TERATOLOGY 50:238–249 (1994)

Behavioral Teratologic Effects of Prenatal Exposure to Continuous-Wave Ultrasound in Unanesthetized Rats

CHARLES V. VORHEES, KAREN D. ACUFF-SMITH, MARY A. SCHILLING, J. EDWARD FISHER, JR., RICHARD A. MEYER, NADINE B. SMITH, D. SCOTT ELLIS, AND WILLIAM D. O'BRIEN, JR.

Divisions of Basic Science Research (C.V.V., K.D.A.-S., M.A.S., J.E.F.) and Cardiology (R.A.M.), Children's Hospital Research Foundation, and Department of Pediatrics, University of Cincinnati, Cincinnati, Ohio 45229; Bioacoustics Research Laboratory, Department of Electrical and Computer Engineering, University of Illinois, Urbana, Illinois 61801 (N.B.S., D.S.E., W.D.O.)

ABSTRACT While there are no known risks associated with diagnostic ultrasound, uncertainty about the safety of prenatal ultrasound exposure remains. The purpose of the present experiment was to evaluate the behavioral teratogenic potential of continuous-wave (cw) ultrasound in rats, in the absence of maternal anesthesia or restraint. Pregnant CD rats, trained to remain immobile in a water-filled ultrasound exposure tank, were scanned with 3 MHz cw ultrasound at levels of 0, 2, 10, 20, or 30 W/cm^2 I_{SPTA} (spatial peak, temporal average intensity) on gestational days 4–20 for approximately 10 min/day. Offspring were examined postnatally for survival, growth, physical landmarks of development, behavioral development, and the adult functions of locomotor activity, learning and memory, and startle reactivity. No effects of prenatal ultrasound were found on maternal characteristics, offspring survival or growth, physical or behavioral landmarks of development, or adult tests of passive avoidance or startle. Effects at the highest intensity were obtained on corner and side locomotor activity and in a multiple-T water maze on measures of errors of commission and time spent finding the goal. The results showed that prenatal cw ultrasound in rats can induce effects on some postnatal neurobehavioral functions at high exposure intensities (30 W/cm^2), but at lower intensities (2–20 W/cm^2) no consistent evidence of neurobehavioral effects was observed.

The widespread use of ultrasonography during pregnancy reflects the absence of known adverse effects and its diagnostic benefits (NIH, '84; Brent et al., '91), although the value of routine prenatal ultrasound screening is being called into question (Ewigman et al., '93). However, recognition of the methodological difficulties inherent in epidemiological studies for detecting subtle effects or low frequency severe effects has prompted continued caution (Ziskin and Petitti, '88). In addition, two developments in clinical practice, the use of diagnostic ultrasound devices with increasing acoustic output intensities and the more frequent use of ultrasound for preconception and early gestational examinations, have prompted a reappraisal of possible reproductive risks (Miller, '91; Martin et al., '91; AIUM, '93; Tarantal and O'Brien, '94).

Experimental efforts to delineate the bioeffects of ultrasound on in utero development have been inconclusive (Carstensen and Gates, '85; O'Brien, '85; Brent et al., '91; AIUM, '93; Tarantal and O'Brien, '94). Some studies have reported increased malformation rates (Mannor et al., '72; Shoji et al., '74; Sikov and Hildebrand, '76; Pizzarello et al., '78; Stolzenberg et al., '80; Sarvazyan et al., '82; Takabayashi et al., '85), while others have found no such effects (McClain et al., '72; O'Brien et al., '82; Child et al., '84, '89; Kimmel et al., '83, '89; Vorhees et al., '91a). Effects of ultrasound exposure on fetal body weight have also been reported (Pizzarello et al., '78; Stolzenberg et al., '80; O'Brien, '83; Tarantal and Hendrickx, '89a; Hande and Devi, '92, '93), but other studies have found no body weight changes (Child et al., '84, '89; Kimmel et al., '83, '89). In addition, reports of behavioral teratogenic effects have appeared (Murai et al., '75a,b; Sikov et al., '77; Tarantal and Hendrickx, '89b; Norton et al., '91).

Received March 1, 1994; accepted July 27, 1994.

Address reprint requests to Charles V. Vorhees, Ph.D., Division of Basic Science Research, Children's Hospital Research Foundation, 3333 Burnet Avenue, Cincinnati, OH 45229-3039.

Karen D. Acuff-Smith is now at the Procter and Gamble Company, Miami Valley Laboratories. P.O. Box 398707, Cincinnati, OH 45239-8707.

J. Edward Fisher, Jr., is now at the Division of Neuropharmacological Drug Products, Center for Drug Evaluation and Research, HFD-120, U.S. Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857.

Part of the difficulty in assessing the developmental effects of ultrasound based on animal investigations lies in the variability of the experimental conditions, exposure parameters, and dependent measures used. Where biological alterations have been found, their significance is often unclear because appropriate dose-effect relationships have not been established. There are also uncertainties with respect to the extrapolation of experimental animal data to humans, particularly for factors such as differences in tissue ultrasound attenuation, target size, species sensitivity, and heating effects which depend on the mass of the exposed organism (NIH, '84; O'Brien, '92).

A limitation common to most animal studies of ultrasound developmental effects involves the use of anesthesia or restraint during insonation. These factors could potentially confound the experimental results, since both are themselves associated with developmental toxicity (Mazze et al., '85; Weinstock et al., '88). Furthermore, anesthesia-induced hypothermia could alter the response to ultrasound-induced heating. In an alternative approach to dealing with these problems, we developed an ultrasound exposure system using rats that had been trained to remain immobile without using anesthetics. This system was previously used to assess the effects of prenatal exposure to continuous-wave (cw) (Vorhees et al., '91a) or pulsed-wave (pw) (Fisher et al., '94) ultrasound on fetal body weight and malformation potential. The present study extends this approach by examining the possible behavioral teratogenic effects of in utero exposure to cw ultrasound using the conditioned immobility procedure.

MATERIALS AND METHODS

Animals

Subjects were nulliparous female Sprague-Dawley CD (VAF) rats (Charles River, Portage, MI), housed according to the guidelines of the American Association for the Accreditation of Laboratory Animal Care. Prior to placing females with males, immobility training was conducted in a water-filled chamber approximately the same size as that of the confinement chamber in the ultrasound exposure tank. Each female received 2 consecutive days of 12 min/day followed by 2 days of 10 min/day confinement in the training tank. This repeated confinement elicited a conditioned immobility, i.e., an attenuation of efforts to escape. Females were housed with males on the day following the last training session. Discovery of a vaginal plug was considered embryonic (E) day 0.

On E0, dams were assigned to one of five treatment groups for cw ultrasound exposure on a weight-matched basis and encoded on the exposimetry computer so that experimenters were blind to treatment group assignment. At least 16 dams were assigned to each group. On E3, each dam received refresher immobility training (12 min) in the exposure tank. After removal from the vessel, the abdomen of each rat was depilated.

Exposure system

For detailed descriptions of the ultrasound exposimetry system which was developed specifically for these experiments see Smith et al. ('90) and Vorhees et al. ('91a). Briefly, the exposure system consisted of a water-filled, rectangular container constructed of acrylic, with a partially focused 4.6 cm diameter, 3 MHz, PZT-4 crystal mounted in a movable transducer assembly platform approximately 30 cm below the water surface. Pregnant rats that had been trained to remain immobile were placed individually in an inner confinement chamber (10 × 15 cm) directly above the ultrasound beam. The ultrasound beam was calibrated under free-field conditions with a calibrated membrane hydrophone (Marconi; see Smith et al. ['90] for details). For a stationary beam, at the focus that would be a location within the floating rat, the free-field 90% and 50% intensity beam widths were 1.6 and 3.2 cm, respectively. At the focus the four cw values of the spatial peak, temporal average intensity (I_{SPTA}) used were 0, 2, 10, 20, and 30 W/cm^2, as calculated from the measured instantaneous pressures. Measurement uncertainty yields an intensity uncertainty of ±15%.

Intensities were selected to span a broad range of exposures in an attempt to establish a clear bioeffect. Accordingly, our lowest exposure intensity of 2 W/cm^2 was chosen to be at or slightly above the upper boundary of diagnostic intensities. At the opposite extreme, 30 W/cm^2 was chosen as one we estimated was likely to increase body temperature and might produce a demonstrable effect. Intermediate values were chosen in equal steps below the highest value in order to describe a dose-response relationship for any effects obtained. The values were also selected based on our previous data showing that these cw intensities were not teratogenic (Vorhees et al., '91a). Duration of exposure was selected somewhat arbitrarily, but was based on the length of time of some clinical examinations and the time rats could be trained to remain relatively immobile.

A modification of an approach described by O'Brien et al. ('82) was used to provide uniform ultrasound exposure to the floating rat's abdominal surface, which was estimated to be approximately 7 (width at the widest point) × 8 cm (xiphoid process to the prepuce) on E17. A raster scan pattern of the movable transducer assembly was set at 8 × 13.5 cm, or approximately 1 cm inside each wall of the confinement chamber, so that the entire enclosure was insonated. The raster scan sequence consisted of sixteen 8 cm rasters separated by 1 cm. Each rat received one raster scan per day on E4–20 for a total of 17 insonation exposures, each lasting 10 min, with 2 min of transducer positioning time at the beginning of the scanning pattern for a total of 12 min/session. Based on the raster scan pat-

TABLE 1. Testing sequence for male/female pairs of offspring in each litter[1]

Test	Offspring pair A	B	C	D	Age (days)
Pinna detachment	+	+	+	+	0→
Incisor eruption	+	+	+	+	7→
Eye opening	+	+	+	+	12→
Vaginal patency (F only)	+	+	+	+	30→
Balanopreputial separation (M only)	+	+	+	+	35→
Olfactory orientation	+	+	−	−	9, 11, 13
Early locomotion	+	+	−	−	10, 12, 14
Air righting	+	+	−	−	15–18
Acoustic startle	+	+	−	−	18–20
Cincinnati water maze	+	−	−	+	50–54 ± 3
Passive avoidance	+	−	−	+	60–74
Locomotor activity	−	+	+	−	20, 60
Acoustic and tactile startle	+	+	−	−	75

[1]There were 16 litters per group and 8 offspring per litter divided into 4 male/female pairs. Pairs were arbitrarily labeled A, B, C, and D. "+" indicates that the pair received the indicated test and "−" indicates that it did not. "→" indicates that rats were tested from the day shown until the criterion was met.

tern and transducer speed, the time that a specific body site was within the 90% intensity beam width during one scanning session was determined to be 8–12 sec. For the 50% intensity beam width the exposure time ranged between 41 and 46.5 sec. Body temperature was not measured because of the complexity of this procedure in unrestrained rats. Our decision was that body temperature would be measured in subsequent experiments only if clear bioeffects emerged from the present experiment.

The exposure tank was refilled daily with fresh deionized water and allowed to degas overnight. Water temperature was maintained at 35°C by a proportional temperature controller (Yellow Springs model 72, Yellow Springs, OH).

Behavioral teratological procedures

Dams were allowed to deliver naturally. On the day of birth (P0), offspring were examined for external defects, sexed, numbered, and four males and four females selected for retention using a random number table. On P9 offspring were individually marked in preparation for reflex testing using black indelible ink. A summary of the testing sequence is provided in Table 1.

Physical landmarks. Daily from P0, each offspring was examined for bilateral detachment of the pinnae. Daily from P7, each offspring was examined for bilateral eruption of upper and lower incisors, noted separately. Daily from P12, each offspring was examined for bilateral eyelid opening to its fullest extent (to outer epicanthus). In addition, day of preputial separation was noted for males and day of vaginal patency was noted for females.

Reflexology. Four tests of early reflex and/or behavior were conducted on all retained offspring per litter: olfactory orientation, air righting, early locomotion (pivoting), and acoustic startle. All of these tests have been described in detail elsewhere with minor modifications as noted below (Vorhees, '83). Olfactory orientation was conducted on days P9, P11, and P13 for 1 min/day by placing animals in a 12 × 38 cm runway midway between equal measured amounts of home cage bedding and clean bedding and scoring movements in either direction based on line crossings marked on the floor ranging from −4 (nearest the clean bedding) to +4 (nearest the home bedding). Evaluation of pivoting was conducted on days P10, P12, and P14 for 1 min/day in a Digiscan activity monitor designed for mice. Air righting was conducted on days P15–18 using a new stop-action photographic method (Vorhees et al., '94). Each offspring received 3 righting trials per day when released from a standard height of 30 cm to a padded landing surface. The proportion of rats successfully righting on either 2/3 or 3/3 trials per day was used to analyze the results of this test. Acoustic startle testing was conducted on days P19 ± 1 and again on P75 in a San Diego Instruments (San Diego, CA) model SR startle apparatus. Each offspring received 51 trials consisting of exposure to the startle stimulus (115 dB(A)), a broad band signal with a predominant frequency of 4 kHz lasting for 20 msec, with 70 dB background noise, an intertrial interval of 8 sec, and a response window of 100 msec, following an initial 5 min test chamber acclimation period. A pressure transducer converted the animal's flinch into a voltage signal that was used to quantify the maximum amplitude (V_{max}), average amplitude (V_{mean}), and latency to maximum response (T_{max}) on each trial. Activity testing on day P20 is described below.

Offspring were weaned on P28 and housed in same sex pairs, then housed individually on P42. After weaning, two male/female pairs (A and D) per litter were assessed on two tests of learning and memory (Cincin

TABLE 2. Effects of cw ultrasound on reproductive outcome after exposure on E4–20

Dependent variable	Group (W/cm^2) I_{SPTA}				
	0	2	10	20	30
No. sperm-positive females	16	16	17	20	18
No. non-parturient dams[1]	0	0	0	2	0
No. litters found dead	0	0	0	1	0
Litters with >6 liveborn	0	0	1	0	1
Gestation length (days)[2]	21.7 (0.1)	21.8 (0.1)	21.9 (0.1)	21.7 (0.1)	21.8 (0.1)
Litter size at birth[2]	15.5 (0.5)	15.5 (0.5)	14.2 (0.9)	14.4 (0.8)	14.3 (0.8)
Offspring mortality at birth	3/248	4/248	3/242	2/254	3/258
Offspring mortality P1–28	2/128	2/128	2/128	3/141	3/136
Offspring mortality P29–90	0/125[3]	0/124[3]	0/126	0/131[3]	0/133

[1]Both non-parturient dams' litters were 100% resorbed.
[2]Values represent the group mean ± SEM (in parentheses).
[3]Numbers do not sum from previous row due to some offspring not being retained after weaning. Only four males and four females were retained after weaning; any extras were euthanized.

nati water maze and passive avoidance), while pairs B and C were tested for activity, and pairs A and B for startle reactivity.

Water maze. The Cincinnati water maze is a multiple-T maze with nine double-ended cul-de-sacs. Rats were tested beginning on the nearest Monday to day P50 for 5 consecutive days. On the first day, rats received 4 trials in a 150 cm straight swimming channel. On the remaining days, rats received 2 trials per day in the maze in path B using an unassisted escape test procedure. Rats were scored for errors and time to complete the maze up to a limit of 5 min/trial. Details of the apparatus (Vorhees, '87) and test procedures (Vorhees et al., '91b) have been described previously. Pairs A and D received this test.

Passive avoidance. Step-through passive avoidance was conducted on days P60 and P74. On P60, each rat was placed in the two-compartment apparatus on the lighted side and allowed up to 3 min to spontaneously crossover to the dark side (rats not crossing on the first trial were retested the next day for crossover, but if they failed a second time they were eliminated from further testing). Upon crossing, the door dividing the two compartments was closed and a single scrambled shock (0.9 mA, 1 sec duration) was administered through the grid floor. On P74 rats were returned to the lighted side and latency to reenter the dark side was assessed for up to 3 min, with no shock for those crossing. Pairs A and D received this test.

Activity. Locomotor activity was assessed using a video tracking system described in detail elsewhere (Vorhees et al., '92). Rats were tested twice, once on P20 and again on P60 for 30 min on each occasion. Computer-generated tracings of the animal's path recorded each 0.1 sec were scored by length in different regions of the 40 × 40 cm field under red lights. Activity in these regions was termed corner, side, and central activity. The combination of corner and side activity was defined as peripheral activity and of all regions as total activity. In addition, the number of section entries was defined as the number of transitions. Pairs B and C received this test.

Startle. Startle was tested on P75 using two stimulus modalities: acoustic and tactile. The acoustic test was identical to that described above prior to weaning. The tactile test consisted of 51 additional trials in which the stimulus used was a mid-chamber air-puff of 12 psi administered for 20 msec on each trial. All other exposure and measurement parameters were as described above for the preweaning acoustic startle test. Pairs A and B were administered this test.

Statistical procedures

All data except mortality were analyzed using fixed-effect factorial analyses of variance (general linear model), with the litter mean used to represent all the subjects within a litter, stratified by sex. Some behavioral tests and body weight measurements involved repeated assessments of the same subjects. In these cases, a split-plot analysis of variance was used with trials as a within-subjects factor in the model. In instances in which a test for sphericity of the variance-covariance matrix of split-plot analyses of variance was significant, the Greenhouse-Geisser correction of F-ratios involving the repeated measure factor was used. Mortality data were analyzed by Fisher's test for uncorrelated proportions. For all analyses of variance, a posteriori group comparisons were conducted using Duncan's multiple range test.

RESULTS

The reproductive outcomes of the experiment are summarized in Table 2. Ultrasound exposure had no demonstrable effect upon the number of nonparturient dams, resorbed litters, number of litters either born dead or with fewer than six live offspring, live litter size, gestation length, or sex ratio. There were no significant effects found on maternal or offspring body weights. No effects on offspring mortality were found (Table 2).

Physical landmarks of development

Physical landmarks of development examined were mean day of pinna detachment, upper and lower incisor

TABLE 3. Physical landmarks of development in offspring exposed prenatally to cw ultrasound[1]

Dependent variable	Group (W/cm^2) I_{SPTA} 0	2	10	20	30	P^2
Pinna detachment	3.1 (0.1)	3.0 (0)	3.3 (0.1)	2.9 (0.1)	3.1 (0.1)	<0.067
Preputial separation	40.9 (0.3)	40.4 (0.2)	41.2 (0.3)	40.6 (0.2)	41.3 (0.4)	0.172
Vaginal patency	33.6 (0.5)	33.4 (0.3)	32.8 (0.2)	32.6 (0.2)	33.0 (0.3)	0.143

[1]Data are expressed as group mean (days) ± SEM (in parentheses).
[2]Based on analysis of variance main effect for treatment group.

eruption, eye opening, preputial separation in males, and vaginal patency in females. No effects were found for upper or lower incisor eruption or for eye opening. $P < 0.2$ in treatment group F-ratios were found for pinna detachment, preputial separation, and vaginal patency. Group means are shown in Table 3. No dose-dependent pattern of effects was seen on any of these measures.

Measures of behavioral development

Two factors were significant for olfactory orientation: day and the day x group interaction ($F(8,154) = 2.2$, $P < 0.04$). Duncan group comparisons on each day revealed a difference only on day 13 of testing, in which the ultrasound 10 W/cm^2 (US10) group had higher orientation scores than controls (Fig. 1).

Analysis of early locomotion, used as an index of pivoting development, revealed no significant treatment group effects.

Analysis of air-righting photographs revealed no significant effect of treatment group on performance of this reflex.

Analyses of preweaning startle habituation revealed no treatment group effects. Separate analyses of the first trial showed a significant treatment group effect for both V_{max} and V_{mean} (both $P < 0.05$), however, the differences were between the US2 group and the US10 group. No treatment group differed from controls and no dose-dependent pattern was observed, suggesting that this effect was due to factors other than ultrasound exposure.

Preweaning locomotor activity was analyzed for number of section transitions, and amount of movement (distance) in the corners, sides, and central regions as well as the sum of these as total activity. No treatment group effects were found for transitions, total, side, or central activity, but a significant group main effect was seen for corner activity ($F(4,76) = 3.9$, $P < 0.01$). Duncan group comparisons revealed that this effect was due to increased corner activity in the US30 group compared to controls or any other ultrasound exposed group (Fig. 2, left). Follow-up analyses of time spent in each region confirmed this effect, i.e., the US30 group spent more time in corners than controls, most notably on intervals 2 and 3, and overall (Fig. 2, middle and inset). Interestingly, the analysis of time spent on the sides showed an opposite pattern. In this case, a significant treatment group effect ($F(4,76) = 2.6$, $P < 0.05$) and Duncan comparisons revealed that the US30 group spent less time along the sides than controls or other ultrasound exposed groups (Fig. 2, right).

Measures of adult behavioral performance

Analysis of swimming times in a straight channel administered prior to maze testing revealed no treatment group effects based on a group x sex x trial analysis of variance. Similar analysis of maze errors, however, revealed a non-significant treatment group main effect ($F(4,77) = 2.2$, $P = 0.076$) and a significant trial x group interaction ($F(28,539) = 1.9$, $P < 0.015$). Maze times showed a similar pattern, except for these data the treatment group main effect was significant ($F(4,77) = 3.0$, $P = 0.022$), while the trial x group interaction was not ($F(28,539) = 1.5$, $P < 0.10$). Errors for each trial and errors averaged across trials and times averaged across trials are shown in Figure 3. Increased errors compared to controls were found on trial 1 in the US2 group and on trial 2 in the US30 group by Duncan comparisons. No differences were seen on trials 3–8. Across all trials, the only significant Duncan comparison was between the controls and the US30 group ($P < 0.05$). A similar pattern was seen on maze times, but the Duncan comparison fell short of conventional significance ($0.05 > P < 0.10$). Again, this trend was for the US30 group to spend more time in the maze than controls.

Analyses of passive avoidance revealed no significant treatment group effect on either training or retention performance.

Adult locomotor activity was analyzed in the same fashion as preweaning activity. No significant treatment group effects were found on any measure.

Adult startle was analyzed in the same fashion as preweaning startle. The only difference was that for adult startle two habituation sessions were conducted: the first for acoustic startle and the second for tactile startle. No significant treatment group effects were found for either acoustic or tactile startle habituation.

DISCUSSION

Interpretation of the current findings

The time-dependent bioheat transfer equation was applied to a circular source to estimate the tempera-

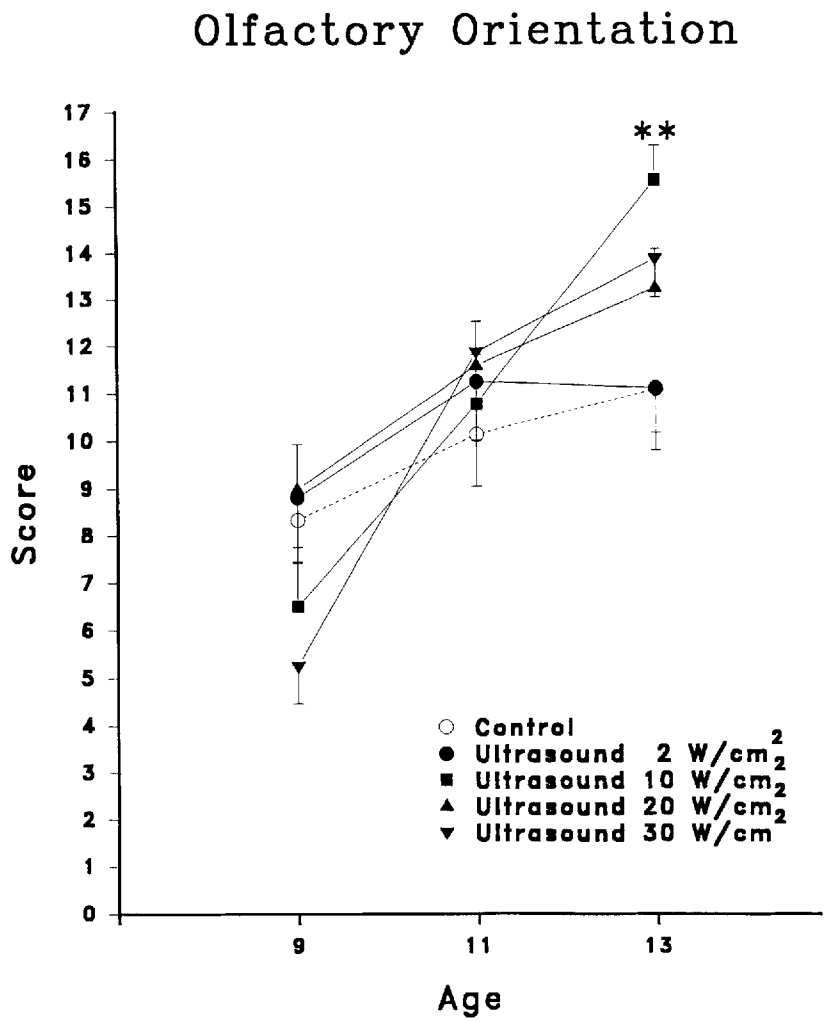

Fig. 1. Mean (±SEM) movement toward the home cage scent during olfactory orientation testing in rats prenatally exposed to cw ultrasound. $^{**}P < 0.01$ compared to control.

ture increase due to an 8 sec ultrasound exposure duration (Pennes, '48; Nyborg, '88; AIUM, '88, '93; Ellis, '91; NCRP, '92). This exposure duration is the minimum time that a specific body site was within the 90% intensity beam width during one scanning session. In this formulation, the source power is the ultrasonic power incident at the rat surface. Using a diameter (D) of 1.6 cm (the free-field 90% intensity beam width), the source powers calculate ($W_{source} = (\pi D^2/4)I_{SPTA}$) to be 4, 20, 40, and 60 W for the I_{SPTA} values of 2, 10, 20, and 30 W/cm^2, respectively. An attenuation coefficient (and absorption coefficient) value of 0.3 dB/cm-MHz for a homogeneous tissue model was used which is the same value used by the U.S. Food and Drug Administration (FDA) for their approval process of diagnostic ultrasound equipment (FDA, '85, '93) and was used in the AIUM ('93) model. A moderate perfusion length value of 1.18 cm was used which was the same as that used in the AIUM ('88) model. The axial profile of the temperature increase exhibited its maximum value at a range of 1.1 cm from the rat's skin surface, which would place the maximum temperature increase within the region of the dam's embryos. At an exposure duration of 8 sec, the maximum temperature increase for the four respective I_{SPTA} values was calculated to be 0.56, 2.8, 5.6, and 8.3°C. If the base temperature of the rat is 38°C, then the potential temperatures of the embryos are predicted to be 38.5, 40.8, 43.6, and 46.3°C for about 8 sec from the ultrasound exposure.

Miller and Ziskin ('89) and AIUM ('93) have reviewed the teratologic hyperthermia literature and plotted the findings as a function of temperature rise vs. duration of exposure (Miller and Ziskin, '89: fig. 9). Fetal temperatures of 41°C for extended periods of time appeared to be a threshold for induction of anomalies. For shorter periods, higher temperatures were re-

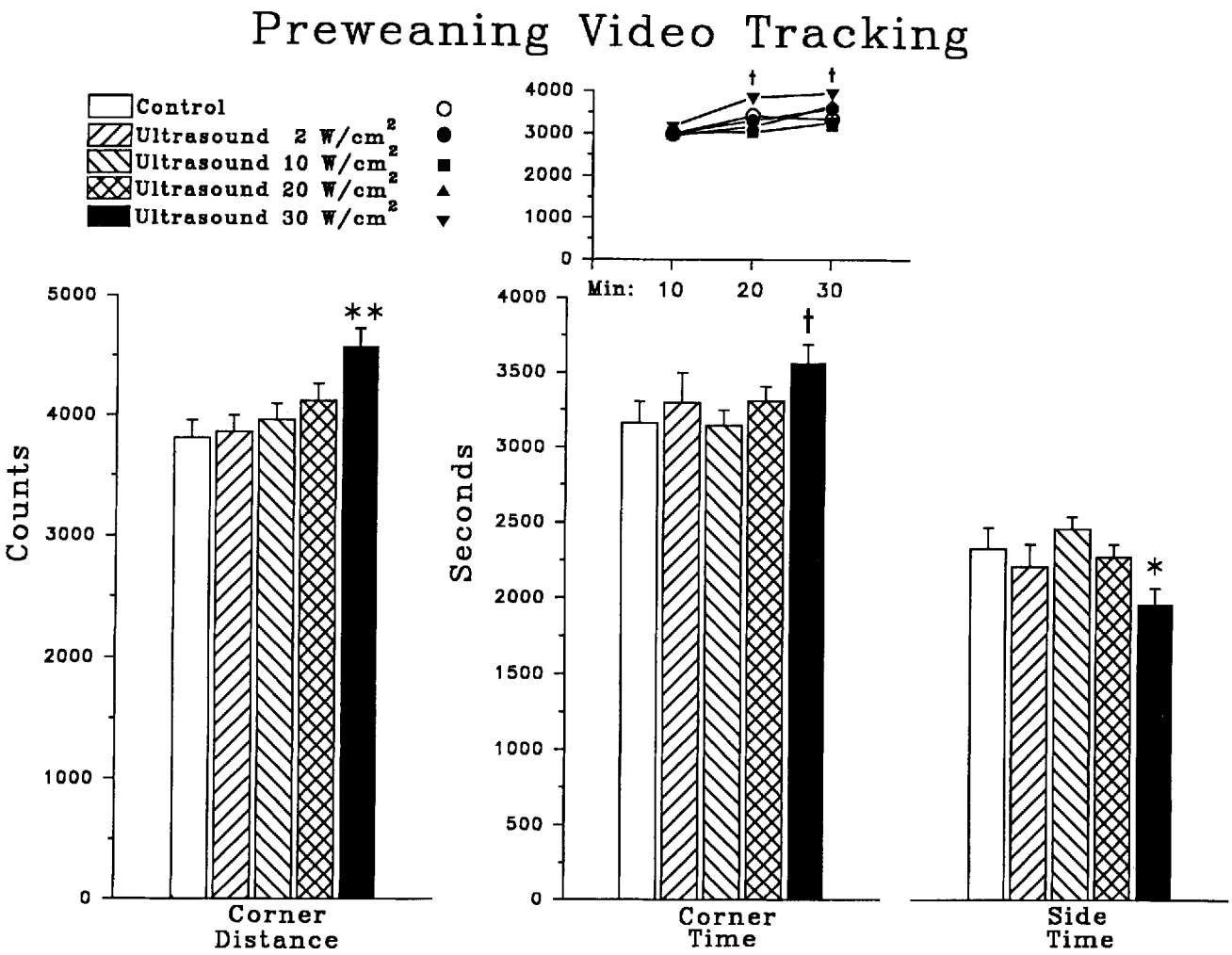

Fig. 2. Mean (±SEM) locomotor activity (distance) and time on day P20 in rats prenatally exposed to cw ultrasound. **Left:** Distance moved in corner regions; **middle:** time spent in corner regions (**inset:** time spent in corners as a function of test interval); **right:** time spent in side regions. $^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

quired to produce anomalies. The temperature *(T)* vs. time at that temperature *(t)* graph yielded a lower boundary below which no observed thermally induced biological effects were reported. This boundary line may be expressed in terms of the following formula

$$t = 4^{(43-T)} \quad (1)$$

where temperature, T, is represented in °C and time, t, in minutes. This function, taken from Miller and Ziskin ('89), shows that there is a trade-off between temperature elevation and duration of exposure for the induction of malformations and that the 41°C threshold traditionally recognized for teratogenesis induction is not absolute, but rather applies only to sustained temperature increases.

The exposure duration reported herein (8 sec) was shorter than any reported previously. At a duration t of 8 sec, Eq. (1) yields a predicted threshold temperature T of 44.5°C. The I_{SPTA} values of 20 and 30 W/cm^2 used herein produce calculated embryonic temperatures of 43.6 and 46.3°C which bracket the temperature of 44.5°C calculated from Eq. (1). Thus, the two highest intensities of ultrasound used herein were near the minimum temperature rise estimated to induce teratogenesis based on an extrapolation to 8 sec of the boundary function developed by Miller and Ziskin ('89). While this extrapolation is theoretical, it is consistent with the large body of data reviewed by Miller and Ziskin ('89). Further, as these authors' point out, this boundary function is for core temperatures, whereas regional temperature elevations would be expected to produce smaller effects. The exposure our system produced was of the latter type, producing localized exposures. We believe that these considerations explain

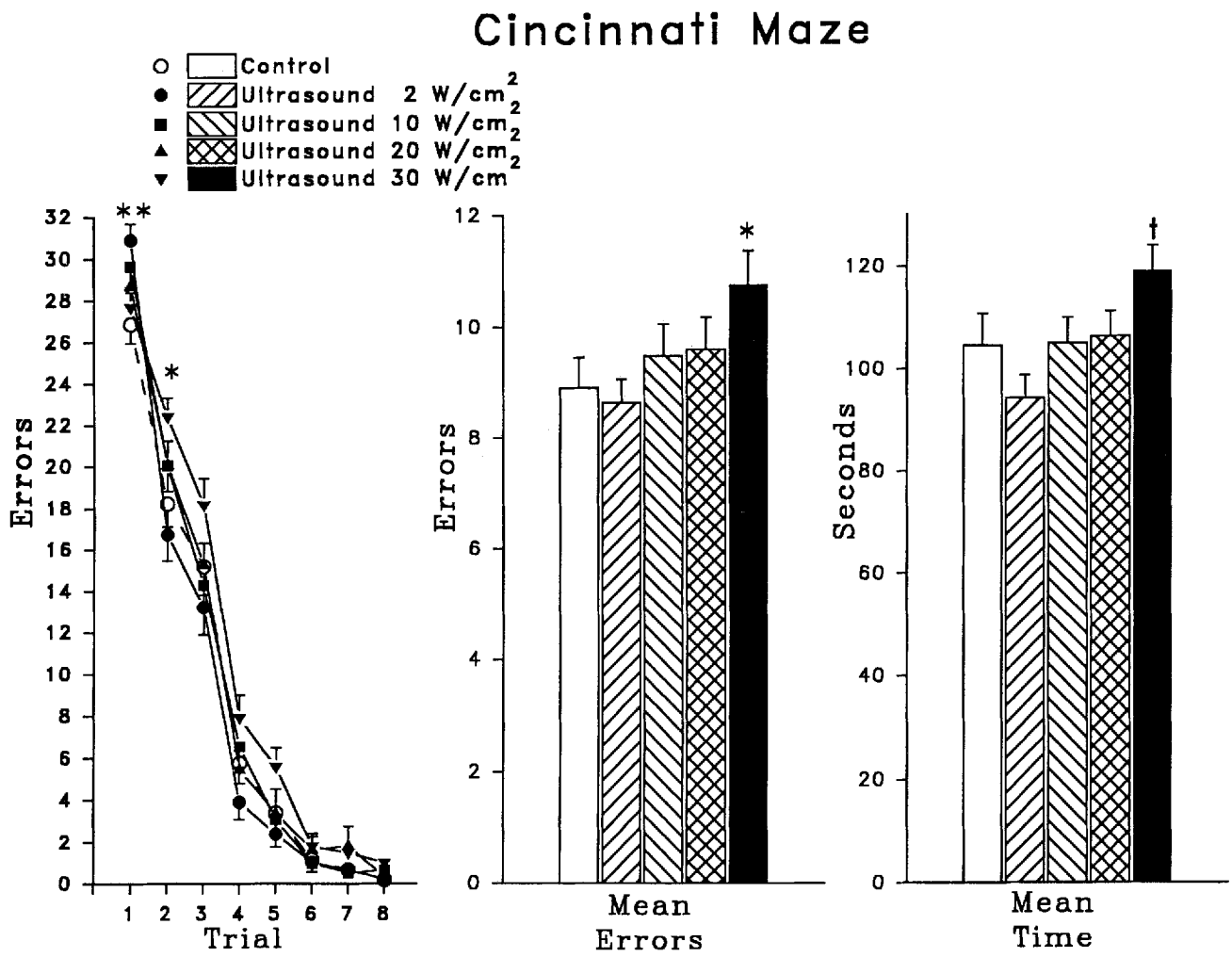

Fig. 3. Left, middle: Mean (±SEM) errors and (**right**) times in the Cincinnati water maze in rats prenatally exposed to cw ultrasound. $^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

why we observed no malformations under our experimental conditions.

In addition, we employed a conditioned immobilization procedure to examine the developmental effects of gestational cw ultrasound exposure in rats without the use of anesthesia or forced restraint. The data showed that such insonation produced no adverse effects on maternal weight or reproductive outcome, nor on postnatal growth or survival of the offspring. There was no convincing evidence for effects on physical landmarks of development nor on most measures of behavioral development. Although an effect was found on olfactory orientation, the pattern of the effect does not support the view that it was treatment-related. This was the case because the effect was only present on one test day and occurred only in the US10 group, but was not seen in either of the higher intensity exposure groups (US20 or US30). A non-linear dose-response pattern would have to be invoked to explain this as being due to ultrasound and there is no compelling evidence to support such an interpretation. Accordingly, the most likely explanation for this effect is that it represents a type I statistical error. The other effect obtained on preweaning behavior was on the test of locomotor activity. Although most of the measures taken on animals during this test were negative, the finding of increased corner distance and time in the US30 group, accompanied by reduced side time in this group, suggests that this pattern may be due to the treatment. The magnitude of the effect (≈10% on corner distance) was near the detection limit for this test, making definitive conclusions impossible. This effect should, therefore, be viewed as tentative until replicated.

No effects were found on most of the neurobehavioral dependent variables measured on the offspring as adults. These included measures of adult locomotor activity, acoustic and tactile startle reactivity, straight channel swimming ability, and passive avoidance re-

tention. One test, however, the Cincinnati maze test of learning, showed evidence of an ultrasound-associated treatment effect. This effect was seen on the number of errors of commission in the maze and on time spent finding the goal. There is often a positive correlation between these two measures based on experiments with other test agents that are developmental neurotoxins (Vorhees, '87; Vorhees et al., '91b). The effect seen, however, was relatively weak, only appearing at the highest exposure intensity, was more clearly seen in aggregate errors averaged across trials than when examined trial-by-trial (Fig. 3, cf. left vs. center), and showed no evidence of a dose-response relationship within the range used here. Because this maze has been one of the most reliable tests of learning impairments found in developmental neurotoxicology, this effect should not be dismissed. It is our interpretation that exposure to high intensities of cw ultrasound induces sufficient central nervous system (CNS) effects to result in long-term complex maze learning impairments in rat offspring following prenatal exposure. The threshold for this effect, under the present experimental conditions, appears to lie between 20 and 30 W/cm^2, since no effects were seen on this test at any of the lower intensities, save one. There was a single trial (trial 1) effect seen in the US2 group for maze errors. That this effect did not appear in aggregate errors averaged across trials or in maze times makes it appear less reliable compared to the effect seen in the US30 group.

In a previous study (Vorhees et al., '91a), we found no evidence of embryotoxicity after prenatal exposure of rats to levels of cw ultrasound up to 30 W/cm^2 or to comparable pw exposures (Fisher et al., '94) equivalent to the highest exposure in the present study in terms of I_{SPTA}. Thus, by comparison of the morphological measures used in those experiments and the functional ones used herein, we conclude that CNS dysfunction may be more sensitive to the effects of high intensity prenatal ultrasound insonation than are measures of malformations or fetal or postnatal body weight, the latter thought by some to be the most sensitive index of prenatal effects.

Previous behavioral teratogenicity studies on ultrasound

There have been relatively few reports on the behavioral teratogenic potential of ultrasound exposure. Murai et al. ('75a) exposed gravid Wistar rats to Doppler ultrasound on E9 for 5 hr to 20 mW/cm^2 at 2.3 MHz. Rats were forcibly restrained by tightly wrapping them in wire mesh. Sham exposed and unrestrained control groups were included and a complex procedure was used which resulted in nine fostering conditions. A 0.3 day acceleration of eye opening was found, but the effect only occurred in relation to unrestrained controls. No effects on limb movement, hindleg movement, walking, surface righting, negative geotaxis, or cliff avoidance were found. Differences were found for the grasp reflex, vibrissa placing, visual placing, and air righting. However, only the delay in the grasp reflex was significant compared to restrained controls.

Murai et al. ('75b) tested the male offspring as adults. No effects on open-field ambulation or defecation were found. However, they reported that on the second and third days a higher percentage of the insonated group vocalized than either restrained or unrestrained controls. It was also found that in shock avoidance the insonated group spent more time on the unshocked side than the unrestrained controls, but not compared to the restrained controls, and the insonated group committed fewer crossovers than either control group. A vertical vs. horizontal stripe shock-escape visual cue discrimination test showed no group differences. While at first glance these data appear suggestive, the experiment reported in these papers has numerous methodological shortcomings: 1) despite nine fostering/crossfostering conditions, fostering was ignored as a factor in the data analyses; 2) the data were analyzed by subject without regard to litter membership, undoubtedly causing overestimations of the number of significant effects (Holson and Pearce, '92); 3) most of the differences were between the insonated and unrestrained controls, which means that these effects were due to restraint rather than ultrasound; 4) rats' abdomens were not depilated, a factor which undoubtedly resulted in an attenuated ultrasound signal; and 5) the few effects which occurred between the insonated and restrained controls were small and of doubtful significance.

Sikov et al. ('77) and Sikov and Hildebrand ('79) anesthetized gravid Wistar rats on E15 and exteriorized the uterus and exposed the fetuses to 0.01, 0.04, 0.71, 0.54, or 1.0 W/cm^2 of 0.93 MHz cw ultrasound for 5 min. They reported a delay in development of the grasp reflex on days 1 and 6, a delay in surface righting on day 6, a delay in head lifting and whole body lifting on day 13, and reduced hanging from a bar on day 15. This experiment had careful characterization of exposure parameters and used multiple groups at different intensities of ultrasound. Controls were appropriately sham treated. The problem with these results is that the findings are only descriptive and are reported using individual offspring as separate data points, with no allowance for litter membership. No tests of significance were provided. Group sizes were not indicated, the insonation method (direct exposure of exteriorized fetuses) was unusual, no tests of more complex functions were included, most of the findings were not dose-dependent, and no control for the separate effects of the anesthetic was included.

Tarantal and Hendrickx ('89a,b) exposed awake cynomolgus monkeys to 12 mW/cm^2 cw ultrasound at 7.5 MHz 5 times/week on E21–35 (10 min/exposure), 3

times/week on E36–60 (10 min/exposure), and once/week on E61–150 (20 min/exposure). Controls were sham exposed. Offspring (13 exposed and 10 controls) were followed for 1 year. The authors reported no effects on Brazelton-like neonatal assessments of behavioral state, reflexes, or habituation, but increased tone in the exposed group. During arena observations, the exposed offspring showed increased quiet activity, primarily sitting, during the first 5 weeks out of 14. No differences between groups were found on tests of object constancy, fine motor coordination, or discrimination-reversal learning. This experiment was carefully performed and the data appropriately analyzed. The insonation device was a clinical instrument and exposure was not fully characterized.

More recently, Norton et al. ('91) have reported on the effects of prenatal exposure to pw ultrasound of 0.78 W/cm^2 (I_{SPTA}) given for 30 min on day E14 (conception as E0) at 2.5 MHz to gravid CD rats. Sham exposed, anesthetic controls, and unexposed controls were included. Ultrasound-exposed offspring had significantly longer negative geotaxis times and longer reflex suspension times than either control group, but no differences in continuous corridor activity. On a test of gait, both the ultrasound group and the sham exposed group had longer stride length and a smaller angle of alternate strides than untreated controls. No histological changes in cortical layers were observed. Findings were based on nested analyses of variance with subject nested within litter. This experiment was well designed and adequate group sizes and data analyses were used. The data suggest that some reflex delays may be attributable to ultrasound, while other effects, such as those for gait, are more closely related to anesthesia than to ultrasound. Norton et al. ('91) also shaved their rats prior to insonation to eliminate hair as a source of signal attenuation; however, only one exposure intensity was used, therefore no dose-response information was obtained.

Finally, Hande et al. ('93) anesthetized Swiss mice with ketamine and exposed them to ultrasound using a clinical device on day 11.5 or 14.5 of gestation for 10 min at I_{SPTP} of 1 W/cm^2 with I_{SATA} of 240 mW/cm^2. The offspring were tested at 3 and 6 months. The day 14.5 exposed group showed reduced preference for the dark side of an open-field at 3 months of age, and both ultrasound groups showed reduced preference at 6 months. On passive avoidance, no differences were seen at 3 months, but at 6 months the day 14.5 group required more trials to remain passive than controls. No differences in 24 hr retention were found. The ultrasound exposure in this experiment was not thoroughly characterized and abdominal hair was not removed prior to exposure. The authors report the data analyzed by subjects, but state that it was also analyzed by litter. The by-litter F-tests were not shown, but given that most of the *P* values were close to 0.05 with degrees of freedom of 2/147, it is unlikely that these would be signficant with reduced degrees of freedom terms of 2/27 in a by-litter analysis.

Two other CNS effects of ultrasound have been reported. Norton et al. ('90) reported that the same exposure described by Norton et al. ('91) also caused increased nucleus sizes in neurons, increased numbers of pyknotic cells, increased numbers of macrophages, and decreased numbers of mitotic figures on E15, 24 hr after ultrasound exposure. In an experiment by Ellisman et al. ('87), postnatal day 3 and 5 Sprague-Dawley rats were exposed to pw ultrasound at 0.135 mW/cm^2 at 3.5 MHz for 30 min and dorsal root nerves from the spinal cord were examined by electron microscopy up to 24 hr later. Vacuoles and evidence of demyelinization were seen in 10 exposed offspring compared to 6 controls. No anesthesia was used, but the neonates were forcibly restrained.

Comparing these data to ours is difficult because either restraint or anesthesia was present in all of these previous studies. The morphological results of Norton et al. ('91) suggest adverse effects, but their neurobehavioral findings are mixed and not clearly indicative of an adverse change. The data of Tarantal and Hendrickx ('89b) are limited and the interpretation is too uncertain to indicate adverse effects, while the data of Murai ('75a,b) and Sikov and Hildebrand ('79) fundamentally show no effects. The data of Ellisman et al. ('87) show effects but the sample size was small, the insonation incompletely characterized, and no effects beyond 24 hr were shown. Therefore, our findings at 30 W/cm^2 on water maze learning and activity and the data of Tarantal and Hendrickx ('89a,b) in primates are the most suggestive that exist for long-term CNS effects from prenatal ultrasound exposure. However, at this juncture, both experimental approaches need replicating prior to any conclusion that these effects are reliable indices of ultrasound's effects on brain development.

The meaning of such data for humans is at this point unclear. Stark et al. ('84) raised concern about ultrasound's effects on the brain when they reported more cases of dyslexia in exposed than in unexposed 7- and 12-year-old children, even though they found no effects on cognitive outcome. Recently, Salvesen et al. ('92) completed two randomized clinical trials in Norway and followed the children to 8–9 years of age and found no association between prenatal ultrasound exposure and dyslexia, nor any effects on reading or spelling attainment or on measures of intelligence. However, this same group (Salvesen et al., '93) has reported a small, but significant increase in non-right-handed dominance in children at this age. While the authors caution that the latter finding might not be meaningful and it is not clear how to interpret a shift in hand dominance, it indicates that the effects of prenatal ultrasound on brain development remain incompletely resolved.

ACKNOWLEDGMENTS

This research was supported by U.S. Public Health Service grants HD21806 and training grant ES07051 (K.D.A.-S., M.A.S., J.E.F.) from the National Institutes of Health.

REFERENCES

American Institute of Ultrasound in Medicine (AIUM) (1988) Bioeffects considerations for the safety of diagnostic ultrasound. J. Ultrasound Med., 7(Suppl.):S1–S38.

American Institute of Ultrasound in Medicine (AIUM) (1993) Bioeffects and safety of diagnostic ultrasound. AIUM Publication, Rockville, MD.

Brent, R.L., R.P. Jensh, and D.A. Beckman (1991) Medical sonography: Reproductive effects and risks. Teratology, *44:*123–146.

Carstensen, E.L., and A.H. Gates (1985) Ultrasound and the fetus. In: Biological Effects of Ultrasound. W.L. Nyborg and M.C. Ziskin, eds. Churchill Livingstone, New York, pp. 85–95.

Child, S.Z., E.L. Carstensen, and H. Davis (1984) A test for the effects of low-temporal-average-intensity pulsed ultrasound on the rat fetus. Exp. Cell Biol., *52:*207–210.

Child, S.Z., D. Hoffman, D. Strassner, E.L. Carstensen, A.H. Gates, C. Cox, and M.W. Miller (1989) A test of I^2T as a dose parameter for fetal weight reduction from exposure to ultrasound. Ultrasound Med. Biol., *15:*39–44.

Ellis, D.S. (1991) The general solution for estimating ultrasonically induced tissue heating. Master of Science Thesis in Electrical Engineering, University of Illinois, Urbana.

Ellisman, M.H., D.E. Palmer, and M.P. Andre (1987) Diagnostic levels of ultrasound may disrupt myelination. Exp. Neurol., *98:*78–92.

Ewigman, B.G., J.P. Crane, F.D. Frigoletto, M.L. LeFevre, R.P. Bain, D. McNellis, et al. (1993) Effect of prenatal ultrasound screening on perinatal outcome. N. Engl. J. Med., *329:*821–827.

Fisher, J.E., Jr., K.D. Acuff-Smith, M.A. Schilling, C.V. Vorhees, R.A. Meyer, N.B. Smith, and W.D. O'Brien, Jr. (1994) Teratologic evaluation of rats prenatally exposed to pulsed-wave ultrasound. Teratology, *49:*150–155.

Food and Drug Administration (FDA) (1985) Guide for measuring and reporting acoustic output of diagnostic ultrasound medical devices. Document 510(k). U.S. Department of Health and Human Services, FDA, Center for Devices and Radiological Health, Rockville, MD.

Food and Drug Administration (FDA) (1993) Revised 510(k) diagnostic ultrasound guidance for 1993. U.S. Department of Health and Human Services, FDA, Center for Devices and Radiological Health, Rockville, MD.

Hande, M.P., and P.U. Devi (1992) Effect of prenatal exposure to diagnostic ultrasound on the development of mice. Radiat. Res., *130:* 125–128.

Hande, M.P., and P.U. Devi (1993) Effect of in utero exposure to diagnostic ultrasound on the postnatal survival and growth of mouse. Teratology, *48:*405–411.

Hande, M.P., P.U. Devi, and K.S. Karanth (1993) Effect of prenatal ultrasound exposure on adult behavior in mice. Neurotoxicol. Teratol., *15:*433–438.

Holson, R.R., and B. Pearce (1992) Principles and pitfalls in the analysis of prenatal treatment effects in multiparous species. Neurotoxicol. Teratol., *14:*221–228.

Kimmel, C.A., M.E. Stratmeyer, W.D. Galloway, J.B. LaBorde, N. Brown, and F. Pinkavitch (1983) The embryotoxic effects of ultrasound exposure in pregnant ICR mice. Teratology, *27:*245–251.

Kimmel, C.A., M.E. Stratmeyer, W.D. Galloway, N.T. Brown, J.B. LaBorde, and H.K. Bates (1989) Developmental exposure of mice to pulsed ultrasound. Teratology, *40:*387–393.

Mannor, S.M., D.M. Serr, S. Tamari, A. Meshorer, and E.H. Frei (1972) The safety of ultrasound in fetal monitoring. Am. J. Obstet. Gynecol., *113:*653–661.

Martin, A.O., E.L. Madsen, A.R. Dyer, L. White, N.P. Bouck, R.E. Sabbagha, M. Hermanoff, J.M. Chen, and L.J. Ludtke (1991) Sister chromatid exchange analysis of human cells exposed to diagnostic levels of ultrasound. J. Ultrasound Med., *10:*665–670.

Mazze, R.I., A.I. Wilson, S.A. Rice, and J.M. Baden (1985) Fetal development in mice exposed to isoflurane. Teratology, *32:*339–345.

McClain, R.M., R.M. Hoar, and M.B. Saltzman (1972) Teratologic study of rats exposed to ultrasound. Am. J. Obstet. Gynecol., *114:* 39–42.

Miller, D.L. (1991) Update on safety of diagnostic ultrasonography. Clin. Ultrasound, *19:*531–540.

Miller, M.W., and M.C. Ziskin (1989) Biological consequences of hyperthermia. Ultrasound Med. Biol., *15:*707–722.

Murai, N., K. Hoshi, and T. Nakamura (1975a) Effects of diagnostic ultrasound irradiated during fetal stage on development of orienting behavior and reflex ontogeny in rats. Tohoku J. Exp. Med., *116:*17–24.

Murai, N., K. Hoshi, C.-H. Kang, and M. Suzuki (1975b) Effects of diagnostic ultrasound irradiated during foetal stage on emotional and cognitive behavior in rats. Tohoku J. Exp. Med., *117:*225–235.

National Council on Radiation Protection and Measurements (NCRP) (1992) Exposure criteria for medical diagnostic ultrasound. I. Criteria based on thermal mechanisms. NCRP Report No. 113, Bethesda, MD.

National Institutes of Health (NIH) (1984) Diagnostic ultrasound imaging in pregnancy. Report of a Consensus Development Conference. NIH Publication No. 84-667. U.S. Government Printing Office, Washington, DC.

Norton, S., B.F. Kimler, E.P. Cytacki, and S.J. Rosenthal (1990) Acute response of fetal rat telencephalon to ultrasound exposure in utero. Exp. Neurol., *107:*154–163.

Norton, S., B.F. Kimler, E.P. Cytacki, and S.J. Rosenthal (1991) Prenatal and postnatal consequences in the brain and behavior of rats exposed to ultrasound in utero. J. Ultrasound Med., *10:*69–75.

Nyborg, W.L. (1988) Solutions of the bio-heat transfer equation. Phys. Med. Biol., *33:*785–792.

O'Brien, W.D. (1983) Dose-dependent effect of ultrasound on fetal weight in mice. J. Ultrasound Med., *2:*1–8.

O'Brien, W.D., Jr. (1985) Biological effects in laboratory animals. In: Biological Effects of Ultrasound. W.L. Nyborg and M.C. Ziskin, eds. Churchill Livingstone, New York, pp. 77–84.

O'Brien, W.D., Jr. (1992) Ultrasound dosimetry and interaction mechanisms. In: Non-Ionizing Radiation: Proceedings of the Second International Non-Ionizing Radiation Workshop. M.W. Greene, ed. Canadian Radiation Protection Association, pp. 151–172.

O'Brien, W.D., Jr., S.J. Januzik, and F. Dunn (1982) Ultrasound biologic effects: A suggestion of strain specificity. J. Ultrasound Med., *1:*367–370.

Pennes, H.H. (1948) Analysis of tissue and arterial blood temperatures in the resting human forearm. J. Appl. Physiol., *1:*93–122.

Pizzarello, D.J., A. Vivino, B. Madden, A. Wolsky, A.E. Keegan, and M. Becker (1978) Effect of pulsed, low-power ultrasound on growing tissues. Exp. Cell Biol., *46:*179–191.

Salvesen, K.A., L.S. Bakketeig, S.H. Eik-Nes, J.O. Undheim, and O. Okland (1992) Routine ultrasonography in utero and school performance at age 8–9 years. Lancet, *339:*85–89.

Salvesen, K.A., L.J. Vatten, S.H. Eik-Nes, K. Hugdahl, and L.S. Bakketeig (1993) Routine ultrasonography in utero and subsequent handedness and neurological development. Br. Med. J., *307:*159–164.

Sarvazyan, A.P., L.V. Beloussov, M.N. Petropavlovskaya, and T.V. Ostrousmova (1982) The action of low-intensity pulsed ultrasound on amphibian embryonic tissues. Ultrasound Med. Biol., *8:*639–654.

Shoji, R., U. Murakami, and T. Shimizu (1975) Influence of low-intensity ultrasonic irradiation on prenatal development of two inbred mouse strains. Teratology, *12:*227–232.

Sikov, M.R., and B.P. Hildebrand (1976) Effects of ultrasound on the prenatal development of the rat. I. 3.2 MHz continuous wave at nine days of gestation. J. Clin. Ultrasound., *4:*357–363.

Sikov, M.R., and B.P. Hildebrand (1979) Effects of prenatal exposure to ultrasound. In: Advances in the Study of Birth Defects, Vol. 2,

Teratological Testing. T.V.N. Persaud, ed. University Park Press, Baltimore, pp. 267–291.

Sikov, M.R., B.P. Hildebrand, and J.D. Stearns (1977) Postnatal sequelae of ultrasound exposure at 15 days of gestation in the rat (work in progress). In: Ultrasound in Medicine, Vol. 3B, Engineering Aspects. D. White and R. Brown, eds. Plenum Press, New York, pp. 2017–2023.

Smith, N.B., C.V. Vorhees, R.A. Meyer, and W.D. O'Brien, Jr. (1990) An automated ultrasonic exposure system to assess the effects of in utero diagnostic ultrasound. IEEE 1990 Ultrasonic Symposium Proceedings. Institute of Electrical and Electronics Engineers, New York, pp. 1385–1388.

Stark, C.R., M. Orleans, A.D. Haverkamp, and J. Murphy (1984) Short- and long-term risks after exposure to diagnostic ultrasound in utero. Obstet. Gynecol., *63:*194–200.

Stolzenberg, S.J., C.A. Tobit, P.D. Edwards, and J.C. Taenzer (1980) Effects of ultrasound on the mouse exposed at different stages of gestation: Acute studies. Radiat. Environ. Biophys., *17:*245–270.

Takabayashi, T., S. Sato, A. Sato, N. Ozawa, S. Sou, A. Yajima, and M. Suzuki (1985) Influence of pulse-wave ultrasonic irradiation on the prenatal development of mouse. Tohuku J. Exp. Med., *147:*403–410.

Tarantal, A.F., and A.G. Hendrickx (1989a) Evaluation of the bioeffects of prenatal ultrasound exposure in the cynomolgus macaque *(Macaca fascicularis).* I. Neonatal/infant observations. Teratology, *39:*137–147.

Tarantal, A.F., and A.G. Hendrickx (1989b) Evaluation of the bioeffects of ultrasound exposure in the cynomolgus macaque *(Macaca fascicularis).* II. Growth and behavior during the first year. Teratology, *39:*149–162.

Tarantal, A.F., and W.D. O'Brien, Jr. (1994) Discussion of ultrasonic safety related to obstetrics. In: Diagnostic Ultrasound Applied to Obstetrics and Gynecology. R.E. Sabbagha, ed. J.B. Lippincott, Philadelphia, 3rd Ed., pp. 45–56.

Vorhees, C.V. (1983) Fetal anticonvulsant syndrome in rats: Dose- and period-response relationships of prenatal diphenylhydantoin, trimethadione, and phenobarbital exposure on the structural and functional development of the offspring. J. Pharmacol. Exp. Ther., *227:*274–287.

Vorhees, C.V. (1987) Maze learning in rats: A comparison of performance in two water mazes in progeny prenatally exposed to different doses of phenytoin. Neurotoxicol. Teratol., *9:*235–241.

Vorhees, C.V., K.D. Acuff-Smith, W.P. Weisenburger, R.A. Meyer, N.B. Smith, and W.D. O'Brien, Jr. (1991a) A teratologic evaluation of continuous-wave, daily ultrasound exposure in unanesthetized pregnant rats. Teratology, *44:*667–674.

Vorhees, C.V., W.P. Weisenburger, K.D. Acuff-Smith, and D.R. Minck (1991b) An analysis of factors influencing complex water maze learning in rats: Effects of task complexity, path order and escape assistance on performance following prenatal exposure to phenytoin. Neurotoxicol. Teratol., *13:*213–222.

Vorhees, C.V., K.D. Acuff-Smith, D.R. Minck, and R.E. Butcher (1992) A method for measuring locomotor behavior in rodents: Contrast-sensitive computer-controlled video tracking activity assessments in rats. Neurotoxicol. Teratol., *14:*43–49.

Vorhees, C.V., K.D. Acuff-Smith, M.S. Moran, and D.R. Minck (1994) A new method for evaluating air-righting reflex ontogeny in rats using prenatal exposure to phenytoin to demonstrate delayed development. Neurotoxicol. Teratol., *16:*in press.

Weinstock, M., E. Fride, and R. Hertzberg (1988) Prenatal stress effects on functional development of the offspring. Prog. Brain Res., *73:*319–331.

Ziskin, M.C., and D.B. Petitti (1988) Epidemiology of human exposure to ultrasound: A critical review. Ultrasound Med. Biol., *14:*91–96.