**Developmental Neurotoxicity Study of Styrene by Inhalation in Crl-CD Rats**

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## Abstract

This study was conducted to assess potential adverse functional and/or morphological effects of styrene on the neurological system in the $F_2$ offspring following $F_0$ and $F_1$ generation whole-body inhalation exposures. Four groups of male and female Crl:CD® (SD)IGS BR rats (25/sex/group) were exposed to 0, 50, 150 and 500 ppm styrene for six hours daily for at least 70 consecutive days prior to mating for the $F_0$ and $F_1$ generations. Inhalation exposure continued for the $F_0$ and $F_1$ females throughout mating and through gestation day 20. On lactation days 1 through 4, the $F_0$ and $F_1$ females received styrene in virgin olive oil via oral gavage at dose levels of 66, 117 and 300 mg/kg/day (divided into three equal doses, approximately two hours apart). Inhalation exposure of the $F_0$ and $F_1$ females was re-initiated on lactation day 5 and continued through weaning of the $F_1$ or $F_2$ pups on postnatal day (PND) 21. Developmental landmarks were assessed in $F_1$ and $F_2$ offspring. The neurological development of randomly selected pups from the $F_2$ generation was assessed by functional observational battery, locomotor activity, acoustic startle response, learning and memory evaluations, brain weights and dimension measurements, and brain morphometric and histologic evaluation.

Styrene exposure did not affect survival or the clinical condition of the animals. As expected from previous studies, slight body weight and histopathologic effects on the nasal olfactory epithelium were found in F0 and F1 rats exposed to 500 ppm and, to a lesser extent, 150 ppm. There were no indications of adverse effects on reproductive performance in either the $F_0$ or $F_1$ generation. There were exposure-related reductions in mean body weights of the $F_1$ and $F_2$ offspring from the mid and high-exposure groups and an overall pattern of delayed development evident in the $F_2$ offspring only from the 500 ppm group. This developmental delay included reduced body weight (which continued through day 70) and slightly delayed acquisition of some physical landmarks of development.

Styrene exposure of the $F_0$ and $F_1$ animals had no effect on survival, the clinical condition or necropsy findings of the $F_2$ animals. Functional observational battery evaluations conducted for all $F_1$ dams during the gestation and lactation periods and for the $F_2$ offspring were unaffected by styrene exposure. Swimming ability as determined by straight channel escape times measured on PND 24 were increased, and reduced grip strength values were evident for both sexes on PND 45 and 60 in the 500 ppm group compared to controls. There were no other parental exposure-related findings in the $F_2$ pre-weaning and post-weaning functional observational battery assessments, the PND 20 and PND 60 auditory startle habituation parameters, in endpoints of learning and memory performance (escape times and errors) in the Biel water maze task at either testing age, or in activity levels measured on PND 61 in the 500 ppm group. Taken together, the exposure-related developmental and neuromotor changes occurred in endpoints known to be both age- and weight-sensitive parameters, and were observed in the absence of any other remarkable indicators of neurobehavioral toxicity. Based on the results of this study, an exposure level of 50 ppm was considered to be the NOAEL for growth of $F_2$ offspring; an exposure level of 500 ppm was considered to be the NOAEL for $F_2$ developmental neurotoxicity.

## Introduction

The reproductive and developmental effects of styrene (CAS # 100-42-5) have been extensively reviewed by Brown et al. (2000). Although few details are provided, Vergieva et al. (1979) reported no dose-related effects on body weights or offspring behavior when rat dams were exposed via inhalation to 163 ppm styrene 4 hours/day 5 days/week on gestational days 2-16 or to 47 ppm on gestational days 2-21. Zaidi et al. (1985) reported that gavage treatment of rat dams with 200 mg/kg/day styrene throughout gestation had no effect on the number of pups born per litter, pup body weight, protein content of the brain, or striatal dopamine receptors of pups. In contrast, Kishi and coworkers have conducted two studies on the effects of prenatal styrene exposure. In the first (Kishi et al., 1992, 1995), pregnant Wistar rats were exposed via inhalation to 0 (14 litters), 60 (3 litters), or 293 ppm (7 litters) styrene 6 hours/day on gestation days 7 to 21. There was no effect on gestation length or average litter size, but pup weights in both styrene exposed groups were reduced compared to the controls. Neurobehavioral evaluation was conducted on 5 control litters, 2 litters exposed at 60 ppm and 5 litters exposed at 293 ppm. They reported differences in a number of developmental landmarks, as well as differences in open field activity, rota-rod activity and operant conditioning response on some but not all tested intervals. In the second study (Katakura et al., 1999, 2001), pregnant female rats were exposed to 0 (ad libitum feed, 14 litters), 0 (pair–fed to 300 ppm group, 12 litters), 50 (9 litters) or 300 (14 litters) ppm styrene by a static inhalation system. Compared to the pair-fed controls, exposure to 300 ppm styrene resulted in increased neonatal death, decreased pup weight on PND 21, increased time to lower, but not upper, incisor eruption, an increased time to development of air righting reflex, and decreased homovanillic acid in the cerebrum.

Effects on the nervous system have been reported for workers in the reinforced plastics industry. Neither the presence of effects nor the level at which the effects were observed have been reported consistently. Effects reported in some studies include central nervous system (CNS) depression, slower nerve conduction velocity, and decreased color discrimination. Some studies report effects at concentrations as low as 10 ppm, while others claim no effects at concentrations as high as 100 ppm (reviewed in IARC, 2002). In laboratory animals, CNS depression was reported following single exposures to 1300 ppm for 4 hours. Changes in dopamine and related metabolites have been reported in the brain of rats exposed to 750 ppm styrene for 3 days. Increases in glial fibrillary acidic protein in the sensorimotor cortex and hippocampus were reported in rats exposed via inhalation to 320 ppm styrene continuously for 3 months (reviewed in IARC, 2002).

Because of the conflicting and limited animal data on the effects of styrene on development of the neurological system, a developmental neurotoxicity study was conducted via whole-body inhalation exposure using current regulatory guidelines. This was accomplished by evaluating dams from the $F_1$ generation and $F_2$ offspring from a two-generation reproduction study (Cruzan et al., in press). In most reproduction studies conducted by the inhalation route, exposure is stopped on day 20 of gestation and not reinstated until lactation day 5 to minimize stress on the offspring from the more than six-hour separation that would occur during the inhalation exposure of the dam. Because high concentrations of styrene affect the CNS and significant development of the CNS occurs during the first few days after birth in rats, dams were treated orally during lactation days 1-4 at doses estimated by PBPK modeling to mimic a six-hour inhalation exposure.

## Materials and Methods

### Study Design

Four groups of $F_0$ and $F_1$ male and female Crl:CD rats (25/sex/group) were exposed to vapor atmospheres of styrene at 0, 50, 150 or 500 ppm for six hours daily (7 days/week) for at least 70 consecutive days prior to mating. Females were paired with males on a 1:1 basis for 14 days or until evidence of mating was observed. The $F_0$ and $F_1$ females continued inhalation exposure throughout mating and gestation through gestation day 20. On lactation days 1 through 4, the $F_0$ and $F_1$ females received styrene in virgin olive oil via oral gavage at dose levels of 66, 117 and 300 mg/kg/day (divided into three equal doses, administered approximately two hours apart). The doses were calculated to mimic the blood level of styrene at 2, 4, and 6 hours in a six-hour inhalation exposure at the target concentration based on the PBPK model of Sarangapani et al. (2002). Inhalation exposure of the $F_0$ and $F_1$ females was re-initiated on lactation day 5 and continued through the day prior to euthanasia. Offspring were weaned on lactation day 21; exposure of $F_1$ pups began on PND 22. $F_2$ pups were weaned on PND 21 and not directly exposed to styrene. Neurobehavioral and neuropathological evaluations were conducted in $F_2$ offspring through PND 72. The details of the two-generation study have been published in the accompanying reproductive toxicity paper (Cruzan et al., in press).

### Test Material

Styrene monomer (inhibited), CAS No. 100-42-5, was provided by Chevron Phillips Chemical Company LLP, St. James, LA. The purity and stability of the styrene were verified by gas chromotography with flame ionization detection. When present in the chromatograms, the percentage of benzene, ethylbenzene, styrene oxide and styrene dimers was also determined. Results obtained indicated the styrene was at least 99.9% pure.

### Exposures

Gas chromatographic analyses of chamber atmospheres demonstrated average exposures of 0, 50, 151, and 499 ppm for $F_0$ styrene concentrations and 0, 50, 153, and 501 ppm for $F_1$ styrene concentrations.

### Animals and Animal Husbandry

Male and female Crl:CD®(SD)IGS BR rats from different barrier colonies were received from Charles River Laboratories, Inc., Raleigh, North Carolina, on July 24, 2001. The methods for acclimation, assignment to treatment groups, animal husbandry and animal welfare are described in the accompanying two-generation reproductive toxicity paper.

### Reproduction Study

All animals were observed twice daily (at least seven hours apart) for moribundity and mortality, appearance, behavior and pharmacotoxic signs (prior to exposure/gavage dosing for the $F_0$ and $F_1$ animals). Individual $F_0$ and $F_1$ male body weights were recorded throughout the study. Further details are provided in the two-generation study (Cruzan et al., in press). After a minimum of 70 days of exposure, each female was housed overnight in the home cage of a randomly chosen male. After mating, the animals were separated and the female was housed in an individual plastic cage with nesting material. All females were allowed to deliver naturally and rear their young to weaning (PND 21). To reduce variability among litter size, 10 $F_1$ and $F_2$ pups of equal sex distribution, if possible, were randomly selected from each litter on PND 4. Pups were individually sexed on PND 0, 4, 7, 14 and 21. $F_1$ pups were individually weighed on PND 1, 4, 7, 14 and 21; $F_2$ pups were individually weighed on PND 1, 4, 7, 11, 13, 17 and 21. The following investigations were used to assess the maturation of the selected $F_1$ and $F_2$ pups: pinna detachment, surface righting response, hair growth, incisor eruption, eye opening, balanopreputial separation, and vaginal patency (Adams, 1986). After litter standardization, one $F_2$ pup/sex/litter (total of 20 pups/sex/group) was assigned to one of two subsets (Subset A and B) for neurobehavioral and neuropathological assessments, and an additional one $F_2$ pup/litter (total of 10 pups/sex/group) was assigned to Subset C for neuropathological assessment.

**Neurobehavioral testing**

Functional Observational Battery (FOB) (Moser, 1991; Irwin, 1968; Gad, 1982; Moser et al., 1988; Haggerty, 1989; O'Donoghue, 1989) testing was performed on all $F_1$ dams on gestation days 6 and 12 and on lactation days 10 and 21; FOB testing was performed on 20 $F_2$ pups/sex/group (Subset A) on PND 4, 11, 22, 45 and 60. Testing (Table 1) was performed by the same trained technicians, whenever possible, who did not know the animal's group assignment. Chatillon Model DPP-1.0 kg or DPP-2.5 kg (as appropriate for the age of the animal) pull-push strain gauges (AMETEK Test and Calibration Instruments Division, Largo, FL) were used for testing fore- and hindlimb grip strength. The test was conducted such that animals were allowed to grasp the pull strain gauge with their forepaws. The observer then pulled the animal until the grip was lost and the hindpaws grasped the push strain gauge and then let go with the continued movement. One pull and push was considered a single trial, and three consecutive trials were conducted on each testing day. The group mean of the averaged trials was reported in grams.

The locomotor activity of the same 20 pups/sex/group assigned to FOB asessments (Subset A) was monitored on PND 13, 17, 21 and 61. Locomotor activity was measured using the SDI Photobeam Activity System (San Diego Instruments, San Diego, California) in a room equipped with a white noise generation system set to operate at approximately 70 dB(A). Each chamber consisted of a series of infrared photobeams surrounding a clear plastic, rectangular cage. Four-sided black enclosures surrounded the clear plastic boxes and decreased the potential for distraction by extraneous environmental stimuli. Each chamber was calibrated before each testing session. The testing of treatment groups was done according to replicate sequence. Each test session was 60 minutes in duration and consisted of 12 five-minute intervals. Each session recorded ambulatory (sequential interruption of two or more photobeams) and total (interruption of one or more photobeams) activity.

An acoustic startle response test was performed on the same 20 rats/sex/group (Subset A) on PND 20 and 60 using the SR-Lab Startle Response System (San Diego Instruments, San Diego, California) in a room equipped with a white-noise generation system set to operate at approximately 70 dB(A). Each isolation chamber was composed of a wood core covered with a laboratory-grade plastic laminate and measured 15 inches x 16 inches x 23 inches. Each cabinet was equipped with an internal light, a fan, two viewing lenses and a complete white-noise generation system. The animal was placed in a cylindrical enclosure of appropriate size, which was then placed into the isolation cabinet. Each enclosure was equipped with a motion sensor, which was calibrated prior to each day's testing. Each test session consisted of a five-minute acclimation period with a 65 ± 5 dB(A) broadband background white noise. The startle stimulus for each trial was a 115 ± 5 dB(A) mixed-frequency noise burst stimulus, approximately 20 milliseconds in duration. Responses were recorded during the first 100 milliseconds following the onset of the startle stimulus for each trial. Each test session consisted of 50 trials, with an eight-second intertrial interval. Startle response data were analyzed in five blocks of 10 trials each. Concurrent with the onset of the startle burst, force data were collected every millisecond for 100 msec. The greatest force data recorded during that 100 msec was considered the maximum response amplitude ($V_{MAX}$) and was reported in mV. The time at which the $V_{MAX}$ was observed was considered the latency (from the onset of the startle burst) to the maximum response amplitude ($T_{MAX}$) and was reported in msec. The average of all 100 response recordings was considered the average response amplitude ($V_{AVE}$) and was reported in mV.

Swimming ability and learning and memory were assessed for 20 rats/sex/group using a water-filled eight-unit T-maze similar to that described by Biel (1940). Those animals tested on PND 62 were the same as used above (Subset A), while those animals tested on PND 24 were used for this test only (Subset B). Animals were placed in the maze and were required to traverse the maze and escape by locating a platform that was hidden 2 cm (adjusted before each trial) beneath the surface of the water. The amount of time required to traverse the maze and the number of errors for all learning and memory trials were recorded. Each testing interval consisted of three phases that were conducted over seven consecutive days. Phase one was an evaluation of swimming ability and motivation to escape from the maze and was performed in four consecutive trials on day one of the Biel maze procedure by measuring the time required for the rat to swim the length of a straight channel. Phase two of the Biel maze procedure evaluated sequential learning on days 2-6. Animals were allowed two trials per day for two days to solve the maze in path A. Animals were then allowed two trials per day for three consecutive days to solve the maze in path B, which was the reverse of path A. For Phases two and three, the minimum intertrial interval was one hour. Phase three, day 7, probed the animal for its memory to solve the maze when challenged in path A. Each animal was allowed two trials to solve the maze in path A. Biel maze data were evaluated as the mean time to escape over all trials for each of the three phases (*i.e.*, swimming ability and motivation, sequential learning and memory) of the Biel maze procedure. Also, the numbers of errors committed were evaluated for phases two and three.

**Neuropathology**

On PND 21, 10 $F_2$ pups/sex/group (Subset C) were perfused *in situ* and the brains processed for microscopic examination. On PND 72, 10 $F_2$ pups/sex/group (selected from Subset A) were perfused *in situ* and central and peripheral nervous system tissues were processed for microscopic examination. The brain and central nervous system tissues were embedded into paraffin; peripheral nervous system tissues were embedded into plastic. At minimum, four coronal sections of the cerebrum and a mid-sagittal section of the cerebellum/pons/medulla and two transverse sections of the remaining half of the cerebellum/pons were prepared. The prepared tissues were sectioned at 4-8 microns, mounted on glass microscope slides and stained with hematoxylin and eosin. A simple, non-blinded morphometric analysis of the brains from these offspring was performed. Two coronal sections of the cerebrum and one midsagittal section of the cerebellum/pons/medulla were used for morphometry. Sections were homologous between animals. Specific levels analyzed were defined as follows: Level 1 was a coronal section taken approximately halfway between the base of the olfactory bulbs and the optic chiasm. This level was just rostral to the point where the corpus callosum bridges across the hemispheres and was characterized by a good representation of the caudoputamen and the presence of the opening of the rostral medial aspect of the lateral ventricle. Level 3 was a coronal section taken just rostral to the attachment of the pituitary gland (infundibular stalk) characterized by a slight separation between the hemispheres of the rostral hippocampus such that CA1 pyramidal neurons from each hemisphere formed only a slight depression before meeting medially. Rostrally there was no depression, and posteriorly there was a more pronounced depression of the medial lines of CA1 neurons between hemispheres. Level 5 was a midsagittal section of the cerebellum and brainstem. Levels 1, 3 and 5 correspond to Figures 11, 32 and 79 of the adult rat brain as depicted by Paxinos and Watson (4th edition, 1998). Measurements were as follows:

Level 1: Total bilateral height of the hemisphere measured just at the beginning of the lateral ventricle, and bilateral vertical thickness of the hemisphere measured at the apex of the corpus callosum and parallel to the height of the hemisphere.

Level 3: Bilateral radial thickness of the frontoparietal cortex; bilateral vertical height of the hemisphere between the layers of hippocampal pyramidal neurons measured along a line that passed through the termination of the dorsal limb of the medial dentate hilus; bilateral height of the medial dentate hilus measured between the termination of the ventral limb perpendicular to the layer of pyramidal neurons, and bilateral length of the ventral limb of the dentate hilus.

Level 5: Thickness of the caudal brainstem, toward lobule #9 (caudal to the cerebellar peduncle), measured at the stalk of the cerebellum and perpendicular to the ventral border of the pons, and distance across the base of cerebellar lobule #9, measured perpendicular to the white matter tract through the middle of the lobule.

These linear measurements were made using a computer imaging system (Pax-It™, Midwest Imaging Systems, Inc., Fraklin Park, IL). The average from the two hemispheres of the coronal sections, and single measurements from the mid-sagittal section of the cerebellum and brainstem, were used in calculations. All brain sections were also examined by light microscopy for any qualitative changes.

**Statistical Methods**

Analyses were conducted using two-tailed tests (except as noted otherwise) for a minimum significance level of 5%, comparing each test article-treated group to the control group. Statistical procedures used in the reproductive phase are covered in detail in the accompanying paper and are not described here (Cruzan et al., in press).

Pup weights through weaning were analyzed separately by sex by analysis of covariance (ANCOVA), with pups weights nested within the litter, with the litter size as the covariate. The number of pups born was used as the covariate. The following assumptions were made regarding the ANCOVA: homogeneity of regression slopes, linear relationship between the pup weights and number of pups born, and additive group and regression effects. These assumptions were not tested. Histopathologic findings in the test article-treated groups were compared to the control group using a two-tailed Fisher's Exact test (Steel and Torrie, 1980). The following FOB data: group mean counts of backings, groomings, urinations and defecations and group means of fore- and hindlimb grip strength, along with ambulatory counts measured in the locomotor activity assessment, average response in the acoustic startle assessment and Biel maze data (mean times to escape in the straight channel, learning and memory phases and the mean errors in the learning and memory phases) were subjected to a parametric one-way analysis of variance (ANOVA) to determine intergroup differences. If statistically significant differences were indicated by the ANOVA, Dunnett's test was used to compare the control and treated groups. FOB parameters which yielded scalar and descriptive data were analyzed by Fisher's Exact Test (Steel and Torrie, 1980). Intrasession total counts measured in the locomotor activity assessment and intrasession peak response and latency to peak response measured in the acoustic startle assessment were analyzed by the univariate repeated measures ANOVA (ReMANOVA, SAS, 1999-2001) to determine the presence of an interaction effect of treatment group by time using a Geisser-Greenhouse adjusted F-statistic. If a significant interaction effect of treatment group by time was indicated by the ReMANOVA, Dunnett's test was used to compare the control and treated groups at each within-session interval using a Geisser-Greenhouse adjusted F-statistic. In addition, the ReMANOVA was used to determine the presence of a main effect of treatment. If a significant main effect of treatment was indicated by the ReMANOVA, Dunnett's test was used to compare the control and treated groups. Repeated measures statistical analyses were performed by BioSTAT Consultants, Inc., Portage, Michigan. All analyses performed by BioSTAT Consultants, Inc., were conducted with the SAS System software (version 8.2).

## Results

### Parental and Reproduction Effects

Briefly (see Cruzan et al., in press), body weight gain was slightly reduced in $F_0$ males and females at 500 ppm, and $F_1$ males and females at 150 and 500 ppm during the premating exposure period. There was no effect on bodyweight or bodyweight gain at 50 ppm in either the $F_0$ or $F_1$ exposure periods. There was no effect of styrene at any exposure level on body weight gain or feed consumption during gestation in either the $F_0$ or $F_1$ dams. At 500 ppm there was increased water consumption during gestation in both $F_0$ and $F_1$ dams. There was no effect at 150 or 50 ppm. There was no effect of styrene exposure on body weight gain or food consumption during lactation in either the $F_0$ or $F_1$ dams. Exposure of $F_0$ and $F_1$ females had no effect on mean estrous cycle length or the mean numbers of days between pairing and coitus. Styrene exposure had no effects on $F_0$ or $F_1$ spermatogenic endpoints (mean testicular and epididymal sperm numbers, sperm production rate, sperm motility and sperm morphology). The mean lengths of gestation were unaffected by styrene exposure. Exposure to styrene did not affect $F_0$ or $F_1$ male or female mating index, male or female fertility index, mean number of pups born, the number of former implantation sites, or the number of unaccounted sites. At the scheduled necropsy of the $F_1$ females, the mean numbers of primordial follicles and corpora lutea were unaffected in females exposed to 500 ppm of styrene. The mean number of $F_1$ and $F_2$ pups born, live litter size, percentage of males per litter at birth and postnatal survival were unaffected by styrene at all exposure levels evaluated. Mean $F_1$ male and female pup body weights were unaffected by parental exposure to styrene. The No-Observed-Adverse-Effect Level (NOAEL) for parental toxicity was 50 ppm and for effects on reproduction was >500 ppm.

**$F_2$ Body Weights**

Mean $F_2$ pup body weight gains and mean body weights in the 500 ppm group were decreased (6.8%-13.3%) throughout the pre-weaning period (PND 0-21). Body weights in these $F_2$ offspring remained reduced though PND 70 (Fig. 1). Mean male and female $F_2$ pup body weight changes in the 150 ppm group were similar to the control group during PND 1-4, but were reduced on PND 7-21. Following weaning, body weights in the $F_2$ offspring of parental animals exposed to 150 ppm of styrene were slightly lower than the controls. Mean body weights and mean body weight gains in the 50 ppm group $F_2$ males and females were unaffected by parental exposure to styrene throughout the pre-weaning and post-weaning periods.

**Developmental Landmarks**

Styrene exposure did not affect pinna detachment, surface righting response, hair growth, incisor eruption, and eye opening. Mean ages of acquisition of vaginal patency (groups means between 34.3 and 36.3 days) and mean body weights (between 108 and 114 grams) on the day of acquisition were unaffected by styrene exposure in $F_1$ females.

In $F_1$ males, the mean ages of acquisition of balanopreputial separation were 45.6, 44.6, 45.7 and 47.0 days in the 0, 50, 150, and 500 ppm groups, respectively. The differences from the control group were not statistically significant, and were within the laboratory's historical control data range (41.6-49.0 days). Mean body weights (grams) on the day of acquisition were 223, 214, 209 and 211 in the 0, 50, 150, and 500 ppm groups, respectively. The slight increase in age at acquisition of balanopreputial separation was judged to be related to the lower body weight at 500 ppm.

In $F_2$ males and females, there were subtle indications of a delay in the acquisition of developmental landmarks, which accompanied the decreased body weights (Table 2). In general, mean ages of acquisition were not statistically significantly increased, but more high exposure animals acquired the landmark later. This included pinna detachment, surface righting ability, initiation of hair growth, eye opening, incisor eruption ($p<0.05$), and balanopreputial separation. The delayed acquisition of these landmarks, in the presence of reduced body weight in offspring indirectly exposed to 500 ppm styrene was suggestive of a slight developmental delay. Vaginal patency in the $F_2$ females was unaffected by styrene exposure.

**Functional Observational Battery (FOB)**

Exposure of $F_1$ dams to styrene had no effect on FOB observations on gestation days 6 and 12 or lactation days 10 and 21. No styrene-related effects were seen in FOB observations in $F_2$ offspring on PND 4, 11, or 22. On PND 45 and 60, forelimb and hindlimb grip strength was decreased in the offspring of parental animals exposed to 500 ppm styrene (Fig. 2). These offspring also weighed less than the control animals at these ages. Given the slight delays in the acquisition of several developmental landmarks in this group, the decreased forelimb and hindlimb grip strength was a further indication of a slight developmental delay. No other FOB parameters were affected on PND 45 or 60.

**Locomotor Activity**

Although there were no statistically significant differences from the control group within the activity sessions conducted on PND 13, 17 and 21 (Fig. 3a,b), there was a slight shift in the ontogeny of locomotor activity in $F_2$ males and females in the 500 ppm group that, in the presence of reduced mean body weights in this group, was suggestive of an exposure-related developmental delay. Campbell et al. (1969) characterized the ontogeny of locomotor activity in the rat and showed that activity typically increases between PND 13 and 17 and then decreases between PND 17 and 21. Cumulative total and ambulatory locomotor activity in the 500 ppm group males and females were decreased slightly on PND 13. Cumulative total and ambulatory motor activity were increased in the male group on PND 17, but were decreased in the female group. By PND 21, mean motor activity in both sexes was increased slightly compared to the controls. On PND 61, motor activity in both sexes in the 500 ppm group was similar to the control group (Fig. 3c,d). Locomotor activity was similar to the control group for males and females in the 50 and 150 ppm groups at all ages tested. No changes in the distribution of within-session activity counts were apparent when the exposure groups were compared with the control group.

**Auditory Startle**

No statistically significant differences or exposure-related trends were apparent in the 50, 150 and 500 ppm group $F_2$ males and females on performance measured in the auditory startle test (Table 3). No changes in the ontogeny and distribution of within-session responses were apparent when compared with the control group.

**Memory and Learning – Biel Maze Swimming Trials**

On PND 24, swimming ability was slightly decreased in the 500 ppm exposure group males and females compared to controls, as evidenced by an increase in the mean time to escape for the straight channel on the first day of Biel maze assessment (Fig. 4). The increased escape times were suggestive of a slight exposure-related developmental neuromotor delay and were consistent with the reduced pre-weaning body weights, slight increases in the ages of acquisition of pre-weaning developmental landmarks, and slight shift in the ontogeny of normal pre-weaning locomotor activity. There were no obvious changes in mean escape times during the swim test for animals of either sex in the 50 or 150 ppm groups on PND 24, or in any animals on PND 62.

Trials 1-4 (conducted on assessment days 2 and 3; PND 25-26 or PND 63-64) of the Biel maze were designed to measure learning and shorter-term memory of Path A, designated the forward path. Trials 5-10 (conducted on assessment days 4-6; PND 27-29 or PND 65-67) were designed to measure learning and shorter-term memory of Path B, designated the reverse path (the exact opposite of Path A). For all treatment groups tested beginning on either PND 25 or PND 62, the mean time to escape was relatively high in Trial 1 and decreased throughout repeated testing in the forward path. For the first trial in the reverse path (Trial 5), mean time to escape was much longer than for the first trial in the forward path but decreased throughout testing in the reverse path. No treatment-related effects on learning in either path direction were noted.

Trials 11 and 12 (conducted on assessment day 7; PND 30 or PND 68) were designed to measure long term memory of Path A, the forward path, which was interrupted by trials 5-10 (Path B). For all exposure groups, the mean time to escape in Trial 11 was generally similar to trial 1 (the first trial of Path A) but relatively longer than Trial 4 (the last trial in the forward path during the learning portion of testing). For all exposure groups, mean time to escape for trial 12 was typically similar to Trials 3 and/or 4. As expected, since animals had already experienced the forward path, the slope of the line between Trials 11 and 12 was steeper than the first four trials. No exposure-related differences in the mean times to escape and numbers of errors (data not shown) were observed in either sex at either age evaluated, indicating that there was no impairment of learning (Trials 1-10) or memory (Trials 11 and 12) following indirect exposure to styrene.

**Neuropathology**

No direct effects of styrene exposure on absolute brain weights of PND 21 or 72 male and female rats were noted (Table 5). However, brain weights relative to final body weights of 500 ppm group females were increased compared to control females in these same animals because mean final body weights were slightly decreased in the 500 ppm group females.

Mean brain lengths, measured at necropsy, in the 150 and 500 ppm group females evaluated at PND 21 were statistically significantly less than (4.0% for both groups) the control group (Table 5). Mean brain lengths in males and females of all three styrene groups were slightly increased when compared to the control group on PND 72. Therefore, the decreased brain lengths on PND 21 were considered unlikely due to parental exposure to styrene. Mean brain width in all exposure groups was similar to the control group value at PND 21 and 72.

No microscopic findings that could be attributed to parental exposure to styrene were noted in the 500 ppm group as a result of the qualitative neuropathologic examination of the brain on PND 21 or central and peripheral nervous system tissues on PND 72.

There were no histomorphologic changes in measurements of brain regions on PND 21 or 72 that could be attributed to parental exposure in the 500 ppm group (Table 4). In female rats of the 500 ppm group evaluated on PND 21, the mean height of the hemisphere on Level 1 (Figure 11 of Paxinos and Watson, 1998) was slightly (6%) increased. However, the cortical thickness was not altered when compared to the control group, and the height of the hemisphere was not altered in offspring evaluated at PND 72. Therefore, the difference noted in PND 21 offspring was considered incidental in nature or potentially an artifactual change due to tissue processing. No other differences from control were observed in any measurement taken from rats on PND 21 or 72.

**Discussion**

As reported in the accompanying two-generation reproduction study (Cruzan et al., in press), the parental systemic toxicity in this study was similar to that previously reported in rats following long-term inhalation exposure to styrene (Cruzan et al., 1997, 1998). Findings included degeneration of the olfactory epithelium that lines the dorsal septum and dorsal and medial aspects of the nasal turbinates of $F_0$ and $F_1$ animals in the 500 ppm group (nasal tissue was not examined in the 50 and 150 ppm groups), decreased mean body weights in the 150 ppm group ($F_0$ and $F_1$ males and $F_1$ females) and 500 ppm group ($F_0$ and $F_1$ males and females). Reproductive performance and offspring postnatal survival prior to weaning were not adversely affected by styrene exposure. Pre-weaning $F_1$ pup weights were unaffected by styrene exposure. Consistent with the lack of effect on pre-weaning body weights in the $F_1$ pups, no styrene-related effects were observed on the $F_1$ preweaning developmental landmarks. Following direct exposure of the $F_1$ weanlings beginning on PND 22, weight gain was reduced in the 500 ppm group which led to reduced mean body weights in this group throughout the generation. As a result of the reduced body weight gain in the 500 ppm group $F_1$ males, a corresponding delay in the acquisition of the age of balanopreputial separation was observed. Ashby and Lefevre (2000) have previously reported that delays in this endpoint are observed in the presence of significant body weight reductions. Therefore, the delay in acquisition of balanopreputial separation was attributed to the reduced male body weight.

As direct exposure to styrene had a greater effect of $F_1$ rats than on $F_0$ rats, styrene had a more pronounced effect on the $F_2$ pups than on the $F_1$ pups. In contrast to the $F_1$ generation, pre-weaning $F_2$ pup weights were reduced in both the 150 ppm and 500 ppm groups (approximately 10 to 13% on PND 21). The weights of the $F_2$ pups selected for neurobehavioral evaluation in the developmental neurotoxicity phase continued to be reduced following weaning in the 150 and 500 ppm groups. A statistically significant delay (approximately ½ day) in the mean age of acquisition of incisor eruption was observed for the $F_2$ pups in the 500 ppm group. Slight delays (not statistically significant) in pinna detachment, surface righting, hair appearance, eye opening and balanopreputial separation were also noted in this group. The magnitude of the delays in each of these endpoints was slight and if they had occurred in isolation the delays would have been attributed to biological variability. However, when evaluated *in toto*, the slight delays in the acquisition of these parameters were suggestive of an overall pattern of slight developmental delay in the 500 ppm group. These pre-weaning developmental endpoints are highly correlated with pup body weight (Lochry, 1987), and the delays in these endpoints were consistent with the reduced body weights observed in this group. Based on this profile, the effects of styrene on the growth and development of the $F_2$ pups was somewhat greater than those effects observed on the $F_1$ pups.

Further evidence of a slight developmental delay in the $F_2$ offspring included an apparent shift in the ontogeny of normal locomotor activity from PND 13 through 21, the reduced swimming ability (presented as longer straight channel escape times) in the water maze on PND 24 and reduced grip strength on both PND 45 and 60 in $F_2$ offspring of $F_0$ and $F_1$ rats exposed to 500 ppm styrene. The ontogeny of swimming ability, the pre-weaning locomotor activity profile and the correlation of body weight with grip strength in rats have been well characterized (Adams, 1986; Kallman, 1994). The general developmental profile in this group demonstrated a pattern of delay that included changes only in age- and weight-sensitive endpoints throughout the entire $F_2$ generation; there were no indications of functional or morphologic effects suggestive of selective neurotoxicity. Therefore, the profile of changes observed in the 500 ppm group of the $F_2$ generation was attributed to a slight developmental delay as a result of parental styrene exposure. The NOAEL for growth of $F_2$ offspring was 50 ppm.

Kishi and coworkers reported deficits in neurological development in rats exposed to styrene, using few litters. The current study, performed according to accepted guidelines with greater statistical power to detect effects, does not support their conclusions. In the first study (Kishi et al., 1992, 1995), they reported (using 5 control and 5 exposed litters) that inhalation exposure of pregnant Wistar rats at 293 ppm styrene on gestation days 7-21 resulted in offspring with decreased open field activity, rota-rod activity, and operant conditioning response. The study reported herein found no significant effects on locomotor activity or learning and memory in offspring following direct exposure of the $F_0$ and $F_1$ generations to 500 ppm styrene throughout growth, mating, gestation, and lactation. In a second study, Katakura et al. (1999, 2001) reported increased neonatal mortality, delayed incisor eruption and delayed air righting reflex in offspring of dams that had been exposed to 300 ppm on gestation days 7 to 21. The authors did not indicate whether there were body weight differences from control at the time these endpoints were determined. In the study reported herein, styrene exposure had no effect on neonatal survival. Surface righting and incisor eruption in offspring of the 500 ppm group were slightly delayed, as noted above, but were judged to be secondary to reduced body weight.

Based on the guideline study reported herein, no specific effect on nervous system development was observed at exposures up to 500 ppm styrene.

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Figure Legends

Figure 1. Bodyweight (g) ± SEM of $F_2$ offspring of rats exposed to styrene in the $F_0$ and $F_1$ generations. A. Males. B. Females (PND 1-70)*Statistically different from control, $p<0.05$. **Statistically different from control $p<0.01$.

Figure 2. Grip strength (g) ± SEM of $F_2$ offspring of rats exposed to styrene in the $F_0$ and $F_1$ generations. A. Males – Forelimb. B. Females – Forelimb. C. Males – Hindlimb. D. Females – Hindlimb. *Statistically different from control, $p<0.05$.

Figure 3. Locomotor activity of $F_2$ offspring of rats exposed to styrene in the $F_0$ and $F_1$ generations; total counts in 60 minute session. A. Males – PND 13, 17, 21. B. Females – PND 13, 17, 21. C. Males – PND 61. Females – PND 61.

Figure 4. Learning and Memory (Biel Maze) of $F_2$ offspring of rats exposed to styrene in the $F_0$ and $F_1$ generations. A. Males. B. Females. Trial indicates the session trial (Swim represents the swimming ability testing conducted on the first day of evaluation). Trials 1-4 were conducted in the forward direction (Path A), trials 5-10 were conducted in the reverse direction (Path B; exact opposite of the forward direction) and trials 11-12 were conducted in the forward direction (Path A). Trials 1-10 tested learning and trials 11-12 tested memory. **Statistically different from control, $p<0.01$

Table 1. Parameters of Functional Observational Battery

| | |
|---|---|
| Ease of removal from cage | Ease of handling animal in hand |
| Lacrimation/chromodacryorrhea | Salivation |
| Piloerection* | Fur appearance* |
| Palpebral closure*+ | Respiratory rate/character |
| Red/crusty deposits | Mucous membranes/eye*+/skin color |
| Eye prominence*+ | Muscle tone |
| Mobility* | General body posture |
| Convulsions/tremors | Gait* |
| Grooming* | Arousal |
| Bizarre/stereotypic behavior | Urination/defecation |
| Pupillary response*+ | Backing |
| Forelimb/hindlimb gripstrength** | |

* Not assessed on PND 4 due to stage of development

+ Not assessed on PND 11 due to stage of development

** Assessed on PND 22, 45 and 60 only

Table 2. Days of acquisition of Developmental Landmarks of $F_2$ offspring of rats exposed to styrene in the $F_0$ and $F_1$ generations – Males and Females Combined

| Landmark: | Pinna detachment | | Surface Righting | | Incisor eruption | | Hair growth | |
|---|---|---|---|---|---|---|---|---|
| | % on pnd4 | Mean (s.d.) | % on pnd5 | Mean (s.d.) | % on pnd10 | Mean (s.d.) | % on pnd13 | Mean (s.d.) |
| Treatment | | Age | | Age | | Age | | Age |
| 0 ppm | 98 | 4.0 (0.07) | 90 | 5.1 (0.10) | 97 | 9.3 (0.30) | 92 | 11.8 (0.82) |
| 50 ppm | 99 | 4.0 (0.03) | 94 | 5.1 (0.11) | 99 | 9.4 (0.38) | 89 | 12.2 (1.31) |
| 150 ppm | 98 | 4.0 (0.07) | 91 | 5.1 (0.14) | 95 | 9.5 (0.42) | 99 | 11.9 (0.83) |
| 500 ppm | 92 | 4.1 (0.22) | 82 | 5.2 (0.18) | 81 | 9.8 (0.57)* | 81 | 12.5 (1.12) |

| Landmark: | Eye Opening | | Vaginal Opening | | | Preputial Separation | | |
|---|---|---|---|---|---|---|---|---|
| | % on pnd17 | Mean (s.d.) | % on pnd36 | Mean (s.d.) | BW | % on pnd51 | Mean (s.d.) | BW |
| Treatment | | Age | | Age | | | Age | |
| 0 ppm | 100 | 15.1 (0.95) | 100 | 33.5 (1.26) | 109.3 | 100 | 45.3 (1.48) | 222.3 |
| 50 ppm | 99 | 15.6 (0.72) | 80 | 34.2 (2.72) | 106.4 | 95 | 46.1 (3.73) | 219.6 |
| 150 ppm | 100 | 15.4 (0.68) | 90 | 34.0 (2.29) | 104.9 | 89 | 46.1 (3.06) | 217.4 |
| 500 ppm | 98 | 15.5 (0.98) | 89 | 34.1 (2.48) | 100.7 | 90 | 47.2 (4.07) | 216.9 |

For each landmark, the first column is the percent of animals in the group that have developed that landmark on the age indicated; the second column is the mean (s.d.) days for development of the landmark in the group. For vaginal opening and balanopreputial separation, the mean body weight at the age of acquisition of the landmark is also included. * Statistically significantly different from control, $p<0.05$.

Table 3. Startle response of $F_2$ offspring of rats exposed to styrene in the $F_0$ and $F_1$ generations.

| $F_0$ and $F_1$ Exposure | $V_{max}$ (millivolts) Males | $V_{max}$ (millivolts) Females | $V_{ave}$ (millivolts) Males | $V_{ave}$ (millivolts) Females | $T_{max}$ (milliseconds) Males | $T_{max}$ (milliseconds) Females |
|---|---|---|---|---|---|---|
| PND 20 | | | | | | |
| 0 | 97.3±46.0 | 102.8±58.5 | 20.3±8.8 | 21.6±11.8 | 27.2±5.7 | 25.4±3.7 |
| 50 | 86.0±31.3 | 98.9±52.8 | 18.0±6.7 | 20.8±10.7 | 28.8±5.9 | 27.5±5.5 |
| 150 | 85.7±36.2 | 88.8±40.0 | 18.5±7.3 | 18.7±8.3 | 28.0±3.8 | 25.4±4.1 |
| 500 | 92.9±32.2 | 95.9±39.5 | 19.4±6.8 | 20.5±7.8 | 27.4±6.0 | 26.8±4.0 |
| PND 60 | | | | | | |
| 0 | 134.0±101.9 | 75.1±41.6 | 29.2±20.7 | 15.8±8.4 | 34.4±5.7 | 34.4±4.5 |
| 50 | 171.7±164.0 | 85.3±49.9 | 38.0±35.1 | 17.3±8.5 | 33.0±5.4 | 34.2±5.0 |
| 150 | 159.7±94.5 | 62.6±34.0 | 34.7±20.6 | 13.8±6.9 | 31.1±4.9 | 35.5±4.2 |
| 500 | 128.5±99.0 | 83.0±46.6 | 27.8±20.3 | 17.3±8.7 | 32.9±5.9 | 33.6±4.3 |

$V_{max}$ is the maximum response to the startle stimulus. $V_{ave}$ is the average response to the startle stimulus, $T_{max}$ is the latency to the maximum response.

Table 4. Selected brain histomorphic measurements of $F_2$ offspring of rats exposed to styrene in the $F_0$ and $F_1$ generations

| | Males | | Females | |
|---|---|---|---|---|
| | O ppm | 500 ppm | 0 ppm | 500ppm |
| Level I | | | | |
| Height of hemisphere (mm) | | | | |
| PND 21 | 0.62±0.05 | 0.61±0.03 | 0.62±0.04 | 0.66±0.04* |
| PND 72 | 0.59±0.03 | 0.61±0.04 | 0.61±0.04 | 0.65±0.04 |
| Cortical Thickness (mm) | | | | |
| PND 21 | 0.16±0.01 | 0.15±0.01 | 0.15±0.01 | 0.16±0.01 |
| PND 72 | 0.15+0.01 | 0.16+0.01 | 0.16+0.01 | 0.15+0.01 |

*Statistically significantly different from control, $p<0.05$.

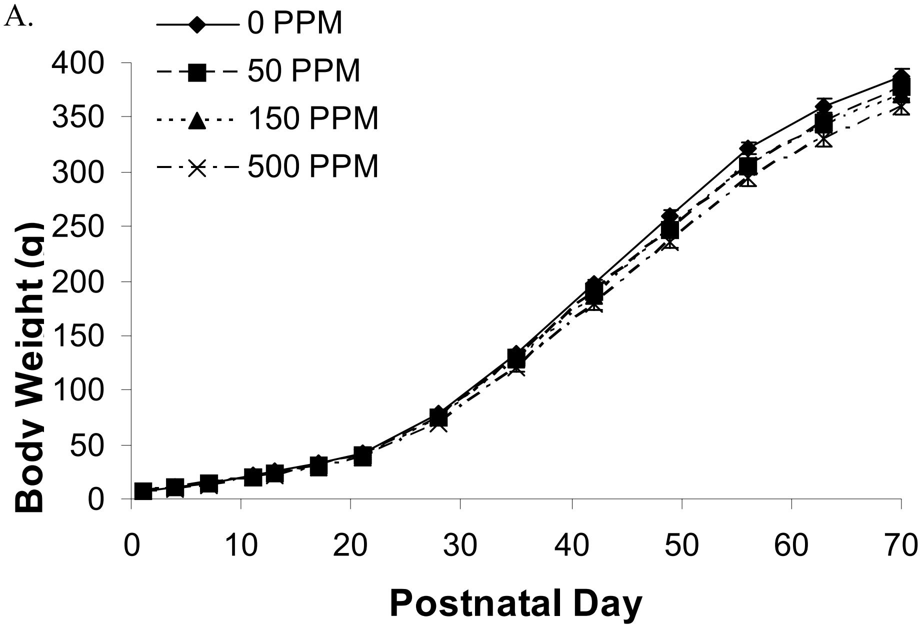
Table 5. Brain weight, length and width of $F_2$ offspring of rats exposed to styrene in the $F_0$ and $F_1$ generations

| | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 50 | 150 | 500 | 0 | 50 | 150 | 500 |
| Body weight (g) | | | | | | | | |
| PND 21 | 43 | 41 | 40 | 39 | 41 | 39 | 36 | 36* |
| PND 72 | 385 | 377 | 372 | 360* | 248 | 235 | 233 | 233 |
| Brain weight (g) | | | | | | | | |
| PND 21 | 1.55 | 1.64 | 1.62 | 1.59 | 1.54 | 1.56 | 1.52 | 1.57 |
| PND 72 | 1.98 | 1.89 | 1.93 | 1.87 | 1.83 | 1.84 | 1.80 | 1.78 |
| Brain Weight/ 100 g body weight | | | | | | | | |
| PND 21 | 3.86 | 3.91 | 4.23 | 4.02 | 3.88 | 4.08 | 4.14 | 4.65* |
| PND 72 | 0.49 | 0.50 | 0.49 | 0.53 | 0.73 | 0.77 | 0.79 | 0.79 |
| Brain length (mm) | | | | | | | | |
| PND 21 | 17.6 | 17.9 | 17.2 | 17.6 | 17.6 | 17.5 | 16.9* | 16.9* |
| PND 72 | 22.6 | 24.2 | 25.4 | 25.6 | 22.2 | 23.8 | 23.6 | 24.4 |
| Brain width (mm) | | | | | | | | |
| PND 21 | 14.6 | 14.6 | 14.0 | 14.5 | 14.0 | 13.9 | 13.9 | 13.6 |
| PND 72 | 15.1 | 14.9 | 15.2 | 14.8 | 14.9 | 14.7 | 14.5 | 14.6 |

*Statistically significantly different from control, $p<0.05$.

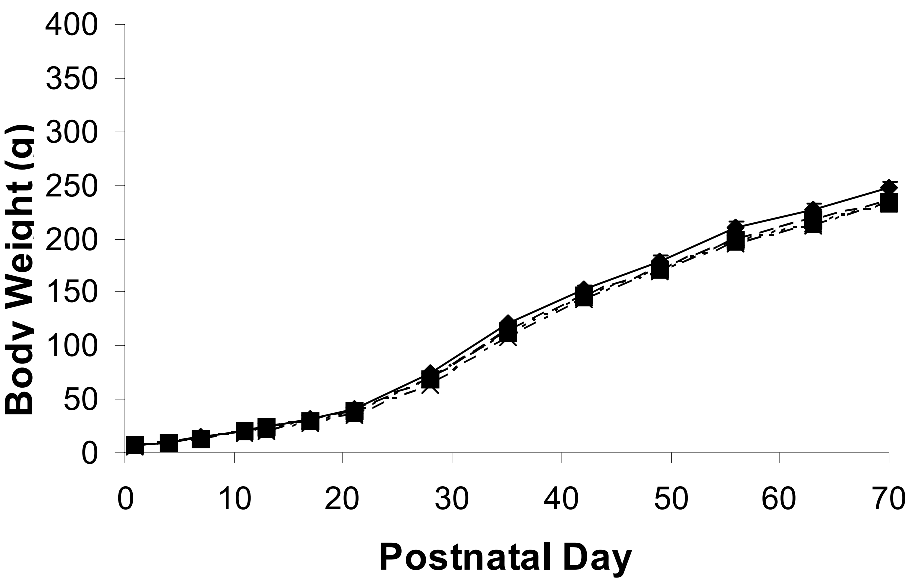
Figure 1.

A.



0 PPM
50 PPM
150 PPM
500 PPM
400
350
300
250
200
150
100
50
0
Body Weight (g)
0
10
20
30
40
50
60
70
Postnatal Day


B.



400
350
300
250
200
150
100
50
0
Body Weight (g)
0
10
20
30
40
50
60
70
Postnatal Day

Figure 2. Grip Strength

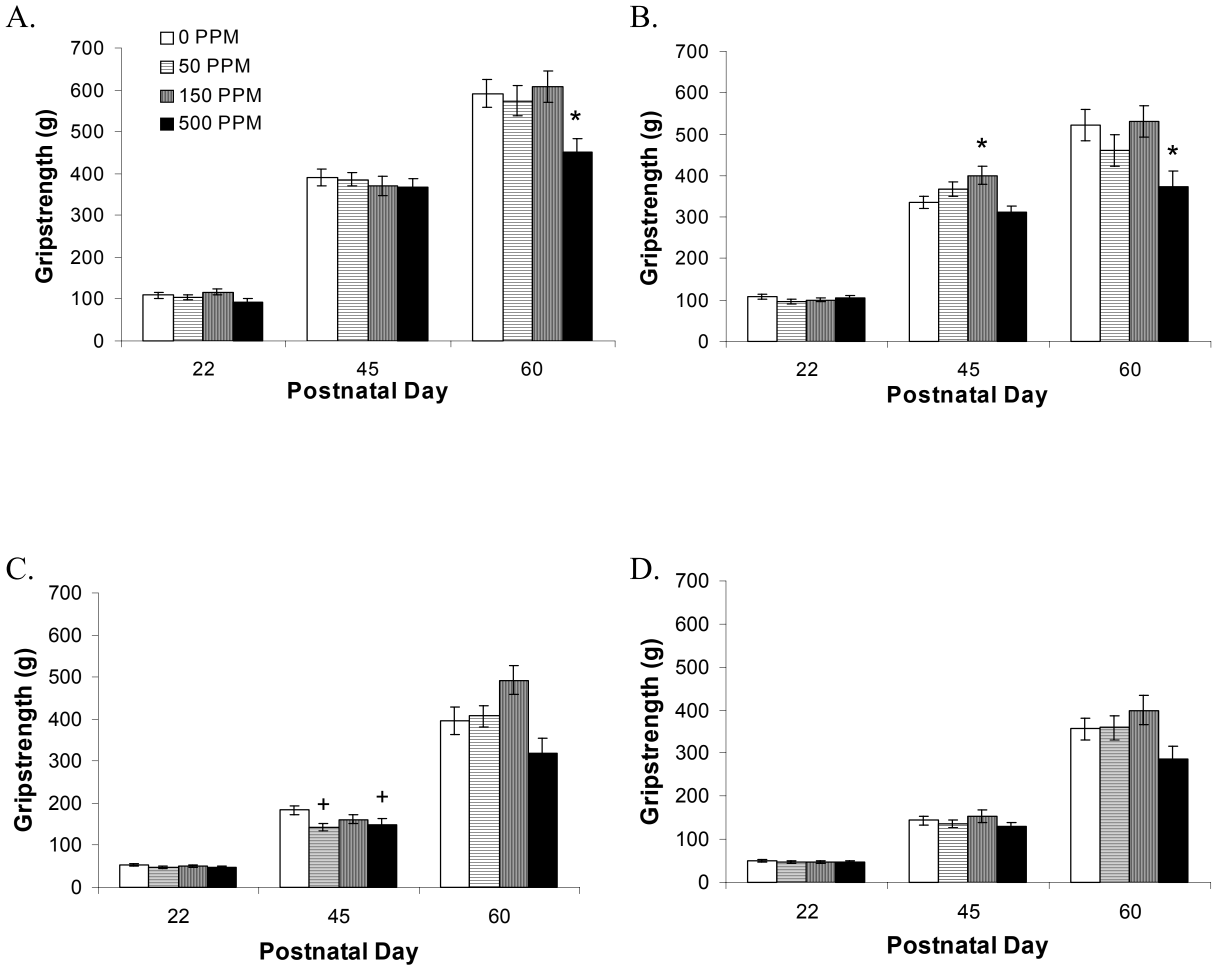
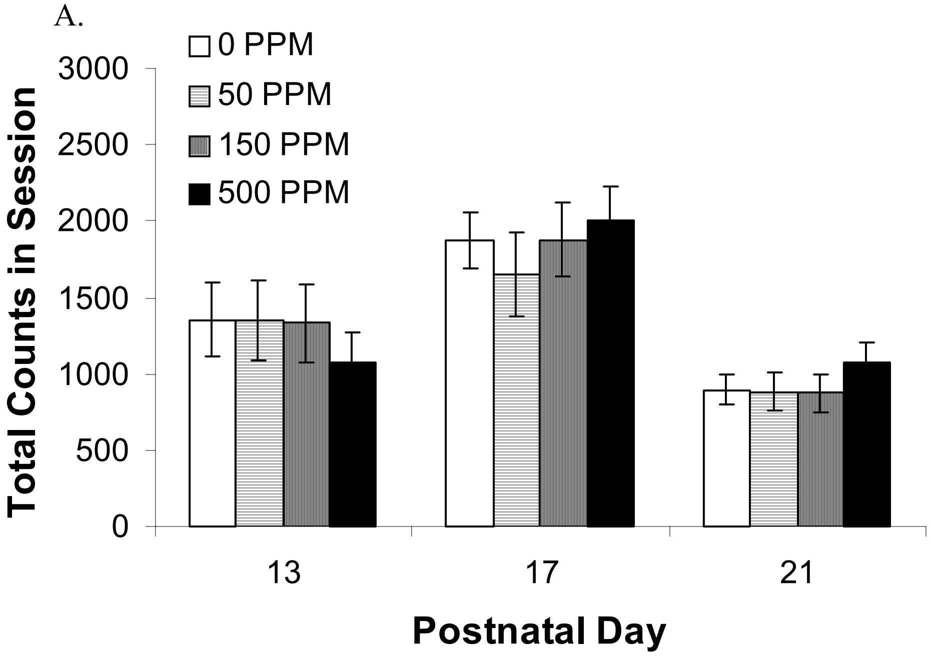
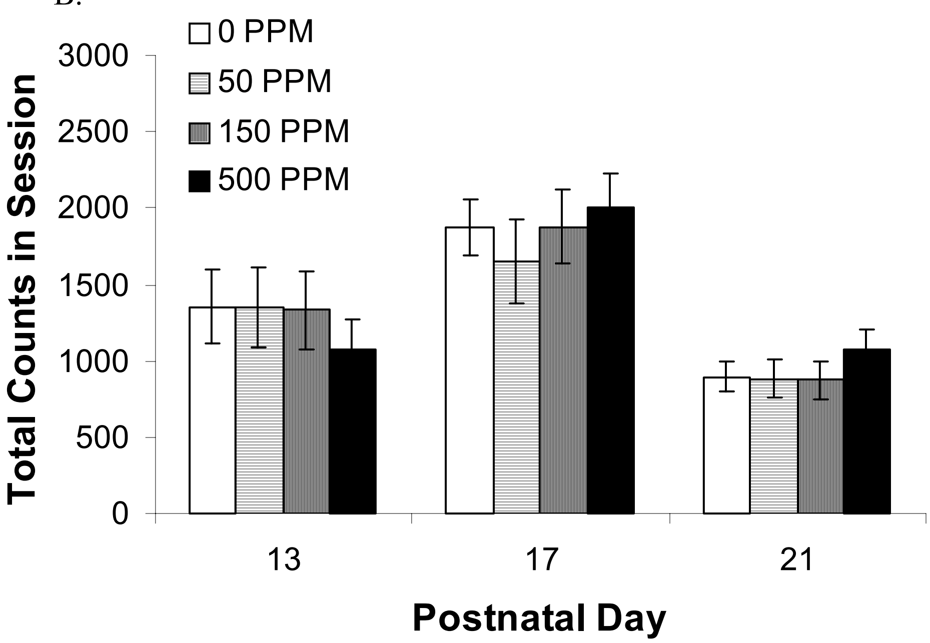


Figure 3. Total locomotor activity

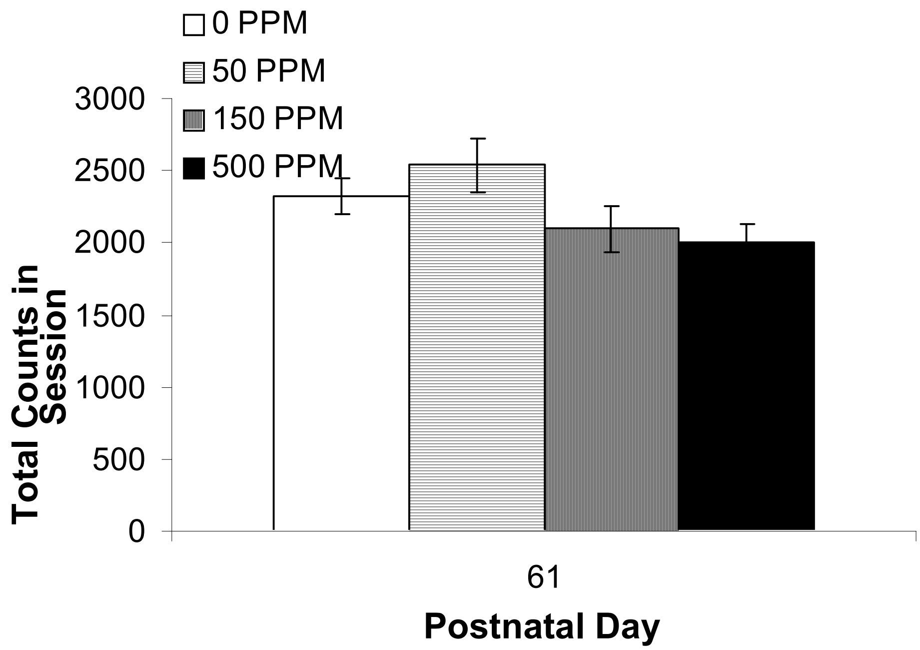
A.




B.



C.


0 PPM
50 PPM
150 PPM
500 PPM
3000
2500
2000
1500
1000
500
0
Total Counts in Session
61
Postnatal Day

D.

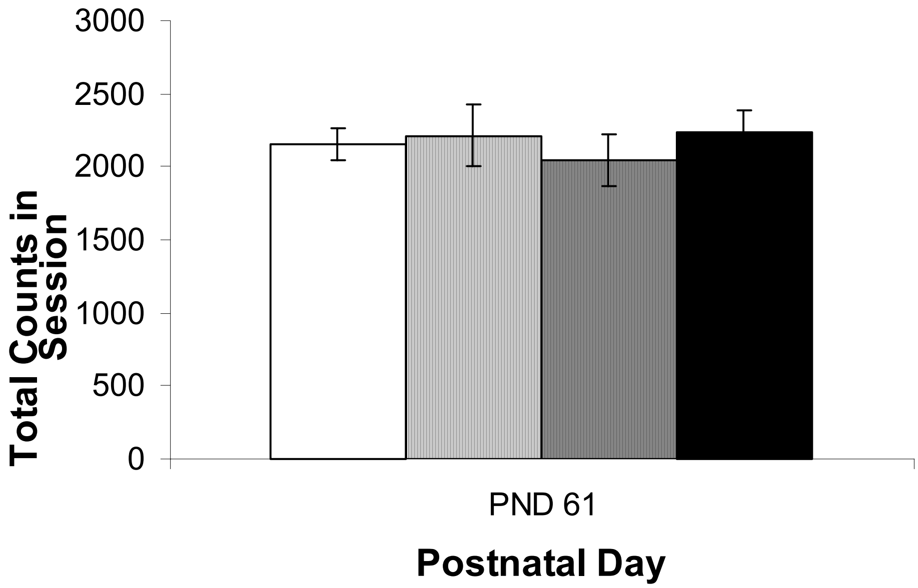
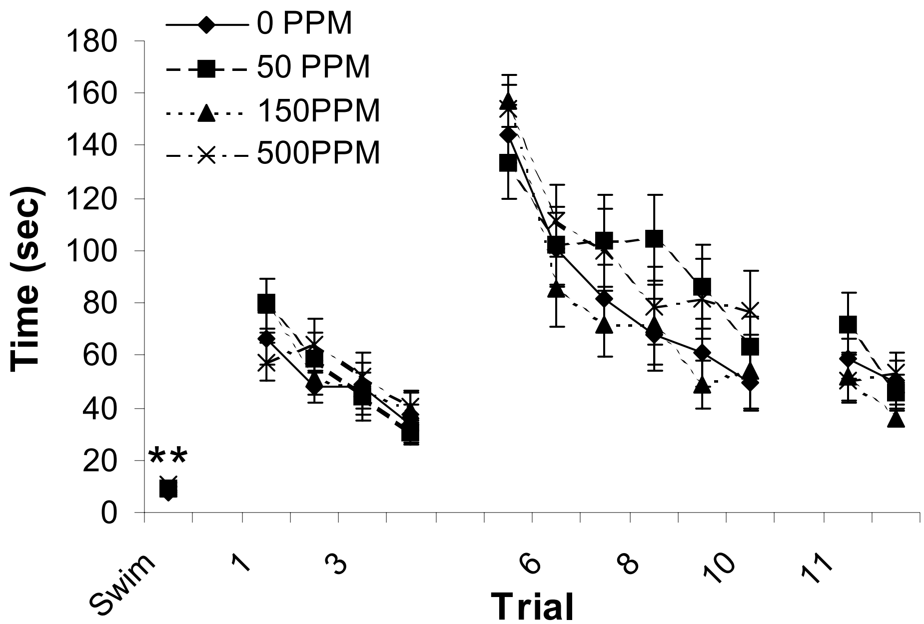

3000
2500
2000
1500
1000
500
0
Total Counts in Session
PND 61
Postnatal Day

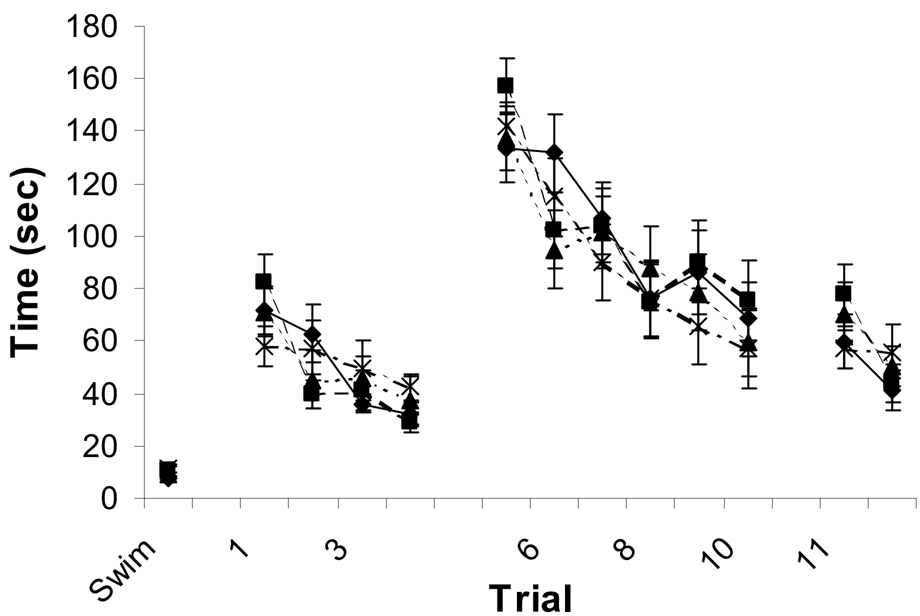
Figure 4.

A.



0 PPM
50 PPM
150PPM
500PPM
180
160
140
120
100
80
60
40
20
0
Time (sec)
**
Swim
1
3
6
8
10
11
Trial


B.



180
160
140
120
100
80
60
40
20
0
Time (sec)
Swim
1
3
6
8
10
11
Trial