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Altered Operant Responding for Motor Reinforcement and the Determination of Benchmark Doses Following Perinatal Exposure to Low-Level 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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Pregnant Holtzman rats were exposed to a single oral dose of 0, 20, 60, or 180 ng/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the 18th day of gestation. Their adult female offspring were trained to respond on a lever for brief opportunities to run in specially designed running wheels. Once they had begun responding on a fixed-ratio 1 (FR1) schedule of reinforcement, the fixed-ratio requirement for lever pressing was increased at five-session intervals to values of FR2, FR5, FR10, FR20, and FR30. We examined vaginal cytology after each behavior session to track estrous cyclicity. Under each of the FR values, perinatal TCDD exposure produced a significant dose-related reduction in the number of earned opportunities to run, the lever response rate, and the total number of revolutions in the wheel. Estrous cyclicity was not affected. Because of the consistent dose–response relationship at all FR values, we used the behavioral data to calculate benchmark doses based on displacements from modeled zero-dose performance of 1% (ED_{01}) and 10% (ED_{10}), as determined by a quadratic fit to the dose–response function. The mean ED_{10} benchmark dose for earned run opportunities was 10.13 ng/kg with a 95% lower bound of 5.77 ng/kg. The corresponding ED_{01} was 0.98 ng/kg with a 95% lower bound of 0.83 ng/kg. The mean ED_{10} for total wheel revolutions was calculated as 7.32 ng/kg with a 95% lower bound of 5.41 ng/kg. The corresponding ED_{01} was 0.71 ng/kg with a 95% lower bound of 0.60. These values should be viewed from the perspective of current human body burdens, whose average value, based on TCDD toxic equivalents, has been calculated as 13 ng/kg. *Key words*: benchmark dose, estrous cycle, operant behavior, prenatal exposure, TCDD, wheel running.
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Polychlorinated dioxins (PCDDs) are ubiquitous and persistent environmental contaminants and powerful developmental and reproductive toxicants. Their detrimental effects have evoked intense public health concerns because they accumulate in the food chain and are retained in body tissues for extended periods. The half-life in humans of their most potent congener, 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) is in the range of 7–10 years. PCDDs are believed to exert their effects through a ligand-activated transcription factor, the aryl hydrocarbon receptor (AhR), which is expressed in most organs and cells in the body. Because ligands for AhR also include polychlorinated dibenzofurans (PCDFs) and "dioxin-like" or coplanar polychlorinated biphenyls (PCBs) and because they typically occur in the environment as mixtures, exposure and body burden estimates are based on summing the relative potencies (toxicity equivalence factors; TEFs) and proportions of these constituents to provide a pragmatic index of toxic potential (total toxicity equivalence; TEQ). In 1995, the average background TEQ body burden was estimated at 13 ng/kg (*1*).

Despite nearly three decades of intensive research, our knowledge of the total health and ecologic risks posed by TCDD and related agents remains ambiguous and incomplete. Adding to the uncertainty, most of the experimental literature is based on exposure regimens using high doses or on *in vitro* studies not directly applicable to risk estimation. Moreover, the relationship between the AhR and its endogenous role in development is unclear (*2,3*). This general lack of understanding has impaired researchers' ability to define the critical period of exposure to TCDD and to describe how such exposures will be expressed functionally.

Functional effects are most pronounced when exposure occurs *in utero*. Such experiments show the developing male rat reproductive system to be sensitive to relatively low doses of TCDD. Perinatal exposure to TCDD has been reported to lower male rat gonadal hormone levels (*4,5*), although other data contradict this finding (*6*) or suggest that the biologic relevance of these reductions is equivocal (*7*). Perinatal exposure clearly interferes with the development of reproductive organs (*4,8,9*), and lowers sperm production (*4,9,10*) and the amount of ejaculated sperm (*6*). In female rats, perinatal TCDD affects the development of the external genitalia and delays puberty (*8,11,12*) in the absence of obvious endocrine changes.

Except for the evidence that *in utero* and lactational exposure to TCDD partially demasculinizes and feminizes sexual behavior in adult male rats (*5*), only a handful of studies (*13–15*) have pursued the neurobehavioral consequences of developmental exposure. These studies show a pattern of both task-specific facilitation and impairment possibly stemming from alterations in dopamine pathways (*16*). The paucity of information about the neurobehavioral toxicity of TCDD led us to undertake the experiment reported here. Behavioral measures provide a diversity of end points for assessing the functional consequences of developmental neurotoxicants. During critical periods of brain development, even minor perturbations in this complex chain of processes can permanently alter behavior. Some of these behavioral changes are often gender specific and may not become apparent until after puberty. A thorough, detailed analysis of how compounds such as TCDD alter behavior, in addition to its intrinsic value as an index of risk, can also yield clues to underlying biologic mechanisms.

Because of the profound effects of TCDD on sexual behavior and reproductive function, we chose to examine behaviors marked by sex differences. One compelling example of gender-specific behavior is the daily amount of gross locomotor activity displayed by the female rat. In running wheels, a staple of experimental psychology for many decades, rodents run spontaneously. The rate of running by adult female rats follows a 4–5 day period, corresponding to the stage of their estrous cycle (proestrus, estrus, $diestrus_1$, $diestrus_2$). In a typical 4-day cycle, serum levels of estradiol, progesterone, prolactin, testosterone, and androstenedione peak during the later half of the day of proestrus (*17,18*). The hormonal and vaginal

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cytologic characteristics of the proestrous phase are associated with behavioral changes such as increased sexual proceptivity and receptivity and increased wheel running. These changes are often referred to as behavioral estrus or the estrous activity cycle. In rats maintained on a 12 hr light:12 hr dark cycle, wheel activity begins to increase during the night of diestrus and peaks during the night of proestrus (*19–24*). The estrous activity cycle does not appear until puberty and disappears during pregnancy, lactation, and after menopause (*23*). Before puberty, there are no differences in wheel-running output between male and female rats (*25*).

Running is such a prepotent activity that a brief opportunity to run can reinforce operant responses such as lever pressing (*26,27*). In the female rat, wheel running might be an intriguing primary reinforcer that, like sexual behavior, is linked to the kind of motor activation associated with behavioral estrus. Favoring such a proposition is the finding that wheel running is sensitive both to acutely administered estrogenic substances and to exposure during development to substances that disturb the sex-specific organization of the brain. For instance, adult female rats exposed chronically to the estrogenic pesticide methoxychlor displayed elevated levels of acyclic wheel-running activity, persistent vaginal estrus, and increased sexual proceptivity and receptivity (*28*). These outcomes were not affected by ovariectomy but were dramatically reduced by exogenous progesterone administration, further demonstrating the estrogenic activity of methoxychlor. Exogenous progesterone is known to inhibit estrogen-mediated wheel running in intact rats or ovariectomized rats receiving estradiol replacement (*29,30*).

In contrast to adult exposure, perinatal exposure to neurotoxicants, even those lacking specific neuroendocrine activity, may reduce wheel-running activity later in the life cycle. For instance, prenatal exposure to ethanol reduced wheel-running behavior in 6-month-old but not in 2-month-old female rat offspring. The vaginal cytology of these animals confirmed that significantly more exposed females were acyclic by 6 months of age, an outcome that may be related to defeminization of leutinizing hormone release (*31*). Developmental lead exposure reduced wheel-running activity in both female and male rat offspring and altered their response to an auditory stressor (*32*). Perinatal exposure to compounds such as the estrogenic pesticide Kepone (chlordecone) has also been shown to disrupt estrous cyclicity following a latent period (*33,34*). Finally, we have shown that wheel-running behavior is increasingly sensitive to prenatal cocaine exposure as rat subjects age (*35*).

Curiously, exposure to TCDD on day 15 of gestation (GD15) did not affect a gross measure of estrous-mediated wheel running, the total number of wheel revolutions per day. Measures of vaginal cytology through 16 months of age confirmed that estrous cyclicity was not altered by TCDD exposure in these animals (*8*), although the same dose did interfere with development of the external genitalia, a finding confirmed later (*11,12*).

Our study was based on whether a detailed behavioral analysis of wheel running would reveal changes of another kind, that is, motivation or disposition to run following perinatal TCDD exposure. The wheels used in the current procedure were designed to detect neurotoxicant-induced motor or motivational effects (*27,35*). Subjects earned brief opportunities to run by pressing a lever on a fixed-ratio schedule of reinforcement. Free-running procedures typically collect a single count of total wheel revolutions during a 24-hr period and provide only an indirect measure of motivation to run, which can be defined as the reinforcing value of access to a wheel. The operant procedure we used is an explicit index of motivation that relies on measuring the reinforcing potency of wheel running. It also records rate, frequency, and interresponse time data for both lever pressing and wheel running in daily 45-min sessions. We hypothesized that this potency would vary over the course of the estrous cycle and interact with prenatal TCDD exposure.

Pregnant female rats were administered a single maternal dose of TCDD on GD18, a time designed to coincide with development of the proximal neural mechanisms that mediate many goal-directed motor behaviors. During the acquisition of new responses, the alerting or attention-gaining properties of these activities are mediated in part by midbrain dopamine systems (*36–38*). In the developing rat brain, substantial increases in catecholamine levels, enzyme activity, and synaptogenesis have been noted on GD18 (*39*); by GD19, differentiated neurons of the ventral tegmental area and nucleus accumbens have increased at the expense of the neuroepithelium (*40–42*). A GD18 exposure onset would also increase lactational transfer of TCDD to offspring, presumably affecting both synaptogenesis and myelinization processes. Lactational exposure alone is sufficient to feminize the sexual behavior of adult male rat offspring (*4*).

Materials and Methods

Breeding and exposure. Male and female Holtzman rats (Harlan Sprague-Dawley, Inc.) were housed in University of Rochester Medical Center Vivarium quarters in a barrier facility containing temperature-controlled rooms with independent, filtered air supplies. Rats were maintained on a 12 hr light:12 hr dark cycle and were allowed to acclimate to the vivarium quarters for 2 weeks before breeding. Females were then placed with males in hanging wire cages and vaginal smears were examined daily. A sperm-positive smear determined gestational day (GD) 0. After detection, dams were placed individually in polycarbonate breeder cages and were assigned to an exposure condition according to a randomized block design. Each block consisted of four assignments: 0, 20, 60, or 180 ng/kg TCDD in olive oil, administered via gavage on GD18. Dams were weighed every 4 days until GD16. They were weighed every other day thereafter until parturition.

All animal care and welfare procedures complied with NIH guidelines. The vivarium is certified by the Association for Assessment and Accreditation of Laboratory Animal Care. Health surveillance of the animals was conducted under the direction of the Laboratory Animal Services Shared Facility of the Environmental Health Sciences Center.

Litters. The first day a new litter was discovered was designated as postnatal day 1 (PND1). We recorded litter sizes, pup weights, and sex distributions on PNDs 1, 4, 8, 12, 16, and 20. Using a randomized procedure, litters were culled to eight offspring on PND4, maintaining equivalent sex distributions when possible. After weaning on PND21, offspring were housed in pairs of same-sex littermates until PND60. After PND60, offspring were housed individually and their body weights, targeted at 220 g, were maintained with a daily feeding schedule.

The breeding and exposure procedure yielded a total of 24 litters. One female rat from each of the seven control, four 20 ng/kg TCDD, six 60 ng/kg TCDD, and seven 180 ng/kg TCDD litters were assigned to the procedure. Remaining offspring were assigned to other behavior procedures not reported here.

Apparatus. The running wheels (Figure 1) were designed to provide a wheel of great enough diameter (60 cm) to permit running on a virtually flat surface (*35,43*). The tracking surface, rather than conventional wire mesh, is constructed of parallel rods spaced at 15-degree intervals. To rotate the wheel, the rat must thrust against one of the rods with a hind limb, while positioning the forelegs on other rods to supplement or support the more powerful hind leg thrust. The rat maintains rotation by coordinating a sequence of similar movements. An electric clutch brake mounted on the axle of the apparatus regulates free rotation of the wheel. An operant response lever and cuelight are located inside the wheel, near the running position. A magnetic reed switch is used to tabulate wheel revolutions and allows a calculation of the revolution rate and distribution in time.

A PDP-11 computer (Digital Equipment Corporation, Maynard, MA) running the SKED-11 state language (State Systems, Kalamazoo, MI) controlled the behavioral procedure and programming system (*44*), which preserves behavioral events in real time with a resolution of 10 msec.

Procedure. Beginning on or near PND77, naïve females were randomly assigned to a wheel apparatus. Each subject then participated in several 12-hr training sessions in the wheels. At the beginning of each session the brake was applied, locking the wheel. At randomized intervals the brake was disengaged and the cuelight illuminated for 30 sec. Over successive training sessions the randomized intervals separating the 30-sec periods of free running were increased. If at any time the subject pressed the response lever, the brake was disengaged and the cuelight illuminated. Subjects typically acquire the lever-press response in 5 sessions or less with this training procedure (*35*).

After subjects acquired the lever press response, they performed under a continuous reinforcement (FR1) schedule of access to wheel running, which unlocked the brake for one 30-sec period for each lever response. Once they achieved a criterion of ≥ 20 lever-presses followed by running (12–15 sessions), the fixed-ratio (FR) requirement was then increased at five-session intervals to FR2, 5, 10, 20, and 30. During these sessions, completion of the FR requirement illuminated the cuelight and released the wheel brake for 20 sec, allowing the subject to run. Each session lasted 45 min or until the subject completed 50 FR series. Sessions were run 5 days a week, Monday–Friday, during the light phase of the subjects' light:dark cycle.

The following variables were used to track individual performance and to compare the exposure groups: total revolutions per session, revolutions per run opportunity, revolutions per minute, and latency to begin running. The following measures provided indices of lever-pressing behavior: the total earned run opportunities per session, lever response rate, and postreinforcement pause (the interval between the end of the 20-sec access period and the resumption of lever responding).

After each session, subjects underwent a vaginal lavage consisting of approximately 250 µL sterile saline applied with the tip of an eyedropper to the vaginal canal. Lavage fluid was placed on a labeled slide, air dried, and later stained with Wright's stain. We scored slides for estrous cycle stage according to the following cytologic characteristics: proestrus, sheets or groups of adherent, small, purple nucleated epithelial cells; estrus, sheets of large, angular, poorly staining, anucleated cornified squamous epithelial cells; and diestrus$_{1,2}$, very few cells, some small epithelial cells, occasional neutrophil and mucus material in the lavage fluid.

Statistical methods. We analyzed data for maternal body weight after TCDD exposure, length of gestation period, number of pups per litter, and sex distribution within litters according to exposure group by one-way analyses of variance (ANOVA). We analyzed pup body weight data during the lactational period by two-way ANOVA, with exposure and gestational day as factors.

We analyzed behavior variables by repeated measures ANOVA. Prenatal exposure was a between-subjects factor, and the six FR values and 18 sessions were within-subjects factors. The 18 sessions selected for analysis included the final 3 FR1 sessions and the first 3 sessions under the other FR values.

We used the Huynh-Feldt adjustment to the degrees of freedom when appropriate (*45*). In addition, each analysis included an examination of residuals as a check on the required assumptions of normally distributed errors with constant variance. For some analyses, we used a square root transformation to stablize the variance because homogeneity of variance is a required assumption of the analysis of variance (*46*). Following appropriate grouping, significant interactions involving the prenatal exposure factor were probed with one-way ANOVAs and Newman-Keuls multiple range tests.

In cases in which behavioral variables were associated with a significant main effect of exposure or an interaction involving the exposure variable ($p \leq 0.05$), the data were examined further with Benchmark Dose Modeling Software (BDMS), version 1.2, provided by the U.S. Environmental Protection Agency (U.S. EPA). For risk assessment, the benchmark approach is a useful alternative to the more traditional no-observed-adverse-effect level (NOAEL). Benchmark calculations consider the entire dose–response relationship and do not involve extrapolations far below experimental observations. The benchmark doses we calculated represent doses that are associated with specific operant behavior performance. Pilot work with this software indicated that the BMDS Continuous Model with second-order polynomial provides an excellent fit to the dose–response data from our wheel-running procedure. With the Continuous Model, we calculated benchmark doses representing the model-estimated control mean minus proportional deviations equivalent to a 10% (ED_{10}) or 1% (ED_{01}) change. The BMDS software also provides a 95% lower bound that can be divided by a standard uncertainty factor, such as 100, to calculate a reference dose or provide a margin of exposure.

Results

Maternal and postpartum data. The body weights of dams assigned to the four exposure groups did not differ from each other on the day of TCDD administration (GD18: $F_{3,19} = 0.59$, $p = 0.63$) or thereafter (GD20: $F_{3,18} = 0.29$, $p = 0.83$). Maternal TCDD administration did not affect the length of the gestation period, the number of pups per litter, or the sex distribution within litters (Table 1).

The body weights of male and female pups across the lactational period (Table 2)

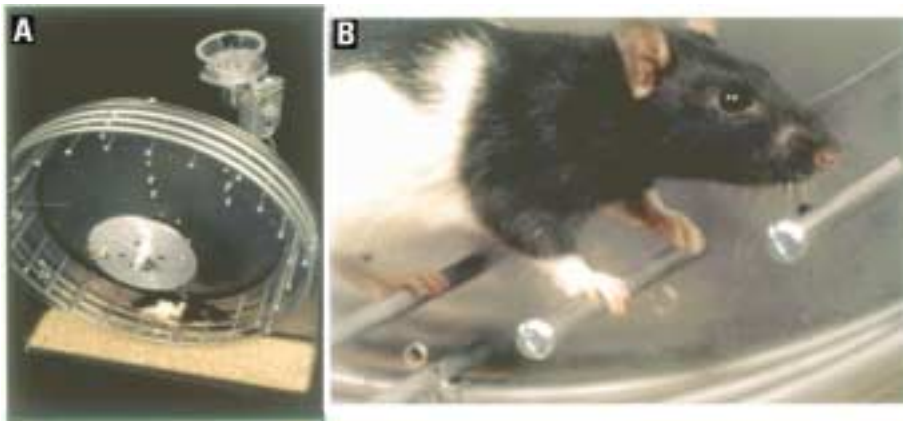

Figure 1. Design of the running wheel used in this study. (*A*) Details of the wheel, showing the lever mounted on the wall of the chamber. (*B*) Typical position of a rat while performing in the wheel.

Table 1. Parturition data showing number of litters, gestation length in days ± SD, and sex distribution ± SD.

Dose	No.	Gestation (days)	Females per litter	Males per litter
Control	7	22.14 ± 0.14	7.3 ± 0.52	6.6 ± 1.0
20 ng/kg	4	22.00 ± 0.00	6.0 ± 0.82	6.5 ± 0.65
60 ng/kg	6	21.67 ± 0.21	7.3 ± 0.80	6.7 ± 0.61
180 ng/kg	7	22.20 ± 0.20	7.0 ± 0.54	5.3 ± 0.57

were examined separately. For both sexes there were significant effects of PND (male, $F_{5,120} = 1378.76$, $p < 0.001$; female, $F_{5,120} = 1562.66$, $p < 0.001$) and exposure (male, $F_{3,120} = 10.88$, $p < 0.001$; female, $F_{3,120} = 11.05$, $p < 0.001$). The PND-by-exposure interactions were not significant for either sex. For each litter, the body weight data were collapsed across PND and probe tests compared the means according to exposure group. Body weight differences showed a curvilinear dose–response trend. Male pups from the 60 ng/kg group weighed significantly more than those in the control group, whereas the female pups in the 60 ng/kg group weighed significantly more than those in the control and 180 ng/kg female groups.

Fixed-ratio wheel running. The ANOVA yielded a significant main effect of TCDD exposure on two of the lever-pressing variables following square root transformations of the raw data: earned run opportunities per session ($F_{3,20} = 4.56$, $p = 0.01$; Figure 2A) and the lever response rate ($F_{3,20} = 4.16$, $p = 0.02$). There was also a significant main effect of exposure following square root transformation of total wheel revolutions per session ($F_{3,20} = 3.43$, $p = 0.04$; Figure 2B). Untransformed data are shown in Table 3. For each of these variables, the data were averaged across the 18 sessions and the exposure groups compared via one-way ANOVA and Newman-Keuls multiple range tests. The probe tests indicated that the 180 ng/kg TCDD group earned significantly fewer run opportunities, pressed the lever at a slower rate, and completed fewer wheel revolutions than the control group.

Significant main effects of the FR value emerged for each variable (earned opportunities, $F_{5,100} = 118.53$, $p = 0.0001$; lever response rate, $F_{4,80} = 10.25$, $p = 0.0001$; total revolutions, $F_{5,100} = 65.31$, $p = 0.0001$) and significant FR-by-session interactions (earned opportunities, $F_{10,200} = 7.62$, $p = 0.0001$; lever response rate, $F_{8,160} = 8.01$, $p = 0.0001$; total revolutions, $F_{10,200} = 8.36$, $p = 0.0001$). The lever response rate variable also yielded a significant main effect of the session factor ($F_{2,40} = 5.22$, $p = 0.01$). There were no other interactions involving the exposure factor. There were no effects on the postreinforcement pause, the revolution rate, or the mean wheel revolutions per opportunity.

Benchmark dose modeling. Benchmark dose calculations were performed on the total wheel revolution and the earned run opportunities data from the five transition sessions, i.e. the first sessions following an increase in the FR value (Table 4). The data upon which these calculations were based are given in Table 3. For earned opportunities (Figure 3), the mean ED_{10} for earned opportunities across all FRs was 10.13 ng/kg, with a 95% lower bound of 5.77 ng/kg. The corresponding ED_{01} was 0.98 ng/kg with a 95% lower bound of 0.84 ng/kg. The mean ED_{10} for total wheel revolutions (Figure 4) was calculated as 7.32 ng/kg with a 95% lower bound of 5.41 ng/kg. The corresponding ED_{01} was 0.71 ng/kg with a 95% lower bound of 0.60 ng/kg. It was not necessary to extrapolate to doses below the fitted dose–response functions. Although, for risk assessment, the U.S. EPA applies uncertainty factors only to the lower bound, Figures 3 and 4 show that both upper and lower bound values would lie relatively close to the benchmark dose.

Estrous cyclicity. To determine if altered estrous cyclicity was related to the behavioral differences observed between the exposure groups, we matched vaginal cytology measures for the control and 180 ng/kg TCDD groups to the daily behavioral data. We grouped the earned run opportunity and wheel revolution data according to FR value and estrous cycle phase (diestrus, proestrus, estrus) and calculated the means. We did not attempt a statistical analysis of these means because the numbers of subjects comprising the various FR value-by-estrous phase groups were unequal. Individual females were free cycling in this procedure and could be in any phase of their cycle during a particular behavioral session. However, visual inspection of the data (Figure 5) suggests that estrous cycle phase did not affect FR responding for wheel-running reinforcement either in control or exposed females. Females in the 180 ng/kg group consistently earned fewer run opportunities than controls, regardless of their phase of the estrous cycle.

We could not directly determine if control and 180 ng/kg females were following typical 4–5-day cycles because vaginal lavage data were collected only during the 5-day test week. However, for each subject we calculated a cyclicity estimate based on the assumption that if a subject was following a 4-day cycle over the course of the entire procedure, then approximately 50, 25, and 25% of the lavage samples should represent days of diestrus, proestrus, and estrus, respectively. A summary of these estimates is presented in Table 5.

Table 2. Mean ± SEM pup body weights (g) across the lactational period.

Group	PND1	PND4	PND8	PND12	PND16	PND20
Males						
Control	8.10 ± 0.15	12.28 ± 0.42	21.29 ± 0.41	32.07 ± 0.82	43.61 ± 0.78	59.18 ± 0.98
20 ng/kg	8.51 ± 0.26	13.81 ± 0.94	24.44 ± 0.77	35.26 ± 1.78	46.42 ± 1.82	62.00 ± 2.25
60 ng/kg	8.81 ± 0.34	13.25 ± 1.78	25.51 ± 1.16	36.04 ± 1.23	47.08 ± 0.94	63.76 ± 1.38
180 ng/kg	8.47 ± 0.31	12.72 ± 0.46	22.86 ± 1.01	33.62 ± 1.40	43.44 ± 1.40	59.32 ± 1.72
Females						
Control	7.48 ± 0.29	11.72 ± 0.54	21.49 ± 0.66	31.53 ± 0.73	42.35 ± 0.74	57.60 ± 1.02
20 ng/kg	7.97 ± 0.26	13.23 ± 0.73	23.34 ± 1.08	34.45 ± 1.12	44.09 ± 1.41	60.68 ± 2.33
60 ng/kg	8.38 ± 0.30	12.72 ± 0.43	24.43 ± 1.26	35.03 ± 1.26	45.78 ± 0.78	62.03 ± 1.11
180 ng/kg	8.07 ± 1.18	12.46 ± 0.43	21.71 ± 0.90	32.38 ± 1.52	42.02 ± 1.16	57.52 ± 1.18

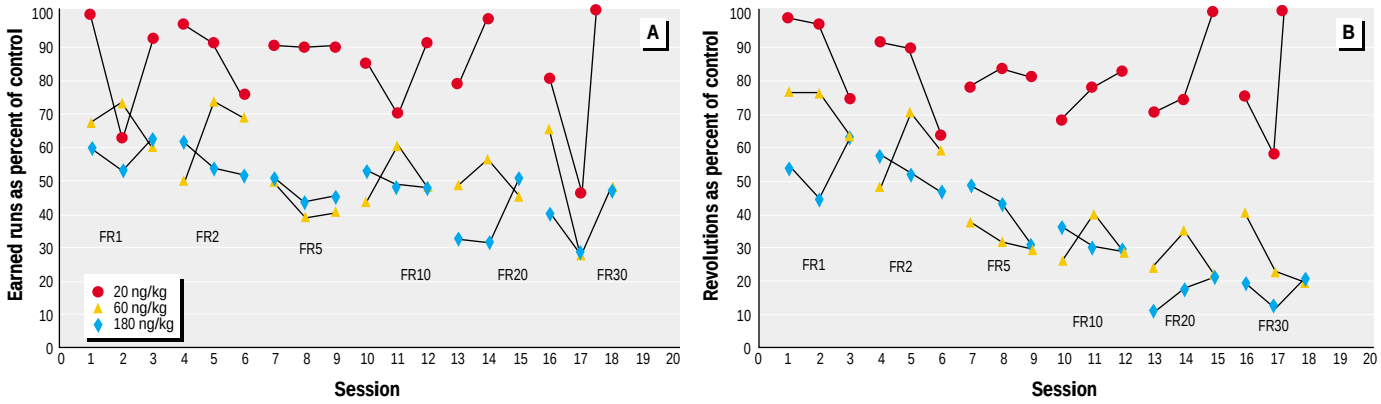

Figure 2. Mean earned opportunities to run per session (*A*) and mean wheel revolutions per session (*B*) expressed as percent of control group performance for the four prenatal exposure groups.

Discussion

Low doses of TCDD administered to pregnant Holtzman rats on GD18 led to a significant reduction of FR responding for access to running wheels in their adult female rat offspring. The reduction of responding was observed across the entire range of programmed FR values and showed a clear relationship with dose. These results suggest reduced responsiveness to environmental contingencies, an effect with extensive implications for many kinds of behaviors, rather than a simple developmental motor deficit. Exposed females were capable of rotating the 60-cm wheel, and, when they did complete a FR, their mean revolutions per 20-sec reinforcement period were no different from those observed for controls. Also, this reduction of operant responding does not reflect an estrous-mediated behavioral change. Females from all of the exposure groups followed similar patterns of vaginal estrous cyclicity. Because young, sexually mature females spend approximately 1 day (or 25% of a 4-day cycle) in proestrus, there is some evidence that control and exposed females were spending an increased proportion of time in the proestrous phase. Our estimates (Table 5) indicate that females were spending 48% of their cycle in the proestrous phase over the course of the behavioral procedure. Because training for the wheel-running procedure began when female rats were 77 days old and concluded when they were 182 days old, our estrous cycle data suggest that females were progressing through typical reproductive life spans with cycles that were lengthening and becoming irregular. As virgin female rats age, they demonstrate a progressive reduction of regular estrous cycling, with many reaching a state of constant vaginal estrus by 10 months of age (*17*). These findings extend work (*8*) showing that maternal administration of higher doses of TCDD on GD15 failed to exert organizational or activational effects on the rat estrous cycle.

Do these behavioral changes represent significant developmental toxicity? We believe that they do and that they signify an important developmental outcome. We view these findings as indicative of persistent motivational deficts following perinatal TCDD exposure. Reduced motivation to respond for incentives may, in fact, be a general phenomenon that extends beyond wheel running. In male rats, the most sensitive behavioral change after prenatal TCDD exposure appears to be the long latencies these animals display before they begin to copulate with sexually receptive females (*5*). The latency to perform the first vaginal intromission has traditionally been considered the most important measure of sexual motivation in the rat (*47*). A single dose of 64 ng/kg TCDD on GD15 significantly increased intromission latency (*5*). This permanent reduction of male sexual motivation, learning deficits in monkeys (*48*), and the disruption of responding for wheel running observed in this procedure are the most sensitive behavioral changes observed to date in the TCDD animal literature.

The range of benchmark doses derived from the current dose–response data approaches or falls below the estimated background human body burden of 13 ng/kg body weight of TEQs (*1*). Furthermore, the maternal dose values used in the benchmark calculations represent applied doses. The actual amount of TCDD delivered to each female rat offspring that participated in the wheel-running procedure is unknown, but recent data indicate that the actual amount of TCDD retained as dam body burden and delivered to each female offspring would be much lower (*49,50*).

The lack of a correlation between estrous cycle phase and responding for access to wheel running was unexpected. Not only does general activity, including wheel running, tend to increase during estrus (*20,23,24*), but, to initiate and maintain wheel rotation requires accurate foot placement on the parallel rods that constitute the running surface. In a different behavioral procedure, the foot placement of female rats required to traverse a narrow beam was more accurate during estrus, while more foot faults were made during diestrus. Estradiol implants directly into the striatum improved the foot placement accuracy of ovariectomized rats (*51*).

The selective effect of low-level perinatal TCDD exposure on FR responding for access to wheel running, in concert with its independence of estrous cycle phase, suggests a mechanism other than estrous-associated

Table 3. Means and SDs used for benchmark dose calculations for the earned run opportunities and total revolutions variables.

Dose	FR2	FR5	FR10	FR20	FR30
Earned run opportunities					
Control	30.86 ± 19.1	26.14 ± 12.28	13.29 ± 8.65	8.29 ± 6.98	5.0 ± 2.99
20 ng/kg	29.75 ± 11.96	23.5 ± 7.04	11.25 ± 5.56	6.5 ± 5.8	4.0 ± 2.3
60 ng/kg	15.17 ± 7.13	12.8 ± 6.17	5.75 ± 3.53	4.0 ± 2.65	3.25 ± 4.04
180 ng/kg	18.86 ± 8.2	13.14 ± 7.14	7.0 ± 6.01	2.67 ± 0.87	2.0 ± 1.53
Total revolutions					
Control	119.29 ± 69.9	123.86 ± 80.51	60.14 ± 50.40	42.00 ± 38.71	21.71 ± 18.34
20 ng/kg	108.50 ± 61.00	96.00 ± 25.6	40.50 ± 15.02	29.25 ± 23.82	16.25 ± 13.12
60 ng/kg	56.50 ± 31.21	46.00 ± 42.11	15.33 ± 14.75	10.00 ± 8.87	8.67 ± 11.78
180 ng/kg	68.14 ± 33.23	59.43 ± 35.56	21.43 ± 27.22	4.29 ± 5.74	4.00 ± 5.77

Table 4. Benchmark doses (BMD; ng/kg) and 95% lower bound (95% LB) calculations based on a 1% or a 10% shift from control group mean (ED_{01} or ED_{10}) for data from sessions immediately following transitions to new FR values.

	FR2		FR5		FR10		FR20		FR30	
	ED_{01}	ED_{10}	ED_{01}	ED_{10}	ED_{01}	ED_{10}	ED_{01}	ED_{10}	ED_{01}	ED_{10}
Earned runs[a]	0.90	9.36	0.91	9.39	0.80	8.22	0.91	9.34	1.39	14.52
95% LB	0.88	5.91	0.90	6.32	0.51	5.17	0.51	5.20	1.39	6.26
Total Revs[b]	0.87	8.97	0.71	7.30	0.59	6.07	0.61	6.19	0.78	8.05
95% LB	0.84	5.73	0.70	6.84	0.58	5.76	0.42	4.08	0.46	4.66

[a]Running opportunities per session. [b]Number of wheel revolutions per session.

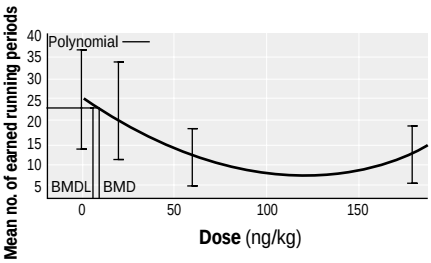

Figure 3. Polynomial model for benchmark dose ED_{10} value and 95% lower confidence level for earned run opportunities calculated from a quadratic fit to the dose–response function. Abbreviations: BMD, benchmark dose; BMDL, 95% lower bound. The dose–response data used to calculate these values came from the first session under the FR5 condition.

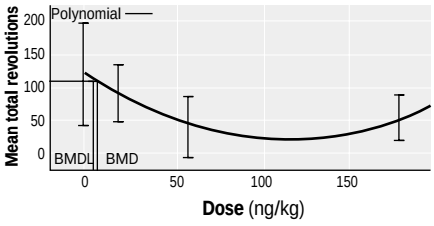

Figure 4. Polynomial model for benchmark dose ED_{10} value and 95% lower confidence level for total wheel revolutions calculated from a quadratic fit to the dose–response function. Abbreviations: BMD, benchmark dose; BMDL, 95% lower bound. The dose–response data used to calculate these values came from the first session under the FR5 condition.

hormonal variations. Instead, perinatal TCDD exposure may have interfered with the organization of brain regions lying outside the hypothalamic–pituitary–gonadal feedback loop that regulates estrous cyclicity. Midbrain monoamine systems are known to mediate various aspects of wheel-running behavior as well as a variety of motivational measures. Systemic administration of dopamine agonists increases free running output while maintaining the cyclic nature of the response (*22*), whereas microinjections of the neurotoxicant 6-hydroxydopamine (6-OHDA) into the nucleus accumbens reduce schedule-induced wheel running (*52*). Conversely, serotonin (5-HT) depletion of the ventrolateral hypothalamus increases wheel running without disrupting the relation between free running and the estrous cycle (*53*). Systemic administration of a 5-HT1c receptor agonist reduces running (*54*). Finally, the rate of norepinephrine and 5-HT turnover is higher in the medial basal hypothalamus of free-running rats compared to sedentary rats (*52*). Collectively, this evidence suggests that hypothalamic serotonin is inhibitory, whereas midbrain dopamine is permissive for wheel-running behavior. Early monoamine activity is present in the developing rat brain during the late gestational period, coincident with the GD18 TCDD exposure used in this procedure. For instance on GD19, amino acid decarboxylase and tyrosine hydroxylase are detectable in both the maturing nucleus accumbens and the ventrolateral hypothalamus. Even higher levels of these monoamine markers are present in the maturing ventral tegmental area after GD17 (*42*). Seo et al. (*15*) noted that the pattern of radial arm maze performance they observed in male rats exposed prenatally to TCDD resembled the pattern, measured as a facilitation of one aspect of performance, also observed by Pearson et al. (*16*) following 6-OHDA lesions in neonatal rats.

In summary, perinatal exposure to TCDD significantly reduced operant responding for wheel-running reinforcement in adult female rat offspring. These results argue for an expanded exploration of behavioral end points in assessing the developmental toxicity of TCDD and related agents. Because the behavioral changes were independent of estrous cyclicity, they also suggest similar assays with male offspring. Although the ultimate cause of other behavioral changes attributed to TCDD may be due to organizational or activational effects arising from gonadal hormone effects, it is also possible that the proximal mechanisms of permanent learning or motivational deficits are attributable to other factors. Thyroid hormone abnormalities are one potential candidate, but other sources of disrupted brain development are another possibility. One example is interference with the potential role of the AhR in developmental processes (*55*). Because the non-cancer risks associated with developmental TCDD exposure in humans are unclear, the examination of a wide range of animal behaviors is necessary for both risk assessment and to provide a context for understanding the often cited changes in male rat sex behavior.

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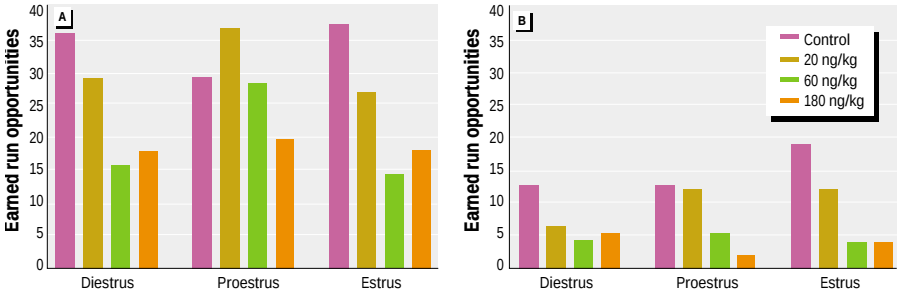

Figure 5. Mean earned run opportunities as a function of estrous cycle stage for the FR2 (*A*) and FR10 (*B*) sessions. Prenatally exposed females consistently earned fewer run opportunities than controls regardless of the stage of the estrous cycle. The same pattern was observed for FR1, FR5, FR20, and FR30 sessions.

Table 5. Percent of time spent in various stages of the estrous cycle over the course of the behavioral testing.

Exposure group	Days of diestrus (%)	Days of proestrus (%)	Days of estrus (%)
Control	24	49	26
20 ng/kg	17	51	32
60 ng/kg	36	42	22
180 ng/kg	23	48	29

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