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Developmental Consequences of Intermittent and Continuous Prenatal Exposure to 1,1,1-Trichloroethane in Mice

HENDRÉE E. JONES, PAUL M. KUNKO,
SUSAN E. ROBINSON AND ROBERT L. BALSTER[1]

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0613

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JONES, H. E, P. M. KUNKO, S. E ROBINSON AND R. L. BALSTER, *Developmental consequences of intermittent and continuous prenatal exposure to 1,1,1-trichloroethane in mice.* PHARMACOL BIOCHEM BEHAV **55**(4) 635–646, 1996.—The effects of 1,1,1-trichloroethane (TCE) on physical and behavioral development were examined in CD-1 mice prenatally exposed under two regimens. In the first study, pregnant mice were exposed to either 2,000 ppm TCE or filtered air for 17 hrs. during gestational days (GD) 12-17. A third group remained untreated. The results revealed no differences on pregnancy outcome. TCE-exposed pups gained less weight, exhibited delays in developmental landmarks and acquisition of the righting reflex, had poorer performance on tests of motor coordination and exhibited delays in negative geotaxis relative to sham or untreated pups. A second experiment was designed to more closely parallel the intermittent, acute, high-concentration pattern of solvent abuse. Pregnant mice were exposed for 60 min. to 8,000 ppm TCE or sham placement in exposure chambers three times/day during GD's 12-17. The results were very similar to what were obtained in the more continuous exposure study. TCE-exposed pups gained less weight, had delays in developmental landmarks and acquisition of the righting reflex and exhibited weaker grip strength, poorer negative geotaxis and less rooting intensity in comparison to sham pups. These data provide evidence for the behavioral and developmental teratogenicity of prenatal TCE exposure late in gestation.

Behavioral teratology CD-1 mice Development Fetotoxicity Haloalkanes Inhalant abuse Pregnancy Prenatal exposure 1,1,1-Trichloroethane

REPORTS of solvent abuse have been noted since the 1950s (20) and continue until the present, both in the United States and throughout the world. Many of the volatile solvents used for intoxication are found in substances widely used in the home and workplace, such as glue, paint thinners, varnishes and aerosols. Solvents in these forms are easily available, inexpensive and legally accessible. These factors contribute to abuse by younger people and those individuals of lower socioeconomic status who cannot as easily gain access to alcohol and other drugs of abuse. Presently, inhalant abuse is one of the most pervasive and least recognized drug problems. In the United States, the prevalence of inhalant abuse among youth is exceeded only by the use of marijuana, alcohol and tobacco (31,32). Since it is the younger population who most often abuse solvents, there is a great concern over the potential negative effects of inhalants on physical and mental development. It is further recognized that youth are also confronted with the added problem of unplanned pregnancy. Thus, it is of importance to examine the possible detrimental effects of abused solvents on the developing embryo and fetus.

Although there is a relatively large research literature on the consequences of prenatal exposure to alcohol (9) and other drugs of abuse (14), there is much less known about the effects of prenatal exposure to commonly abused inhalants. A few epidemiological investigations have been conducted in which central nervous system (CNS) deficits were reported in infants born to women who were occupationally exposed to organic solvents (29). On the other hand, solvents examined in another study failed to reveal an association with abnormalities (49). A case-referent investigation found evidence that cases of cleft palate were associated with prenatal organic solvent exposure

[1]To whom requests for reprints should be addressed.

(28). In another report, deliberate sniffing of paint, composed mostly of toluene, was associated with renal tubular acidosis and growth retardation in the offspring of women who abused the substance during pregnancy (26).

Clinical experience with babies born to inhalant-abusing women has led some to describe a fetal solvent syndrome which includes CNS defects such as mental retardation, scaphocephaly and hypertonia (30). Greater rates of preterm delivery, perinatal death, growth retardation, delayed development, hyperactivity, microcephaly and mental retardation have also been reported in children of mothers who abused toluene while pregnant (65). These incidents further support the possibility of a fetal solvent syndrome (27,59). The facial dysmorphia of these children was reported to be comparable to that described after prenatal alcohol exposure (33). These case studies of adverse outcomes in offspring of inhalant abusers is in contrast to results of another investigation which compared the neurobehavioral development of 41 children whose mothers were occupationally exposed to solvents during pregnancy with a group of matched, unexposed children. No differences were found between the two groups on any of the measures (17).

1,1,1-Trichloroethane (TCE) is found in dry cleaning products, spot removers, rubber cements, adhesives, typewriter correction fluids, fabric cleaners and paint removers (42, 58). TCE is so widely used because it is generally less toxic than other halohydrocarbons (55). For the present experiment, TCE was chosen due to its prevalence in commonly abused products, its low systemic toxicity and previous data demonstrating its shared behavioral effects with abused depressant drugs (46,47,50,51).

Animal studies of prenatal exposure to TCE have focused on concentrations relevant to occupational use and have produced conflicting results. Exposure of pregnant mice and rats to 875 ppm TCE for 7 hrs/day during days 6-15 of gestation did not result in maternal or fetal toxicity, nor was teratogenicity observed (52). In another experiment, Long–Evans rats were exposed to 2,100 ppm TCE 6 hrs/day before mating (5 d/wk, for 2 wk), during pregnancy (7 d/wk, for 20 days), or both before mating and during pregnancy (66). Decreases in fetal body weight were noted following exposure during gestational exposure alone. Skeletal and soft tissue abnormalities were seen following exposure before and during gestation. Evaluations of adult offspring found no differences in activity or in response to an amphetamine challenge test. Taken together, the results suggested a developmental delay rather than teratogenicity. In a multigenerational study, ICR Swiss mice were given TCE in drinking water at concentrations of 0, 583, 1749 and 5833 ppm continuously for the duration of the experiment. TCE exposure did not produce reproductive toxicity in the parental generation nor did it adversely effect pup development or reproductive capacity in the offspring (40). Sprague–Dawley rats administered 3, 10 or 30 ppm in drinking water 20 days before conception and through gestation day 20 produced offspring that were similar to controls on measures of cardiac morphology, prenatal viability and growth (25). In contrast, cardiac abnormalities were observed in offspring of female Sprague–Dawley rats given TCE 10 ppm in drinking water before conception and then throughout the gestational and lactational period (13). Fetal mortality and abnormal development have also been reported in chicks exposed to 5 to 100 μM TCE injected into the air space of the egg (16).

To further explore the consequences of prenatal exposure to TCE, we conducted two studies using mice exposed during the last week of gestation. One study used 17 h/day exposure to a low, but behaviorally active (18), concentration of TCE (2,000 ppm) and the second study used multiple daily intermittent exposures to a high, grossly intoxicating concentration of TCE (8,000 ppm) to more closely simulate the exposure pattern of inhalant abusers. Offspring were studied intensively from postnatal day (PND) 1 to 21 using a modified version of a battery of tests for mice (10). They were also assessed post-weaning for residual effects on motor activity and learning and memory.

MATERIALS AND METHODS

Experiment 1: Continuous 17 h/Day Exposure

Subjects. Timed-pregnant female CD-1 mice weighing an average of 30 g were obtained on gestation day 11 (sperm = day 0) from Charles River Laboratories (Wilmington, MA). All mice were singly housed in 28.5 × 17.5 × 12 cm clear plastic cages with wood chip bedding material and food and water available ad lib. The vivarium in which the mice were housed was a light (12 h on/ 12 h off) and temperature (21–24C) controlled, American Association for Accreditation of Laboratory Animal Care - approved animal facility. The following day after arrival to the vivarium, dams were assigned to one of six groups (N = 10): TCE exposed (TCE), sham exposed (Sham), untreated control (untreated), foster TCE, foster sham or foster untreated. All foster animals remained undisturbed, except for routine cage cleaning, throughout the exposure period.

Animal husbandry during exposures. In order to insure that initial dam weights were equivalent among groups, dams in the TCE, sham and untreated groups were weighed and then TCE dams were matched with dams of similar weight in the sham and untreated groups. Dams were pair-fed for the duration of the experiment in an attempt to control for possible differences in maternal food consumption and resulting maternal weight gain. Pair-feeding consisted of measuring the amount of food consumed by the TCE dam and giving that amount to the sham and untreated dams to which the TCE dam was matched. Sham exposed and untreated dams received the amount of food that the TCE exposed dams had consumed in the prior 24 h. During the exposures, all dams had access to their food allotment and 3 cc of water. At the end of the daily exposure period, the mice were removed from the exposure chamber, weighed, and returned to their home cage. Any remaining food and water was returned with the dams to their home cage. Food and water intake were monitored for each exposure. Foster dams were kept in the vivarium throughout gestation with food and water ad lib.

Test chemicals and exposures. Exposures to 2,000 ppm TCE were conducted daily for 17 h/day during gestation days 12–17. Animals in the sham-exposed control group were placed in an identical exposure chamber at the same time as the TCE exposure group, but exposed to filtered air only. The untreated control group was brought from the vivarium to the exposure room for the same hours as the exposed groups each day but was left undisturbed in their home cages except for daily weighing. Exposures were conducted overnight (1600–0800 h) in a 20.8-l sealed glass exposure chamber with a stainless steel grid floor and fitted with a Teflon lined lid. In order to control for a possible difference in dam activity, to insure equivalent exposure of each animal to air or vapor, and to accurately monitor food and water intake during the 17-h exposure period, dams were placed in individual compartments (7 × 5 × 22 cm) made from wire mesh and fitted with individual water bottles.

To expose the subjects, air containing the TCE vapor concentration was continually passed through the chamber. To generate the TCE vapor, filtered air controlled by a flow regulator (R7630 series, Matheson Co., Dorsey, MD) was passed through a gas dispersion tube inserted in a 2-l flask containing TCE. The vapor-laden air exiting the flask was diluted with filtered air controlled by another flow regulator. The two flow rates were adjusted to produce the test concentrations at a constant 10 l/min flow rate which allowed the chamber concentration to reach 95% of the plateau concentration or return to 0 ppm within 10 min.

Chamber TCE concentrations were continuously monitored by single wavelength-monitoring IR spectrometry (Miran IB, Foxboro Analytical, North Haven CT). The spectrometer was equipped with a 10-cm flow-through stainless steel cell (Model 424, Foxboro Analytical) and BaF_2 windows. The IR spctrometer was calibrated using a closed-loop system which consisted of injecting known volumes of solvent calculated for each concentration into the closed systtem and taking the average of absorbance readings as representative of the absorbance for a particular concentration (6). TCE was monitored at the analytical wavelength of 9.3 microns. Flow regulators were readjusted manually to maintain test concentrations, although this was rarely needed after proper settings had been established. Recordings of chamber concentrations on an x-y plotter showed that concentrations did not vary by more than 10% from one exposure to the next. The mean concentration over the six exposure days was 2000 ± 52 ppm. The mean temperature and humidity were 21.7 ± 0.5°C and 30.5 ± 5.4 %, respectively. TCE (99% ; T-391, inhibited) was purchased from Fisher Scientific Co. (Pittsburgh, PA.)

Parturition procedures. Following the termination of the last exposure on gestation day 17, dams were returned to their home cages and given a paper towel for nest construction. The subjects were checked twice daily (0830 h and 1630 h). for parturition. Litters born before 0830 h and before 1600 h were considered day 0 of life. For litters born after 1600 h the next day was considered day 0. As soon as possible after birth, the dam was weighed and the following litter data were collected: entire litter weight, size and sex ratio and the number of malformed or dead offspring. Litters were culled to four of each sex, with partial adjustment of sex ratios (5:3) if needed. Litters having less than 7 pups were discarded. All litters were fostered to surrogate mothers who had delivered within 24 h of the treatment dams.

Observations. One male and one female pup from each litter were randomly assigned to one of four test categories: (i) physical development and motor activity; (ii) reflex development; (iii) muscle strength; and (iv) motor coordination. In the litters which had unequal numbers of males and females, the extra male or female was assigned to the remaining test category. The pups were identified by using an indelible marker to label the pup with the designated number (1–4) of the behavioral test category to which it was assigned. One litter was tested for all measures appropriate for that day before moving to the next litter. All pups were removed from the nest and placed on a heating pad to provide warmth before and after testing. Testing was conducted by an individual uninformed about group assignment. After all of the tests were administered to the designated pups, all pups were returned to the nest. The order of testing the litters was determined initially by chance but then reversed on alternate days for the remainder of the study. Each pup was given all of the tests from that testing category before the next pup was tested.

Physical development. One male and one female pup from each litter was randomly assigned for observation of physical landmarks. Pup body weights were recorded daily until day 14. The pups were checked daily from postnatal day (PND) 1 to 14 and the days that both eyes were opened, pinnae detached and incisors erupted were recorded. These same pups (male only) were tested at PND 23–25 for spontaneous motor activity .

Six behavioral tests were selected to evaluate various CNS functions including reflex development, muscle strength, motor coordination and spontaneous activity.

Reflex development. Animals were tested on a standardized surface of a surgical blue pad with the environment (temperature, lighting and time of day) held constant. For the righting reflex, the pup was placed on its side and the latency to right itself recorded in seconds. The criterion used for successful righting was the animal's turning over to a position in which all four paws were in direct contact with the test surface and the limbs in a position so that walking was possible. A maximum of 60 s was given for the pup to right. For the rooting reflex, the pup was placed with all four paws on the test surface and the facial region of the snout was stimulated bilaterally by placing the thumb and index finger on both sides of the head and pressing very lightly for up to 5 s. The most complete response was demonstrated with up and down and pushing movements of the head of the mouse as it was stimulated. If the experimenter moved her hand in a linear path, measured over a marked piece of tape, away from the animal while continuing to stimulate the snout, it was expected that the animal would follow the hand while continuing the rooting. Rooting distance (cm) was used to measure rooting reflex intensity.

Forelimb grip strength. Forelimb grip strength was assessed by the use of a modified strain gauge (DFG-2, Chatillon, Greenburough, NC). Modifications included the mounting of a horizontal bar (0.15 cm diameter) supported by a 9 × 10 cm steel bar frame. The horizontal bar was suspended 28.5 cm from a surface covered with cotton gauze. The animal was held by the nape of the neck and its forepaws placed on the bar. The animal was then pulled gently by the tail and the strength of the grasp measured in grams. The peak strength of five determinations was used for data analysis.

COORDINATION

Coordination. Coordination was examined in two behavioral tests. For the negative geotaxis test the pup was placed, head down, 20 cm from the bottom of a wire mesh screen (0.5 cm^2 holes) that was tilted 45°. The latency (up to 60 sec) for the pup to turn 180° (upright) was recorded. For the inverted screen test, the pup was placed on a horizontal wire mesh square which was rotated 180° so that the mouse was upside down. The latency (up to 60 sec) to climb on the top side of the screen was recorded. The apparatus consisted of a metal rod 1.4 m long, to which were attached 6 evenly spaced, 13-cm^2 square pieces of wire screen mesh (0.5 cm^2 holes). Cotton gauze was provided under the apparatus to cushion the pup's fall.

Motor activity. On PNDs 23, 24 & 25 mice assigned to this test were placed in individual 28.5 × 17.5 × 12 cm chambers (Omnitech, Columbus, OH) at approximately 1000 h. The horizontal movements of the mouse were recorded as the number of infrared beam interruptions for 10 min. Testing was done on three successive days to include a measure of habituation.

Statistical analyses. Since 2 pups per litter (one male, one female) were tested for each measure, a litter mean score for

each developmental and behavioral measure was obtained in order to avoid inflation of the sample size. Following the statistical methods recommended by the Collaborative Behavioral Teratology Study which suggests ANOVA as the basic analysis tool (11) and those methods employed by others (48), the litter was utilized as the unit of measure and treatment group was the main effect for all models. In order to control for the use of repeated ANOVA's, the repeated measures significance levels were adjusted in the negative direction using the Greenhouse-Geisser adjustment of the F-ratio (24,35). The maternal weight and food consumption during exposures were analyzed using a 2 factor (group x day) repeated measures ANOVA. Pup weight was also analyzed using a 2-factor repeated measures ANOVA. Litter size, litter weight and developmental landmarks were analyzed by a one-factor ANOVA's. The behavioral tests of righting reflex, rooting reflex, grip strength, negative geotaxis, inverted screen and activity were analyzed using a 2-factor repeated measures ANOVA. If the treatment factor was significant in the repeated measures ANOVA's, further day specific ANOVA models were conducted. When the day-specific models reached the criterion for significance, Scheffe's post-hoc test was used. Preliminary analysis of the data included sex as a variable and, in the absence of a significant sex by treatment interaction, further analyses were conducted on the data collapsed across sex. The significance level was set at 0.05.

Experiment II: Intermittent High Concentration Exposure

Subjects. Twenty four timed-pregnant female CD-1 mice weighing an average of 30 g were obtained on gestation day 11 from Charles River Laboratories (Wilmington, MA). Upon arrival, all mice were singly housed with food and water available ad lib as described for Experiment I. Twenty-four hours after arrival at the vivarium, mice were randomly assigned to either the TCE exposed (TCE) or sham exposed (Sham) group. An untreated control group was not used in the present experiment based on the results of the previous experiment showing no effect of sham exposure and consensus suggesting against their routine use (11).

Animal husbandry during exposures. Dams in the TCE and sham groups were weighed and then overall group means were compared in order to insure similar initial weights. Over the course of exposures, maternal weight was recorded twice daily—before the first and after the last exposure. Food and water was continuously available ad lib except for the brief periods of exposure. Food and water intake of the dams was monitored each day before the first exposure.

Test chemicals and exposures. Exposures to 8000 ppm TCE were conducted daily during gestation days 12–17. The production and monitoring of TCE vapor was similar to that described for Experiment I. The 8000 ppm TCE concentration did not vary over any of the exposures. The mean temperature and humidity were 22 ± 0.6°C and 36 ± 1.0 %, respectively, for the entire six days of exposure. The sham exposed control group (N = 12) was placed in an identical exposure chamber at the same time as the TCE exposure group, but exposed to filtered air only. Exposures were conducted 3 times a day for 60 min (beginning at 08:30 h, 1030 h, and 1230 h) in a 20.8-l sealed glass exposure chamber with a stainless steel grid floor and fitted with a Teflon lined lid. Based on pilot experiments in which cessation of activity was observed during exposures to this concentration of TCE for short periods of time, mice were not placed in individual wire mesh compartments nor were they given access to food and water during the brief exposure periods.

Parturition procedures. With the exception that fostering was not used in this experiment, all parturition procedures were identical to Experiment I.

Observation of dams. To obtain some information on the effects of the acute high concentration TCE exposures on the dams, they were assessed following the first exposure each day. Assessment was conducted by using a subset of tests from a functional observational battery for neurobehavioral toxicity testing developed for rats (44,45) and modified for mice (56). Dams in the TCE and sham exposure groups were removed from the exposure chamber following termination of exposure and evaluated. Two experimenters scored different dams to insure that the latency between cessation of exposure and evaluation was less than 5 min. Scoring was according to criteria described previously (56) on the following measures listed in the sequence in which the observations were performed.

I. Response to handling (scored immediately after removal from exposure chamber)
   A. Ease of removal from exposure chamber (5-point scale)
   B. Salivation (3-point scale)
   C. Lacrimation (3-point scale)
   D. Piloerection (presence or absence)
II. Observations made after placement on a smooth horizontal surface
   A. Gait (8-point scale)
   B. Gait abnormalities (4-point scale)
   C. Clonic movements (8-point scale)
   D. Tonic movements (6-point scale)
   E. Stereotypies (narrative description)
   F. Bizarre behavior/other (narrative description)

Observations. The testing categories (i.e. physical development, activity and learning, reflex development, muscle strength and motor coordination), the assignment of pups to categories and the protocol for each test were identical to the procedures described for Experiment I. The only changes for the present experiment were the evaluation of the pups until weaning (PND 21) and the pups examined for physical development and activity were retained and later tested for learning and memory when they reached the age of 85 days.

One-trial Passive Avoidance Conditioning. The apparatus was a nontransparent chamber with a clear Plexiglas front (28 × 24 × 30 cm) equipped with a metal grid floor (0.5 cm diameter bars). A dark compartment (15 × 24 × 15 cm) with an 8 × 8 × 8 cm opening was placed in the larger illuminated chamber. Training consisted of placing the mouse in the light compartment facing away from the opening. When the mouse entered the dark compartment, a 1-mA electric current was delivered via the grid floor continuously for 3 s or until the mouse escaped the dark compartment. After the training trial, the mouse was returned to the home cage. Retention was tested 24 and 48 h after the acquisition trial by placing the mouse in the lighted chamber and recording the latency to enter the dark compartment. Upon entering the dark compartment on the retention days the mouse received a second foot shock, whereas if the mouse did not enter the dark compartment within 300 s, the retention test was terminated and a maximal score of 300 s was assigned.

Statistical analyses. Data analyses were carried out as described in Experiment I. The passive-avoidance learning and retention data were also analyzed using a 2-factor (groups by days) repeated measures ANOVA. If the treatment factor

TABLE 1

EFFECTS OF PRENATAL EXPOSURE TO TCE (17 HR/DAY AT 2,000 PPM) ON MATERNAL AND LITTER CHARACTERISTICS

	Untreated	Sham Exposure	TCE Exposure
A. Maternal characteristics			
Maternal weight gain (g)*	6.1 ± 0.7	6.5 ± 1.3	7.7 ± 0.8
B. Litter characteristics			
Number of litters/number of dams	9/10†	8/10‡	8/10§
Gestation length (days)	20.0 ± 0.5	20.5 ± 0.1	20.0 ± 0.0
Litter size	11.4 ± 0.7	11.8 ± 0.8	11.4 ± 0.5
Number of live pups/litter			
Male	5.8 ± 0.3	5.7 ± 0.3	5.8 ± 0.3
Female	5.7 ± 0.3	5.7 ± 0.3	6.0 ± 0.3
Litter weight (g)	16.3 ± .07	15.5 ± 1.0	13.3 ± 1.2

*Mean (± SE) maternal weight gain from gestation days 12–17 for those dams which produced litters. Maternal weight before exposure on day 12 subtracted from maternal weight before exposure on day 17.

†Only 9 of 10 dams delivered—one dam died before delivery on gestation day 19. Only 8 litters were used in behavioral testing—one dam cannibalized her litter by PND 1.

‡Only 8 of 10 dams delivered—one dam died during exposure and one dam died before delivery on G20. Only 7 litters were used in behavioral testing—one litter died by PND1.

§Only 8 of 10 dams delivered—one died during exposure and one was found dead on G19 before delivery. 7 TCE litters were used for litter data analysis since one dam had only two pups and there was evidence of pup cannibalization in the home cage. Only 6 TCE litters were used in behavioral testing since one litter died on PND 1.

was significant in the repeated measures ANOVAs, further day specific ANOVA models were conducted and post hoc evaluations conducted.

RESULTS

Experiment I: Continuous 17 h/Day Exposure to 2000 ppm TCE

Maternal results. Of the sixty-five dams that arrived from the supplier a total of six mice were not pregnant. Five non-pregnant mice were discovered upon arrival and were not assigned to groups, thus reducing the sample size for each of the treatment groups. Later, one mouse in the foster sham group was deemed not pregnant on gestation day 14. Although no systematic investigation was undertaken to examine the behavioral effects in the dams exposed to 2000 ppm TCE, subjective observations of these dams relative to the sham-exposed animals show that they did not appear to differ in activity or gross physical appearance. Maternal and offspring data for the dams included in the three experimental groups are presented in Table 1. For reasons described in the footnotes to Table 1, the results include only dams that delivered in each group. Maternal weight gain during gestation did not significantly differ among groups and no significant differences were found in the length of gestation, litter size or litter weight. The number of litters evaluated with developmental tests were as follows: untreated (N = 8), sham exposed (N = 7) and TCE exposed (N = 6).

Body weight and developmental landmarks. There was a significant main effect for treatment on pup weight (Fig. 1) [$F(2, 18) = 15.5, p < 0.05$]. Significant weight reductions were observed from PND 2–7, 9 and 11–14 in the TCE-exposed pups relative to sham exposed pups ($p < 0.05$). TCE-exposed pups were significantly lighter than untreated pups on PNDs 2–4, 8–9 and 11–14. No differences were obtained between sham-exposed and untreated control pups.

Table 2 shows that TCE had significant effects on ontogeny of development. A one way ANOVA indicated significant effects of treatment on the timing of pinnae detachment [$F(2, 18) = 127, p < 0.01$] incisor eruption [$F(2, 18) = 13.8, p < 0.01$] and eye opening [$F(2, 18) = 45.2, p < 0.01$]. Post hoc tests revealed that TCE-exposed offspring had significant delays in all three developmental landmarks relative to both sham-exposed and untreated control pups.

Righting reflex. There was a significant effect for treatment [$F(2, 18) = 58.8, p < 0.01$] on the latency to perform the righting reflex (Fig. 2a). TCE-exposed pups had longer laten-

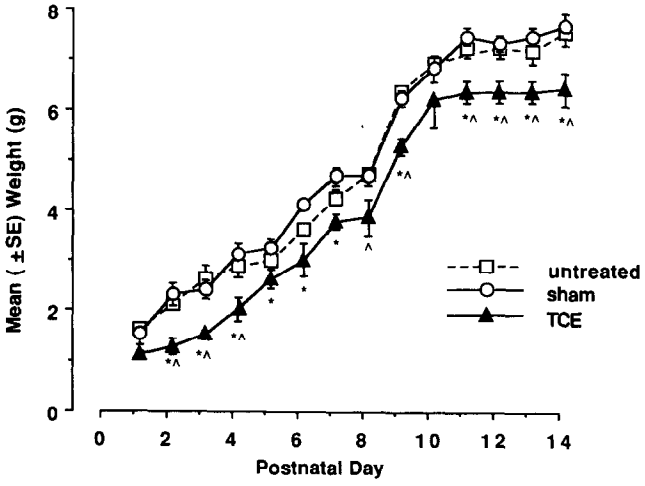

FIG. 1. Effects of 17 h/day prenatal 2000 ppm TCE exposure on pup weight gain. Mean (+SE) of pup weights for PND 1-14. *TCE group significantly different from sham exposure group ($p < 0.05$). ^TCE group significantly different from untreated control group ($p < 0.05$).

TABLE 2

EFFECTS OF PRENATAL EXPOSURE TO TCE (17 HR/DAY AT 2,000 PPM) ON DEVELOPMENTAL LANDMARKS

Developmental Landmark	Untreated	Sham Exposure	TCE Exposure
Pinnae detachment	4.0 ± 0.0	3.9 ± 0.3	7.0 ± 0.0*
Incisor eruption	12.0 ± 0.0	12.1 ± 0.1	12.8 ± 0.2*
Eye opening	13.8 ± 0.2	13.7 ± 0.2	15.8 ± 0.2*

Shown are mean (± SE) days until landmark was achieved for each group.

*Significantly different from sham and untreated control ($p < 0.05$).

cies to right relative to both sham-exposed and untreated control pups from PND 2–11. Most mice in the TCE-exposed group could not perform the righting response within the 60-sec cutoff until PND 6 whereas all sham-exposed and untreated control pups exhibited the righting reflex by PND 3.

Rooting reflex. There was no effect of treatment [$F(2, 18) = 2.31, p = 0.128$] on the rooting reflex measured as distance traveled (Fig. 2b). The rooting reflex shows a clear ontogeny with a progressive increase in rooting distance from PND 5 peaking at PND 9–10 and lost by PND 13–14.

Grip strength. There was a main effect of treatment [$F(2, 18) = 13.2, p < 0.01$] on forelimb grip strength (Fig. 3). TCE-exposed pups displayed weaker forelimb grip strength compared to both sham exposed and untreated groups on PNDs 4–9, 11–12 and 14. The forelimb grip strength measure shows an ontogeny with TCE pups lagging behind sham-exposed and untreated control pups.

Negative geotaxis. There was a main effect of treatment [$F(2, 18) = 14.1, p < 0.01$] on latency to orient towards the top of an inclined screen (Fig. 4a). TCE-exposed pups had significantly longer latencies to turn 180° relative to both sham-exposed and untreated pups on PNDs 9-12. TCE-exposed pups were different from untreated control pups but not sham-exposed pups on PND 8. By PND 9 both sham exposed and untreated pups were able to orient towards the top of the screen within 20 sec; however, it was not until PND 12 that all TCE-exposed pups completed the negative geotaxis task in under 20 sec.

Inverted screen. There was a main effect of treatment [$F(2, 18) = 17.3, p < 0.01$] on the latency to climb to the top of the screen (Fig. 4b). TCE-exposed pups had longer latencies to climb on top of the screen relative to either sham exposed or untreated pups on PNDs 13–14. By PND 14 not one of the TCE-exposed pups was able to successfully complete the inverted screen test whereas all of the control pups were climbing to the top of the screen in less than 60 sec.

Motor activity. Activity data were collected for one male from each litter during 3 successive days of the post-weaning period. There was no effect of treatment [$F(2, 18) = 1.44, p > 0.05$] but there was a significant effect of days [$F(2, 18) = 4.43, p < 0.05$]. Over the 3 days of testing, there was a decrease in the number of photocell beam breaks, evidencing habituation.

Experiment II: Intermittent Exposure to 8,000 ppm TCE

Maternal observations. Maternal and birth result data are presented in Table 3. All dams produced litters and all data shown in Table 3 are based on 12 dams and litters per exposure condition. No significant differences between the TCE-exposed and sham-exposed groups were found in maternal weight gain,

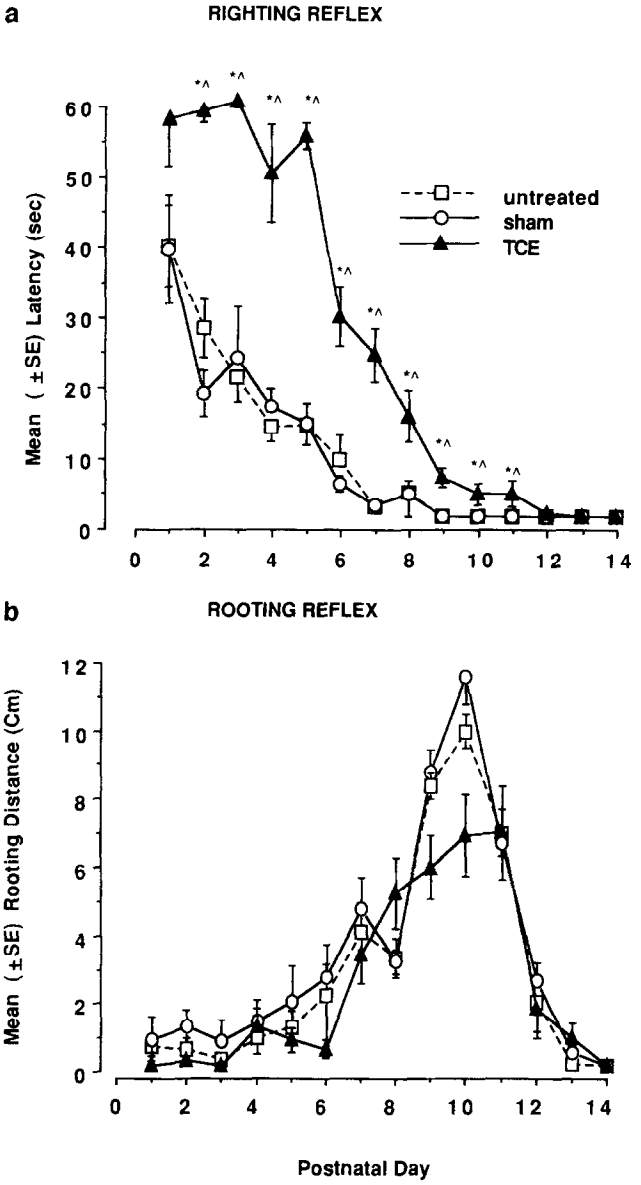

FIG. 2. Effects of 17 h/day prenatal 2000 ppm TCE exposure on reflex development (a) Effects on righting reflex. Mean (±SE) of pup righting times (in seconds) for PND 1-14. *TCE group significantly different from sham exposure group ($p < 0.05$). ^TCE group significantly different from untreated control group ($p < 0.05$). (b) Effects on rooting distance (in centimeters). Mean (±SE) of pup rooting reflex for PND 1-14.

food or water consumption throughout the exposures. Upon parturition, no differences were observed in the length of gestation, litter size, entire litter weight or number of male and female pups. The number of litters evaluated with developmental tests were as follows: TCE exposed ($N = 10$) and sham exposed ($N = 12$).

Functional observational battery. During the six days of exposure, dams were assessed on selected measures from the Functional Observational Battery (FOB) immediately following the first exposure of that day. Numerical data from this battery are not shown, but the pattern of effects is described here. Dams

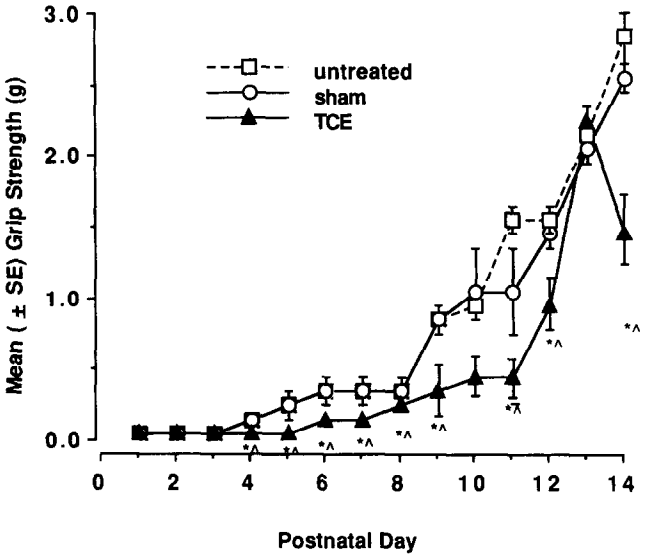

FIG. 3. Effects of 17 h/day prenatal 2000 ppm TCE exposure on forelimb grip strength. Mean (±SE) of pup grip strength (grams) for PND 1-14. *TCE group significantly different from sham exposure group ($p < 0.05$). ^ TCE group significantly different from untreated control group ($p < 0.05$).

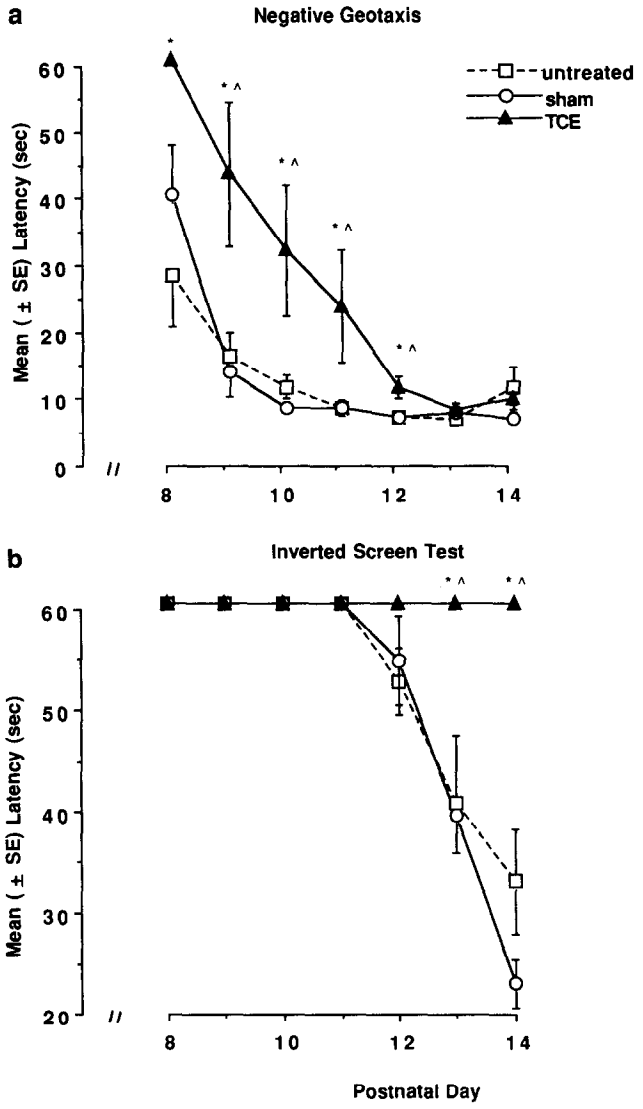

FIG. 4. Effects of 17 h/day prenatal 2000 ppm TCE exposure on negative geotaxis and the inverted screen test. A 60-sec cutoff was used for each measure. (a) Effects on negative geotaxis. Mean (±SE) latency (in seconds) to turn 180 for PND 8-14. *TCE group significantly different from sham exposure group ($p < 0.05$).^ TCE group significantly different from untreated control group ($p < 0.05$). (b) Effects on inverted screen test. Mean (±SE) latency (in seconds) to climb on top of the screen for PND 8-14. *TCE group significantly different from sham exposure group ($p < 0.05$). ^TCE group significantly different from untreated control group ($p < 0.05$).

exposed to TCE 8,000 ppm were easily removed after each exposure for all of the six days, being nearly anesthetized by this high concentration. The dams were completely recovered by the next exposure. The lack of tolerance to TCE is similar to what others have reported (37). In contrast, sham-exposed dams were more difficult to remove from the exposure chamber during the first three days of the exposure regimen. They emitted rearing, flinching or vocalization when the experimenter attempted to remove them from the chamber. By the last three days the sham-exposed dams were habituated to removal from the exposure chamber. Compared to the normal gait of the sham animals during the six days of exposure, the gait of the TCE-exposed dams demonstrated splayed hindlimbs, severe sway and ataxia, which resulted in high scores representing gait abnormalities. Clonic movements such as mild tremors and jerks were observed in TCE-exposed dams whereas sham dams exhibited none of these movements. No observations of tonic movements were noted nor were any of the dams in either group found to exhibit salivation, lacrimation, piloerection, stereotopies or other bizarre behaviors.

Body weights and developmental landmarks. There was a significant main effect for treatment [$F(1, 20) = 23.4, p < 0.05$] on pup weight (Fig. 5) Significant weight reductions were observed from PND 2-19 in the TCE-exposed pups relative to sham exposed pups. No differences in weight were observed on PND 20. Table 4 illustrates that TCE had significant effects on ontogeny of development. Significant effects of treatment were obtained on the timing of pinnae detachment [$F(1, 20) = 10.2$, $p < 0.01$], incisor eruption [$F(1, 20) = 14.6, p < 0.01$] and eye opening [$F(1, 20) = 31.5, p < 0.01$].

Righting reflex. There was a significant effect of treatment [$F(1, 20) = 36.5$, $p < 0.01$] on the latency to perform the righting reflex (Fig. 6a). TCE-exposed pups had a longer latency to right relative to the sham exposure group on PNDs 2-11. Most of the mice in the sham-exposed group could right themselves by PND 3 whereas those in the TCE-exposed group did not until PND 7.

Rooting reflex. There was a main effect of treatment [$F(1, 20) = 60.2, p < 0.01$] on the rooting reflex measured as distance traveled (Fig. 6b). TCE-exposed pups displayed decreased rooting distance relative to sham exposed pups on PNDs 6-10 and 13. Overall, TCE-exposed pups displayed a similar curvilinear pattern of rooting distance as that of sham-exposed pups, yet the distance TCE-exposed pups rooted was significantly less compared to sham-exposed offspring.

Grip strength. There was a main effect of treatment [$F(1, 20) = 43.5, p < 0.01$] on forelimb grip strength (Fig. 7). TCE-

TABLE 3

EFFECTS OF PRENATAL INTERMITTENT EXPOSURE TO TCE (8,000 PPM) ON MATERNAL AND LITTER CHARACTERISTICS

	Sham Exposed	TCE Exposed
A. Maternal characteristics		
Maternal weight gain (g)*	10.3 ± 0.8	9.4 ± 1.4
Food consumption (g)†	6.3 ± 0.5	8.4 ± 1.1
Water consumption (g)‡	4.0 ± 0.5	5.0 ± 0.3
B. Litter characteristics		
Number of litters/number of dams	12/12	12/12§
Gestation length (days)	10.0 ± 0.5	12.5 ± 0.1
Number of pups/litter		
Male	5.1 ± 0.3	4.75 ± 0.3
Female	4.9 ± 0.2	5.0 ± 0.3
Litter weight (g)	15.6 ± 0.8	13.3 ± 0.9

*Maternal weight gain from gestation days 12–17. Maternal weight before exposure on day 12 subtracted from maternal weight before exposure on day 17.

†Total amount of food eaten (g) divided by 5 days of exposure.

‡Total amount of water consumed (g) divided by 5 days of exposure.

§Only 10 litters were tested—one litter only produced six pups and another had 4 pups die by PND 1.

exposed pups had decreased forelimb grip strength relative to sham pups on PNDs 4-9, 11-14 and 16-18. TCE-exposed offspring demonstrated a similar pattern of increased grip strength over days as sham pups yet the average strength of the TCE-exposed pups was less than that of similar aged sham pups.

Negative geotaxis. There was a main effect of treatment [$F(1, 20) = 27.63$, p . TCE-exposed pups had longer latencies to turn 1800 on PNDs 9, 11-13 and 16 (Fig. 8a). TCE exposed offspring significantly lagged behind sham-exposed control pups in the speed to appropriately orient the body.

Inverted screen. The effects for treatment [$F(1, 20) = 3.37$, $p = .08$] were not significant for latency to climb on top of the screen (Fig. 8b).

Motor activity. No significant treatment differences [$F(1, 20) = 0.09$, $p > 0.05$] were observed for number of beam breaks. There was a significant effect of days [$F(2, 20) = 17.4$, $p < 0.05$]. Over the three days of testing, there was a decrease in the number of photocell beam breaks, evidencing habituation.

Passive avoidance conditioning. No significant treatment differences [$F(1, 20) = 0.91$, $p > 0.05$] were observed for latency to enter the dark compartment on the training session nor on latency to avoid the dark compartment on the retention session [$F(1, 20) = 0.12$, $p > 0.05$].

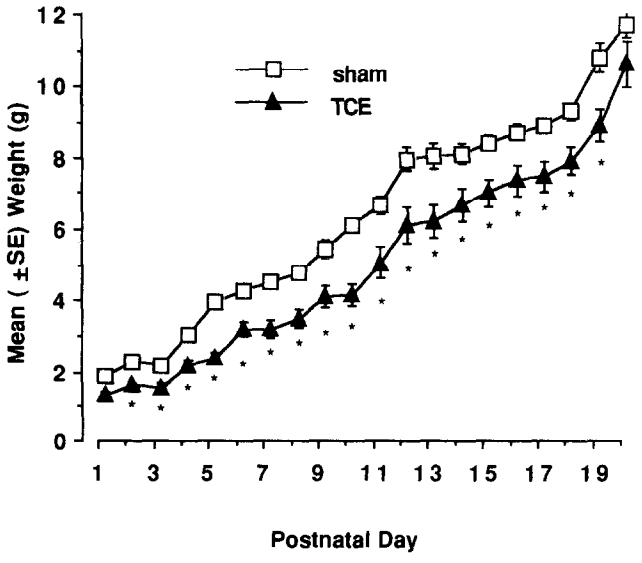

FIG. 5. Effects of intermittent prenatal 8000 ppm TCE exposure on pup weight gain. Mean (±SE) of pup weights for PND 1-21. *TCE group significantly different from sham exposure group ($p < 0.05$).

DISCUSSION

TCE was evaluated for possible developmental effects in offspring of mice exposed during the last week of gestation. The present study represents the first experimental report on the pre-weaning behavioral effects of in utero exposure to TCE. Offspring of dams exposed either continuously (2,000 ppm for 17 h/day) or intermittently (8,000 ppm for 1 hour, 3

TABLE 4

EFFECTS OF PRENATAL INTERMITTENT EXPOSURE TO TCE (8,000 PPM) ON DEVELOPMENTAL LANDMARKS

Developmental Landmark	Sham Exposed	TCE Exposed
Pinnae detachment	5.3 ± 0.3	6.5 ± 0.2*
Incisor eruption	10.3 ± 0.1	11.0 ± 0.1*
Eye opening	14.2 ± 0.1	15.5 ± 0.2*

Shown are the mean (± SE) days until landmark was achieved for each group.

*Significantly different from sham exposed ($p < 0.05$).

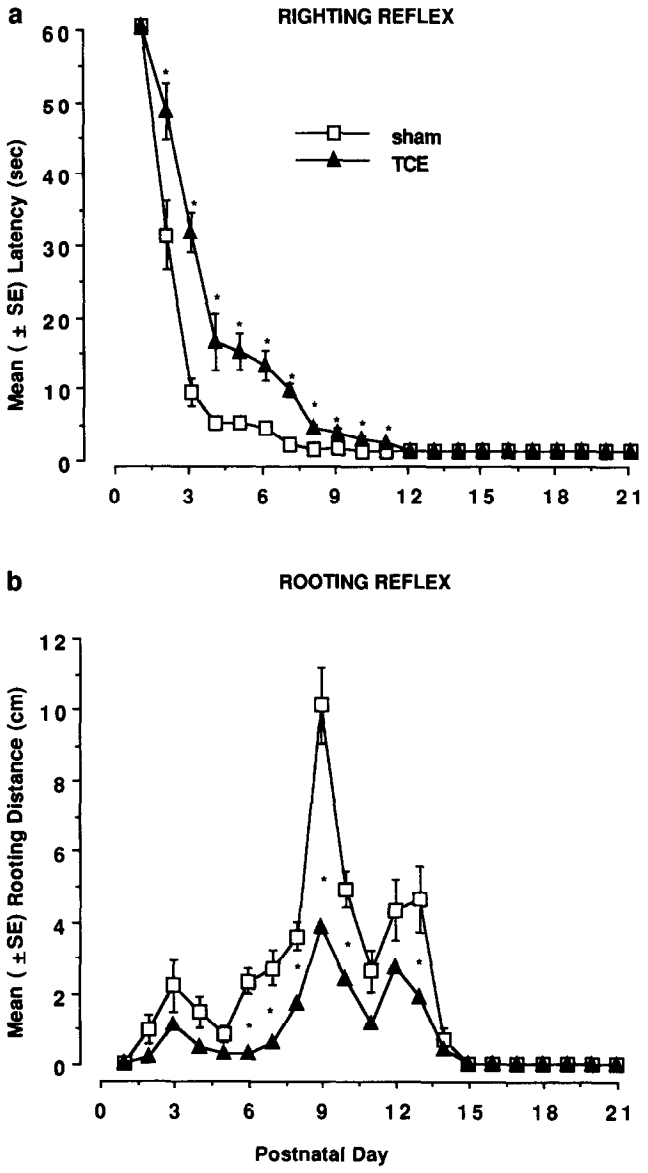

FIG. 6. Effects of intermittent prenatal 8000 ppm TCE exposure on reflex development. (a) Effects on righting reflex. Mean (±SE) of latencies to right (in seconds) for PND 1-21. *TCE group significantly different from sham exposure group ($p < 0.05$). (b) Effects on rooting distance (in centimeters). Mean (±SE) of pup rooting for PND 1-21. *TCE group significantly different from sham exposure group ($p < 0.05$).

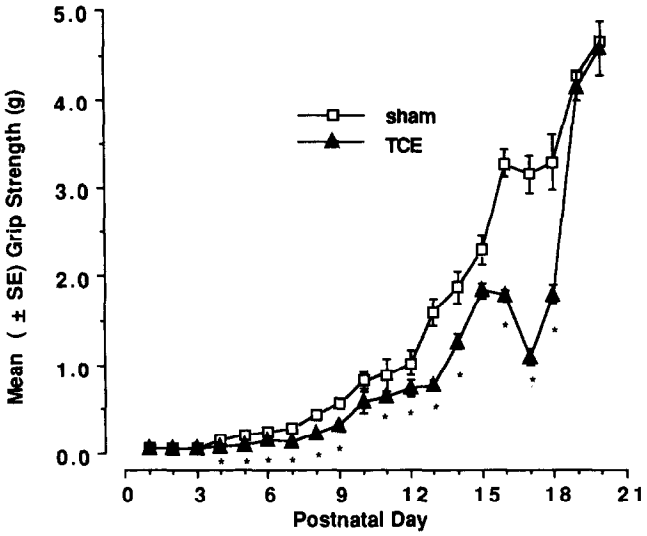

FIG. 7. Effects of intermittent prenatal 8000 ppm TCE exposure on forelimb grip strength. Mean (±SE) of grip strength (grams) for PND 1-21. *TCE group significantly different from sham exposure group ($p < 0.05$).

times/day) to TCE during days 12-17 of gestation demonstrated delays in maturational and behavioral endpoints commonly assessed in the developmental neurotoxicology field. Significant effects were observed compared with offspring of both untreated and sham exposed dams.

Two vapor concentrations and regimens known to alter behavior were selected for the present study. For the continuous exposure experiment, a 2,000 ppm concentration was chosen based on previous data which had demonstrated behavioral effects at this concentration that are similar to those produced by abused depressant drugs (37,46,47) and continuous exposure (23 hrs/day for 4 days) to this concentration can produce physical dependence (18). Further, 2,000 ppm TCE was selected since continuous exposure to 2,100 ppm TCE did not impair mating or seriously decrease weight gain in dams or fetuses (65). In order to more closely mimic the exposure regimen of a solvent abuser in a second experiment, dams were exposed to a higher concentration of TCE for shorter periods of time. The 8,000 ppm TCE concentration and 60-min exposure period were selected based on previous investigations of behavior following exposure to 8,000 ppm TCE which have found that it severely impairs motor function without inducing death (47).

Taken together, the results of both experiments with exposure during the last week of gestation suggest that TCE is a behavioral teratogen in that it produces a pattern of developmental and behavioral delays similar to the pattern produced by established teratogens such as alcohol. Among its effects, fetal alcohol exposure has been associated with decreased birth weights and retarded development (2). Problems with balance and gait and motor ability have been identified as effects of neonatal exposure to alcohol and are suggestive of ethanol acting on the cerebellum (43). Additionally, the effects of delayed motor development produced by prenatal ethanol exposure are similar to those observed with TCE in the present study (39). These similarities between the results of animal tests of prenatal alcohol exposure and TCE exposure raise a concern that children born to mothers exposed to TCE during their pregnancy may be susceptible to a Fetal Solvent Syndrome (59,27) which may share features with the Fetal Alcohol Syndrome. The principle rationale for conducting these experiments was based on the likelihood of TCE exposure to pregnant women engaging in inhalant abuse. The fact that a series of short (1-h) multiple daily exposures to a concentration of TCE which produced clear intoxication of the dams could produce a behavioral teratogenic effect in mice provide concern for inhalant-abusing women who may engage in a similar pattern of high-concentration intermittent exposure. Behavioral teratogenic effects in mice were also seen with more continuous exposure to lower concentration which might be

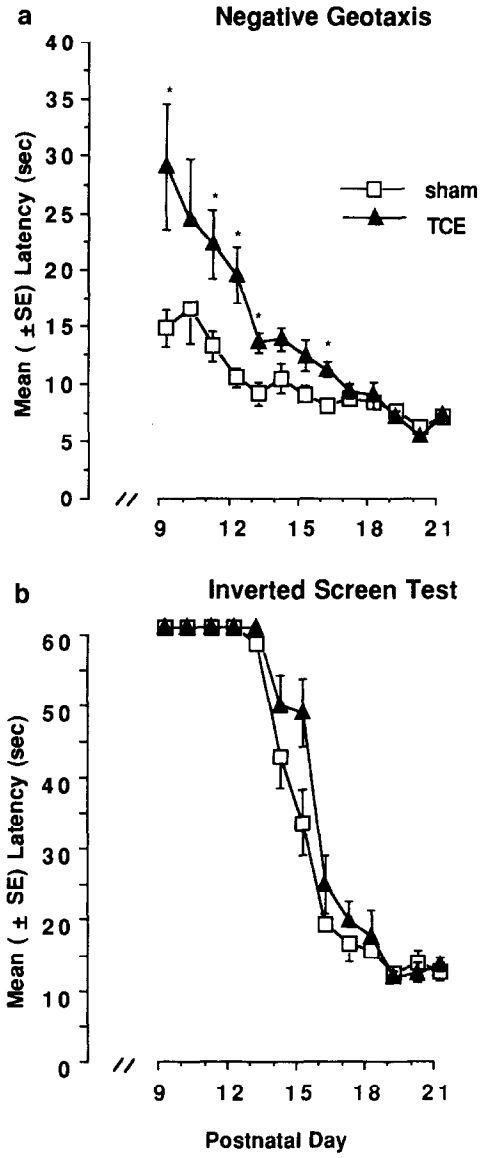

FIG. 8. Effects of intermittent prenatal 8000 ppm TCE exposure on negative geotaxis and the inverted screen test. A 60-sec cutoff was used for each measure. (a) effects on negative geotaxis. Mean (+SE) of latency (in seconds) to turn 180 for PND 8- 21. *TCE group significantly different from sham exposure group ($p < 0.05$). (b) Effects on inverted screen test. Mean (+SE) of latency (in seconds) to climb on top of the screen for PND 9-21. No significant differences were seen in TCE pups relative to sham exposed pups.

more similar to what might occur in a workplace exposure, suggesting cause for concern here as well.

The developmental delays in TCE-exposed pups are also somewhat similar to those of another classic behavioral teratogen, malnutrition. Malnutrition generally results in impaired growth and physical maturation and induces delays in reflex ontogeny; however, it is important to note that rat pups malnourished during the suckling period are more affected by malnutrition than pups malnurished in utero (53). Like with TCE, in utero exposure to malnutrition has been associated with delays in pinnae detachment (63). In contrast to our results with TCE, prenatal malnutrition has not been observed to alter reflex ontogeny in measures of righting (53).

The present experiment attempted to control for the possibility of fetal undernutrition by initially weight matching groups and pair-feeding (in the chronic exposure regimen only) which resulted in similar maternal weights and litter weights among groups. It is known that there are other ways that fetal undernutrition might have occurred. For instance, radiolabeled TCE was observed in the placenta and fetus following short periods of inhalation exposures with pregnant mice (12). The effect that fetal and placental exposure to TCE has on placental uptake and transport capacity of essential minerals and nutrients is not known. Another possibility for the detrimental effects of TCE may be a result of fetal hypoxia which has also been implicated in the etiology of alcohol-induced growth retardation (3) and demonstrated in several species (19,34). If nutrient availability is reduced to the fetus during the period of exposure, then this might have contributed to the reduced pup weight observed at least 24 h after birth. However, this is doubtful since it would be expected that birth weight would also be reduced.

Another possible indirect basis for the effects of TCE is the maternal stress associated with exposure. We attempted to control for this as much as possible by including a sham-exposed group in both studies, and no differences between sham exposed and untreated groups were noted. Nonetheless, both concentrations of TCE used were undoubtedly highly odiferous, a property which is difficult to disassociate from direct biological effects. Several studies have reported delayed behavioral and motor development following in utero exposure to restraint stress (7,8,21). However, in light of the data demonstrating that restraint stress in pregnant mice reduced food and water intake and body weight gain (36), it appears necessary to include pair-fed and ad-lib control groups in the experimental design in order to show that the effects cannot be explained solely by stress-induced maternal malnutrition. Later studies employing this experimental design found that pups stressed in utero did not differ from pair-fed pups suggesting that most of the delays were a result of stress-induced maternal undernutrition (62). Furthermore, it has been demonstrated that, although pair-feeding is suitable for a nutritional control group, the paradigm itself must be treated as an experimental condition. Restricted meal feeding may alter adrenal and placental weights (64), maternal hormones, activity and circadian rhythms in addition to altering behavioral and physiological responses of the progeny (22,38). Two other sources of stress which may have had an impact on the offspring were 1) the in utero exposure to stress via maternal handling and 2) postnatal exposure to stress encountered during behavioral testing. Prenatal exposure to maternal handling has resulted in increased responsivity to stressors in adulthood (4), attenuation of typical responses to acute ethanol and increased ethanol-induced swim test performance in adulthood (15). Although the stressing of pregnant rats has been shown to influence later adult behavior of the offspring (5,57), postnatal handling produces alterations in the opposite direction (61) suggesting that the detrimental effects of prenatal stress may be offset postnatally.

The weight gain in TCE-exposed animals was less than that of controls in both chronic and intermittent exposure experiments. The slow rate of weight gain may be a result of poor lactational performance by the dam. An alternative explanation may lie in an altered sucking behavior of TCE-exposed offspring. Delayed development of sucking behavior has been reported in offspring exposed to ethanol in utero

(60). Although suckling behavior was not directly assessed in the present study, rooting reflex was examined. In utero intermittent exposure to TCE 8,000 ppm resulted in decreased rooting distance relative to sham exposed whereas continuous 17 hrs/day exposure to 2,000 ppm did not significantly alter rooting distance. One explanation for the lack of differences on the rooting reflex in the continuous exposure paradigm is that TCE had no effect; however, an alternative explanation may lie with the employment of the cross-fostering procedure that was used only in the continuous exposure paradigm. Fostering the pups exposed continuously to 2,000 ppm TCE may have been beneficial especially since it is not known what impact TCE may have on maternal behavior, the behavior of the pup to elicit adequate maternal attention or on the complex maternal-infant interaction. It would be of interest in the future to examine parameters of this intricate relationship as well as behavioral aspects specific to the pup such as sucking performance characterized by sucking pressure, suckling attachment latencies and length of time attached (54).

Many of the anomalies observed early in life following exposure to TCE appear to be reversible suggesting that they are due to a delay in CNS maturation rather than an irreversible effect. In addition, no effects of TCE exposure were seen post-weaning in motor activity or passive avoidance learning. The lack of observed differences in activity and learning between groups later in life may be due to one or several factors. One possibility may be that the TCE-exposed offspring learn to compensate for their initial deficits. Another possibility is that more sensitive measures of learning and activity are needed to elucidate the differences between TCE and unexposed offspring observed after weaning. Conceivably, exposing the animals to postnatal stimulation via handling may have affected development. Such an alternative was found to have an impact following prenatal exposure to alcohol. Rats exposed to alcohol in utero were handled or not handled until 35 days of age at which time all were tested in a step-down passive avoidance task. Nonhandled animals exhibited deficits in inhibiting responding relative to controls. In contrast, handled offspring did not differ from controls (23). Since handling animals prior to weaning has been reported to accelerate development and affect subsequent behavior (1, 41) and prenatal TCE exposure may effect behavior by causing a delay in maturation, it will be important in future research to establish if there are long term effects associated with prenatal TCE exposure disassociated from early handling experience.

In conclusion, the results of two experiments have shown that prenatal exposure to TCE can produce developmental toxicity in the offspring of exposed mice. These studies were done for the primary purpose of assessing possible adverse consequences of abuse of TCE-containing products by pregnant women. It is difficult to model, in animals, the highly variable exposure conditions that would likely occur in inhalant abusers. We attempted to approach this problem by studying two exposure regimens, one using a 17 hour/day exposure to 2000 ppm TCE, a concentration that produces acute behavioral effects in mice and is likely close to concentrations used by TCE abusers. The other used 60-min exposures, three times a day, to a maximally tolerated concentration of 8,000 ppm which produced significant intoxication of the dams. We believe that these two conditions bracket the types of exposure conditions that might be relevant to exposures from inhalant abuse. The fact that both exposure regimens resulted in a very similar pattern of developmental delays in the offspring exposed mice suggest real cause for concern for possible harmful effects of TCE abuse by pregnant women. Further research is needed to determine if other abused inhalants display a similar potential for behavioral teratology.

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