The developing nervous system has been identified as a potential target of pesticide exposure. Heptachlor is a cyclodiene pesticide that was widely used for many years, and for which inadvertent exposure to children and fetuses took place in the early 1980s; yet little is known regarding the developmental neurotoxicity of it and other cyclodiennes. The aim of this study was to determine whether perinatal heptachlor exposure results in persistent alterations in nervous system function. Pregnant Sprague-Dawley dams were dosed from gestational day (GD) 12 to postnatal day (PND) 7, whereupon the rat pups were dosed directly until PND 21 (group A) or PND 42 (group B). Dose levels were 0, 0.03, 0.3, or 3 mg/kg/day, po. There were no dose-related effects on maternal weight, litter size, or pup growth. GABAA receptor binding (using \([\text{S}^35\text{S}]\) tert-butylbicyclophosphorothionate; TBPS) and GABA-stimulated Cl flux were evaluated in control and high-dose brain tissues taken on PND 7, 21, and 43. The \(B_{\text{max}}\) values for \([\text{S}^35\text{S}]\)-TBPS binding in brainstem, but not cortex, were decreased in female rats across all ages tested. There were no such changes in male rats, nor were \(K_{\text{d}}\) values altered in either tissue or gender. GABA-stimulated Cl flux was decreased in female cortex synaptoneurosomes only on PND 21. The ontogeny of the righting response (PND 2–5) was delayed in the high-dose females. All subsequent testing took place a week to months after dosing ceased. The functional observational battery (FOB) showed treatment-related, but not necessarily dose-related, changes in different aspects of the rat’s reactivity and activity levels. Group-A rats also showed altered within-session habituation of motor activity. There were no heptachlor-related differences in motor activity following challenge with a range of chlor Diazepoxide doses. Cognitive assessments were conducted in both groups of rats. There were no statistically significant differences among treatment groups in a one-trial passive avoidance test, although there was a trend toward less learning. In group B, rats (both sexes), heptachlor altered spatial learning in the Morris water maze during two weeks of daily training (2 trials/day). On probe trials, heptachlor-treated rats did not show significant preference for the correct quadrant (all dose groups in males, high dose in females). These rats did not show alterations on subsequent working-memory training (where the platform position was relearned each day). Thus, perinatal exposure to heptachlor produced neurochemical and persistent neurobehavioral changes, including alterations in spatial learning and memory.

**Key Words:** heptachlor; developmental neurotoxicity; Morris water maze; pesticide exposure.

Specific human populations may be more susceptible to the neurotoxicity of certain chemicals. Recently, there has been growing scientific and public concern over the issue of increased susceptibility of infants and children to the toxic effects of chemicals in the environment. The nervous system was identified as a potential target of pesticide exposure in the 1993 National Research Council report entitled *Pesticides in the Diets of Infants and Children* (NRC, 1993). The young developing organism may be affected more adversely by chemical exposure than adults, due to the unique stage of development of the nervous system at the time of exposure. Development of the nervous system is quite protracted, and specific processes of migration, proliferation, and differentiation occur from gestation throughout childhood and into adolescence. These processes follow an orchestrated schedule of development, and disruption by chemical exposure at any point along this time line may adversely affect both current and subsequent processes. The temporal schedule for development leads to critical periods of vulnerability, which differ depending on the neuronal target (for review, see Rice and Barone, 2000).

Different neurotransmitter systems act in specific ways to influence these developmental processes. One such transmitter is γ-aminobutyric acid (GABA), the actions of which have been reviewed recently (Lauder et al., 1998). GABA appears in the nervous system very early in gestation, and is widespread in the developing brain (Lauder et al., 1986). It is evident that GABA serves as a trophic signal which influences the develop-
opment of almost all neurotransmitter systems, including serotonergic, dopaminergic, and cholinergic; this action is mediated through GABA<sub>\alpha</sub> receptors (Kenigsberg et al., 1998; Lauder et al., 1986, 1998; Liu et al., 1997a). Thus, exposure to chemicals that act on the GABAergic system via the GABA<sub>\alpha</sub> receptor may well alter development of many different neurotransmitter systems.

The GABA<sub>\alpha</sub> receptor is a ligand-gated Cl<sup>-</sup> channel which, when activated by GABA, increases Cl<sup>-</sup> conductance into the neuron (Sieghart, 1995). Cyclodiene pesticides have been shown to act on the GABA<sub>\alpha</sub> receptor, by binding at the Cl<sup>-</sup> channel portion of the receptor and thereby blocking the inhibitory actions of GABA (Abalis et al., 1986; Cole and Casida, 1986; Gant et al., 1987; Lawrence and Casida, 1984). Acute actions of cyclodiene pesticides include excitation, hypotension, and convulsions (Cole and Casida, 1986; Fendick et al., 1990). Evidence is accumulating that nervous system development is influenced by cyclodiene pesticides. For example, gestational exposure to dieldrin and lindane decreases GABA<sub>\alpha</sub> receptor expression (Braunen et al., 1998; Lauder et al., 1998; Liu et al., 1997b; 1998). Furthermore, perinatal exposures to lindane and aldrin have been reported to lower seizure threshold (Albertson et al., 1985; Castro et al., 1992), and exposure to endrin and chlordane causes neurobehavioral alterations (Cassidy et al., 1994; Gray et al., 1981).

Heptachlor is a cyclodiene pesticide that was widely used for many years, until concerns regarding carcinogenicity potential and environmental persistence limited its use in the U.S. (Fendick et al., 1990). In 1980–1982, residents on the Hawaiian island of Oahu were inadvertently exposed to high levels of heptachlor in the milk supply; the source was found to be dairy cows fed with green chop gathered from pineapple fields which had been treated with heptachlor (Baker et al., 1991; Smith, 1982). Concern has been raised regarding the developmental outcomes of the offspring from that time, including those exposed in utero and children who consumed the dairy milk. Even though heptachlor has not been used in the U.S. for over a decade, the most recent report of the Pesticide Data Program (USDA, 1997) cited up to 29% of certain food samples with detectable levels of heptachlor epoxide, the most stable metabolite of heptachlor (Fendick et al., 1990). Thus, the potential for adverse neurological effects following developmental heptachlor exposure is still a concern in the U.S. In addition, there are other cyclodiennes (e.g., lindane, dieldrin, and endosulfan) that are currently in use for agricultural and medicinal purposes, for which only limited data regarding potential developmental outcomes are available.

The design of this study was to simulate, in the rat, the exposure period of concern in humans, which ranges from late gestation to approximately age 18 (NRC, 1993). Doses were set so that the low dose (0.03 mg/kg/d) produced heptachlor and heptachlor epoxide levels in rat dam milk that matched the 95th percentile of human milk values on Oahu, Hawaii in 1981 (Siegel, 1988). The aim of these studies was to determine if exposure of rats, during the last trimester of gestation through weaning or puberty, affects adversely the adult function of the nervous, immune, and/or reproductive systems. Results of the immunological and reproductive evaluations are reported elsewhere (Smialowicz et al., in press). The neurotoxicological studies included screening evaluations (functional observational battery, motor activity, righting reflex ontogeny), cognitive tests (associative and non-associative learning, spatial learning and memory, and working memory), and measures of GABA<sub>\alpha</sub> receptor function (chloride flux, benzodiazepine challenge) and expression (receptor binding).

**MATERIALS AND METHODS**

**Chemicals.** Heptachlor (99% purity, verified by GC/FID; Radian Corp., Austin, TX) was dissolved in corn oil. Dosing solutions were prepared and analyzed during a Midwest Research Institute under contract to NIEHS (NBS-ES-55385). All dosing solutions were found to be ±2.5–5% of target values. Chlordiazepoxide HCl (USP purity) was purchased from RBI (Natick, MA).

For the neurochemical assays, [35S] tert-butylbicyclophosphorothionate ([35S]-TBPS; 114 Ci/mmole) and [3]Cl (13.39 µCi/mg) were obtained from New England Nuclear (Boston, MA). Unlabeled TBPS was obtained from RBI (Natick, MA). All other chemicals were obtained from commercial sources and were of the highest available grade.

**Animals.** Pregnant Sprague-Dawley rats (Tac:N(SD)FBR) were received from Taconic Farms (Germantown, NY) on gestational day 4–5. Upon receipt, dams were acclimated for ~7 days before dosing began. Dams (n = 15–20/dose) were assigned to treatment groups by stratified randomization, prior to dosing, to assure equivalent body weight means across groups. Some rats were either not pregnant or did not deliver (incidence not dose-related), and a total of 67 littles were used for the remainder of the study: 16 controls, 19 at 0.03 mg/kg/d, 15 at 0.3 mg/kg/d, and 17 at 3 mg/kg/d.

During dosing, rats were housed in polycarbonate cages in the NIEHS animal facility with a 12:12 h light:dark cycle, and were maintained at 20 ± 1°C and 55 ± 10% humidity. They were allowed ad libitum access to NIH-07 certified feed (Zeigler Bros., Inc., Gardners, PA) and deionized water. The day of birth was designated as PND 0, and all rats were weaned on PND 21. After weaning, group B rats were group-housed with same-sex littermates while dosing continued. After dosing ended (on either PND 21 or PND 42), 2 sets of offspring were transferred to the U.S. EPA facility, which had similar environmental conditions, except that they were switched to Purina Rat Chow #5001 (Ralston Purina Co., St. Louis, MO) and singly-housed. These rats were allowed at least one week to acclimate after moving. Thus, all dosing and the righting reflex testing took place in the NIEHS facility, and all subsequent tests took place at the U.S. EPA.

**Experimental design.** The general design of this collaborative study is described in Chapin et al. (1997). Pregnant F<sub>0</sub> dams were orally dosed with either 0, 0.03, 0.3, or 3 mg/kg/day from gestational day (GID) 12 to postnatal day (PND) 7. The pups were then dosed directly using the same dose levels, until either PND 21 (group A) or PND 42 (group B). Litters were standardized to 5 males and 5 females on PND 7. On that day, a separate set of dams and litters (n = 5–6/dose level) were used to determine the amounts of heptachlor and heptachlor epoxide in milk, plasma, and liver of dams, and in brain, plasma, and liver of pups. The analytical methods have been described in Clark et al. (2000). The day after birth (PND 1), each pup was marked with a toe tattoo, and an individual identification number was made by tail tattoo at weaning on PND 21. For neurobehavioral evaluations, one male and one female from 10 litters were used for each dose group; due to experimental constraints, some litters provided pups for both groups A and B. Other pups (littermates) and litters were sacrificed on certain days for evaluation of the GABA system, for lactational assessment, or for the study of immunotoxicity and reproductive effects (Smialowicz et al., in press).
strength, landing foot splay, rectal temperature, and body weight were quantified using appropriate devices. The same observer conducted all tests, and was blind with respect to the dose levels.

Water maze testing, a chlordiazepoxide dose-response curve for motor activity was determined during 30-min sessions. All rats received all chlordiazepoxide doses (0, 4, 10, and 18 mg/kg i.p.), in a counterbalanced order, 40 min before testing. Dosing occurred on Tuesdays and Fridays for 2 weeks.

Neurochemical evaluations. GABA<sub>A</sub> receptor expression and GABA-stimulated Cl⁻ flux were measured in control and high-dose rats. Male and female pups were sacrificed on separate days, but at the same postnatal age. The brain was removed quickly after decapitation and dissected freehand (Glowinski and Iversen, 1966) into brainstem (pons and medulla; cerebellum removed) and cortex. The Cl⁻ flux assay was conducted on fresh tissue (the same day as the tissue collection), whereas brain regions for receptor binding studies were frozen at –80°C for later analysis.

Synaptoneurosomes (n = 6/sex, control and 3 mg/kg/day) were prepared using the protocol of Schwartz and coworkers (Schwartz et al., 1986). Unless otherwise noted, all procedures were carried out at 4°C. Preliminary experimen-
ments indicated that GABA-simulated levels of Cl⁻ flux in the brainstem was too low to be measured reliably; therefore, only cortex was used. Two cortices/sex/litter from PND 7 rat pups were weighed and pooled for the flux assay, whereas, one cortex/sex/litter and 1 cortex/sex/litter were used for the flux assay on PND 21 and 43, respectively. Cortices were homogenized by hand in a glass-glass dounce with a tight pestle insert (12 strokes) in 10–15 volumes of flux buffer solution (pH 7.4) containing HEPES, 20 mM; NaCl, 118 mM; KCl, 4.7 mM; MgSO₄, 1.18 mM; CaCl₂, 2.5 mM; and glucose, 10 mM. The homogenate was filtered through 3 layers of 100 μm Nitex screen and centrifuged 15 min at 1000 × g. The pellet was gently resuspended in 30 ml flux buffer using a glass-glass dounce with a loose pestle insert (6 strokes). The synaptoneurosomes were pelleted as above and washed twice more in flux buffer. The final pellet was resuspended in flux buffer at approximately 5 mg protein per ml, and an aliquot was saved for protein determination (Bradford, 1976).

Synaptoneurosomes were aliquoted (300 μl; ~1 mg protein) into test tubes and preincubated for 12 min in a 30°C water bath. At the end of the preincubation period, 200 μl ³⁵Cl⁻ (0.2 μCi per sample) ± GABA (50 μM) was added to the synaptoneurosomes, vortexed, and incubated 5 s, as measured by a metronome. Influx of ³⁵Cl⁻ was stopped by adding 4 ml ice-cold flux buffer, and filtering immediately under vacuum through S&S #30 filters, presoaked in 0.1% BSA in flux buffer. Each sample tube and filter were rinsed with 2 additional 4-ml aliquots of ice-cold flux buffer. The filters were placed into scintillation vials with UltimaGold™, and the radioactivity on the filters was determined using a Wallac Model 1410 Liquid Scintillation Counter. Net GABA-stimulated flux (nmol Cl⁻/mg protein) was determined by subtracting Cl⁻ flux measured in the absence of GABA (basal flux) from GABA-stimulated flux.

For receptor binding, membrane fractions were prepared by modifications on the method of Ito et al. (1989). The brain sections were homogenized in 10 volumes of ice-cold 0.32 M sucrose and centrifuged at 1000 rpm for 10 min at room temperature. Thereafter the sample was re-cooled on ice and all subsequent steps conducted at 4°C (unless otherwise stated). The pellet was resuspended in 10 vol. of distilled water, allowed to stand for 10 min, and centrifuged for 30 min at 35,000 × g. The pellet was resuspended in assay buffer (KH₂PO₄, 40 mM; KCl, 100 mM; pH 7.4, 25°C), incubated at 35°C for 30 min, and again centrifuged at 35,000 × g for 30 min. The pellet was resuspended in assay buffer and frozen at –80°C until needed.

The binding assay was carried out in assay buffer containing TBPS (2 nM radiolabeled TBPS plus 2, 4, 10, 20, 40, 100, 200, or 400 nM nonlabeled TBPS; one tube/concentration) and membrane solution (0.1–0.5 mg protein). The solution was incubated for 2 h at 24°C. Incubation was terminated by filtration onto glass fiber filters and rinsing rapidly with 1.5 ml ice-cold assay buffer using a Skatron filtration apparatus. Nonspecific binding was determined by the presence of 100 μM picrotoxin.

Protein content was determined using the Bradford method (Bradford, 1976). Specific binding was determined by subtracting nonspecific binding from the total. The values for Kᵣ and Bₘₐₓ were calculated by fitting the data (specific binding vs. free TBPS) with a nonlinear regression curve (all r² values > 0.95) using GraphPad Prism software (San Diego, CA).

Data analysis. For analysis of most behavioral tests (to the extent possible, with sample size and available procedure limitations) littermates were taken into account in the statistical model. In general, analyses of variance (ANOVA) were conducted using grouping factors of dose, repeated testing across days, and sex nested as a within-litter factor. Step-down ANOVAs were conducted as warranted. For the righting reflex, pups within a litter were considered as one observation. Data from groups A and B were considered as separate experiments. Continuous data were analyzed by a general linear model (GLM; SAS, 1990). Rank-order data were analyzed using a categorical modeling procedure (CATMOD; SAS, 1990) that fits linear models to functions of response frequencies, which is then analyzed by weighted regression. In all cases, resulting probability values < 0.05 were considered significant.

For the righting reflex, pups within a litter were considered as one observation. Data from groups A and B were considered as separate experiments. Continuous data were analyzed by a general linear model (GLM; SAS, 1990). Rank-order data were analyzed using a categorical modeling procedure (CATMOD; SAS, 1990) that fits linear models to functions of response frequencies, which is then analyzed by weighted regression. In all cases, resulting probability values < 0.05 were considered significant.

The FOB (which included motor activity) data were summarized using a severity scoring procedure that combines scores for the specific domains of neurological function. The purpose of this procedure was to focus attention on the overall functions affected, and to minimize the number of overall analyses conducted and thus the likelihood of false positives. This method of analysis mathematically normalized the individual data for all endpoints to a 1-to-4 scale (procedure described in McDaniel and Moser, 1993; Moser, 1995; Moser et al., 1995). Score assignments were based on the mean and standard deviation of the control groups for continuous data, and derived from the frequency of occurrence of each rank for ordinal variables. These scores were then summed across measures that comprised each domain, and subjected to analysis; square root-transformed scores were used when variances were not homogeneous across dose groups. Only if the domain scores were significant overall were the individual measures that comprise that domain then analyzed. Analysis of habituation of motor activity (counts across intervals of the session) were analyzed independently of the FOB parameters.

Two-way ANOVA was used to analyze the chloridiazepoxide challenge data, with heptachlor treatment as a group factor and repeated-measures on chloridiazepoxide dose (all rats received all doses). Passive avoidance latency (in s) to cross into the darkened side were analyzed using the Kruskal-Wallis one-way ANOVA for non-parametric data (NPARIWAY; SAS, 1990), for both the training session and the 24-h retention trial. The frequency of nose pokes and half-body crosses during the 24-h test were also analyzed (FREQ; SAS, 1990).

Acquisition in the water maze was analyzed with an ANOVA, using dose and platform position between subjects, repeated measures of trial nested within daily blocks, and sex nested within litter. There were no trial-by-block interactions, so subsequent analyses utilized block data. For most endpoints, there was no influence of platform position. Dependent variables were: latency and path length taken to find the platform, swim speed, and percent time spent in the outer and middle zones (tank divided into 3 concentric zones, platform located in the middle zone). Working memory was evaluated by latency in trials nested within daily blocks.

For the probe trials, the dependent variables were percent path length and proximity measure in each of the 4 quadrants. Quadrant measures were analyzed using a within-subject ANOVA for each dose group; significant quadrant bias indicated that the rats localized their swim, and did not randomly swim throughout the tank.

TBPS binding data (Kᵣ and Bₘₐₓ) for each brain region were analyzed with a 2-way ANOVA of treatment and age, followed by step-down analyses as warranted. Chloride flux experiments were analyzed using a split-plot design, with the experimental day as the main-plot and the subject as the sub-plot. The effects of age and sex could not be tested directly, since each age (PND 7, 21, and 43) and sex combination was used on only one day, and thus the experiment had only one replication of the main-plot treatments. Within each main-plot, the treatment effect and interactions with treatment and age were tested.

RESULTS

Developmental and growth indices. Heptachlor had no influence on maternal weight gain or any pregnancy outcomes. Litter size, pup survival, and growth (body weight pre- and post-weaning) were not significantly altered. These data are detailed in Smialowicz et al. (in press). In addition, there was no effect of treatment on body weight at any time throughout the course of neurobehavioral testing.

Brain levels. Heptachlor was not detected above the limit of quantitation in rat pup brain. The concentration of heptachlor epoxide was proportional to dose (levels in ng/g tissue for ascending dose groups, mean ± SEM, n = 5–6/dose: 20.4 ± 2.7, 184.5 ± 11.1, and 1927.2 ± 214.1). Thus, there was a
significant exposure of the rat pups even before direct dosing began.

**Righting reflex.** Righting was slowed by perinatal exposure to heptachlor in female, but not male, rats. The data revealed significant interactions between sex, dose, and day of testing (all \( p \)-values < 0.03), so ANOVAs were conducted for males and females separately. These analyses revealed no dose effect in males, but an overall significant main effect of dose in females (no interaction with time). Comparison of dose groups with the control indicated that the high-dose group had slower latency to right across all days. The data on each day of testing for both males and females are presented in Figure 1. It is apparent that even the high-dose group was righting at a greater speed each day, implying that heptachlor caused a developmental delay in the ontogeny of the behavior rather than the inability to perform the task.

**FOB/motor activity.** Neurobehavioral alterations were detected with the FOB and motor activity tests in both groups of heptachlor-treated rats, but they were differentially affected depending on gender and the length of dosing. Group A, whose exposure ended on PND 21, showed changes in the activity domain, whereas group B showed alterations in the excitability, autonomic, and neuromuscular domains. Note that sensorimotor function was not altered, convulsive or involuntary movements were never detected, and there were no differences in body weight, temperature, or general health indicators (e.g., piloerection, body tone, etc.).

Rats dosed until PND 21 showed a significant dose-by-sex interaction on the activity domain; thus, individual endpoints were analyzed separately by sex. In male rats, increased open-field activity was evident in the high-dose group (\( p = 0.001 \)); 50% of the high dose group received the highest activity score (mean rank = 4.4) compared to controls, for which only 20% received the highest score (mean rank = 3.8). Females rats showed increased activity in the figure-eight chambers (\( p = 0.004 \)), although the finding was not dose-dependent. Only the low-dose group had significantly higher counts (121% of control values), although the mean values for all dose groups were numerically higher than controls.

Group-B rats, dosed until PND 42, showed no changes in activity measures, but instead had alterations in other functional domains—autonomic (dose-by-sex interaction), neuromuscular (main effect of dose), and excitability (main effect of dose). The only change in autonomic measures in males was increased levels of urination (overall dose \( p = 0.01 \)), which was significant only in the lower-dose groups (0.03 and 0.3 mg/kg/day). These differences were somewhat small (65% of rats showing little or no urination during the 3-min open-field observation, compared to 95% of controls and 85% of high-dose rats). There were significant gender differences in one neuromuscular measure, landing foot splay. Step-down analyses revealed increased splay (31% higher than controls) in males, but only in the 0.03-mg/kg/day dose group. Finally, there were no gender differences in the increased score for removal reactivity, although again only the lowest-dose group was significant (25% of rats showing high reactivity, compared to 0–5% in the other treatment groups).

Habituation of activity (change in activity over the 1-h session) was significantly altered in the group-A rats. The pattern of habituation was gender-specific (significant dose-by-sex-by-interval), and high-dose males showed a faster decline in activity, but low-dose females showed less habituation compared to controls. There were no effects on this measure in group-B rats.

**Passive avoidance.** Latencies to cross into the darkened chamber in the initial training trial were similar across all treatment groups (data not shown), and the 24-h retention test data are presented in Figure 2. These data showed that 3 to 4
rats in the treated groups (0.3, 3 mg/kg/day) crossed in less than 300 s, which was a higher number than in controls (usually one rat). This trend was most evident in female rats, and in male rats treated to PND 21. There were, however, no significant differences across treatment groups (Kruskal-Wallis p-values all > 0.1).

Female rats in group B did show an increase in the number of nose pokes during the 24-h trial. This finding was significant in all heptachlor-treated groups compared to controls (Fisher’s exact test, p = 0.04); 70–90% of treated rats displayed nose pokes, compared to only 30% of controls. No differences in the occurrence of nose pokes or half-body crosses were obtained in males or in group-A females.

Morris water maze. In general, control rats learned the position of the platform within 4–6 days of spatial training. Latency data across daily training blocks are shown in Figure 3. Control mean latencies at asymptotic performance (last 2 days of training) were, for males and females, respectively, 8.5 and 16.0 s for group A, and 9.4 and 13.2 sec for group B; thus, control performance was comparable between the 2 groups of rats.

In rats treated with heptachlor until PND 21, there were no significant differences on any of the dependent measures. The only exception was time spent in the middle zone (block-by-sex interaction, p = 0.005); however, analyses of each gender separately did not reveal any significant effect.

There were significant differences on water maze performance in rats treated until PND 42. The latency data (Fig. 3) showed a significant main effect of treatment (p < 0.05) and no interaction with sex, and post hoc comparisons revealed differences in the high-dose group (male and female data combined). The data for path length were similar, except that it did not reach overall significance (p < 0.10).

Analysis of the time spent in the concentric zones of the round tank indicate that treated rats showed a different spatial search strategy (significant block-by-sex interaction). Male rats in both the middle- and high-dose groups spent significantly (overall dose p = 0.003) more time in the outer zone compared to control, indicating that they were circling the perimeter instead of venturing into the middle zone where the platforms were located. This was most apparent at the beginning of the second week of training, when controls spent 44.6% of the time in the outer zone, compared to treated rats (0.3 mg/kg/day: 65.7%; 3 mg/kg/day: 67.8%). This pattern did not reach significance (p < 0.10) in females, who spent more time overall in the outer zone; in the last week of training, controls spent 60–70% of time in that area compared to high-dose rats at 66–80%.

Swim speed showed a dose-by-platform interaction, indicating that speed was not similar across groups for the 2 platform positions. In males, the data for one position indicated that all dose groups (overall means 15.2–17.0 cm/s) were significantly lower than control (20.7 cm/s). However, the mean control value for that subset was higher than controls for the other platform (16.6 cm/s), and indeed the values for the treated groups were consistent with the other control group. The data for female rats revealed that only one dose group (low dose; 0.03 mg/kg/day) showed faster swim speed (20.7 cm/s) com-
pared to control (17.3 cm/s), and for one platform position only. Thus, the effect on swim speed was equivocal.

Control performance in the probe trials improved from the first to the second week for both groups of rats (A and B). In the first probe trial, control values for the percent of path length in the correct quadrant was 28–32% (25% would indicate random chance), whereas the percentage increased to 35–46% in the second probe. Thus, at the end of the first week, the rats were still in the acquisition phase of learning the platform location. In group A, there were no differences between control and treated groups on any of the measures of performance on either probe trial.

A significant influence of heptachlor was detected on probe performance in group B. Quadrant analysis of the first probe data indicated that control males did show a significant bias for both proximity \((p = 0.02; \text{ data shown in Fig. 4})\) and percent path length \((p = 0.04)\). Within-subject analysis of the quadrant distribution showed that all treated males (including the low-dose group) did not show a significant bias, indicating random searching across quadrants and platform locations. None of the female rats (control or heptachlor-treated) showed significant quadrant differences in the first probe trial. In the second probe trial, male rats in the middle- and high-dose groups, and females in the high-dose group, still did not show a significant quadrant bias.

During the working memory paradigm, rats learned the new position of the platform each day. For both groups, statistical analyses revealed an effect of trial, indicating that the first and second trials differed each day, and an effect of day, implying that performance changed from day to day; there was no interaction with treatment on either of these factors. There was, however, a significant overall dose effect in the group-A male rats, in that the low-dose group showed higher latency and longer path length compared to control, across all trials (mean latency: control, 20.5 s; low dose, 27.9; mid dose, 22.8; high dose, 25.9). There were no differences in group-A females, or group-B rats. Likewise, swim speed was not altered.

Chlordiazepoxide challenge. Chlordiazepoxide produced a dose-dependent decrease in activity over the 30-min session, as shown in Figure 5, for the group-B rats. The heptachlor-treated females in group B appeared to be less affected at the highest chlordiazepoxide dose; however, there were no significant differences overall in the dose-response to chlordiazepoxide (i.e., no heptachlor treatment-by-chlordiazepoxide dose interactions).

Chloride flux. GABA-stimulated \(^{36}\)Cl\(^-\) flux was examined in synaptoneurosomes on PND 7, 21, and 43 in control and high-dose animals; net \(^{36}\)Cl\(^-\) flux data are presented in Table 2. There was a significant age-by-treatment interaction \((p = 0.02)\), and separate ANOVAs indicated that exposure to heptachlor significantly reduced \(^{36}\)Cl\(^-\) flux only in PND 21 females. At this age, GABA-stimulated \(^{36}\)Cl\(^-\) flux was approximately 28% lower in treated females than in controls. Heptachlor was without significant effect at any other age.

Receptor binding. GABA binding characteristics were assayed in tissues on PND 7, 21, and 43 in control and high-dose animals. There was a significant overall treatment effect on
B_{max} in the brainstem tissue of females (overall dose \( p = 0.003 \)), but not males. Averaged across the age groups, there was approximately a 22% decrease in B_{max}. Female B_{max} data for cortex tissue showed an age-by-treatment interaction (\( p = 0.03 \)), but univariate analyses at each age did not reveal a significant treatment effect. These data are presented in Table 3. There were no significant effects on K_D in either sex (K_D control values, mean ± SD, averaged across time: brainstem—males: 128 ± 53 nM; females: 113 ± 36 nM; cortex—males: 111 ± 71 nM; females, 99 ± 68 nM).

**FIG. 4.** Water maze memory trial, as proximity score, during 60-s probe trial (platform removed) in rats dosed until PND 42 (group B). Data presented as mean (±SEM) proximity score for each quadrant during the first probe (end of first week) and second probe (end of second week) trials.

**FIG. 5.** Dose-response curves for activity decreases produced by chlordiazepoxide (ip, 40-min pretest) in group-B rats. All rats received all doses of chlordiazepoxide. Data presented as mean (±SEM) total activity counts during 30-min sessions.
TABLE 2

GABA-Stimulated Chloride Flux (nmol chloride/mg protein) in Rats Treated with Vehicle (control) or Heptachlor (3 mg/kg/day) from Gestation to PND 42

<table>
<thead>
<tr>
<th></th>
<th>PND 7</th>
<th>PND 21</th>
<th>PND 43</th>
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<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.2 ± 2.2</td>
<td>9.9 ± 0.4</td>
<td>8.4 ± 0.6</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>9.4 ± 1.3</td>
<td>9.8 ± 1.1</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.6 ± 1.1</td>
<td>16.9 ± 1.0</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>8.6 ± 1.0</td>
<td>12.6 ± 1.1*</td>
<td>8.4 ± 0.5</td>
</tr>
</tbody>
</table>

*Significantly lower than control.

**DISCUSSION**

The present study examined potential behavioral and neurochemical alterations following perinatal heptachlor exposure. Heptachlor did not produce obvious toxicity in the dam or the offspring (Smialowicz et al., in press), as evaluated by maternal weight gain and health, litter size, and pup survivability and weight gain. However, the ontogeny of the righting response was slowed in females, indicating a developmental delay in these pups. The pesticide was readily available to the fetus and pup, even before direct dosing began on PND 7. Tissue analyses of heptachlor and heptachlor epoxide levels showed a dose-dependent increase in concentration in the dam plasma and milk (Smialowicz et al., in press). The epoxide, but not heptachlor itself, was also detected in the brain, liver, and plasma of PND 7 pups. The brain levels reported here represent the second-highest store of heptachlor epoxide in the neonatal tissues; only fat had higher concentrations at each dose level (10–20-fold higher). Littermates of the rats from this study were used for other toxicological evaluations as well (see Smialowicz et al., in press). In the adult male offspring, immunological challenge uncovered suppression of the primary and secondary response to sheep red blood cell exposure. There were no effects of heptachlor on any of the reproductive endpoints examined.

Cyclodiene insecticides, including heptachlor and heptachlor epoxide, bind to GABA<sub>A</sub> receptors with high affinity, and the acute toxicities of these compounds in animals are related to their affinity for this receptor (Cole and Casida, 1986). In addition, cyclodienes are reported to alter expression of the GABA<sub>A</sub> receptor during development (Lauder et al., 1998), and thus may produce long-lasting alterations in brain function. In this study, evaluations were conducted while the rats were being exposed (righting reflex, Cl<sup>-</sup> flux, and receptor binding) as well as weeks to months after exposure ended (FOB, motor activity, cognitive tests). Thus, some of the changes reported herein could be due to the direct interactions at the GABA receptor, and others may be due to changes in GABA’s influence on neuronal development.

The number of GABA<sub>A</sub> binding sites was decreased in female rats, an effect that was consistent across the ages tested. This change was significant in the brainstem, but not the cortex. A similar decrease in TBPS binding, as well as decreased GABA<sub>A</sub> receptor subunit mRNA levels, was reported in the brainstem, but not in “rest of brain” for GD 17 fetuses following in utero exposure to dieldrin or lindane (Brannen et al., 1998; Lauder et al., 1998; Liu et al., 1997b, 1998). These previous papers did not examine gender differences. The present findings with heptachlor extend the generality of this alteration of TBPS binding sites. At the present time, it is not clear how the results of these binding or flux experiments relate to the behavioral effects of heptachlor exposure. Despite these biochemical changes, the functional response to chlordiazepoxide, a pharmacological agent that acts on the GABAergic receptor, was not significantly altered.

A number of investigators have demonstrated that, in vitro, both heptachlor and heptachlor epoxide significantly reduce GABA<sub>A</sub>-stimulated <sup>36</sup>Cl<sup>-</sup> flux into synaptoneurosomes (Abalis et al., 1986; Bloomquist et al., 1986) and in cortical neurons (Pomes et al., 1994) with IC<sub>50</sub> values for inhibition of <sup>36</sup>Cl<sup>-</sup> flux ranging from 400 nM to 121 µM for heptachlor and 70 nM to 18 µM for heptachlor epoxide.

**TABLE 3**

GABA Receptor B<sub>max</sub> (pmol/mg protein) in Rats Treated with Vehicle (control) or Heptachlor (3 mg/kg/day) from Gestation to PND 42

<table>
<thead>
<tr>
<th></th>
<th>PND 7</th>
<th>PND 21</th>
<th>PND 43</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brainstem</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.24 ± 0.25</td>
<td>1.00 ± 0.13</td>
<td>1.22 ± 0.39</td>
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<tr>
<td>Heptachlor</td>
<td>1.20 ± 0.09</td>
<td>0.91 ± 0.12</td>
<td>0.68 ± 0.20</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.32 ± 0.13</td>
<td>1.14 ± 0.09</td>
<td>0.89 ± 0.10</td>
</tr>
<tr>
<td>Heptachlor*</td>
<td>0.79 ± 0.01</td>
<td>1.01 ± 0.10</td>
<td>0.70 ± 0.09</td>
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<tr>
<td><strong>Cortex</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Males</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.49 ± 0.22</td>
<td>2.62 ± 0.18</td>
<td>2.18 ± 0.12</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>1.41 ± 0.52</td>
<td>2.16 ± 0.21</td>
<td>2.37 ± 0.20</td>
</tr>
<tr>
<td>Females</td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>1.27 ± 0.48</td>
<td>2.50 ± 0.28</td>
<td>2.53 ± 0.16</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>2.94 ± 0.91</td>
<td>2.68 ± 0.28</td>
<td>2.20 ± 0.18</td>
</tr>
</tbody>
</table>

*Significant effect across ages.

Note. n = 3–6/sex/dose at each age. Data are presented as mean ± SEM.
compounds, the dose of technical chlordane used was also higher (5 mg/kg/day) and exposure was more prolonged (dams were dosed from GD 4 through PND 21, offspring dosed from PND 22–80). Thus, there are several differences that may account for the lack of effect of heptachlor treatment in males, or at other ages, in the present study.

Gestational or perinatal exposure to other cyclodiene has been reported to produce changes in motor activity and other behavioral endpoints. Specifically, increased motor activity levels were reported in offspring following gestational exposure to aldrin, perinatal endrin exposure, and postnatal dosing with lindane (Castro et al., 1992; Gray et al., 1981; Rivera et al., 1990, 1998). In the present study, rats treated with heptachlor until PND 21 also showed increased activity levels in either the open field or the figure-eight activity chambers on PNDs 27–28 and 40–41. The rats dosed until PND 42 did not display such activity changes; however, they were not tested at the same age as group A. Relatively few other behavioral changes were observed in the heptachlor-treated offspring. Rats dosed until PND 42 showed consistently increased levels of reactivity (excitability) in both sexes, and increased foot splay and urination in males. These changes were not large in magnitude, nor were they dose-dependent, indicating that these effects may not be biologically important.

The most pronounced effects of heptachlor were on the measures of cognitive function. In the rats dosed until PND 42, heptachlor slowed acquisition of the spatial task and impaired recall during probe trials. These effects on the probe trials were significant even at the lowest dose in males. Differences in search strategy may account for the effect on spatial training parameters. An efficient search strategy was not developed by heptachlor-treated rats, who spent more time circling the outer zone of the tank, even during the second week of training when control rats had learned to venture into the zone where the platform was located. The findings from the passive avoidance task also suggested slower learning in heptachlor-treated rats; however, the differences were not statistically significant. Previous papers on cyclodiene report different conclusions. Cassidy and colleagues (Cassidy et al., 1994) reported better performance in a Cincinnati maze in females treated with chlordane, although no dose-response was evident. Quicker learning in a variation of the passive avoidance task was also reported in lindane-treated rats (Rivera et al., 1998). It is difficult to compare these studies directly, since the cognitive tasks, experimental treatments, and dosing regimens were all different.

Delayed acquisition in a Morris water maze has been reported following perinatal exposure to a variety of other chemicals, including lead, ethanol, and toluene (Hass et al., 1999; Kuhlmann et al., 1997; Matthews and Simson, 1998). Since cognitive deficits in lead- and alcohol-exposed children are well known, this indicates that alterations in water maze performance may be predictive of these human outcomes. The similarity of heptachlor effects in the present study to the effects of chemicals such as lead raises concern for potential subtle deficits in human-exposed populations.

There could be several possible explanations for the lack of effects in the water maze in the group-A rats. Group-B rats were exposed to heptachlor for an additional 3 weeks (total dose administered, approximately 1.56, 15.6, or 156 mg/kg) compared to group A (total dose, 0.93, 9.3, or 93 mg/kg). Heptachlor epoxide is extremely persistent in the body (especially in fat; Fendick et al., 1990), and it can be expected that the higher-dose groups in group B had a greater body burden, which may influence the test measures. An alternative explanation is that the group-B dosing regimen covered a critical period of development for the spatial task. However, neuronal growth and differentiation are essentially complete by PND 21 (Rice and Barone, 2000), although it is possible that heptachlor delayed this maturation process. There may exist a critical window of vulnerability for this persistent effect of heptachlor. Finally, group-B rats were tested at an older age than group-A rats, and the effects detected may not have been evident at the earlier age.

The data from these studies provide some implications for exposed populations, such as those drinking tainted milk in Hawaii (Baker et al., 1991; Smith, 1982). A review of Hawaiian birth records following that incident revealed no obvious malformations or adverse birth outcomes (Le Marchand et al., 1986). The present data also confirm that heptachlor, at environmentally relevant doses and even higher, did not alter pregnancy or general health of the offspring. Furthermore, the behavioral effects observed occurred without obvious toxicity, suggesting the possibility of subtle neurological changes that could have implications for human health.

In summary, the data from this perinatal study of heptachlor suggest developmental delays, alterations in GABAergic neurotransmission, and neurobehavioral changes, including cognitive deficits. Females were somewhat more affected, as were rats dosed until PND 42. The present data support previous findings of ex vivo receptor changes following developmental exposure to cyclodiene, and extend those results by elucidating persistent neurobehavioral effects. The connection between the neurochemical and behavioral changes cannot be established here, but is an area of research that would improve understanding of the health consequences of perinatal exposure to cyclodiene pesticides.

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