 235 and 245 g for the females and between 275 and 285 g for the males.

Apparatus. Experimental sessions were conducted in four identical standard operant conditioning chambers (Coulbourn Instruments, Model E10-10TC), with aluminum front, rear, and top panels and clear acrylic side walls, enclosed in a sound-attenuating enclosure (Coulbourn Instruments, Model E10-20) with an exhaust fan in continuous operation. The experimental space was 25 cm wide, 27 cm long, and 30 cm high. The front panel contained three response levers (Coulbourn Instruments, Model E21-03). The side levers were located approximately 2.5 cm above the floor and 3 cm from the left and right wall, and the center lever was equidistant between them. A minimum force of 0.25 N was required to operate the response levers. A horizontal bank of three 1-cm-diameter stimulus lamps, separated center-to-center by 1.5 cm, was located 4.5 cm above each lever. Reinforcement consisted of the delivery of a single 45-mg Noyes Standard Lab Animal Food Pellet (Improved Formula A) dispensed into a recessed food well located 4 cm above the floor and centered on the rear panel (Coulbourn Pellet Dispenser Model E14-12). Pellet delivery was accompanied by a 0.5-sec buzzer and flashing of a lamp located in the food well.

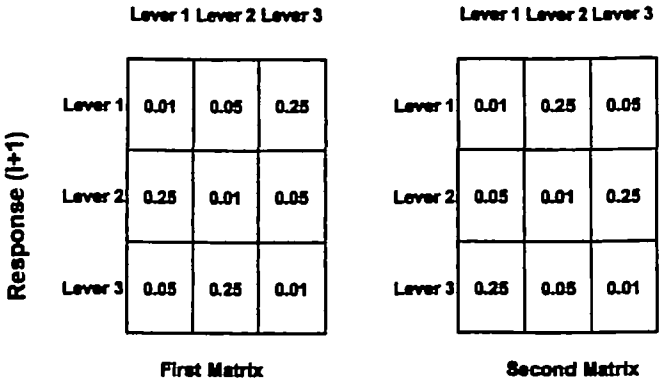

FIG. 1. Reinforcement probabilities for the stochastic spatial discrimination test. Each time a rat pressed one of three levers, a random number generator determined, on the basis of the location of the previous lever press and the indicated probabilities, whether a pellet would be delivered following the response.

A photodiode sensor located in the food well detected when the rats procured each pellet. General chamber illumination was provided by a single houselight centered on the back panel and 2 cm from the ceiling.

Data recording and scheduling of experimental events were controlled from a nearby room by a PDP-11 computer operating under SKED-11 software (Snapper *et al.*, 1982).

Preliminary training. Each rat was trained to respond on each of the three levers separately during preliminary training. In each training session, one lever only (left, center, or right) was designated the active lever. Every response on the active lever produced a food pellet, but responses on the other two levers had no programmed effect. Each session ended after either 100 pellets were earned or 45 min elapsed. In the first session, the left lever was the active lever. Left-lever training sessions were repeated until 100 pellets were earned within a session. This procedure was then repeated for both the center and right levers in that order. Then left, center, and right lever training sessions, with the same criterion for changing levers, was repeated two more times for each rat.

Stochastic Spatial Discrimination Procedure

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 relationship 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 Fig. 1, certain sequences maximized the probability of pellet delivery. Each time the rat pressed a lever, a random number generator determined, on the basis of the location of the previous lever press and the associated probability, whether a pellet would be delivered for the response. For example, in the first condition, Matrix 1, reinforcement probability was 0.25 for a left lever press (Response i + 1) if the previous response (Response i) was on the right lever, but only 0.01 if Response (i) occurred on the left lever. After responding stabilized under the first payoff matrix (Matrix 1), the matrix entries were changed as shown in the second payoff matrix (Matrix 2). Cohort 1 rats responded for approximately 46 sessions under Matrix 1 and 75 sessions under Matrix 2; Cohort 2 for 51 and 41 sessions under Matrix 1 and Matrix 2, respectively; Cohort 3 for 47 and 54 sessions; and Cohort 4 for 43 and 32 sessions under Matrix 1 and Matrix 2.

The primary measure of performance is the degree to which the rats' lever-press sequences were sensitive to the different probabilities of reinforcement, i.e., the degree to which high reinforcement-probability se-

quences were more likely than low reinforcement-probability sequences. The efficiency index (percent of maximum reinforcements earned) was calculated as the number of reinforcements (pellet deliveries) actually earned during a session divided by the maximum number of reinforcers possible during the session (given the number of responses emitted by each rat). The response measure, the percentage of maximum reinforcers earned, was calculated without including repeated (i.e., successive) responses on the same lever, even though there was a low probability of reinforcement for such responses. This definition of the response unit thereby precluded counting double responses, most of which appear to be caused by response topographies or position biases that are not directly controlled by the reinforcement schedule (e.g., Sidman, 1956; Laties *et al.*, 1965), and, therefore, are considered as sources of measurement error. For the first condition, shown by the entries in the First Matrix of Fig. 1, payoffs would be maximized by repeating the sequence LEVER 1–LEVER 2–LEVER 3–LEVER 1 and so forth, corresponding in the test chambers to left, center, right, left, etc. Following the change to the Second Matrix of Figure 1, which occurred after 45 sessions on the First Matrix, the optimal sequence became LEVER 1–LEVER 3–LEVER 2–LEVER 1, and so forth.

Statistical Methods

Data for this series of experiments were analyzed primarily by repeated measures analysis of variance. Each such analysis included both between animal (grouping) factors and within animal factors. Between animal factors in these analyses were Treatment (Methanol/Control) and Cohort (corresponding to replicate experiments); Cohort was not included in one of the suckling test analyses due to insufficient sample size, as noted below. Within animal factors represented mainly repeated measurements on each animal (either the same variable over time or 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 factor (in which case the unit of analysis was really the litter). Any other within factors were crossed with the Gender factor. Complete ANOVA tables are available in Appendix A in Weiss *et al.* (1996). Statistically significant p values, i.e., ≤ 0.05, and others of particular interest are identified and discussed here. The BMDP statistics software (BMDP2V) was used for all such analyses.

TABLE 2
Suckling Test: Latency (SD) and Proportion of Subjects Successfully Attaching to the Dam's Nipple

	Methanol (ppm)			
	0		4500	
	Latency in seconds (SD)			
Cohort 2	47.1 (40.2)		35.3 (28.4)	
Cohort 3	35.7 (13.6)		42.8 (51.6)	
Cohort 4	25.0 (17.9)		34.6 (21.9)	
	Proportion attaching			
	Male	Female	Male	Female
Cohort 2	0.71	0.57	1.00	1.00
Cohort 3	0.25	0.50	0.43	0.14
Cohort 4	0.30	0.10	0.18	0.27

TABLE 3
Olfactory Aversion Test: Cohorts 2, 3, and 4

	Time spent in each region of the test chamber		
	Paired	Center	Unpaired
Control mean	21.44	117.33	41.23
Control SEM	3.67	9.84	6.47
4500 ppm mean	28.99	102.75	48.26
4500 ppm SEM	2.18	4.28	2.10

RESULTS

Exposure to 4500 ppm methanol vapor during the developmental period between GD 6 and PND 21 altered motor activity tested at PND 18 and 25, and it altered both operant fixed-ratio running-wheel and stochastic spatial discrimination performances in the adult offspring. Methanol effects were not observed in neonates on the other two tests, i.e., suckling behavior on PND 4 and conditioned odor aversion on PND 10.

Neonatal Results

Suckling Test

Table 2 shows the latencies to nipple attachment and the proportion of subjects that attached to the nipple. Considerable variability within exposure conditions is apparent. There was no effect of methanol exposure on the pup's latency to attach to the nipple of the test dam ($p = 0.59$). 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. Attachment proportions 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 seen for females ($p = 0.03$) was not consistent across cohorts ($p = 0.24$).

Olfactory Aversion Test

Time spent in the three regions of the test chamber are summarized in Table 3. The label "Paired" designates the test chamber area above the pad containing the odor which was present during electric shock delivery. "Unpaired" designates the test chamber area, at the opposite end, above the pad containing the neutral odor. For all cohorts, the 10-day-old pups spent most of their time in the center area. When

out of the center area there was a tendency to spend more time in the presence of the Unpaired rather than the Paired region, indicating that the association of the Paired odor with shock had been learned.

Two questions were addressed in the statistical analysis. First, "Did exposure to methanol affect the distribution of times spent in the shock-paired, CS^+, and neutral odor, CS^-, regions of the test apparatus?" The analysis indicated that exposure to methanol did not affect the relative preferences ($p = 0.82$); it had no effect on performances among cohorts, nor on males vs females, which performed similarly under the procedure.

The second question asked is "Did exposure to methanol affect the amount of time spent in the center region?" Methanol exposure might have affected the overall level of 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 time spent in the center region ($p = 0.32$).

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 in this preparation is demonstrated by more time being spent in the Unpaired than the Paired region; i.e., the pups learn to move away from the odor that was paired with the shock. As time spent in the center region decreased, more subjects spent more time in the Unpaired region ($p < 0.01$). The PND 10 pups, therefore, learned to move away from the stimulus paired with the shock, although exposure to 4500 ppm methanol did not alter their performances.

Activity

Methanol exposure affected performance on the activity test. The same effects were seen in offspring from both Cohorts 3 and 4, which were tested on PND 18 and 25. Figure 2 shows that the motor activity for both groups decreased across the 15-min test, with the greatest decline occurring during the first 5 min. Table 4 and Figure 3 summarize these performances for all 15 min.

No main effect of methanol was observed across the entire 15-min period ($p = 0.50$) nor between cohorts ($p = 0.97$). There was a significant interaction between Methanol Exposure and Postnatal Day ($p < 0.01$). An interaction is shown by the lack of parallelism between the treatment doses as other variables change. Figure 3 shows that the activity levels of methanol-exposed pups fell below those of control pups on PND 18 and above them on PND 25.

Activity levels changed across Session Time ($p < 0.01$), as expected. Neither the Session Time × Gender interaction ($p = 0.55$) nor the Session Time × Postnatal Day ($p = 0.25$) interaction was significant.

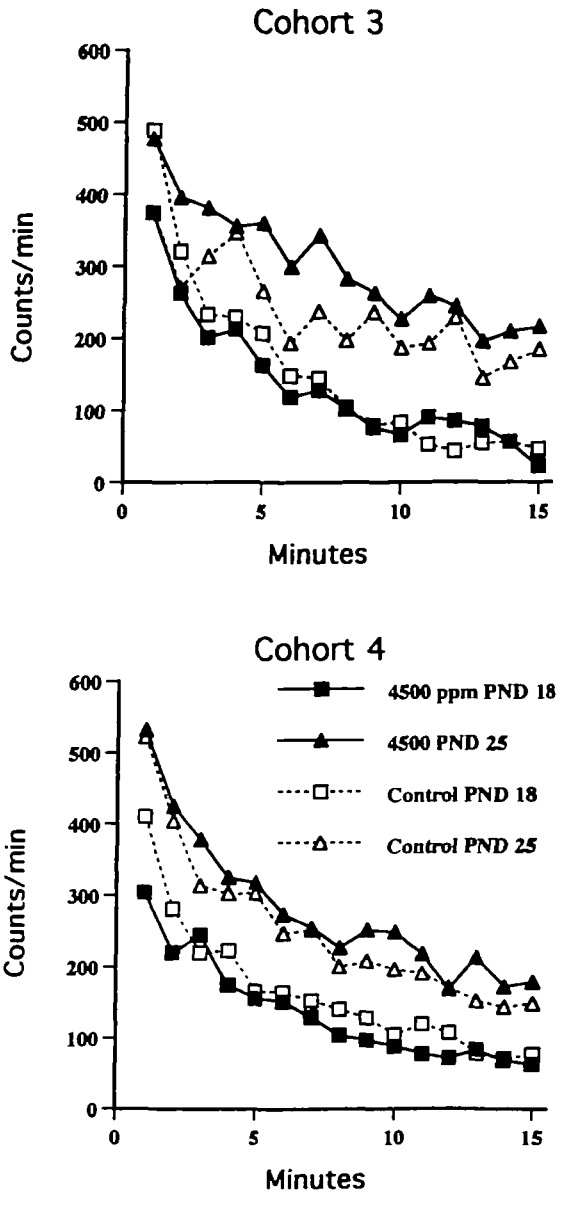

FIG. 2. Activity counts/min (mean) during the 15-min test penod for exposed and control males and females in cohorts 3 and 4.

Adults

Fixed Ratio Wheel Running

Figure 4A shows the mean responses per minute for all four cohorts across FR conditions. Overall rate of responding (total responses/session duration in minutes) was obtained for the following conditions: the mean of the first 5 sessions of the baseline FR values; the mean of the last 15 sessions of the baseline; the rate for individual challenge sessions in which the FR criterion became the baseline value multiplied by the factors 1.25, 1.50, 1.75, and 2.00; and the rate for

TABLE 4
Activity Test: Mean and Standard Deviation of Total Counts for All 15 Minutes on Postnatal Days 18 and 25 for Cohorts 3 and 4

	Cohort 3, mean	Cohort 3, SD	Cohort 4, mean	Cohort 4, SD
PND 18: Control	2292	899	2441	747
PND 18: 4500 ppm	2039	467	2029	678
PND 25: Control	3542	524	3753	1156
PND 25: 4500 ppm	4511	1060	4183	814
PND 18: 4500-Control	−253		−412	
PND 25: 4500-Control	969		430	

Note. The difference in total counts (4500-Control) for each postnatal test day also is shown.

the fifth session of Extinction, during which pellets were never presented. Table 5 provides variability data for both Fig. 4A and Fig. 4B. The data for Cohort 1 were based on only two animals in each group, which probably accounts for the large variability shown.

Three separate repeated measures analysis of variance were conducted. The first examined all of the data. There was no main effect of methanol on rate of running (p = 0.62). Males differed from females ($p < 0.01$), and those differences interacted with Cohort ($p = 0.04$). Performances differed across the Fixed-Ratio conditions ($p < 0.01$), indicating that the subjects were sensitive to the change in conditions. The Fixed Ratio condition × Cohort interaction was also significant ($p < 0.01$). The interaction, Fixed-Ratio condition × Cohort × Treatment was significant ($p = 0.04$), indicating that methanol exposure history altered performances across the FR conditions, but that the effect was not consistent across cohorts. There was a significant interaction between Gender × Fixed Ratio condition × Cohort (p = 0.04). Sensitivity to methanol exposure history also was shown in the Gender × FR condition × Treatment interaction ($p < 0.01$). This result also interacted with Cohort ($p < 0.01$).

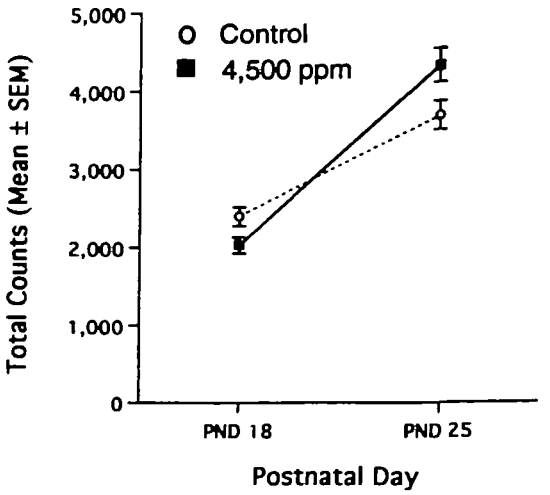

FIG. 3. Total counts (mean ± SEM) for the entire 15-min activity test averaged across both cohorts 3 and 4 and both genders for the exposed and control groups on PND 18 and PND 25.

In the second analysis, we focused more closely on performance changes occurring in response to changes in FR value from the baseline value. That analysis examined the data from FR conditions 3, 4, 5, and 6, corresponding to successive 25% increments in the FR criterion for single sessions across 4 weeks. Although no main effect of methanol appeared ($p = 0.40$), there was a significant interaction between Cohort performances and methanol exposure history (p = 0.05). The Gender difference was seen again ($p < 0.01$) but not the Gender × Cohort interaction ($p = 0.10$). A significant interaction of exposure with Gender ($p = 0.04$) appeared again, as shown in Fig. 4A. Duplicating the first analysis, performances differed across FR conditions ($p < 0.01$), as did the interaction with Cohort ($p < 0.01$). As in the first analysis, the Gender × FR condition × Treatment interaction was significant ($p = 0.02$), but, in this case, that result did not interact with Cohort ($p = 0.06$).

In a different analysis, the initial values (the first five sessions) were subtracted from the later measures as shown in Fig. 4B. Again, there was no main effect of methanol exposure ($p = 0.57$). Performances differed among Cohorts ($p < 0.01$ and among FR conditions ($p < 0.01$), and the FR condition × Cohort interaction was significant ($p < 0.01$). The main effect of Gender here was not significant (p = 0.37), but there was a significant interaction between Gender and Treatment ($p < 0.01$). The interaction Gender × FR condition × Treatment was also significant ($p = 0.03$).

Overall, the results show that the rate of responding of males differed from that of females; that, as the Fixed-Ratio conditions changed, the behavior changed; and that the changes across conditions depended jointly upon sex and methanol exposure history.

Stochastic Spatial Discrimination Learning

The primary measure of performance was an index of efficiency chosen to ascertain the degree to which rats emit-

ted optimal sequences of lever presses. Figure 5 shows how both control and methanol-exposed groups approached the maximizing criterion during training with both matrices. The data shown are from the first 41 sessions in each matrix for each of Cohorts 1, 2, and 3. Insufficient data were obtained from Cohort 4 for including it in the analysis. The general pattern of acquisition was similar for both groups and both matrices. Response sequences rapidly became more efficient over the first 10 sessions or so and then began approaching asymptotic performance more slowly. 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, RS/1 Documentation, Bolt Beranek and Newman Inc., 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 an ED_{50}). The parameter n is a rate change parameter comparable to a straight-line slope constant. The curves in Fig. 5 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. Parameter estimates for the two groups and two matrices are shown in Table 6.

Four separate repeated-measures ANOVAs (treatment group by matrix for each of four parameters) were conducted to determine the significance of group differences in the parameter estimates (Weiss *et al.* 1996, present complete ANOVA results tables). Consistent with the apparent effects in Fig. 5, control and methanol-treated animals differed only in the level of asymptotic performance reached, d ($p = 0.02$). There was no significant effect of treatment on the intercept, a, learning rate, n, or sessions to half asymptote, i. Further analysis of the simple main effects showed that control and methanol-exposed rats differed primarily in the asymptote obtained in Matrix 2 ($p = 0.03$) where the asymptotic performance of the exposed rats fell below that of the controls.

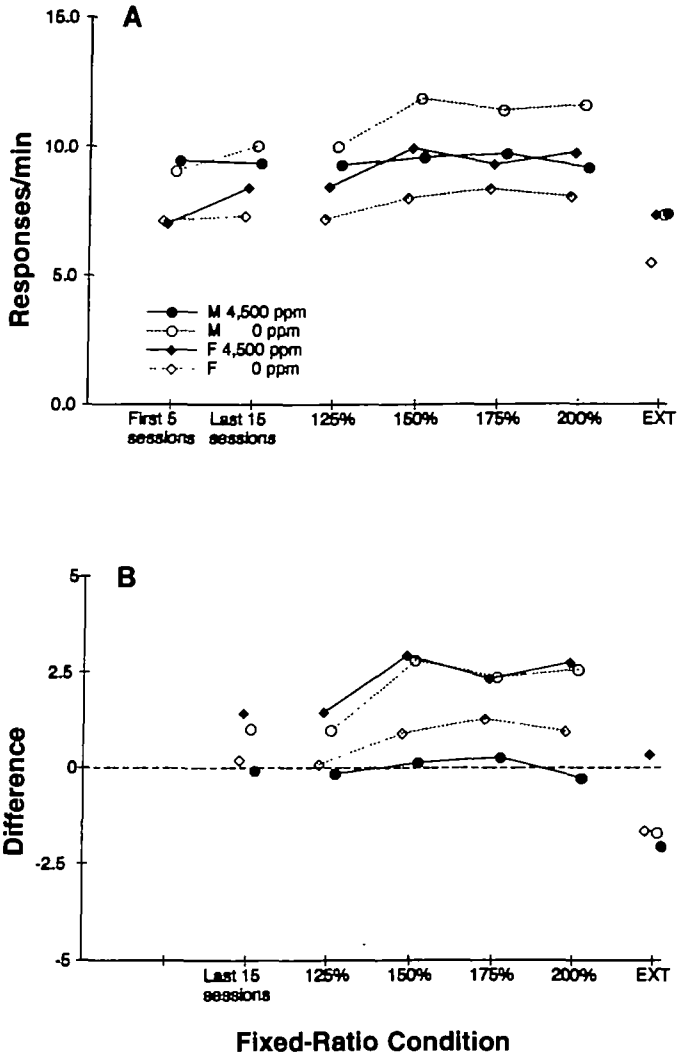

FIG. 4. (A) Mean rate of running in the wheels for food reinforcement for the four cohorts. Females were required to rotate the wheel 20 times (FR 20) and males 28 times (FR 28) to produce food pellet delivery. Shown here are data from the first 5 sessions on the final schedule parameters; the last 15 sessions; performance during single sessions when the FR requirements were raised by 25, 50, 75, and 100%, respectively; and during the final session of extinction when running did not produce food pellets. Variability measures appear in Table 5. (B) Mean difference in rate of fixed-ratio wheel running for the four cohorts. The mean rate of running during the first five sessions of FR 28 for males and FR 20 for females was subtracted from the other conditions shown. The data were obtained from the same sessions shown in A. Variability measures appear in Table 5.

DISCUSSION

Exposure of rats to 4500 ppm methanol via inhalation, from GD 6 to PND 21, did not alter suckling or olfactory conditioned behavior in the young neonate, but did affect the level of motor activity in the older neonate. Methanol effects also were detected in the two operant behaviors studied in the adults, but the results were subtle. Each effect emerged as a statistically significant interaction in the absence of a significant main effect. This indicates that they depended on other experimental conditions. In humans, factors such as age, sex, race, etc. are frequent covariates in

TABLE 5

Variability (SEM) of the Mean Rate of Running (resp/min) during Each Fixed Ratio Running Wheel Condition Shown in Fig. 4A and the Variability (SEM) in the Difference between the Mean Rate of Running for Each of Those Conditions Minus the Rate for the First Five Sessions of Final Baseline Training Shown in Fig. 4B

	Fixed ratio running wheel condition						
	Final FR		Behavioral challenges				
Group	First 5 sessions	Last 15 sessions	125%	150%	175%	200%	EXT
			SEM mean rate				
Male control	0.847	1.438	1.710	2.413	2.450	2.167	0.897
Male 4500 ppm	0.723	1.035	0.741	0.985	1.268	1.090	1.610
Female control	1.821	1.017	1.391	1.055	0.976	1.079	0.715
Female 4500 ppm	1.306	0.781	0.716	1.102	0.939	1.202	0.986
			SEM difference				
Male control		0.990	1.206	1.591	1.736	1.408	1.150
Male 4500 ppm		0.725	0.543	0.668	1.180	0.976	1.315
Female control		1.031	1.053	0.810	0.996	0.837	1.341
Female 4500 ppm		0.767	0.786	0.585	0.696	0.620	1.050

environmental toxicity, particularly at lower exposure levels. With methanol one might anticipate similar results, which cannot be ignored either in pursuing questions about mechanisms or setting standards for exposure.

Methanol-induced alterations in motor activity depended on age; in methanol-exposed pups total activity was decreased on PND 18 and increased on PND 25. Methanol did not affect the pattern of the decline in motor activity seen during the 15-min test period. The decline, commonly observed in tests of this nature, is generally interpreted as habituation, which is often viewed as a form of learning (Stanton, 1994). The interpretation of this result most consistent with the literature is that methanol exposure modified normal developmental levels of activity, which vary systematically through the neonatal period (Kellogg *et al.*, 1980). The observation is complicated by the fact that, on PND 18, the pups were being tested within 24 hr 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 Treatment × PND interaction. Blood methanol concentrations at the end of the 6-hr exposures, however, were similar or declining throughout the neonatal period (Stern *et al.*, 1996), yet exposure to methanol affected neither suckling behavior in the PND 5 pups nor olfactory aversion conditioning in the PND 10 pups.

In contrast to the present experiment, Stanton *et al.* (1996) detected no methanol-induced alterations in motor activity, even though the offspring had been exposed to higher (15,000 ppm) concentrations of methanol vapor. Numerous differences between experiments, including the obvious factor of vapor concentration, could have contributed to the different outcomes: (1) *Response definitions:* Stanton *et al.* used a figure-eight maze with eight photodetectors spaced at distances around the maze that far exceeded the 1.3 cm spacing used by us. (2) *Subject variables:* The same subjects were tested at different ages, and more frequently, with each test being longer in duration in the Stanton *et al.* study, an outcome which might lead to more rapid habituation to the test environment than in the present study. (3) *Schedule of exposures:* Exposures were conducted from GD 7–19 in the Stanton *et al.* study; thus, in contrast to the present experiment, the rat pups were not exposed to methanol during the developmental period that is equivalent to the third trimester in the human. Postnatal exposures to ethanol in the rodent may produce effects on cell proliferation and neuronal migration that differ from those seen during prenatal only exposures (cf., Miller, 1992; Pierce and West, 1987; West *et al.* 1986); differential prenatal vs postnatal exposure effects on behavior also have been reported (e.g., Middaugh and Gentry, 1992; Wigal and Amsel, 1990). Comparable studies for methanol have not been conducted, although, as noted earlier, there is evidence that the period of developmental exposure to methanol can determine the emergence of abnormalities (Bolon *et al.*, 1993). (4) *Adaptation procedures:* No chamber adaptation procedure was used by Stanton *et al.* Timed-pregnant females were received in the laboratory on

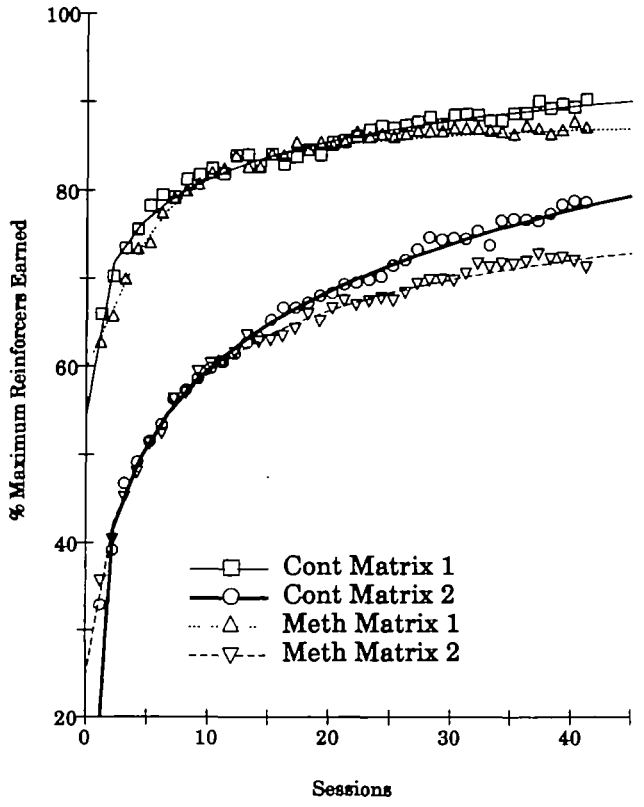

FIG. 5. Performance on the stochastic spatial discrimination test. Mean efficiency scores, i.e., the degree to which optimal performances were reached, are shown for each group for each session. A fitted hyperbolic function is also plotted for each group. Matrix 1 refers to the first set of transition probabilities on which the rats were trained (see Fig. 1). Matrix 2 refers to the second set. Variability measures appear in Table 6.

GD 2 with exposures beginning on GD 7. The absence of preexposure handling most likely resulted in both the methanol-exposed and control groups being stressed during at least the first few sessions of exposure due to the novelty of the situation. Since stress itself may be a potent variable during pregnancy, such an effect could have masked effects produced by the methanol treatment. Although addressing a different issue, Cooper *et al.* (1991) showed that prior adaptation to handling and the chamber altered neuroendocrine effects of exposure to methanol.

Developmental exposure to methanol also affected operant running in the adult offspring. The effect of methanol was shown as an interaction with two other factors. One was gender. One possible source of that interaction was the smaller size of the female offspring, and, accordingly, the lower FR requirement selected as the baseline value. Although fixed-ratio running wheel baseline performances were maintained under baseline values of FR 20 for females and FR 28 for males, we included both sexes in the same analyses. The value of this approach is that it allowed us to examine possible interactions of methanol exposure history with gender, just as was done for the other procedures used in this study. Although varying interpretations of such interactions may be entertained, we would have been faced with a similar problem had the FR values for males and females been equal, since they would not have been functionally equivalent. It is more difficult to sustain responding under FR 28 in females than males with these running wheels.

The other factor 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 (i.e., methanol vs control) × FR condition interaction. In the first five days of responding under the final baseline (FR 20 for females and FR 28 for males), males from both groups responded similarly but at rates greater than those 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 control rats. Viewed another way, methanol exposure moved males and females in opposite directions. When the FR criterion was increased during the single-session challenges, the changes in rate of wheel turning again depended on both gender and methanol exposure history.

Sexual dimorphism is frequently observed in response to other chemical agents on several behavioral endpoints including activity, spatial behaviors, and schedule-controlled behavior (e.g., Goodlett and Peterson, 1995; Miller and Seidler, 1994; Navarro *et al.*, 1994; van Haaren, 1994; van Haaren and Andersen, 1994). The effect of prenatal exposure to ethanol on subsequent maternal behaviors depends on both gender (some maternal behaviors can be observed in males) and age of testing (Barron and Riley, 1985). Greater female responsiveness to cocaine is a common finding; for example, adult females exposed perinatally to cocaine were less active than controls while males showed no effect of exposure (Dow-Edwards, 1989). In the case of methylmercury, male children seem to be more sensitive than females (McKeown-Eyssen *et al.*, 1983).

One implication of the significant Methanol × FR condition interaction is that transitional behaviors that occur in response to *changes* in contingencies may provide sensitive indices of neurobehavioral impairment even when performances attained under steady-state conditions are not (e.g., Weiss, 1970; Moerschbaecher *et al.*, 1979; Newland *et al.*, 1994). Both Riley *et al.* (1980) and Middaugh and Gentry (1992) made a similar suggestion in interpreting the effects of prenatal exposure to ethanol on fixed-ratio bar pressing. In the present study, compensatory adjustments in attaining the steady-state baseline performance, i.e., responding under FR 20 for females and FR 28 for males, might have masked potential underlying functional deficits. The behavioral chal-

TABLE 6
Stochastic Spatial Discrimination Parameter Estimates for Percentage of Maximum Reinforcements Earned for Matrix 1 and Matrix 2

	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)

Note. Parameters: *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 sessions required for the efficiency scores to increase to the midpoint between a and d; and *n*, rat change comparable to a straight-line slope constant.

lenges that changed the contingencies provided an opportunity to assess operant performances during transition periods when such adjustments would not have occurred. Although this laboratory has previously demonstrated that steady-state operant running itself was altered by exposure to methanol (Youssef *et al.*, 1993), as well as to ozone (Tepper and Weiss, 1986), those reactions occurred immediately following acute exposures.

Under the stochastic spatial discrimination procedure, the performance efficiency of control animals significantly exceeded that of methanol-exposed animals only after extended training in the reversal condition of Matrix 2. Since a balanced design was not used in the sequence of studying performances under the two matrices, further studies are required for elucidating the roles of some of the variables, e.g. task complexity vs change in task. The differences demonstrated between groups implies that developmental exposure to methanol produced rather subtle cognitive deficits. Weiss and Heller (1969), in their original investigation of this task, viewed it as a means of ascertaining the ability of subjects to discriminate serial contingencies in reinforcement schedules.

Many investigators specializing in the experimental analysis of behavior have turned to quantitative models to describe how reinforcement schedules control behavior. The Matching Law (Herrnstein, 1970; Davison and McCarthy, 1988) is 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. 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 right-most lever). 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 an additional 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 response positions. Figure 5 shows that adjustment to this procedure was slow and apparently difficult. Even at the end of 41 training sessions, performances had not yet attained the estimated asymptotic values. It seems likely that the difficulty of the task may have played a critical role in revealing the neurotoxic effects of methanol. Other studies have shown that as task difficulty increases due to weak stimulus control of behavior (e.g., Laties, 1975) and/or increased complexity in response sequences (e.g., Polidora, 1963; Thompson, 1975; Moerschbaecher *et al.*, 1979), sensitivity increases to disruption by other variables including environmental toxicants and drugs. That methanol effects were statistically significant only for the asymptote of Matrix 2 indicates the ease with which neurotoxic effects might be overlooked with less-complex behavioral tasks.

Many behavioral adjustments to changes in the environment are considered forms of learning. For both the operant running wheel and the stochastic spatial discrimination procedures, developmental exposure to methanol produced subtle alterations in such adjustments. Deficits in adjusting to changes in the environment might be considered adverse effects which are included in the US EPA definition of neurotoxicity (Tilson *et al.*, 1995). Such an interpretation ultimately depends on a preponderance of evidence, currently not available for methanol at concentrations up to that studied here.

Neither suckling behavior nor olfactory learning in the

neonates were sensitive to methanol exposure. The two behavioral functions had been selected as potentially useful because of previous findings by ourselves and others. Several studies (e.g., Chen *et al.,* 1982) found changes in suckling in pups exposed to ethanol, but only with rather high blood levels in the dams resulting from substituting ethanol for 35% of total calories. Infurna and Weiss (1986) found differences due to prenatal methanol in latency to suckling and in locating odors from the home nest. These findings, however, were based on giving pregnant rats a 2% (v/v) solution of methanol as their drinking fluid. Under those conditions, the dams most likely attained blood methanol levels greater than 1 mg/ml (Weiss *et al.,* 1996), a value well above those seen in the present study, which might account for the difference in behavioral outcomes.

Olfactory discrimination training also has proven sensitive to other prenatal treatments (e.g., Crofton *et al.,* 1993; Stanton, 1991; Spear *et al.,* 1989). Spear and her collaborators demonstrated impairment of olfactory discriminations in rats exposed prenatally to high levels of cocaine.

Since methanol did not affect olfactory conditioning in the present experiment, two questions may be raised for the current study: (1) Did the offspring from either group acquire, or learn, the discrimination? (2) Were potential differences between groups masked by a floor or ceiling effect established by the selected training and testing values? A preliminary study conducted as part of this project (see Weiss *et al.,* 1996) demonstrated (1) that the pups in the present study spent more time away from the stimulus paired with the shock than untrained pups, and (2) that by adjusting parameter values, other pups would spend even more time away from that stimulus than those of the present study. In the present study, therefore, olfactory discrimination learning occurred in the 10-day-old pup, and performances were not bounded by either a ceiling or a floor effect that would have precluded detecting an effect of methanol exposure.

The results of the current project showed some subtle, functional consequences resulting from exposure to 4500 ppm during early development. In addition, we recently reported finding morphological changes in other subjects from the same cohorts (Weiss *et al.,* 1996; Stern *et al.,* 1996). The changes appeared in the form of a molecular mechanism governing cell–cell connections (in this case, neural cell adhesion molecules) rather than as overt pathology. Although estimated levels of exposure for the general population are far below the concentration selected for the research reported here, further investigations are warranted for addressing questions about lower concentration exposures, potentially interacting variables, and, ultimately, mechanisms.

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