Toxicology and Industrial Health **(1999) 15, 356–369**


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A rat model of neurobehavioral sensitization to toluene

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Some individuals report that, following either a single high-level or repeated lower-level exposures to chemicals (initiation), subsequent exposure to very low concentrations of chemicals (triggering) produces a variety of adverse effects, including disruption of cognitive processes. Our objective was to model this two-step process in a laboratory animal. Two groups of 16 rats, eight male and eight female, received whole-body inhalation exposure to toluene, either at 80 ppm for 6 h/day for 4 weeks (Repeat group) or to 1600 ppm for 6 h/day on one day only (Acute group). Two other groups (Trigger group and Clean group) of 16 were sham-exposed. After 17 days without toluene exposure, the Acute, Repeat and Trigger groups began a series of daily toluene 'trigger' exposures (10 ppm for 1 h) followed immediately by testing on an operant repeated-acquisitions task requiring learning within and across sessions. The Clean group was sham-exposed prior to operant testing. Trigger or sham exposures and operant testing continued 5 days/week for 17 sessions. Analysis of variance revealed a variety of statistically significant ($P<0.05$) differences between treatment groups. Furthermore, the patterns of differences between groups differed ($P<0.05$) for female and male rats. For example, male rats of the Trigger group made the most responses, and female rats of the Repeat group responded most slowly. The observation of important changes in the operant behavior of female and male rats previously exposed to toluene, at relatively low concentrations (80 or 1600 ppm) and then later re-exposed at very low concentrations (10 ppm), is consistent with the experiences of humans reporting cognitive difficulties following acute or chronic exposures to chemicals.

Introduction

Evidence is accumulating that exposure to relatively low levels of common solvents, combustion products, pesticides and mixed volatile organic compounds (VOCs) found in indoor air can be associated with long-term cognitive and neurophysiologic sequelae (Ashford and Miller, 1998). These effects appear to result from a two-step process, initiation and triggering. The initiating exposure can be either an acute high-level exposure, as in a chemical spill, or a chronic low-level exposure, as in a sick building. After a period of time, very-low-level exposures, at concentrations previously tolerated, reportedly trigger symptoms. These effects appear to occur most clearly in a subset of the population; additionally, chemical intolerances are reported more frequently by females (Miller, 1994). Miller (1997) has described this two-step process as toxicant-induced loss of tolerance (TILT). Although possible mechanisms have been proposed, e.g., VOCs might sensitize the olfactory–limbic pathway (Bell et al., 1992) and/or elicit a kindling-like process (Rossi, 1996), leading to heightened reactivity to very-low-level chemical exposures, efforts to demonstrate the effect in animal models are just beginning. Sorg et al. (1996, 1998) have demonstrated that repeated low-level formaldehyde exposure produces cross-sensitization to cocaine-induced increases in rearing activity.

It is important to determine whether sensitization to low-level VOC exposures can occur in some individuals and whether this adversely affects cognitive performance. An animal model that could predict effects of chemicals on cognitive function of humans would be of practical value, especially for situations where impaired attention, memory or decision-making ability could have serious consequences, such as when operating a vehicle or making complex decisions.

Behavioral scientists have developed a variety of ways to assess the neurobehavioral capabilities of laboratory animals (NAS, 1975). Some methods entail quantitative observation of natural behaviors; the subject is not required to learn a novel behavior. Operant methods, in which subjects are given rewards contingent upon completion of a learned behavior, have proven to be excellent tools for the assessment of learning and memory (NAS, 1977). For a given reinforcement contingency, patterns of responding are both stable and very similar across species, including mouse to human (Ferster and Skinner, 1957). Task difficulty can be increased to examine higher-order cognitive functions (Weiss and O'Donoghue, 1994). Operant behavior is affected in known ways by agents, including chemicals

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(Laties, 1978) and drugs (Dews, 1972) that affect the function of the central nervous system.

Our objective was to compare the acute effects of exposure to very low concentrations (10 ppm) of toluene following either repeated low-level (80 ppm) or single high-level (1600 ppm) exposure. For the low-level exposure, we selected a concentration equal to 80% of the human occupational exposure standard for toluene of 100 ppm. The primary hypothesis was that an 'initiating' exposure to toluene would sensitize the subjects so that subsequent 'trigger' exposures would produce neurobehavioral deficits. The secondary hypothesis was that females would be affected more than males, consistent with observations in humans (Cullen et al., 1992; Fiedler et al., 1994; Lax and Henneberger, 1995).

A single experiment with two essentially identical replicates was completed: one replicate used female rats as subjects, and the other used males. Cognitive capability was assessed using a three-lever operant task requiring subjects to transition between levers. The subjects had to determine which of three levers would produce food rewards; after 30 rewards were delivered, another randomly selected lever became correct. After 30 rewards had been earned responding on the second lever, the third lever became correct for 30 rewards. No experimentally controlled cues signaled the effective lever. Beginning on the 18th day after initial exposures ended, the experimental group subjects began receiving a series of daily toluene trigger exposures (10 ppm$\times$1 h) immediately followed by 60-min operant sessions. The control group was sham-exposed prior to testing.

Methods

Subjects

Thirty-six male and 36 female Sprague–Dawley rats (*Rattus norvegicus*, Crl:CD BR {SD}) were obtained from Charles River Laboratories (Wilmington, MA). When received, the subjects were 54–58 days of age and weighed 180–200 g. Subjects were caged individually in polycarbonate cages with hardwood bedding. (Stainless steel wire mesh cages were used in the exposure chambers.) Temperatures were maintained at 64°–79°F, with a relative humidity of 40–70%. Lighting was timer-controlled to provide a 12-h light–dark cycle, with light onset at 7:00 a.m. Laboratory Rodent Diet 5001 (PMI Feeds, St. Louis, MO) was used.

Caloric regulation is required for controlled performance on an appetitively motivated operant task. Body weights were measured daily. Beginning on the third day after arrival, the subjects were fed sufficient food intended to maintain their weight at between 75% and 80% of the age-adjusted, free-feeding weight of this strain and sex, based on vendor-supplied growth curves. Food allotments were given at the end of the day. Tap water was provided *ad libitum* with water bottles. For the 6-h periods in the exposure chambers, subjects did not receive food or water. Food, but not water, was available in the operant apparatus.

Experimental Design

Four treatment groups, each with eight subjects, were used in each of two replicates; one used female rats and the other used male rats (Figure 1). The Repeated Exposure Group ('Repeat') was exposed to 80 ppm toluene for 6 h/day for a period of 20 days. The Acute Exposure Group ('Acute') received sham exposures for 19 days and then a single 6-h exposure to 1600 ppm toluene on the 20th day. For both the Acute and Repeat groups, total toluene exposure was 9600 ppm$\times$h. Both the Trigger Control Group ('Trigger') and the Clean Air Control Group ('Clean') were sham-exposed for 6 h/day for 20 days.

Exposure Chambers

Toluene exposure was by whole-body inhalation: this route of administration is typical of most human toluene exposure. Certified ACS (99.9% pure) toluene (CAS Number 108-88-3) obtained from Fisher Scientific (Hampton, NH) was used. Laboratory quantities of toluene were kept in tightly closed brown glass jugs (4 l) placed in

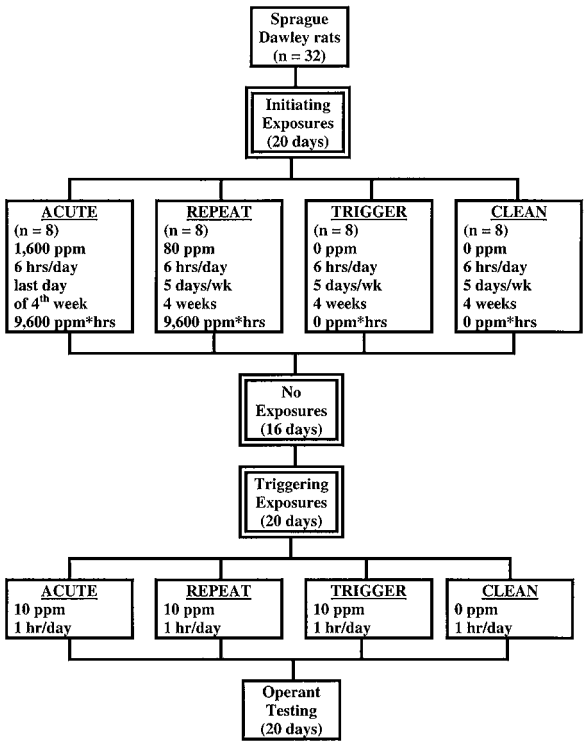

Figure 1. Flow chart summary of the experimental design used with two replicates. The first replicate used 32 female rats and the second used 32 male rats.

protective plastic enclosures. When not in use, they were stored in a fire-proof cabinet located in a cool, well-ventilated area away from oxidizing agents and sources of heat or ignition. The air exhaust system in the inhalation facility operated at negative pressure from the point where the solvent entered the air stream until the air was exhausted from the building. This ensured that any leaks in the system resulted in fresh air being drawn into the ducting rather than allowing solvent vapors to escape into the laboratory. The exposure laboratory also had a negative pressure exhaust system with an air inlet near floor level for collection of vapors heavier than air.

The five 1.5-m^3 exposure chambers (Figure 2) were constructed of glass and stainless steel and were operated at a flow rate sufficient to ensure 12–15 conditioned, HEPA-filtered air changes per hour. A positive-displacement flowmeter located at the inlet side of each chamber monitored the airflow rate and provided a signal to the control computer. This flow was displayed and recorded every 3 min. An audible warning alarm was activated if the flow went above or below programmed 'acceptable' values (±5%); a second alarm indicated flows above or below 'unacceptable' values (±10%). The specific mean flow values for the alarms depended upon the toluene concentration being produced. Chamber temperature also was displayed and recorded every 3 min. An audible warning alarm signaled temperatures below 68°F or above 75°F. A second alarm signaled when the temperature fell below 64°F or exceeded 79°F.

The test atmosphere generation system was designed specifically for solvents. Conditioned input air passed through an in-line heating unit that dispensed vaporized toluene into the airstream. The physical attributes of toluene were resident in a spreadsheet program that calculated pump and temperature settings required to produce a given concentration. Toluene was introduced into the evaporator by adjustable peristaltic pumps; the type of pump and flow rate depended upon the toluene concentration being generated. A microprocessor-based temperature controller heated the evaporators. The vapor was delivered through a stainless steel duct system to the animal chamber inlets. An acoustic filter on each chamber minimized noise in the chamber.

Actual toluene concentrations for both control and exposure groups were determined by monitoring the chamber atmospheres using a dedicated M200 gas chromatography system. The gas chromatograph readings were stored in a computer for analysis. Air from each chamber was sampled in sequence every 3 min, meaning a reading was taken from each of the five chambers every 15 min.

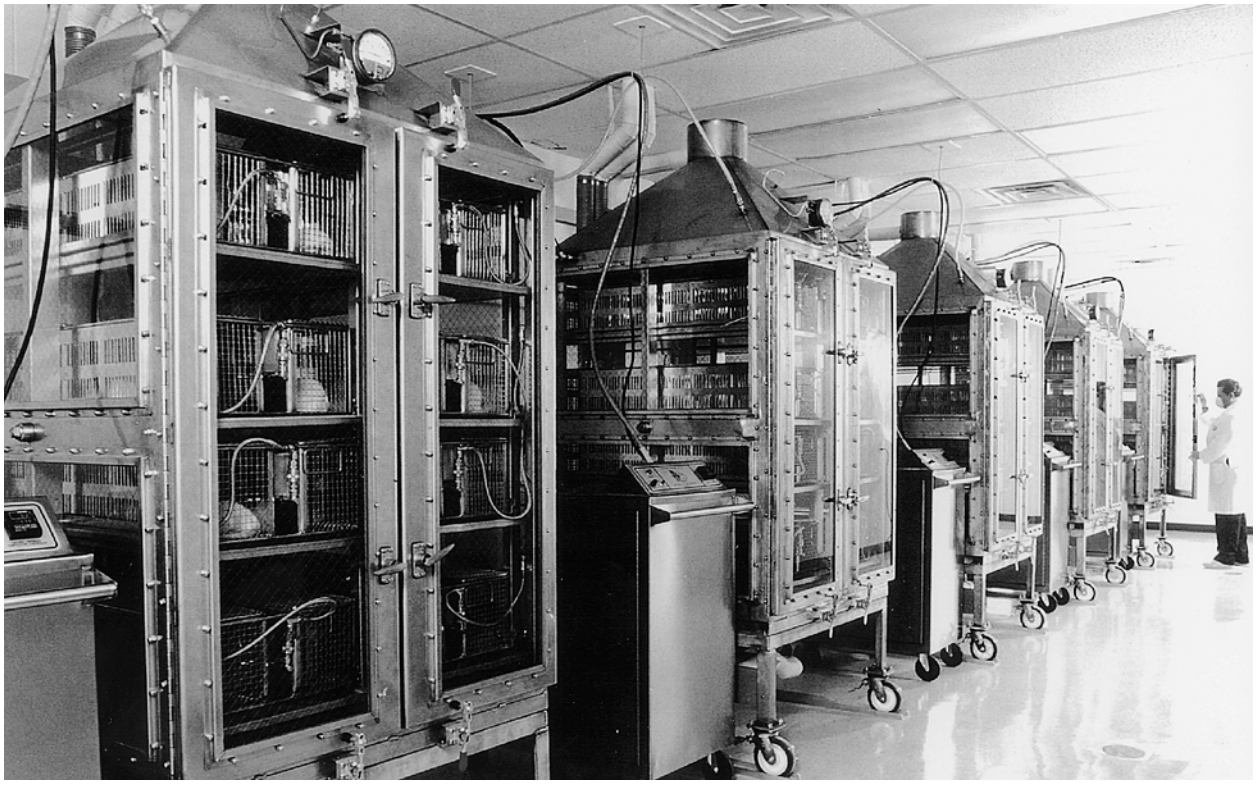

Figure 2. Photograph of a portion of the interior of the Inhalation Neurotoxicology Laboratory showing the five 1.5-m^3 exposure chambers, each with solvent vapor generation unit.

Alarms sounded if the toluene concentration fell below or rose above programmed set points. At 10 ppm, the 'acceptable' set points were 9 and 11 ppm (±10%) and the 'unacceptable' set points were 8 and 12 ppm (±20%). At 80 ppm, the pairs of acceptable and unacceptable set points were 75 and 85 ppm (±6%) and 70 and 90 ppm (±13%), respectively. At 1600 ppm, the pairs of set points were 1500 and 1700 ppm (±6%) and 1400 and 1800 ppm (±13%).

To verify chamber operation before starting the study, a series of tests was completed using acetone. To verify spatial homogeneity, samples were drawn at 5-min intervals from 36 points, nine on each of four planes within each chamber. To verify temporal stability, samples were drawn at 5-min intervals over 6-h periods from a reference location within each chamber. Spatial and temporal variations were minimal; the coefficients of variation (CV) were less than 1% for all chambers. (The CV is the standard deviation [SD] divided by the mean, expressed as percent.) Other 6-h tests assessed steady-state accuracy and stability. For example, with a target of 500 ppm, the mean concentration was 495 ppm, the SD was 3.24, and the CV was 0.65%. At 1000 ppm, the mean concentration was 1007 ppm, and the CV was 0.73%. At 5000 ppm, the mean concentration was 50001 ppm and the CV was 0.88%. In validation of chambers for inhalation toxicology, 10% variation is regarded as good and 5% variation is regarded as excellent (Cheng and Moss, 1995).

Operant Task

Using four Coulbourn Instruments (Lehigh Valley, PA) operant chambers, each equipped with three levers and a food pellet receptacle located beneath the middle lever, we implemented a three-lever task, the 'response transition procedure'. This task has been used successfully to study the effects of both scopolamine and a synthetic cholinergic antagonist on repeated acquisitions (Cohn et al., 1992). Both within and between daily sessions, the subjects must learn when each lever provides food reinforcement. The reinforced lever is not signaled, i.e., no cue lights or other stimuli are provided as cues.

At the beginning of each session, one of the three response levers was selected randomly as the 'correct' lever; responses on this lever produced 45-mg food-reward pellets (P.J. Noyes; Lancaster, NH). Responses on the two other 'incorrect' levers did not deliver food rewards. After 30 reinforcements were received for responses on the initially correct lever, additional responses on that lever no longer produced food rewards, i.e., it became incorrect. One of the two remaining levers then was selected randomly to become correct, producing food rewards until 30 reinforcements had been delivered. Then the third lever became correct. A session continued until either 90 rewards had been delivered or 60 min had elapsed.

Although the session consisted of 90 trials, trials 1, 31, and 61 were the 'transition' trials at which subjects had to learn a new behavior: the subject must learn to stop responding on one lever and to start responding on another. Subjects tend to perseverate in responding on the formerly reinforced lever. Once a shift has been made, subjects should continue to respond primarily on the newly correct lever with a minimum of wandering to the other two levers.

A compound schedule of reinforcement consisting of variable interval (VI) and fixed ratio (FR) components was used. On a VI, the first correct lever press occurring after a variable elapsed time interval produces a food reinforcement. On a VI5 schedule, the first response occurring, on average, 5 s after the previous response is reinforced. (From reinforcer to reinforcer, the length of the VI varies in a random manner about a selected mean value, given in seconds.) On an FR5, the reinforcer is provided after the fifth response. On the response transition task used here, an FR contingency was employed in which responses on either incorrect lever successively increased the FR on the correct lever from one to five consecutive responses for delivery of a food reward. Upon completion of both the FR and VI requirements on the correct lever, food delivery occurred and the FR component was reset to one. The titrating FR schedule increased the cost of errors, discouraging frequent lever switching and encouraging persistent responding on the correct lever.

Training Stages Training proceeded gradually through a series of 12 progressively more difficult stages intended to effectively train nearly all subjects. To proceed to the next stage, a subject had to earn 90 food pellets in a session (the maximum achievable) with a predetermined minimum overall percent correct score (Table 1). Percent correct was defined as the total number of presses on the correct levers divided by the total number of responses made on all levers. No subject was advanced beyond stage 12.

Table 1. Summary of training stages.

Stage	FR	VI	Percent correct
1	1	0	85
2	2	0	50
3	3	0	65
4	5	0	50
5	5	1	50
6	5	2	65
7	5	3	65
8	5	10	50
9	5	15	65
10	5	20	50
11	5	25	65
12	5	30	–

Shaping Subjects were received 11 days prior to initiation of exposure to allow time for them to adapt to the laboratory after shipping and to acquire appropriate performance on the basic operant task before group assignment. Initially, all 36 subjects for each replicate were trained for 3 days on stage 1. With FR=1 and VI=0, the subjects learned to determine the lever giving pellets, to respond on that lever, and to go to another lever when the payoff stopped. If a subject earned 90 rewards on the first two training days, it was not run on the third day, which was a Friday. (The intent was to minimize dispersion among subjects in the number of rewards received.) Following this initial training period, the 32 subjects comprising each replicate were assigned, in equal numbers, to one of the four treatment groups. Then, after a weekend, the exposure phase of the experiment began.

Randomization A performance-stratified subject-assignment method was used to determine group assignments in each replicate. Based on total number of rewards earned on the first two training days, the four subjects performing most poorly were eliminated from each study. The remaining 32 subjects were ranked from best to worst; ties were acceptable. In descending order, subjects from each set of four were assigned randomly to one of the four experimental groups. Within sets of four, both subject selection and group selection were random, without replacement.

Exposure Procedures

Initiating Exposures The initiating exposure was for 6 h/day, Monday through Friday, for four consecutive weeks (Figure 1). During the first 19 days of exposure, the Repeat group (80 ppm) was placed in one chamber and the remaining animals were placed in a second chamber (0 ppm). On the 20th day of exposure, the Repeat group (80 ppm) was placed in one chamber, the Acute group (1600 ppm) was placed in a second chamber, and the remaining two groups were placed in a third chamber (0 ppm). Once the subjects were placed in the exposure chambers, it took about 15 min for the toluene to reach the desired concentration. After 6 h, the toluene supply was turned off; it then took about 15 min for the toluene concentration to drop to near 0 ppm. To allow a period of time in which neurobehavioral sensitization could develop, the subjects were not placed in the exposure chambers during a 16-day 'rest period'.

Trigger Exposures and Operant Testing On the Monday following the 16-day rest period, an initial 1-day test of ability to perform on Stage 1 was completed. On this day, which was identified as 'day 0', there were no trigger (or sham) toluene exposures. Instead, the ability of all subjects to perform the basic task (FR1, V0) was measured: (1) to confirm that the subjects had not forgotten how to barpress in the 46 days since their last shaping session; and (2) to determine if acute or repeated toluene exposure during the initiation phase had produced major, lasting deleterious effects on the operant performance of the subjects.

On the next day (day 1), subjects began receiving 1-h 10 ppm toluene (or sham) exposures just before behavioral testing. (The chamber was brought to 10 ppm before exposing the first set of subjects; opening and closing the chamber door to remove and insert subsequent sets of subjects had minor effects on chamber toluene concentration.) In replicate 1, conducted with female subjects, operant testing sessions were completed on 18 successive weekdays, beginning on Monday. In replicate 2, completed with male subjects, operant testing sessions were completed on 23 successive weekdays, beginning on Monday. The addition of an extra week of triggering with the males is the only difference between replicates 1 and 2. For the data analyses reported here, only data from the first 18 operant testing sessions of the male rats were used.

At the beginning of each day of trigger exposure and operant testing, three subjects (one each from the Acute, Repeat and Trigger groups) were placed in the 10 ppm chamber. The subjects were exposed to toluene for 1 h and then removed for immediate operant testing. At the same time, a subject from the Clean group was sham exposed for 1 h and then placed in an operant chamber. After a 10-min delay, a second set of four subjects was placed in the appropriate exposure chambers while the first set of four subjects was in the operant boxes. This procedure was continued for eight sets of four subjects per day, using a fixed order of exposure and testing.

On days 1 through 3 of operant training, no subjects were moved to stage 2, even if they achieved 85% correct. Day 4 was the first day on which progression to stage 2 could occur.

Locomotor Activity

A pilot study involving assessment of locomotor activity was completed at the termination of the operant study. On the Thursday and Friday of the fourth week of replicate 1 and of the fifth week of replicate 2, trigger exposures were given, but the subjects were tested for locomotor activity instead of operant performance. The 30-min motor activity tests, based on the Environmental Protection Agency neurotoxicity test guidelines, were conducted using Figure 8 maze systems manufactured by San Diego Instruments (San Diego, CA). A 7-W light bulb suspended over the center of each of the 16 mazes provided uniform lighting. Wide-band noise of 65–75 dBA, measured at the center of the shelving holding the mazes, provided an appropriate background sound level. For each subject, total beam breaks during six 5-min periods were recorded in each session.

General Statistical Analysis Methods

The five operant performance measures (dependent variables) analyzed were: (1) overall percent correct lever responses, (2) total number of responses made, (3) total number of correct lever responses, (4) total number of incorrect lever responses, and (5) time (seconds) to complete a trial. The training stage, operant measures, and locomotor activity scores were analyzed using the SPSS (Chicago, IL) statistical package to conduct mixed, repeated-measures analysis of variance (ANOVA).

In the language of ANOVA, an experimental design can include 'within-subject' and/or 'between-subject' factors (Winer, 1971). A within-subject factor, such as Trial, includes repeated measurements from a subject. In this experiment, Trial had three 'levels': Trial 1, Trial 31 and Trial 61. On a between-subjects factor, such as Sex, a subject can be in only one level of the factor, Female or Male in this case. A 'mixed design' includes both between- and within-subject factors. A 'one-way' ANOVA contains only one factor, such as Group (Acute, Repeat, Trigger or Clean), and a 'three-way' ANOVA includes three factors such as, in this experiment, Trial (1, 31, and 61), Sex (Female or Male), and Group (Acute, Repeat, Trigger or Clean). Specifications of the ANOVA models used are provided in the Results section.

ANOVA provides tests of both 'main effects' and 'interactions'. A statistically significant ($P<0.05$) main effect of, e.g., Group, indicates that at least one pair of the four means (Acute, Repeat, Trigger and Clean) differs statistically. In a test of main effects, scores on other factors, such as Sex and Trial, are combined. ANOVA also assesses interactions of two or more variables. For example, if the Group×Sex interaction is significant, it indicates that the patterns of differences among the four treatment groups differ for male and female rats.

Results

Body Weight

Because appetitive (food-reinforced) motivation is a critical variable in most operant experiments, it is important to demonstrate good control of feeding and body weight. There were no important differences in body weight among groups. The female rats averaged 77.2% of the age-adjusted free-feeding body weight of female Sprague–Dawley rats. The intra-day CVs averaged 1.3%, and the inter-day CVs averaged 1.8%. The subjects' weight averaged 219 g over the period of operant testing. Part of the inter-day variation is a result of normal weight gain. Over this period, the expected free-feeding weight of female rats would average 284 g (SD=3.4; CV=1.4%), with subjects growing from 277 to 294 g.

The male rats averaged 76.9% of the age-adjusted free-feeding body weight of male Sprague–Dawley rats. The intra-day CVs averaged 2.1%, and the inter-day CVs averaged 4.2%. The subjects' weight averaged 352 g over the period of operant testing. Part of the inter-day variation is a result of normal weight gain. The inter-day CV is greater for the male rats because the period was 1 week longer and because males rats were growing more rapidly at this age than female rats. Over this period, the expected free-feeding weight of male rats would average 458 g (SD=11; CV=2.4%), with subjects growing from 440 to 475 g.

Toluene Exposure

The average toluene concentration during the 20 80-ppm exposures of replicates 1 and 2 was 79.5 ppm, with a mean CV of 4.9%. The average toluene concentration during the trigger exposures of replicates 1 and 2 was 11.0 ppm, with a mean CV of 8.0%. The average during the 1600 ppm exposures was 1588 ppm, with a mean CV of 4.4%. The actual concentrations achieved were close to the planned levels, i.e., within 0.02% at 80 ppm, within 2% at 1600 ppm, and within 10% at 10 ppm. The intra-day (mean CV=5.9%) and inter-day (mean CV=6.7%) variations were small, and there were no systematic trends in measures of within-day and between-day variation.

Operant Behavior

Training Stage The progression of subjects through training stages did not appear to be affected by toluene exposure. The numbers of subjects in stage 12 on trigger day 17 did not differ significantly by group ($\chi^2=1.19$, $df=3$, NS). Ten of 16 subjects in the Clean group reached stage 12, as did 8/16 in the Acute group, 8/16 in the Trigger group, and 7/16 in the Repeat group.

The daily training scores of the four groups were very similar (Figure 3). The daily training stage data were analyzed using a two-factor, mixed ANOVA with the between-factor of Group (Acute, Repeat, Trigger, and Clean) and the within-factor of Day ($n=14$, days 3 through 17). Statistically significant main effects of Day ($F=2412$; $df=13, 30$; $P<0.001$) and Sex ($F=29.96$; $df=1, 56$; $P<.001$) and a Day×Sex interaction ($F=5.71$; $df=13, 39$; $P<0.001$) were found. For the 14-day period of operant data analyzed, the average training stage for male rats was 6.40 (SEM=0.17) and that of female rats was 5.51 (SEM=0.16); the males' mean was 16% greater than did the females'. On days 4 through 17, the mean training stage for male rats was greater, by about one stage, than for female rats (Figure 4).

First Four Operant Days Because there was no trigger toluene exposure on the first day of operant testing, these

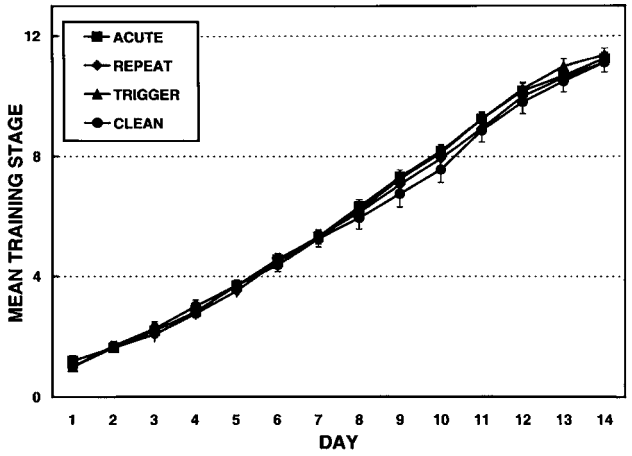

Figure 3. Mean training stage by group for days 4 through 17 of operant testing. The error bars, which are standard errors of the means, are only slightly larger than the symbols used to plot data points. In this figure only, data from days 4–17 of operant testing are numbered as days 1–14.

data were analyzed using a one-way, between-groups design. The factor called Group had four levels (Repeat, Acute, Trigger and Clean). Separate ANOVAs were completed for each of the five operant dependent variables. As expected, all subjects performed the basic operant task (FR=1, VI=0) well, even though they had not been in the operant chambers for 46 days; there were no statistically significant ($P<0.05$) differences among the four groups in either replicate. Because all subjects were kept in stage 1 for the first 3 days of triggering, these operant data were analyzed using a mixed two-way design: the within subjects factor was Day ($n=3$), and the between-subjects factor was Group ($n=4$). No statistically significant differences involving Group were found.

Remaining 14 Operant Days Initial analyses indicated no group differences when data from all 90 trials were included. After earning two or three rewards on a new lever, the subjects consistently performed with high reliability, making most of their responses on the correct lever. Examination of perseveration on the three successive trials after lever selection did not reveal differences among groups. During stage 1, on the first trial after a transition, about 50% of responses occurred on the formerly correct lever; on the third trial after a transition, 20% of responses occurred on the formerly correct lever. In stage 9, on the first trial after a transition, about 75% of responses occurred on the formerly correct lever; on the third trial after a transition, less than 5% of responses occurred on the formerly correct lever. The subjects became well-trained. In summary, good performance on the vast majority of the 90 trials obscured events occurring on the three transition trials requiring learning of a new response. Thus, the analyses reported here focus on trials 1, 31 and 61, those on which the subjects had to identify a newly correct lever.

The operant data from stage 9 were analyzed using a mixed, three-factor ANOVA with the within-factor Trial ($n=3$) and the between-factors of Group ($n=4$) and Sex ($n=2$). Only two female rats, one from the Repeat group and one from the Clean group, failed to complete this training stage. All male rats completed stage 9. When conducting an ANOVA, SPSS deletes any case for which there is missing data. Thus, the analysis was completed with data from 62 subjects, 30 female and 32 male. The analysis of data from stage 9 was a compromise, providing the most advanced degree of training with minimal loss of subjects. Scores for each subject by stage were means across the total number of days a subject was within a given stage.

Sixteen statistically significant ($P<0.05$) main effects or interactions were found (Table 2). By themselves, the five statistically significant effects of Trial are not of particular interest; they reflect the nature of the operant task used in this experiment. The data on percent correct responses provide one general view of the results (Figure 5). As expected, performance was best on Trial 1 (40% correct) and worst on Trial 61 (9% correct). This pattern did not vary significantly among groups. In terms of accuracy, male and female rats performed in a statistically equivalent manner: females averaged 19% correct (SEM=1.8), and males averaged 21% correct (SEM=2.0).

Differences in the operant performance of female and male rats are of somewhat more interest. Three statistically significant Sex effects were observed: (1) total number of responses, (2) number of incorrect responses, and (3) time to complete a trial (Table 2). Male rats (mean=82, SEM=11) averaged 17% more total lever responses than female rats (mean=70, SEM=12). Male rats (mean=73, SEM=11) also averaged 20% more incorrect lever responses than female rats (mean=61, SEM=6). Interestingly, female rats (mean=146 s, SEM=7) required 30% longer to complete a

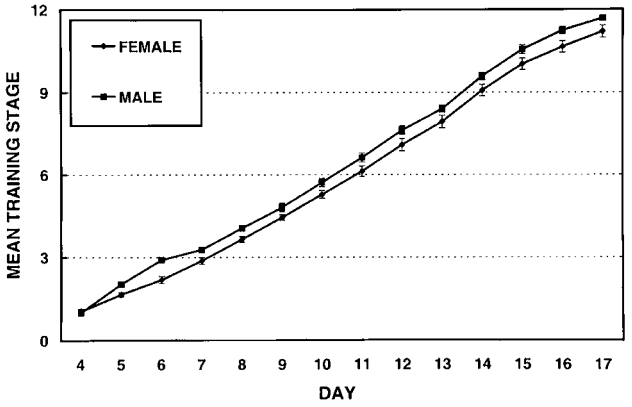

Figure 4. Statistically significant ($P<0.05$) Sex×Training Stage interaction. The error bars, which are standard errors of the means, are only slightly larger than the symbols used to plot data points.

Table 2. Summary of probability values from ANOVA of operant data from stage 9. Bold=indicative of an effect from toluene inhalation.

Factor	*df*	Percent correct responses per trial	Number of responses per trial	Number of correct responses per trial	Number of incorrect responses per trial	Time to complete a trial
Trial	2,108	0.001[a]	0.001	0.004	0.000	0.000
Sex×Trial	6,108	0.502	0.032	0.276	0.033	0.924
Group×Trial	6,108	0.796	0.310	0.110	0.228	0.510
Group×Sex×Trial	6,108	0.233	0.250	0.789	0.174	0.349
Sex	1,54	0.411	0.046	0.706	0.039	0.045
Group	3,54	0.792	**0.006**	0.708	**0.007**	**0.030**
Group×Sex	3,54	0.175	**0.014**	0.163	**0.011**	**0.029**

[a]As reported by SPSS.

trial than did male rats (mean=111 s, SEM=11). Female rats responded more slowly than males: trial duration was greater, but number of responses was less.

In addition, two statistically significant ($P<0.05$) Sex× Trial effects were detected. On both total responses (Figure 6) and number of incorrect responses, the males performed slightly better on Trial 1 and much worse on Trial 61. As compared with Trial 1, Trial 61 requires two to three times as many responses to earn a reward. At Trials 1 and 31, performance by female and male rats was similar, but on Trial 61, male rats averaged 38% more responses than female rats. The pattern for number of incorrect responses was the same as total responses: the female rats averaged 26 (SEM=1.3), 72 (SEM=3.3), and 85 (SEM=4.4) incorrect responses for Trials 1, 31 and 61; the male rats averaged 18 (SEM=1.3), 82 (SEM=2.8) and 118 (SEM=6.8). On trial 1, male rats made fewer total responses and fewer incorrect responses than did female rats.

Group differences are of primary interest: there were three main effects ($P<0.05$) for Group on the dependent variables: (1) number of responses per trial, (2) number of incorrect responses per trial, and (3) time to complete a trial (Table 2). Relative to the Clean (control) group, all three toluene-exposed groups, Acute, Repeat, and Trigger, made more responses (Figure 7). Together, they averaged 81 responses, 45% more than the Clean group, which averaged 59 responses. Here, the differences among the Acute, Repeat, and Trigger groups appear modest, suggesting that this particular effect was mediated primarily by the 10-ppm trigger exposure. For number of incorrect responses, the pattern was very similar. The Acute (mean=75, SEM=6.3), Repeat (mean=73, SEM=5.8), and Trigger (mean=69, SEM=7.5) groups averaged 72 incorrect responses, 24% more than the Clean group (mean=51, SEM=3.4).

The Group effect for duration also was statistically significant (Table 2). The Acute and Repeat groups averaged 152 s to earn a reward on a transition trial, 46% longer than the Trigger and Clean groups, which averaged 104 s (Figure 8).

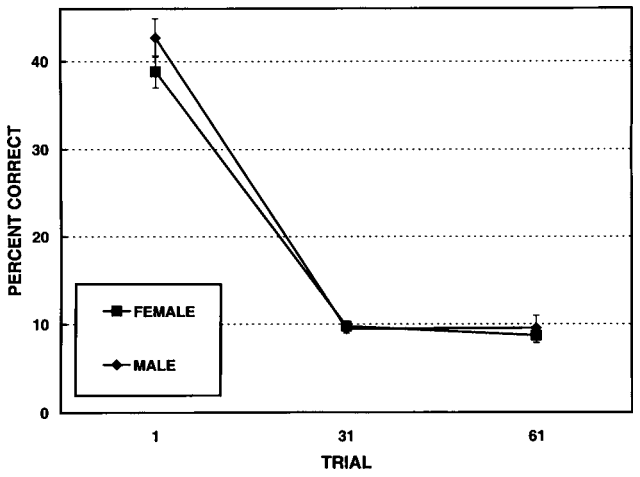

Figure 5. Mean percent correct lever responses for trials 1, 31 and 61 for female and male rats. The error bars are standard errors for the means. Analysis of variance uniformly indicates statistically significant ($P<0.05$) Trial effects.

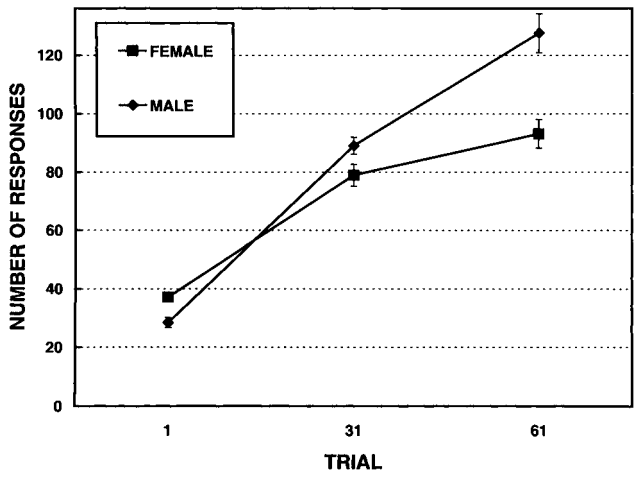

Figure 6. Mean number of responses required to earn a reward on trials 1, 31 and 61 for female and male rats. The error bars are standard errors for the means. Analysis of variance uniformly indicates statistically significant ($P<0.05$) Trial effects. For this variable, the Sex×Trial interaction also is statistically significant.

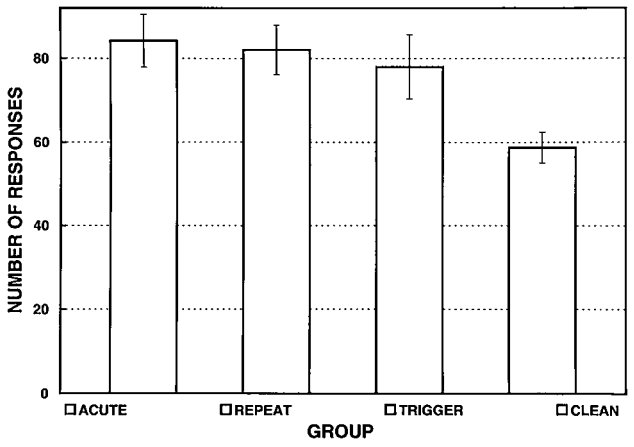

Figure 7. Statistically significant ($P < 0.05$) Group effect for total responses of female and male rats. The error bars are standard errors for the means. Data from trials 1, 31 and 61 are averaged together. All three toluene-exposed groups made more responses than the control group.

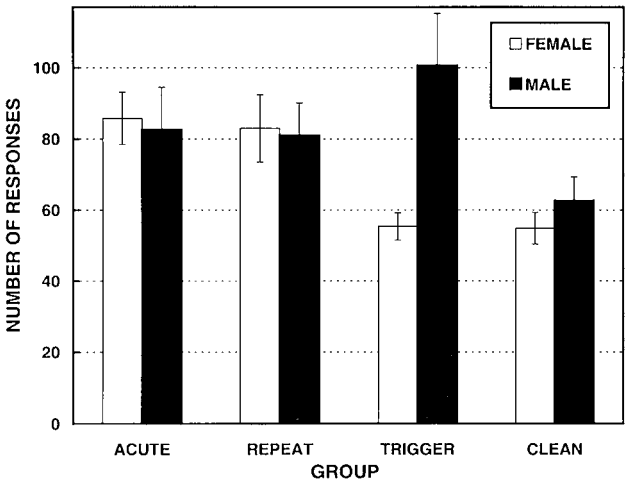

Figure 9. Statistically significant ($P < 0.05$) Group×Sex interaction for number of responses. Data for female and male rats across trials 1, 31 and 61 were combined. The error bars are standard errors for the means. For both females and males, the Acute and Repeat groups were adversely affected by initiating toluene exposure. For males, but not for females, Trigger exposure alone affected operant responding.

Percent correct did not differ statistically among groups (Table 2); in general, the ratio of correct-to-incorrect responses remained relatively constant. Overall, the Clean groups averaged 21.3% correct (SEM=3.4), and the Trigger groups averaged 19.5% (SEM=3.9). The Acute groups averaged 19.5% (SEM=4.0), and the Repeat groups averaged 19.1% (SEM=3.9).

Three statistically significant ($P<0.05$) Group×Sex interactions also were detected (Table 2). First, female rats in the Acute and Repeat groups made an equivalent number of total responses, averaging 54% more responses than the Clean and Trigger groups (Figure 9). On this variable, all effects are not attributable to the 10-ppm trigger exposure.

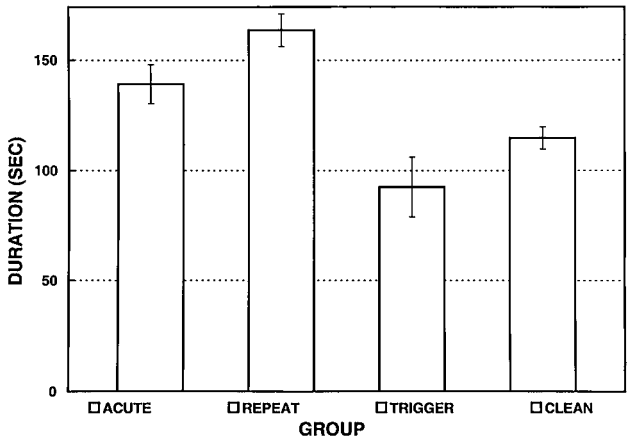

Figure 8. Statistically significant ($P < 0.05$) Group effect for the average duration required by female and male rats to earn a reward on transition trials. The error bars are standard errors for the means. Data from trials 1, 31 and 61 are averaged together. The Acute and Repeat groups performed more slowly than the control groups.

As indicated by interaction effect, the pattern was different for male rats, primarily because the Trigger group performed very poorly. The male Acute and Repeat groups were equivalent, requiring 30% more responses than the Clean group. Notably, as compared to their control group, female rats of the Acute and Repeat groups were affected more severely than were the male rats of these groups. However, among male rats, the Trigger group's mean for total responses was 60% greater than the Clean group's mean. (In female rats, the Trigger and Clean groups were equal on this variable.) As noted before, in the Clean groups, male rats made more responses than female rats.

Second, the overall pattern of results for the Sex×Group interaction ($P<0.05$) in number of incorrect responses was very similar to that shown for correct responses. For female rats, the means for the Acute, Repeat, Trigger and Clean groups were 76, 75, 47, and 46 incorrect responses. The respective means for male rats were 74, 71, 91, and 55.

However, on duration to complete a trial, the only group of female rats to differ appreciably from the Clean control group was the Repeat group (Figure 10). The Repeat group required 52% more seconds to complete transition trials than did the Clean group. For male rats, the Acute group was most adversely affected; their mean was 74% greater than that of the Clean group. As noted before, female rats responded more slowly: the female Clean group averaged 66% more seconds per trial than did the male Clean group. These differences in patterns are reflected in the statistically significant Group×Sex interaction for duration in the ANOVA. The effects on duration for male Acute rats and female Repeat rats cannot be attributed solely to the influence of exposure to 10 ppm of toluene for 1 h.

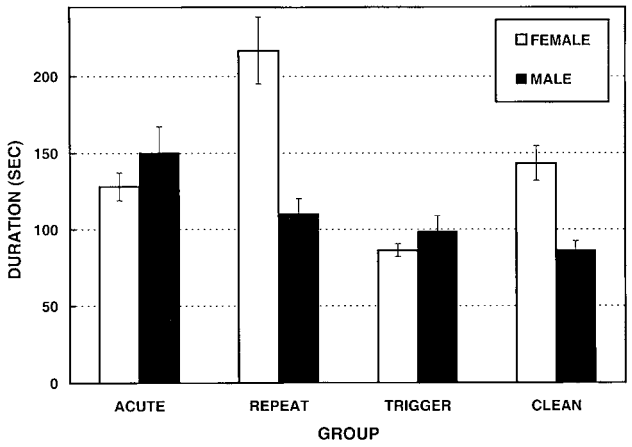

Figure 10. Statistically significant ($P < 0.05$) Group×Sex interaction for duration required to earn a food reward. Data for female and male rats across trials 1, 31 and 61 were combined. The error bars are standard errors for the means. Repeat exposure adversely affected

Because the mean scores for female and male rats in the Clean groups differ, interpretation of statistically significant Group×Sex interactions is complex. For total number of responses, the ratio of the male and female means was 1.15, and the male-to-female ratio was 1.20 for number of incorrect responses. Conversely, for duration to complete a trial, the female-to-male ratio was 1.76. Another way to view the data on Group×Sex interactions is to express group means for each sex as a ratio to the Clean group mean for that sex (Table 3). First, for total responses and incorrect responses, female rats had higher ratios than males for both Acute and Repeat groups. Overall, males averaged 1.31×Clean and the females averaged 1.59×Clean, an increase of 21%. Second, for duration to complete a transition trial, the female Repeat group (1.65×Clean) and the male Acute group (1.74×Clean) were affected most strongly; the male Repeat group was less affected (1.29×Clean), and the female Acute group responded somewhat more rapidly (0.90×Clean). Third, for total responses and incorrect responses, the male Trigger groups were affected (mean=1.63) and the females were not (mean=1.01). Fourth, for duration to complete a transition trial, female rats of the Trigger group respond considerably more rapidly (0.60×Clean) and male rats of the Trigger group respond somewhat more slowly (1.15×Clean).

Summary Four general conclusions describe the effects of *operant task characteristics*: (1) total number of responses and number of incorrect responses showed very similar results for both sexes in all groups; (2) number of correct responses and percent correct did not vary among groups or conditions; (3) trial strongly affected numbers of responses; and (4) duration to complete a trial was not determined simply by number of responses made.

Five general conclusions describe statistically significant *sex differences* in operant behavior on this task: (1) male rats proceeded through the training stages 16% faster than females; (2) male rats made 17% more total responses and 20% more incorrect responses; and (3) even though making fewer responses, female rats required 46% longer to complete the three transition trials. Two conclusions describe the statistically significant Sex×Trial effects: for both total responses and incorrect responses, male rats performed 25% better than female rats on trial 1, 13% worse than female rats on trial 31, and 38% worse than female rats on trial 61. However, because the number of total responses and number of incorrect responses were so strongly related, the ratio of correct responses to total responses, i.e., percent correct, was equivalent for female and male rats.

Three conclusions describe the statistically significant *Group effects*. First, the Acute, Repeat, and Trigger groups all were equivalent; overall, they made 37% more total responses and 41% more incorrect responses than did the Clean group. These differences appear to be related primarily to the 10-ppm toluene Trigger exposure. Second, with respect to duration required to complete a trial, the Repeat group averaged 43% longer than the Clean group; the Acute group averaged 21% longer and the Trigger group averaged 19% less. Thus, the prior history of the initiating exposure affected time to complete the task following trigger exposure. Repeated 80 ppm exposure had greater consequences, following 10 ppm trigger exposures, than did a single 1600 ppm exposure. These effects were not mediated solely by the 10-ppm trigger exposure. Third, in the absence of an initiating exposure, 60-min trigger exposures to 10 ppm of toluene reduced the time required to earn a reward.

Three major conclusions summarize the statistically *Group and Sex interactions*. First, Group×Sex significantly influenced number of responses and number of incorrect responses. The major difference was that trigger exposure to 10 ppm did not affect female rats (1.0×Clean) but greatly affected male rats (1.6×Clean). Second, duration of responding was strongly affected, somewhat more in the female Repeat group (1.5×Clean) than in the male Repeat group (1.3×Clean). Conversely, Acute exposure affected males (1.7×Clean) without affecting females (0.9×Clean).

Table 3. Group means as ratios to control for female and male rats.

Variable	Sex	Acute	Repeat	Trigger
Number	Female	1.56	1.51	1.00
Responses	Male	1.32	1.29	1.60
Incorrect	Female	1.65	1.63	1.02
Response	Male	1.35	1.29	1.65
Trial	Female	0.90	1.52	0.60
Duration	Male	1.74	1.28	1.15

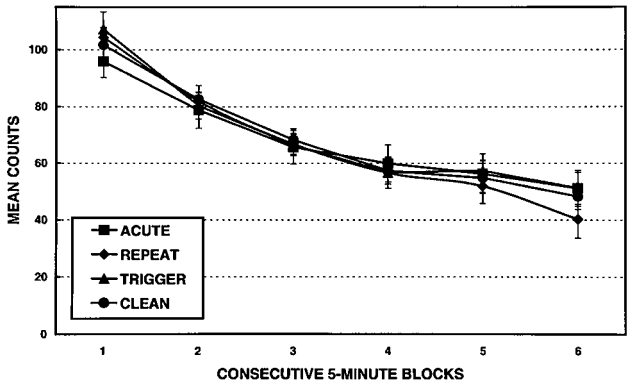

Figure 11. Locomotor activity by group at 5-min intervals for four groups of 16 rats. Means and standard errors are indicated. The downward trend in activity shown by all groups is an expected feature of exploratory behavior.

Locomotor Activity

Locomotor activity was not affected by toluene exposure. Figure 8 maze data, counts of infrared beam breaks during 5-min periods ('blocks'), were analyzed by a three-factor, mixed ANOVA. The within-subjects factor was Block (n=6), and the between-subjects factors were Sex (n=2) and Group (n=4). As expected, Block produced a statistically significant effect (F=127; df=5,280; P<0.001). Animals normally show habituation, with activity declining after initial exploration of a maze (Figure 11). During the first 5-min period, the rats averaged about 100 beam breaks; during the last 5-min period, they averaged about 50 beam breaks. There were no statistically significant differences involving either main effects or interactions of Group or Sex.

Discussion

Effects Observed at Very Low Levels

When tested following 1-h, 10-ppm trigger exposures, the Acute (initiated with 1600 ppm × 6 h × 1 day), Repeat (initiated with 80 ppm×6 h×20 days) and Trigger (sham-exposed during initiation) groups all showed equivalent increases in numbers of responses, as compared to the Clean control group. This suggests that these observed changes were caused by the 1-h 10-ppm toluene exposures. By itself, 10 ppm exposure shortened the time required to complete a trial, suggesting a mild stimulatory effect. However, as compared to the Clean group, duration required to complete a trial by the Repeat group was 43% longer; duration was 21% longer for the Acute group. These changes represent persistent effects of the initiating exposures. Furthermore, the two different patterns of initiating exposure, both with a total dose of 9600 ppm×h, had different consequences: the resultant effect is not a linear product of initiating exposure Concentration×Time. Contrary to what might have been expected based on a conventional threshold concept, a series of 80 ppm exposures had more effect on several measures than did a single 1600 ppm exposure.

The results of this experiment, which showed changes in operant behavior following repeated exposure to 10 and to 80 ppm toluene, differ from the conventional toxicology literature on toluene. Based on 23 estimates, from studies of toluene using a diverse set of exposures parameters and measuring a wide variety of endpoints (USDHHS, 1994a), the mean no-observed-effect level for toluene is 1350 ppm (SD=2400; minimum=200; maximum=12,000). From 43 estimates, the mean lowest-observed-adverse-effect level for toluene is 2967 ppm (SD=5272; minimum=80; maximum=30,000), respectively. The American Conference of Governmental Industrial Hygienists, the National Institute of Occupational Safety and Health, and the Occupational Safety and Health Administration all have occupational (8 h/day, 5 days/week) exposure guidelines or limits for toluene of 100 ppm (USDHHS, 1994a).

Studies of the effects of toluene on operant performance often have involved relatively high toluene concentrations. For example, Rees et al. (1989) used 4500 ppm, Miyake et al. (1983) used 7000 ppm, and Wada et al. (1989) used 8000 ppm in experiments with rats. In all cases, subtle operant effects were reported. Taylor and Evans (1985) reported that 4500 ppm affected match-to-sample performance of macaques. Such exposures are not highly aversive, and they even can be rewarding: Weiss et al. (1979) reported that squirrel monkeys would perform an operant task to self-administer toluene at 10,000 ppm. Inhalation of solvents, including toluene, is a common form of substance abuse among humans.

Von Euler et al. (1993, 1994) reported that 17 days after toluene exposure at 80 ppm (6 h/day, 5 days/week, for 4 weeks), locomotor activity of apomorphine-treated rats was increased and spatial learning of rats in a water maze was decreased. (Without apomorphine, locomotor activity of toluene-exposed rats was not changed.) These findings are the lowest concentration of toluene producing a behavioral effect of which we are aware. We also observed effects associated with repeated 80 ppm toluene exposure in our study, adding to their observations. However, our observation of behavioral effects elicited by repeated exposure to 10 ppm toluene is a novel finding. It might be inferred that as more cognitively demanding behavioral tasks are used, effects will be found at lower exposure levels. The observation of effects at 10 ppm in this study, in contrast to the higher thresholds reported in previous studies, might reflect differences in the tasks used. The operant task used here was difficult, and repeated learning was required within each test session. Toluene-related effects were most apparent for the third problem of the day (trial 61), which was the most difficult for the subjects.

These considerations also could have relevance for human testing. Simple neurobehavioral tasks might not

reveal subtle, but important and highly relevant, cognitive deficits detectable with more demanding and sophisticated tasks. For example, Bunegin et al. (1998) studied two groups of Gulf War veterans, those reporting illness and those not reporting illness. When briefly exposed to acetone at 40 ppm, a concentration well below the occupational exposure limit of 750 ppm (USDHHS, 1994b), the 'ill' subjects believed that they had performed more poorly on the cognitive assessment battery. The accuracy of their performance on the series of computerized cognitive tasks did not differ after sham and acetone exposures, but they performed more slowly after acetone exposure. Perhaps with more demanding tasks, accuracy deficits might have become manifest. Bunegin et al. (1998) also measured alterations in middle cerebral artery blood flow. The ill group had higher baseline flows, and flow did not increase with cognitive challenge, as it did in the healthy controls, suggesting functional consequences of low-level acetone exposure might occur. Small-magnitude changes in cognitive function, which might be difficult to measure, could be quite noticeable to a human subject and might have serious consequences. For example, Gulf War veterans who are ill report cognitive difficulties, such as confusion or inability to concentrate while driving (Miller and Prihoda, 1999). Kang and Bullman (1996) found a small but significantly increased risk of accidental deaths among Gulf War veterans, principally from motor vehicle accidents.

Differences between Females and Males

Female and male rats exhibited different behavioral styles while performing this operant task. The male rats progressed through the stages more rapidly. In stage 9, male rats made more responses than did females rats. Female rats made fewer responses and worked more slowly than did male rats. Both groups showed equivalent percent accuracy. As compared to female rats, male rats made somewhat fewer responses on trial 1 and many more responses on trial 61. Characterization of 'better' performance depends upon the choice of dependent variable.

The sexes also differed in their responses to toluene. With duration of responding as the dependent variable, prior repeated 80 ppm initiating exposures followed by 10 ppm triggering exposures affected female rats somewhat more than male rats. Conversely, a single 1600 ppm exposure affected male rats without affecting female rats. With number of responses as the dependent variable, 10 ppm exposures alone did not affect female rats but strongly affected male rats.

The observation that female and male rats responded differently to toluene exposure suggests that physiological differences could underlie both the increased frequency and severity of chemical intolerances, and the reduced frequency of addiction, reported by women as compared to men (Miller, 1999). Abdiction (avoiding a substance) and addiction (seeking a substance) might well be complementary aspects of a single basic process. Of those reporting chemical sensitivities, 80% are women. Conversely, addiction occurs twice as frequently in men (Miller, 1999). These interesting findings, obtained in an animal model in which the psychological and social factors so important in human behavior are not operative, offer the possibility of important, new insights into sex-related differences with respect to TILT. It is possible that VOCs interact differentially with the subtly different, neurologically mediated factors affecting cognitive performance. Although pre-existing differences between female and male rats are not a logically necessary pre-condition for differences in responses to toluene exposures, the existence of sex-based differences prior to exposure does make it easier to envision differences in responses to toluene exposure.

Neurobehavioral Similarities between Humans and Rodents

In human performance studies, such as experiments conducted with NASA astronauts, investigators report that accuracy is rarely reduced, but time to complete a task often is increased (e.g., Eddy et al., 1998). Human subjects report that they can complete cognitive tasks while fatigued, but a greater than normal effort is required, and humans with chemical intolerances often report that thinking requires increased effort (Miller and Mitzel, 1995). In our experiment with rats, percent correct was not affected, but total numbers of responses and time to complete a task were increased, without being directly linked. As with humans, the VOC-exposed animals experienced a reduction in throughput, the number of tasks completed per time unit.

Initiating or Trigger Exposures

These data suggest that there are residual changes, following either a single 6-h toluene exposure at 1600 ppm in male rats or twenty 6-h 80 ppm toluene exposures in female rats, that are not manifest without a 10-ppm trigger exposure. However, to fully assess this possibility, a future experiment should include groups receiving initiating exposures without subsequent trigger exposures. Additionally the effects described here were assessed at stages 1 and 9. It appears as though effects became apparent as the requirements of the operant task were made more difficult, both across stages and over transition trials within a stage. However, there is at least one alternative explanation. Perhaps, the group differences at stage 9 resulted from a progressive effect of the multiple 10 ppm trigger exposures experienced over a series of days. (Most male rats were in stage 9 on triggering day 10, and most female rats were in stage 9 on triggering day 11.) This possibility could be tested in subsequent experiments by using other combinations of training days and triggering days. For example, rats with initiating exposures could be trained to stage 9 before

beginning trigger exposures. These are but two examples of the research required to clarify the possible independent and inter-dependent effects of past and current toluene exposures on cognitive performance.

Other Considerations

Whenever a treatment produces changes in operant behavior, it becomes important to understand the mechanisms involved (e.g., Weiss and O'Donoghue, 1994). Changes in operant performance can reflect changes in cognitive, motivational, motor or sensory functions. Ideally, all of these possibilities would be investigated in subsequent experiments. The absence of locomotor effects in this experiment is a small but reassuring step in that direction. Additionally, the differences in effects of toluene inhalation on female and male rats could reflect pharmacokinetic differences between females and males. The male rats weighed 61% more than the female rats, and differences in body composition could have affected the uptake, distribution, and clearance of toluene.

An operant experiment with four groups of 16 subjects providing data with relatively small variability should provide robust and meaningful results. However, before unwarranted emphasis is placed on the pattern of results observed here, replication and extension of these results are required. Further experiments are needed to confirm that effects of the type reported here do occur and to examine the underlying mechanisms which produce them.

Summary

Our dual observations that (1) repeated initiating exposure to 80 ppm of toluene over a 1-month period can produce measurable neurobehavioral consequences following triggering exposures to 10 ppm of toluene, and that (2) repeated 1-h 10-ppm toluene exposures alone can produce measurable neurobehavioral consequences provide a new view of the possible effects of VOC exposures. Most conclusions about the presence or absence of toxic effects of VOCs and other toxicants are based on high-dose exposures, and the temporal patterns relevant to the initiating and triggering concepts of TILT are not tested. Furthermore, the observation of neurobehavioral changes from repeated, low-level exposures of rats to toluene might have important implications for human performance. Our findings suggest some sort of persistent change occurs, presumably in the central nervous system, so that following an initiating exposure, measurable reductions in cognitive performance can be produced by subsequent very low-level triggering exposures. Understanding the effects of chemical exposures on higher-order cognitive processes might require (1) the use of more complex and demanding cognitive tasks, (2) measurement of time to complete a task as well as accuracy, and (3) employment of repeated and intermittent exposure designs allowing time for the development of TILT.

Acknowledgments

This experiment was completed in accordance with the requirements and recommendations in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996, National Academy Press, Washington, DC) and the Animal Welfare Act (1970). Other applicable federal, state and local laws, regulations, and policies also were followed. This animal experiment was completed in the Inhalation Neurotoxicology Laboratory of Southwest Research Institute (SwRI), where Dr. Rogers was employed at the time. The experiment was funded by a grant from the Office of Naval Research (N0014-96-1-0500) and supported by SwRI Internal Research project (12-9732). The authors thank SwRI employees Gus Allo, Robert Garay, and Mary MacCallum for their dedicated performance during this experiment. The authors also thank Eric Rogers for setting up and testing equipment, summarizing data, and preparing figures.

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